DIETARY STRATEGIES FOR HEALTHY AGING – CALORIC RESTRICTION AND BEYOND

EDITED BY: Sebastian J. Hofer and Sergio Davinelli

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DIETARY STRATEGIES FOR HEALTHY AGING – CALORIC RESTRICTION AND BEYOND

Topic Editors:
Sebastian J. Hofer, University of Graz, Austria
Sergio Davinelli, University of Molise, Italy

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Editorial: Dietary Strategies for Healthy Aging—Caloric Restriction and Beyond

Sebastian J. Hofer[1] *and Sergio Davinelli*[2]*

[1] *Institute of Molecular Biosciences, NAWI Graz, University of Graz, Graz, Austria,* [2] *Department of Medicine and Health Sciences "V. Tiberio", University of Molise, Campobasso, Italy*

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Editorial on the Research Topic

Dietary Strategies for Healthy Aging—Caloric Restriction and Beyond

Ample evidence generated over the past two decades underlines the crucial role of nutrition to achieve healthy aging. Numerous dietary strategies and food components have increasingly been investigated as interventions to preserve function and compress morbidity and disability to a period later in life. Findings from preclinical investigations, as well as cross-sectional, longitudinal, and intervention studies indicated that well-established 'holistic' healthy diets [e.g., DASH (1) and Mediterranean diets (2, 3)] and specific dietary components [e.g., polyamines (4), and polyphenols (5)] may exert health-promoting effects during aging.

Among these dietary strategies, caloric restriction (CR), an ambiguous term often synonymously used with energy restriction (ER), has proven to be a very robust and broadly applicable intervention that affects aging-relevant molecular pathways and induces a range of beneficial effects involved in the maintenance of physiological functions throughout the life course (6, 7). Generally, CR is defined as a reduction in daily caloric intake without malnutrition. Different dietary alternative strategies have been developed to produce CR-like effects. Prominent examples include intermittent fasting (IF) and alternate-day fasting (ADF) (8). However, since the real-life applicability of CR is hardly sustainable in the long-term, the development of pharmacological mimetics of CR has gained considerable attention (9, 10). Multiple screenings have been performed or are currently taking place to identify synthetic or natural molecules that can be used to mimic CR phenotypes.

This Research Topic contains 11 articles covering the specific parts of the above-mentioned aspects.

Frailty is a prevalent age-related clinical condition generally recognized as a state of high vulnerability to adverse health outcomes (11). Although its etiology remains poorly understood, specific dietary regimens may prevent, delay, or even reverse this condition. Liu et al. summarize evidences on the impact of CR and alternative approaches on frailty syndrome, highlighting potential underlying mechanisms. Besides frailty, older adults are particularly vulnerable to cardiometabolic disease and unhealthy diet is a leading risk factor for the development of cardiometabolic disease (12). Perry et al. have examined the response of a caloric-restricted DASH (Dietary Approaches to Stop Hypertension) diet on parameters of cardiometabolic health in a cohort of sedentary obese older adults. They found that a DASH-like diet with restricted calories is an effective approach to improve cardiovascular, metabolic, and inflammatory biomarkers. Besides calories, macronutrients play an important role in shaping a diet's effects on health and aging (13). Dietary fiber is considered to contribute no calories to our diet (14) and its intake is associated with lower risk of type 2 diabetes, cardiovascular disease, and weight gain. However, the antihypertensive

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Jasminka Z. Ilich,
Florida State University, United States

***Correspondence:**
Sergio Davinelli
sergio.davinelli@unimol.it

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effect of dietary fiber intake has not been elucidated until now. Results from the SWAN study published in this e-collection revealed that intake of dietary fiber from grains contributes to a lower risk of hypertension in midlife women.

In this scenario, Voglhuber et al. provide an excellent overview of alternatives to CR (e.g., IF, ADF, and the Mediterranean diet) that could mimic the cardiometabolic benefits of CR in animal models and humans. The authors also summarize emerging potential CR mimetics, such as spermidine, NAD^+ precursors, and metformin, highlighting the mechanism underpinning their cardiometabolic and health-promoting effects. Similarly, Stadler and Marsche provide an insightful overview, detailing how dietary strategies and various nutritional components may improve the atheroprotective functions of high-density lipoproteins (HDL). They discuss the clinical efficacy of Mediterranean diet, CR, and IF to increase HDL functionality and promote cardiovascular health. Furthermore, these authors address this topic by focusing on phenolic compounds that could exert positive effects on HDL function.

As briefly discussed in the articles by Voglhuber et al., as well as Stadler and Marsche, CR and fasting applications in real-life contexts harbor significant challenges. Hence, the concept of CR mimetics (CRMs) was developed to identify bioactive substances that mimic the molecular effects induced by CR (9, 10, 15). Recently, this research field has gained an increasing scientific interest. Therefore, in Hofer et al. we have performed a comprehensive literature review describing a wide range of naturally occurring substances with CR-mimicking properties, along with their dietary sources, intake levels and the current state of clinical research. Despite challenges in assimilating experimental findings into clinical treatments, CRM candidates, such as polyamines, polyphenols, NAD^+ precursors, and glycolytic inhibitors, appear to prolong life- and healthspan in model organisms. Accordingly, the consumption of CRMs may elicit numerous disease-protecting activities, including neuroprotective effects. In line, the paper from de Vries et al. presents a systematic review and meta-analysis on the brain-enhancing effects of polyphenols, a promising source of potential CRMs. They included 66 randomized clinical trials with participants aged 40 years or older. The results indicate that short- and moderate-term supplementation with some classes of polyphenols may improve working and episodic memory in non-pathological and pathological aging.

Although religious fasting is practiced by millions of believers around the world, scarce data exist on its impact on human health. For centuries, humans have adopted various forms of religious fasting as regimens of spiritual purification. Bahá'í fasting is a specific form of intermittent dry fasting characterized by abstaining from food and liquids during daylight hours every year in March for 19 consecutive days. Koppold-Liebscher et al. conducted a prospective exploratory cohort study in Bahá'í volunteers to evaluate safety and effects of Bahá'í fasting on hydration, metabolism, and circadian clock. The study revealed that this form of fasting is safe with no negative effects on hydration biomarkers. Furthermore, Bahá'í fasting appears to improve fat metabolism and cause only transient alterations of circadian rhythms. Despite the relatively small number of studies on this topic, fasting treatments are also associated with detoxifying properties. Grundler et al. explored the effects of long-term fasting (10 days) on the excretion of heavy metals and glyphosate in 109 healthy subjects. The results of the study provide first insights into the changes in heavy metal excretion after fasting and show a reduction in the urinary levels of arsenic and nickel, as well as a reduction in hair lead levels.

The opinion article from Ostojic discusses the importance of the creatine transporter (CT1), which is located on the plasma membrane of various energy-demanding cells and whose dysfunction is associated with movement and behavior disorders. A potential but barely explored cellular effect of CR could be an increase in CT1 activity, facilitating creatinine uptake.

Finally, Wahl and LaRocca present a detailed review of the transcriptomic effects of healthy nutritional interventions, including CR, IF, ADF, prolonged fasting, time-restricted feeding, and protein and amino acid restriction. They describe gaps in the research, highlighting the importance of transcriptome/multi-omic studies to better understand the effects of these dietary interventions.

Despite the richness and importance of the topics covered in the papers published in this e-collection, the complex impact of CR and CR-alternatives on aging and health is still insufficiently understood and there are many aspects to be clarified in this fascinating research field.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Cardiometabolic Changes in Response to a Calorie-Restricted DASH Diet in Obese Older Adults

Cydne A. Perry[1], Gary P. Van Guilder[2], Mosharraf Hossain[3] and Alyssa Kauffman[3]*

[1] Department of Applied Health Science, Indiana University Bloomington School of Public Health, Bloomington, IN, United States, [2] High Altitude Exercise Physiology Department, Western Colorado University, Gunnison, CO, United States, [3] Department of Health and Nutritional Sciences, South Dakota State University, Brookings, SD, United States

Objective: To examine the response of a calorie-restricted Dietary Approaches to Stop Hypertension diet on indicators of cardiometabolic health in a cohort of sedentary obese older adults.

Design: This was a controlled-feeding trial with a parallel design. Each participant consumed either 3 oz (85 g; $n = 15$) or 6 oz (170.1 g; $n = 13$) of lean fresh beef within a standardized calorie-restricted DASH-like diet for 12-weeks. Fasted blood samples were collected and used to measure conventional biomarkers of cardiovascular, metabolic and inflammatory health.

Participants: Caucasian older (70.8 years), obese (BMI: 32 ± 6.9 kg/m^2; WC: 101 ± 16.4 cm) females ($n = 17$) and males ($n = 11$) from the rural community of Brookings, South Dakota.

Results: 28 participants completed the 12-week feeding trial, with no differences ($p > 0.05$) among the biomarkers of cardiometabolic health between the 3 and 6 oz beef intake groups. However, when the beef intake groups were combined, all biomarkers changed concentration in response to the intervention diet. Total cholesterol ($p < 0.001$), LDL-C ($p = 0.004$), HDL-C ($p < 0.0001$), insulin ($p = 0.014$), glucose ($p = 0.008$), HOMA-IR ($p < 0.05$), IL-12 ($p < 0.001$), and CRP ($p = 0.006$) all decreased in response to the study diet. IGF-1 ($p < 0.001$) and IL-8 ($p = 0.005$) increased in response to the intervention. Correlations among cardiometabolic biomarkers and body composition measures were observed. By study end, the decrease in insulin ($R^2 = 0.22$; $P = 0.012$) and HOMA-IR ($R^2 = 0.22$; $P = 0.01$) was positively correlated with the decrease in waist circumference. The increase in IGF-1 was significantly correlated with the decrease in waist circumference ($R^2 = 0.21$; $p = 0.014$). The increase in IGF-1 was significantly correlated with the increase in sit-to-stand ($R^2 = 0.21$; $p = 0.016$). The increase in IL-8 was significantly correlated with decreases in total cholesterol ($R^2 = 0.24$; $P = 0.008$), LDL-C ($R^2 = 0.17$; $P = 0.031$) and glucose ($R^2 = 0.44$; $P = 0.0001$).

Conclusions: These findings suggest that a DASH-like diet with restricted calories may potentially improve biomarkers of cardiometabolic health in sedentary obese older

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Edited by:
Sebastian J. Hofer, University of Graz, Austria

Reviewed by:
Pao-Hwa Lin, Duke University Medical Center, United States
Helen Hermana M. Hermsdorff, Universidade Federal de Viçosa, Brazil

****Correspondence:***
Cydne A. Perry
cydperry@iu.edu

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adults. These results also point to interrelationships between body composition changes and changes in cardiometabolic biomarkers. Lastly, regardless of meat intake amount, positive impacts on cardiometabolic biomarkers were observed in this cohort of older adults with an obese phenotype.

Keywords: older adults, calorie restriction, DASH diet, obesity, cardiometabolic health

INTRODUCTION

Cardiometabolic disease is an umbrella term that describes a cluster of modifiable risk factors (i.e., hypertension, abdominal adiposity, dyslipidemia, and increased fasting glucose and triglycerides) that increase a person's risk for developing cardiovascular disease, type-2 diabetes, and metabolic syndrome (1, 2). The older adult population is particularly vulnerable to cardiometabolic disease as they are more likely to experience co-existing risk factors (3). Currently, 41% of adults aged 65 years and older in the United States are obese and 80% have at least one chronic disorder related to cardiometabolic disease (4, 5). Furthermore, the older adult population is projected to increase to 98 million by the year 2060 (6). With 47 million Americans experiencing cardiometabolic disorders (7) and the health risks associated with the growing older adult population, it is important to begin implementing targeted intervention strategies that decrease risk factors associated with cardiometabolic disease that will in turn result in the reduction of cardiovascular disease and type-2 diabetes in older adults.

Diet quality is an influential factor in the development of cardiometabolic disease (8, 9) and dietary patterns are vital to the quality of life and survival in older adults (10). Unhealthy diet is one of the leading risk factors for cardiometabolic disease in the United States (11) and accounts for at least 45% of all cardiometabolic deaths (12, 13). With the current diet-related cardiometabolic disease health costs estimated to be $50.4 billion and individual costs being highest among men >65 years (14), effective diet therapies need to be implemented to address this health issue in older adults that in turn reduce the economic burden that ensues. In a 12-week controlled-feeding study examining body composition and muscle strength changes in response to a DASH-like diet in obese older adults, we observed improvements characterized by reductions in body fat, waist circumference, and blood pressure (15). Additionally, handgrip strength was well-maintained with an increase in strength-to-weight ratio (15). Extending the scope of these findings and given the role that abdominal adiposity and blood pressure play on cardiometabolic health, our objectives for this study were two-fold: (i) to evaluate changes in blood biomarkers of cardiovascular, metabolic, and inflammatory health in response to a calorie-restricted DASH study diet in obese adults 65 years and older; and (ii) to assess associations between cardiometabolic biomarkers and body composition measures in this cohort of older adults.

MATERIALS AND METHODS

Study Participants

Participant characteristics, recruitment and study diet were previously reported (15). Briefly, sedentary adults aged 65-years and older were recruited from Brookings, South Dakota from June 2017 to August 2018 (15). Interested volunteers completed a questionnaire that included date of birth, medication use, vitamin and mineral use, and drug and alcohol use prior to the start of the study. Participation on this study depended on the following: (1) age; (2) upward mobile ability; (3) eating one meal per day at the on-site location; (4) not consuming foods and beverages outside of those provided by research personnel; and (5) provide blood samples at 5 timepoints throughout the intervention. A full characterization of body composition measurements and outcomes has been previously published (15). The study was conducted in accordance with the Declaration of Helsinki. The protocol was reviewed and approved by the Institutional Review Board for Human Study Participant Use at South Dakota State University (Approval #: IRB-1712006-EXP) and informed consent was obtained from all participants before entry into the study (ClinicalTrials.gov Identifier: NCT04127240).

Study Design and Diet Intervention

This was a human controlled-feeding trial with a parallel design in which females ($n = 17$) and males ($n = 11$) aged 65-years and older were assigned to consume either 3 oz (85 g; $n = 15$) or 6 oz (170.1 g; $n = 13$) of lean fresh beef per day within a standardized DASH-like diet as they entered the study. As previously described, the study diet was created using Nutritionist Pro software and based on the DASH eating plan by the National Heart, Lung and Blood Institute, National Institutes of Health (16) and the 2015–2020 *Dietary Guidelines for Americans* for daily caloric intake for older sedentary adults (17). All participants consumed the same standardized DASH-like diet with the exception of the meat intake amounts. The 3 or 6 oz amounts were equally provided among the three major meals: breakfast (1 or 2 oz), lunch (1 or 2 oz), and dinner (1 or 2 oz) for a total of 3 or 6 oz for the entire day. All foods were purchased by research personnel from the local grocery store. All food items were weighed out to the nearest gram and prepared at the South Dakota State University food's laboratory. All participants were required to eat at least one meal per day in the food's laboratory Monday through Friday; all other meals, snacks, and beverages were provided as takeaways. The caloric intake for this study was based upon the 2015–2020 *Dietary Guidelines for Americans* for daily caloric intake for

sedentary adults aged 61 years and older (17). The participants that were assigned 3 oz of beef consumed 1,700 calories per day. The 6 oz beef intake group consumed 1,900 calories per day. With beef intake groups combined the participants consumed on average 1,800 calories per day. As previously described, the composition of the study diet included the following estimated (based upon Nutritionist Pro software) daily servings: 7 servings of grains (all whole grains); 5 servings of vegetables; 4 servings of fruits; 3 servings of dairy (low-fat); 4.5 servings of lean meat (average of 3 and 6 oz intakes); 4 servings/week of legumes; 0 servings of sweets. These serving sizes were within the DASH eating plan by the National Heart, Lung and Blood Institute, National Institutes of Health (15, 16). Additionally, as an average of the two meat intake groups, the study diet provided an estimated 1,895 mg/d of sodium, 585 mg magnesium, 4,395 mg potassium, and 1,187 mg calcium, 59% carbohydrates, 21% fat, 20% protein, and 8% saturated fat (15). Since all participants consumed a daily multivitamin/multimineral supplement an additional 50 mg magnesium, 80 mg potassium, and 220 mg calcium was provided.

Since participants were required to eat one meal in the food's laboratory Monday–Friday, investigators had consistent interactions with study participants throughout the study period, which enhanced the compliance to the dietary regimen. In addition, participants verified consumption of each food item by completing a daily checklist provided by the investigators. A multivitamin/multimineral supplement for seniors was provided daily to ensure adequate micronutrient intake.

Blood Sample Collection and Analytical Measurements

Fasting blood samples were collected in two 10-mL serum separator clot activator tubes (SST Vacutainer; Pulmolab) and two EDTA-coated tubes (Pulmolab) by a trained phlebotomist. The two 10-mL EDTA-coated tubes were put on ice immediately after blood collection and centrifuged within 90 min at 1,055 × g for 15 min at 4°C. The SST tubes were kept at room temperature, allowed to clot, and centrifuged at 650 × g for 15 min at room temperature. All of the samples were aliquoted into 1.8-mL cryostat vials (CryoTube; NUNC) and stored at −80°C.

Quantification of total cholesterol, LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), insulin, glucose, insulin-like growth factor 1 (IGF-1), and C-reactive protein (CRP) were performed by the Human Nutritional Chemistry Service Laboratory at Cornell University (Ithaca, NY). The Dimension Xpand plus integrated chemistry automated analyzer (Siemens Healthineers) was used to measure total cholesterol (intra- and interassay CV 1.9 and 8.2%, respectively), LDL-C (intra- and interassay CV 1.3 and 1.0%, respectively), HDL-C (intra- and interassay CV 1.4 and 2.6%, respectively) and glucose (intra- and interassay CV 0.8 and 1.3%, respectively). The Immulite 2000 automated immunoassay system (Siemens Healthineers) was used to measure CRP (intra- and interassay CV 4.5 and 4.1%, respectively), IGF-1 (intra- and interassay CV 4.2 and 7.0%, respectively) and insulin (intra- and interassay CV 5.5 and 3.4%, respectively).

Meso Scale Discovery (Meso Scale Diagnostics, LLC, USA) measured interleukin-8 (intra- and interassay CV 4.7 and 4.4%, respectively) and interleukin-12 (intra- and interassay CV 5.8 and 6.8%, respectively) using the V-plex proinflammatory panel 1 human kit on the Meso QuickPlex SQ 120 with electrochemiluminescence detection.

The homeostatic model assessment of insulin resistance (HOMA-IR) was used to quantify insulin sensitivity using the following formula: fasting plasma glucose (mmol/l) times fasting serum insulin (μIU/mL) divided by 22.5.

Body Composition and Muscle Strength Measurements

Body composition and muscle measurements were previously detailed and reported (15). Briefly, body mass index was calculated as total body weight in kilograms divided by height in meters squared. A Gulick tape was used to measure abdominal waist circumference. The measurement was taken at the smallest part of the abdomen, above the umbilicus and below the xiphoid process to the nearest 0.1 cm at the end of normal expiration using standard procedures. Total body weight and percent body fat were measured by bioelectrical impedance (InBody 270, InBody USA, Cerritos, California). Handgrip strength (kg) was quantified by the maximum grip force of the right and left hand using a hand-held dynamometer (Smedley III analog). Right and left grip strength data were summed to provide a composite score. Grip strength relative to total body weight was calculated by dividing grip force by the body mass (kg) of the participant at each time point.

Statistical Analysis

Differences in baseline characteristics between males and females and for beef intake groups were determined by Independent Samples *T*-test. Differences in cardiometabolic and body composition characteristics between beef intake groups at week 12 was determined by Independent Samples *T*-test. Linear mixed models with a random intercept for each participant and Time (weeks 0, 3, 6, 9, and 12) as the fixed effect was used to determine changes in the primary outcome variables across the intervention. An unstructured covariance matrix was assumed. The primary outcome of interest was the difference between baseline and week 12. When indicated by a significant Time effect, pairwise differences at specific time points were identified using the Bonferroni adjustment for multiple comparisons. To adjust for the influence of changes in body weight across the intervention on cardiometabolic variables, we repeated the analyses of the time effect by including body weight as a covariate in linear mixed model. To check the robustness of the primary outcomes, we performed sensitivity analyses with the exclusion of the three normal weight BMI participants. In addition to pooling data for males and females, data are displayed separately by sex. Relations between variables of interest were determined by Pearson's correlation coefficient. Stepwise multiple regression analysis was performed to identify the independent determinants of the change from baseline in wait circumference, IGF-1 levels, and IL-8 levels. In each multiple regression model, variables with a related probability of >0.10 were removed. Statistical

significance was set at $p < 0.05$. Data are presented as means (SD) and analyzed with SPSS version 24 (IBM Inc., Armonk, NY, USA).

RESULTS

Baseline (Week 0) Characteristics of Study Participants

Twenty-eight participants aged 70.8 years (range = 65–84 years) completed the 12-week controlled-feeding study and were included in the final analysis. Baseline characteristics of cardiometabolic and body composition measures separated by beef intake amounts are presented in **Table 1**. There were no statistically significant differences ($p > 0.05$) detected between the 3 oz (85 g) and 6 oz (170.1 g) meat intake groups at baseline for age, cardiometabolic biomarkers or body composition measures. Baseline characteristics of cardiometabolic biomarkers separated by sex are presented in **Table 2**. At baseline, females had statistically higher total cholesterol ($p = 0.02$) and HDL-C ($p < 0.0001$) compared to males. No statistical differences ($p > 0.05$) were detected for LDL-C, insulin, glucose, HOMA-IR, IGF-1, IL-8, IL-12, and CRP between females and males. Baseline body composition characteristics separated by sex has been previously reported (15). Briefly, at baseline males had greater ($p < 0.05$) body fat, waist circumference and grip strength compared to females.

Prior to entry into the study, participants provided information regarding medication use. Self-reported medication use is shown in **Supplementary Table 1**.

TABLE 1 | Baseline characteristics of study participants separated by meat intake group.

Variables	3 oz meat intake group ($n = 15$)	6 oz meat intake group ($n = 13$)	*p*-value
Age (years)	70.6 (5.9)	71.1 (6.0)	0.8341
Female	8	9	–
Male	7	4	–
Cardiometabolic markers			
Total cholesterol (mg/dL)	189.5 (37.3)	171.0 (37.9)	0.2053
LDL-C (mg/dL)	109.4 (29.4)	98.7 (28.2)	0.3364
HDL-C (mg/dL)	53.4 (14.7)	55.0 (19.7)	0.8058
Insulin (μIU/mL)	13.7 (7.0)	13.9 (9.8)	0.9622
Glucose (mg/dL)	105.8 (20.9)	110.2 (26.4)	0.6277
HOMA-IR	3.70 (2.14)	4.24 (4.29)	0.6684
IGF-1 (ng/mL)	96.1 (22.9)	93.3 (17.9)	0.7221
IL8 (pg/mL)	6.20 (2.52)	6.35 (3.18)	0.8887
IL12 (pg/mL)	0.99 (1.12)	0.79 (0.71)	0.7624
CRP (mg/L)	2.69 (2.46)	4.26 (5.36)	0.3173
Body composition			
Total body weight (kg)	92.7 (16.8)	87.1 (18.2)	0.4417
Body mass index	32.1 (6.1)	30.4 (6.5)	0.4883
Waist circumference (cm)	100.2 (13.7)	96.0 (14.8)	0.4147
Body fat (%)	36.2 (10.1)	36.2 (10.7)	0.9860
Handgrip strength (kg)	68.4 (22.1)	59.0 (15.2)	0.2032
Sit-to-stand (reps)	11.9 (1.8)	10.5 (2.0)	0.0640

Data are presented as means and standard deviations with the exception of the number of females and males. Independent samples T-test was performed to determine group differences in the baseline characteristics by meat intake group.

TABLE 2 | Baseline cardiometabolic characteristics separated by sex.

Variables	Females ($n = 17$)	Males ($n = 11$)	*p*-value
Total cholesterol (mg/dL)	194.3 (36.1)	160.1 (32.3)	**0.02**
LDL-C (mg/dL)	108.3 (31.4)	98.5 (24.9)	0.39
HDL-C (mg/dL)	62.6 (16.2)	41.2 (6.8)	**<0.0001**
Insulin (μIU/mL)	13.3 (9.4)	15.2 (6.4)	0.57
Glucose (mg/dL)	104.7 (23.3)	112.7 (24.0)	0.39
HOMA-IR	3.9 (3.9)	4.3 (2.1)	0.78
IGF-1 (ng/mL)	91.2 (24.0)	100.4 (12.2)	0.25
IL8 (pg/mL)	6.4 (3.4)	6.0 (1.5)	0.73
IL12 (pg/mL)	0.86 (0.6)	1.0 (1.3)	0.71
CRP (mg/L)	3.4 (2.5)	1.8 (1.2)	0.09

Baseline body composition characteristics separated by sex has been previously reported (15). Data are presented as means and standard deviations. Independent samples T-test was performed to determine group differences in the baseline characteristics between females and males. The bold values indicate the biomarkers that are statistically significant.

Cardiometabolic and Body Composition Changes in Response to the Study Diet

Effects of meat intake on cardiometabolic and body composition outcomes at week 12, are presented in **Table 3**. By week 12 of the intervention there were no statistically significant differences ($p > 0.05$) between the 3 oz (85 g) and 6 oz (170.1 g) meat intake groups on cardiometabolic outcomes or body composition measures.

Cardiometabolic changes in response to the intervention diet with both meat intake groups combined are shown in **Table 4**. Throughout the 12-week intervention period, significant changes in response to the study diet across the intervention period were detected. Significant decreases were observed in all participants within the 12-week intervention period for total cholesterol ($p < 0.001$); LDL-C ($p = 0.004$); HDL-C ($p < 0.001$); insulin ($p = 0.014$); glucose ($p = 0.008$); HOMA-IR ($p < 0.05$); IL-12 ($p < 0.001$) and CRP ($p = 0.006$). Significant increases in response the study diet was detected for IGF-1 ($p < 0.001$); and IL-8 ($p = 0.005$). For all the biomarkers listed in **Table 4**, observed power for the effect of diet across time was high. Power for the favorable changes across time for total cholesterol, LDL-C, HOMA-IR, IL-12, IGF-1, IL-8, and CRP were >90%. Observed power for glucose was 80 and 75% for insulin. After performing sensitivity analyses with the exclusion of the 3 normal weight BMI participants, it can be concluded that the statistically significant decreases for total cholesterol ($p < 0.001$); LDL-C ($p = 0.001$); HDL-C ($p < 0.003$); insulin ($p = 0.02$); glucose ($p = 0.0037$); HOMA-IR ($p < 0.0111$); IL-12 ($p < 0.002$) and CRP ($p = 0.02$) remained. Sensitively analyses also revealed that the significant increases in IGF-1 ($p < 0.001$) and IL-8 ($p = 0.007$) remained after excluding the normal weight BMI participants.

TABLE 3 | Cardiometabolic and body composition characteristics of obese older adults at week 12.

Variables	3 oz meat intake group ($n = 15$)	Percent change from baseline	6 oz meat intake group ($n = 13$)	Percent change from baseline	*p*-value
Cardiometabolic markers					
Total cholesterol (mg/dL)	176.5 (30.9)	−6.9 (17.2)	164.9 (42.6)	−3.6 (12.4)	0.4203
LDL-C (mg/dL)	104.7 (31.7)	−4.3 (7.8)	94.6 (27.6)	−4.2 (2.1)	0.3796
HDL-C (mg/dL)	49.5 (10.5)	−7.3 (28.6)	49.7 (17.0)	−9.6 (13.7)	0.9757
Insulin (μIU/mL)	10.2 (6.2)	−25.5 (11.4)	11.5 (7.1)	−17.3 (27.6)	0.5994
Glucose (mg/dL)	96.2 (19.2)	−9.1 (8.1)	100.4 (27.5)	−8.9 (4.2)	0.6364
HOMA-IR	2.43 (1.64)	−34.3 (23.4)	3.25 (3.24)	−23.3 (24.5)	0.3961
IGF-1 (ng/mL)	104.1 (21.0)	8.3 (8.3)	100.2 (17.1)	7.4 (4.5)	0.5913
IL-8 (pg/mL)	7.30(4.00)	17.7 (58.7)	7.00 (3.42)	10.2 (7.5)	0.8318
IL-12 (pg/mL)	0.82 (0.98)	−17.2 (12.5)	0.80 (0.65)	1.3 (8.5)	0.9502
CRP (mg/L)	2.76 (2.60)	2.6 (5.7)	3.11 (5.03)	−27.0 (6.2)	0.8159
Anthropometric measures					
Total body weight (kg)	86.7 (14.4)	−6.5 (14.3)	82.1 (17.0)	−5.7 (6.6)	0.4495
Body mass index (kg/m^2)	30.1 (5.8)	−6.2 (4.9)	28.6 (6.0)	−5.9 (7.7)	0.5194
Waist circumference (cm)	96.4 (12.3)	−3.8 (10.2)	92.0 (14.0)	−4.2 (5.4)	0.3815
Body fat (%)	33.4 (11.3)	−7.7 (11.9)	35.0 (9.7)	−3.3 (9.3)	0.7026
Handgrip strength (kg)	68.7 (19.7)	0.4 (10.9)	62.1 (15.1)	5.3 (0.7)	0.3356
Sit-to-stand (reps)	13.9 (2.7)	16.8 (50.0)	13.9 (2.6)	32.4 (30.0)	0.9920

Data are presented as means and standard deviations. Independent samples T-test on absolute data was performed to determine group differences in the cardiometabolic and body composition characteristics by meat intake group.

TABLE 4 | Cardiometabolic biomarker changes in obese older adults consuming the DASH diet for 12-weeks.

	Weeks of intervention					
Variables	**0**	**3**	**6**	**9**	**12**	***p*-value**
Total cholesterol (mg/dL)	180.9 (38.1)	163.5 (30.7)	164.4 (30.9)	164.2 (34.3)	171.4 (36.3)*	<0.001
LDL-C (mg/dL)	104.5 (28.9)	95.7 (25.6)	95.6 (25.6)	96.3 (27.7)*	100.0 (29.8)	0.004
HDL-C (mg/dL)	54.2 (16.9)	50.1 (13.8)	49.8 (14.0)	48.0 (13.6)	49.6 (13.6)*	<0.001
Insulin (μIU/mL)	14.1 (8.2)	12.4 (8.3)	12.2 (7.7)	10.6 (6.5)	11.3 (6.5)*	0.014
Glucose (mg/dL)	108.5 (23.4)	102.4 (14.9)	100.9 (18.6)	98.1 (17.8)	101.3 (20.8)*	0.008
HOMA-IR	4.0 (3.3)	3.3 (2.3)	3.3 (2.7)	2.7 (2.2)	3.0 (2.5)*	<0.05
IGF-1 (ng/mL)	94.8 (20.4)	103.6 (22.9)	104.7 (21.9)	106.6 (22.1)	102.1 (19.3)*	<0.001
IL8 (pg/mL)	6.3 (2.8)	6.7 (4.3)	5.9 (3.2)	9.8 (5.5)*	8.1 (4.9)	0.005
IL12 (pg/mL)	0.79 (0.6)	0.65 (0.5)*	0.70 (0.5)	0.56 (0.4)*	0.69 (0.5)	<0.001
CRP (mg/L)	2.8 (2.2)	1.9 (1.8)*	2.3 (2.0)	2.5 (2.5)	2.3 (2.1)*	0.006

*Body composition changes in response to the study diet was previously reported (15). Data are presented as means and standard deviations, Linear mixed models with a random intercept for each participant and Time (weeks 0, 3, 6, 9, and 12) as the fixed effect was used to determine changes in the primary outcome variables across the intervention. *p < 0.05 vs. baseline.*

As previously reported, total body weight decreased by 6.3% in all participants by week 12 in response to the intervention diet (15). When adjusting for changes in total body weight, the significant decreases in total cholesterol (adjusted $p < 0.001$), LDL-C (adjusted $p = 0.002$), glucose (adjusted $p = 0.049$) and HOMA-IR (adjusted $p = 0.045$) remained. Changes in body composition measures in response to the intervention diet has been previously reported (15).

By week 12, in all participants, total cholesterol decreased by 4.9% ($p < 0.001$); HDL-C decreased by 8.5% ($p < 0.001$); insulin decreased by 13.1% ($p = 0.014$); glucose decreased by 8.4% ($p = 0.008$); HOMA-IR decreased by 25% ($p < 0.05$); IGF-1 increased 10% ($p < 0.001$); IL-8 increased by 39% ($p = 0.005$); and CRP decreased by 18% ($p = 0.006$). By week 9, in all participants, LDL-C decreased by 4% ($p = 0.004$) and IL-12 decreased by 12.7% ($p < 0.001$).

At baseline, 46% (8 females; 5 males) of the participants entered the study with features of metabolic abnormalities identified by large waist circumference (males: >40 inches; females >35 inches), low HDL-C (males: <40 mg/dL; females

<50 mg/dL), high blood pressure (>130/85 mmHg), and high fasting glucose (>100 mg/dL). As a result of the intervention, by week 12, 17.8% of the participants displayed the above characteristics, representing a significant reduction ($p = 0.008$ for McNemar Chi-square test).

All participants that self-reported medication use at baseline remained on their medications throughout the intervention period except for one male participant whose physician had taken him off of his statin medication.

Correlations Between Cardiometabolic Biomarkers and Body Composition Measures

Correlations between insulin sensitivity markers and waist circumference are presented in **Figure 1**. As previously reported, waist circumference decreased by 3.7% in response to the study diet (15). This change in waist circumference was associated with decreases in insulin ($R^2 = 0.22$; $p = 0.012$; **Figure 1A**) and HOMA-IR ($R^2 = 0.22$; $p = 0.01$; **Figure 1B**). Additionally, there was an inverse relationship between waist circumference and grip strength, such that the decrease in waist circumference was associated with the increase in grip strength ($R^2 = 0.28$; $p = 0.004$; **Figure 1C**). Age, and the percent change from baseline in insulin, HOMA, total cholesterol, HDL-cholesterol, LDL-cholesterol, glucose, body weight, percent body fat, BMI, IGF-1, IL-8, IL-12, CRP, grip strength, and 30-s sit-to-stand were included in the multiple regression model to predict the change in waist circumference. The prediction model was statistically significant ($F = 13.441$; $p < 0.001$) and accounted for 50% of the variance of the decrease in waist circumference (Adjusted $R^2 = 0.499$). Percent reduction in body weight ($\beta = 0.481$; $p = 0.004$) and the percent increase in grip strength ($\beta = -0.405$; $p = 0.014$) were the only independent predictors of the decrease in waist circumference. All other variables were excluded.

Associations between IGF-1 and body composition measures are shown in **Figure 2**. An inverse relationship between IGF-1 and waist circumference was observed, such that the increase in IGF-1 was associated with the decrease in waist circumference ($R^2 = 0.21$; $p = 0.014$; **Figure 2A**). The increase in IGF-1 was associated with the increase in the sit-to-stand test ($R^2 = 0.21$; $p = 0.016$; **Figure 2B**). Age, and the percent change from baseline in insulin, HOMA, total cholesterol, HDL-cholesterol, LDL-cholesterol, glucose, body weight, percent body fat, BMI, IL-8, IL-12, CRP, grip strength, and 30-s sit-to-stand were included in the multiple regression model to predict the change in IGF-1. The prediction model was statistically significant ($F = 6.331$; $p = 0.019$) and accounted for 18% of the variance of the increase in IGF-1 (Adjusted $R^2 = 0.176$). Percent increase in the 30-s sit-to-stand test ($\beta = 0.385$; $p = 0.019$) independently predicted the increase in IGF-1. All other variables were excluded.

Relationships between IL-8 and cardiometabolic biomarkers are presented in **Figure 3**. The increase in IL-8 was associated with decreases in total cholesterol ($R^2 = 0.24$; $p = 0.008$; **Figure 3A**); LDL-C ($R^2 = 0.17$; $p = 0.031$; **Figure 3B**); and glucose ($R^2 = 0.44$; $p = 0.0001$; **Figure 3C**). Age, and the percent

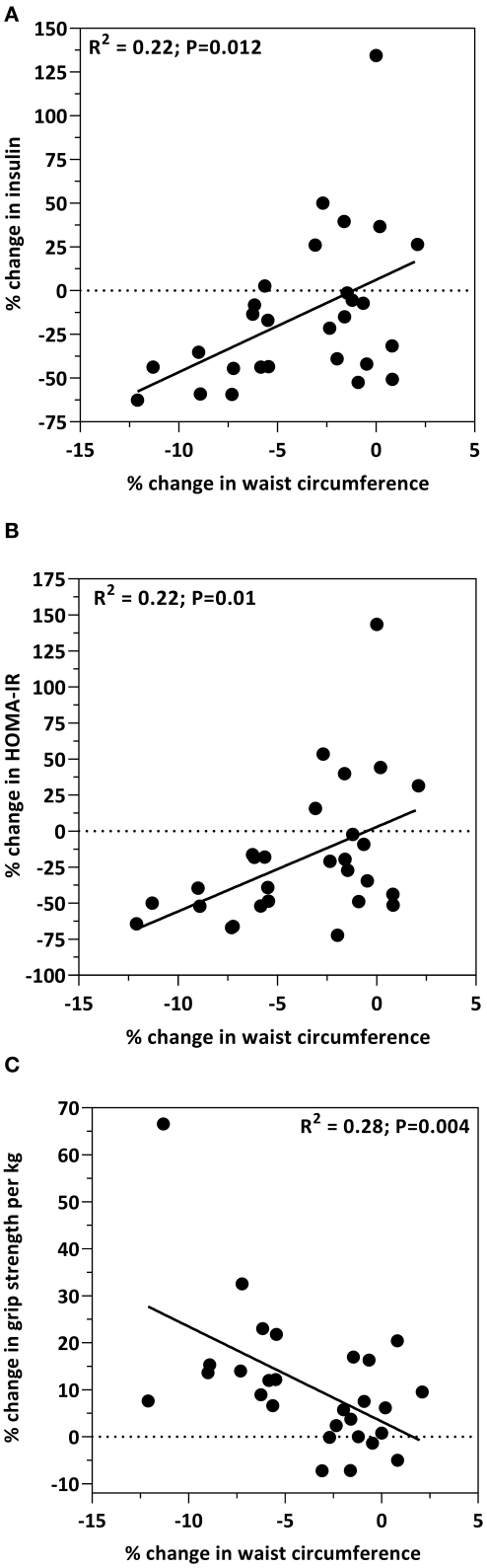

FIGURE 1 | Bivariate correlations between the percent change from baseline in waist circumference and the percent change from baseline in insulin levels **(A)**, HOMA-IR **(B)** and grip strength **(C)**.

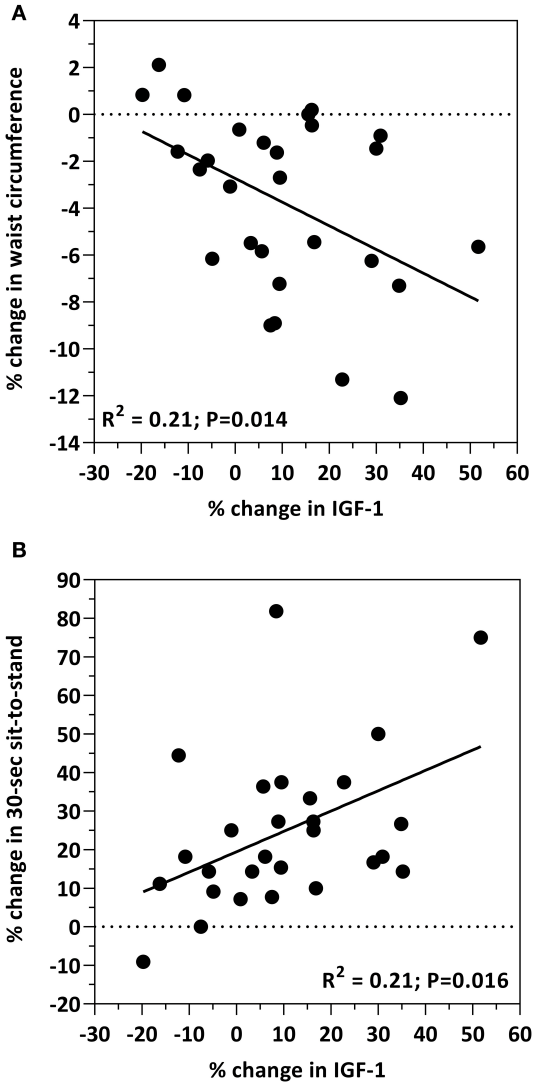

FIGURE 2 | Bivariate correlations between the percent change from baseline in IGF-1 levels and the percent change from baseline in waist circumference **(A)** and the 30-s sit-to-stand test **(B)**.

change from baseline in insulin, HOMA, total cholesterol, HDL-cholesterol, LDL-cholesterol, glucose, body weight, percent body fat, BMI, IGF-1, IL-12, CRP, grip strength, and 30-s sit-to-stand were included in the multiple regression model to predict the change in IL-8. The prediction model was statistically significant ($F = 14.421$; $p < 0.001$) and accounted for 52% of the variance of the increase in IL-8 (Adjusted $R^2 = 0.518$). Percent reduction in glucose levels ($\beta = -0.763$; $p < 0.001$) and the percent decrease in IL-12 concentrations ($\beta = 0.327$; $p = 0.033$) independently predicted the increase in IL-8. All other variables were excluded.

DISCUSSION

This highly controlled feeding study sought to evaluate the impact of the DASH diet on changes in biomarkers of

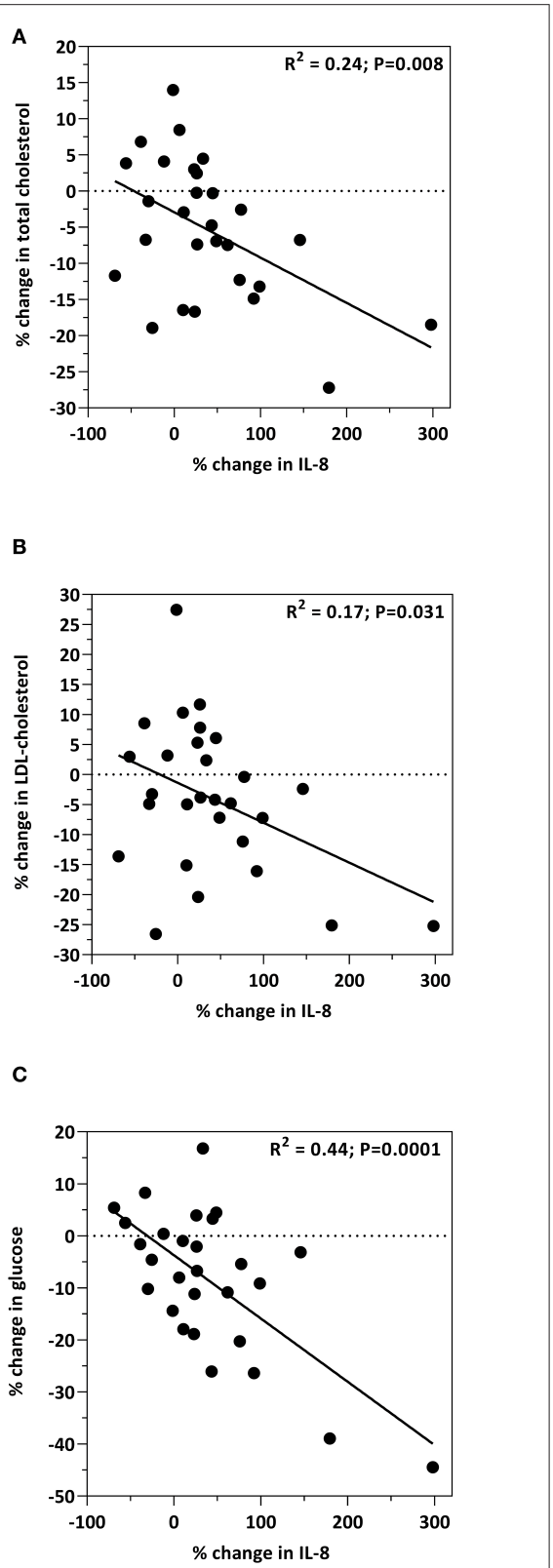

FIGURE 3 | Bivariate correlations between the percent change from baseline in IL-8 levels and the percent change from baseline in plasma total cholesterol **(A)**, LDL-C **(B)** and glucose **(C)**.

cardiometabolic health in a cohort of sedentary obese older adults. Although the results of the present study did not differ between the meat intake groups, there were cardiometabolic changes in response to the 12-week diet intervention on measures of cholesterol, insulin sensitivity, and inflammation when the meat intake groups were combined. Additionally, associations were observed between changes in cardiometabolic biomarkers and changes in body composition measures.

In Response to the DASH Diet, Total Cholesterol, LDL-C, and HDL-C Decreased in Obese Older Adults

In the present study under controlled-feeding intakes, total cholesterol was significantly reduced in all participants by 4.9% ($p < 0.001$) and LDL cholesterol was reduced by 4% ($p = 0.004$). There were no differences in cholesterol outcomes when separated by meat intake groups. The study diet provided on average an estimated 195.5 mg of dietary cholesterol per day that was consistently consumed throughout the 12-week intervention period. A review by Grundy, showed that several metabolic studies have reported a linear relationship between dietary cholesterol intake and serum cholesterol levels (18). In fact, Keys et al. (19) showed that a dietary cholesterol intake of about 200 mg per day should result in a decrease in blood cholesterol levels by 5%, an observation shown by the present study in a cohort of obese older adults. The DASH dietary pattern does not include recommendations for dietary cholesterol intake and the 2015–2020 *Dietary Guidelines for Americans* no longer recommends limiting dietary cholesterol intake to 300 mg per day. Dietary cholesterol, however, is not the only factor that influences serum cholesterol concentrations. Saturated fat, when consumed above current recommendations, also affects blood cholesterol and saturated fat has a greater negative impact on the development of cardiovascular disease (20). As previously reported 8% of the total calories from the study diet was saturated fat (15), which is within the 2015–2020 *Dietary Guidelines for Americans* to limit saturated fat intake to <10%, but above the American Heart Association recommendations of <7%. Cholesterol and saturated fat, however, are only two components of a whole-diet and other factors, such as total calories and dietary fiber need to be considered with changes in cholesterol levels. The diet for the present study provided on average 1,800 calories per day as recommended by the USDA for sedentary older adults (15, 17). At 1,800 calories, the DASH diet recommends substantial amounts of whole-grains, fruits and vegetables, all of which the study diet provided (15, 16). Adhering to these recommendations, the study diet provided 29.9 g of total dietary fiber, which is a major dietary factor that aids in further lowering cholesterol levels. The cholesterol lowering benefits of the DASH diet have been previously reported (21) and findings from the present show similar results. Due to the well-established cardiovascular benefits of the DASH dietary pattern and cardiovascular disease remaining as the number one cause of death in the United States, more aggressive implementation strategies may need to be established to begin a population-wide adherence to the DASH diet.

Previous reports have documented that the DASH diet lowers HDL cholesterol (HDL-C) (21–24). In the present study, HDL-C decreased by 8.5% ($p < 0.001$) from baseline to study-end in all participants; no differences in HDL-C levels were observed based upon meat intake groups. In a cohort of middle-aged overweight adults, Chiu et al. compared the DASH diet (27% total calories from fat) to a high-fat DASH diet (HF-DASH; 40% total calories from fat) in which HDL-C decreased with consumption of the DASH diet (24). This outcome was not observed with consumption of the HF-DASH diet. Similarly, the diet for the present study provided 21% total calories from fat (15) and the outcomes were similar in this cohort of obese older adults. Although it has been previously reported that HDL-C concentrations drop with consumption of a low-fat diet (25), it is unknown of the impact of the DASH diet on functional changes to HDL. There are several functions of HDL-C that are vital for cardioprotection such as reverse cholesterol transport, anti-inflammatory action, modulation of glucose metabolism, and endothelial protection (26). Although HDL-C concentrations decreased in the present study, it is unknown whether this decease impacted HDL-C functionality that in turn may have negatively impacted cardiovascular health. To gain a better understanding of this relationship, future DASH diet intervention studies should include measures of HDL-C functionality and ascertain the impact on cardiovascular health.

Markers of Insulin Sensitivity Improved and Were Associated With Reductions in Abdominal Adiposity in Older Adults With an Obese Phenotype Consuming the DASH Diet

Outcomes of the present study show that consumption of the study diet for 12-weeks, under controlled feeding conditions, among a cohort of obese older adults resulted in reductions in insulin, glucose and HOMA-IR. Insulin was significantly reduced in all participants by 13.4% ($p = 0.014$) and glucose was reduced by 8.4% ($p = 0.008$). Furthermore, HOMA-IR decreased by 25% ($p < 0.05$) by study end. Although recent systematic reviews have reported that the DASH diet has no beneficial effect on fasting blood glucose and HOMA-IR (27, 28), similar findings of the present study have been reported among type 2 diabetics and women with gestational diabetes (29–31). Shirini et al. concluded that the DASH diet may improve insulin sensitivity independent of weight loss (27). We previously reported that the participants in the present study reduced total body weight by 6.3% (15). After adjusting for changes in total body weight, the significant decreases in glucose (adjusted $p = 0.049$) and HOMA-IR (adjusted $p = 0.045$) remained.

One modifiable risk factor associated with cardiometabolic disease is abdominal adiposity (2). Abdominal adiposity is the hallmark of the obese phenotype in older adults and is the primary contributing factor to chronic disease risk. Waist circumference serves as a measure of abdominal adiposity and a surrogate indicator of cardiometabolic disease risk (32, 33). We previously reported a 3.7% reduction in waist circumference in this cohort of older adults and that this reduction may in

part be due to the decrease in body fat as a result of the intervention (15). In the present study we report that the decrease in waist circumference is correlated with the decrease in insulin ($R^2 = 0.22$; $p = 0.012$; **Figure 1A**) and HOMA-IR ($R^2 = 0.22$; $p = 0.01$; **Figure 1B**). The association between abdominal adiposity and insulin resistance is well-established, specifically in populations with obesity and type 2 diabetes (34–36) as well as healthy populations (e.g., women and adolescents) (37, 38). Interestingly, Díez-Fernández et al. recently reported that in young adults, waist circumference mediates the relationship between muscular strength and cardiometabolic risk (39). What is important to appreciate is that increased abdominal adiposity results in a redistribution of ectopic adipose tissue within skeletal muscle. Ectopic fat deposited in skeletal muscle contributes to poor skeletal muscle function characterized by reduced muscle mass and strength, and impaired glucose tolerance. This is crucial given that skeletal muscle is the largest consumer of glucose and plays a central role with insulin sensitivity. Indeed, the outcomes of the present study show that the decrease in waist circumference was inversely related to muscle strength ($R^2 = 0.28$; $p = 0.004$; **Figure 1C**) suggesting a favorable change in skeletal muscle function may be the result of reduced abdominal obesity. Although previous reports as well as the outcomes from the present study show that an interrelationship between insulin sensitivity, abdominal adiposity, and muscle health exists, more studies are needed in various populations to better understand the role that diet, dietary patterns or dietary components play within this interrelationship.

Insulin like growth factor-1 (IGF-1), a growth hormone produced by the liver, exerts its effects on glucose regulation and is positively correlated with insulin sensitivity and muscle health (40, 41). For example, IGF-1 has several anabolic properties (i.e., cell growth and differentiation, mitochondrial biogenesis, reduced inflammation, neuromuscular junction stability) on skeletal muscle that counteract the development of sarcopenia by activating AMPK and PGC1α. Low IGF-1 concentrations are associated with several cardiometabolic risk factors including obesity, insulin resistance, diabetes and inflammation (40–45). Low circulating IGF-1 levels may also predict for increased risk of heart disease and myocardial infarction (46–48). Conversely, increased IGF-1 levels are paralleled with improvements in insulin sensitivity in premenopausal obese women with insulin resistance consuming a calorie-restricted diet (49). In the present study, IGF-1 concentrations increased by 10% ($p < 0.001$) in all participants as a result of the intervention. Moreover, this increase was negatively associated with waist circumference ($R^2 = 0.21$; $p = 0.014$; **Figure 2A**), a similar finding reported by Succurro et al. in a cohort of non-diabetetic adults (50). Although several clinical studies report associations between IGF-1 and muscle strength in various human populations (e.g., healthy adults, older women and sarcopenic obese elderly) (51–53), they primarily focused on aging and/or exercise. In the present study, in which the focus was diet, we observed a positive relationship between IGF-1 and muscle strength ($R^2 = 0.21$; $p = 0.016$; **Figure 2B**). While these findings collectively are suggestive of a relationship between diet, IGF-1, abdominal adiposity and muscle, this area remains highly unexplored and more studies are required to elucidate these relationships and impact on cardiometabolic outcomes.

Inflammatory Biomarkers Were Influenced by Consumption of the DASH Diet in Older Adults With an Obese Phenotype

Interleukin 8 (IL-8) is a chemokine involved in ischemic tissue repair and thought to exert beneficial effects on cardioprotection (54). Circulating IL-8 concentrations, however, are elevated in obese individuals and are considered to be a factor relating obesity to increased cardiovascular risk (55). Results of the present study, in a cohort of obese older adults showed that by week 9 of the intervention, IL-8 levels increased by 38.8% ($p = 0.005$) as a result of the study diet. Most notably, this increase was associated with decreases in total cholesterol ($R^2 = 0.24$; $p = 0.008$; **Figure 3A**), LDL-C ($R^2 = 0.17$; $p = 0.031$; **Figure 3B**), and glucose ($R^2 = 0.44$; $p = 0.0001$; **Figure 3C**), pointing toward a more cardioprotective role for IL-8. Reports of circulating concentrations of IL-8 on heart function have resulted in conflicting outcomes. A case study investigating serum levels of IL-8 on myocardial infarction (MI) showed that concentrations were associated with increased MI risk in men, but reduced occurrence of MI in women (56). In rodents treated with IGF-1, increased IL-8 displayed a proangiogenic effect with protection against ischemic myocardium (57). Indeed, the concentrations of IGF-1 in the present study increased by 10% ($p < 0.001$). Although previous reports showed an association of IL-8 with BMI, fat mass, and waist-to-hip ratio in obese individuals (55), the results of the present study did not observe such associations. With conflicting outcomes from previous reports, including the present study, many more studies are required to fully uncover the role of IL-8 in heart health, specifically with regard to cardioprotection and risk of cardiovascular disease. Future studies are needed to determine specific populations that benefit from increased IL-8 and which populations are negatively affected.

Interleukin 12 (IL-12) is a proinflammatory cytokine involved in the pathogenesis of numerous inflammatory disorders such as psoriasis, crohn's disease, ulcerative colitis, multiple sclerosis, and rheumatoid arthritis (58). IL-12 production is a contributing factor in obesity-related inflammation and insulin resistance (59). In rodent studies involving older mice, treatment with an anti-IL-12 monoclonal antibody alleviated the inflammatory bowel disease, colitis (60). Additionally, mice consuming a low-carbohydrate, soy, fish oil diet showed reduced IL-12 concentrations in bronchial tissue, leading to decreased inflammation and DNA damage in the lungs (61). Indeed, results of the present study showed that in response to the study diet circulating IL-12 levels decreased by 12.7% ($p < 0.001$) by week 9. Although these findings are suggestive of an improved inflammatory state or reduced risk for the onset of IL-12 induced disorders, more human studies are required to determine whether diet-induced reductions in IL-12 prevent or alleviate inflammatory disorders in which IL-12 has a role.

In the present study C-reactive protein (CRP) decreased by 11.3% ($p = 0.006$) in all participants over the course of the

intervention. CRP is a widely used biomarker of inflammation and is elevated in individuals with an obese phenotype (62). When elevated, it serves as a marker for insulin resistance (63) and is associated with coronary artery disease and total mortality (64, 65). CRP may also serve as a possible biomarker for infection and pneumonia in geriatric patients (66). Furthermore, CRP has been associated with HOMA-IR and inversely related with grip strength (63, 67). Indeed, HOMA-IR decreased ($p < 0.05$) in the present study and strength-to-weight ratio increased as previously reported (15). A recent meta-analysis reported that the DASH diet had no effect on CRP concentrations (28). However, consumption of the DASH diet among type-2 diabetics reduced CRP levels by 26.9% (68) and the DASH diet was associated with a reduction in CRP in women (69). It is possible that the DASH diet may not be effective in reducing CRP levels in healthy adults, but rather in individuals with obesity and/or diabetes.

LIMITATIONS

Limitations of this study include the following: (i) a non-intervention control group was not included in this study; (ii) cohort of participants in the present study were all white which is representative of the dominant racial background in the state of South Dakota; (iii) all participants were upwardly mobile and lived in their own homes; (iv) no participants required support for daily living activities; no one resided in assisted living facilities; (v) overall, regardless of removing the three participants with a normal weight BMI significant differences in the cardiometabolic responses remained. Due to these limitations great caution should be taken when generalizing the outcomes of the present study to various populations of older adults with diverse ethnic/racial and demographic backgrounds as well as different living conditions.

CONCLUSIONS

Results from the present study confirm that the DASH diet with restricted calories beased upon the U.S. Dietary Guidelines for Americans is an effective approach to improve blood biomarkers of cardiovascular, metabolic, and inflammatory health in obese older adults. The outcomes of the present study also show positive cardiometabolic improvements with daily lean beef consumption. Because older adults are especially vulnerable to cardiometabolic disease and unhealthy diet is a leading risk factor for the development of cardiometabolic disease strategies for dietary behavioral change may be need implemented to increase the adoption of the DASH diet.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board for Human Study Participant Use at South Dakota State University (Approval #: IRB-1712006-EXP). The study was conducted in accordance with the Declaration of Helsinki and informed consent was obtained from all participants before entry into the study. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CP: conceptualization/study design, methodology, manuscript preparation, review, editing, supervision, project administration, and funding acquisition. GV: statistical analysis, review, and editing. MH and AK: data curation and review. All authors contributed to the article and approved the submitted version.

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OPINION
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Modulation of CT1 Function: From Klotho Protein to Ammonia and Beyond

*Sergej M. Ostojic**

FSPE Applied Bioenergetics Lab, University of Novi Sad, Novi Sad, Serbia

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INTRODUCTION

The creatine transporter (CT1 or SLC6A8) is a sodium- and chloride-dependent multi-pass membrane protein required for the cellular uptake of creatine, a key high-energy phosphate-storage molecule. This substrate-specific carrier (**Table 1**) is located across the plasma membrane of various energy-demanding cells and organs, including the brain, skeletal muscle and myocardium, gastrointestinal tract, kidney and urinary bladder, male and female organs, skin, bone marrow, and granulocytes. CT1 defect or malfunction is characterized by a severe depletion of the intracellular creatine pool, accompanied by intellectual disability, seizures, and movement and behavior disorders (1, 2). Transferring creatine through biomembranes thus represents an essential component of normal high-energy metabolism, with CT1 often recognized as a possible therapeutic target for the modulation of creatine homeostasis (3). This perspective paper explores several agents and vehicles that could switch CT1 upregulation and facilitate creatine uptake, and discusses the pros and cons of this strategy for experimental and clinical nutrition.

CT1 MODULATION BY SUBSTRATE AVAILABILITY

The activity of creatine carriers appears to be partially regulated by the levels of intracellular creatine, with the amount perhaps modulating CT1 function by a feedback mechanism. The upregulation of CT1 expression by low creatine concentrations probably happens at the post-transcriptional level and may involve alternative splicing (4) and/or CT1 phosphorylation and glycolysation (5). A group from Columbia University was arguably the first who showed that creatine-starved myoblasts increased creatine transport activity for up to 3-fold above the levels observed in the cells maintained in a medium containing creatine (6). The authors reported that creatine must enter the cell to exert its regulatory activity on creatine transport by either regulating the number or turnover of CT1. Similarly, creatine uptake activity was significantly augmented in skeletal muscle membrane vesicles of rats who were subjected to 4-day creatine starvation (7) and in isolated hearts from creatine-free guanidinoacetate N-methyl transferase knockout mice (5), suggesting that fostering low creatine concentrations may upregulate CT1 and facilitate creatine assimilation. On the other hand, the creatine uptake can be reduced by the addition of exogenous creatine and consequent downregulation of CT1 expression, as seen in the skeletal muscle of rats supplemented with creatine for up to 6 months (8). Whether exposure to low creatine and concomitant upregulation of CT1 have any clinical potential remains unknown at the moment. Theoretically, if a creatine-free diet that instigates CT1 upregulation is followed by creatine loading, this could potentiate cellular uptake of creatine above normal amounts, a phenomenon that might be referred to as "creatine super-compensation." This event is supposed to be transitory since the augmented intracellular creatine pool downregulates its own transport by 50% within 3–6 h (6).

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Sebastian J. Hofer,
University of Graz, Austria

Reviewed by:
Maurizio Balestrino,
University of Genoa, Italy
Yoshitaka Iwazu,
Jichi Medical University, Japan

***Correspondence:**
Sergej M. Ostojic
sergej.ostojic@chess.edu.rs
orcid.org/0000-0002-7270-2541

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TABLE 1 | Basic structural and functional characteristics of the creatine transporter.

	Description
Protein	Sodium- and chloride-dependent creatine transporter 1 (CT1)
Taxonomic ID	9606 (NCBI)
Gene*	Solute carrier family 6 member 8 (*SCL6A8*), locus Xq28
Organism	Homo sapiens
Size	635 amino acids (70.5 kDa); highly conserved (97%) between species
Subcellular location	Plasmalemma; possibly mitochondria
Tissue specificity	Skeletal muscle and kidney, brain, heart, colon, testis, prostate, etc.
Coupling ratio	2 Na^+: 1 Cl^-: 1 creatine
K_m	15–77 μM
Sequences	4 Isoforms produced by alternative splicing

**Several studies reported the existence of another human creatine transporter gene on chromosome 16p11.2; mRNA transcripts from this gene may only be expressed in the testis.*

STIMULATION OF CT1 BY KLOTHO PROTEIN

Klotho protein (Clotho; HFTC3) exists in both full-length membrane form and soluble secreted form, playing a modulatory role in aging, bone metabolism, and endothelial dysfunction (9). Klotho, an enzyme and hormone, has been reported to participate in the regulation of cellular transport processes across the plasma membrane either indirectly through inhibiting calcitriol [1,25(OH)2D3] formation or other mechanisms or by directly affecting transporter proteins, including ion channels, cellular carriers, and Na(+)/K(+)-ATPase (10), and this might include CT1. The researchers from the University of Tübingen explored the effect of Klotho protein on CT1 modulation in the Xenopus oocyte experimental model (11). The authors found that the co-expression of Klotho protein increases a creatine-induced current in CT1-expressing oocytes, suggesting a Klotho-driven upregulation of creatine carriers, presumably by stabilizing the carrier protein in the cell membrane. The increase in creatine-induced current was reversed by a β-glucuronidase inhibitor (D-saccharic acid 1,4-lactone monohydrate), implying that upregulation of CT1 requires the β-glucuronidase activity of Klotho protein. In addition, Klotho protein levels required were within the range of concentrations encountered *in vivo*, which indicates that the stimulation of CT1 by Klotho likely exists in physiological conditions. Since Klotho protein can be activated by phosphate restriction, curcumin, or vitamin D [for a review see (12)], targeting the Klotho-CT1 axis by specific dietary interventions might therefore expedite creatine uptake and contribute to high-phosphate bioenergetics balance.

GLUCOCORTICOID-INDUCIBLE KINASES AND CT1

The serum and glucocorticoid-regulated kinases are among the candidates involved in the regulation of CT1. These protein kinases are mainly expressed in the gut, brain, and endocrine tissues and play an important role in cellular stress responses by activating potassium, sodium, and chloride channels (13, 14). It appears that creatine transporter activity can be stimulated by glucocorticoid-inducible kinases in Xenopus oocytes heterologously expressing human CT1. Shojaiefard et al. (15) demonstrated that the serum and glucocorticoid-regulated kinases SGK1 and SGK3 stimulate CT1 by increasing the maximal transport rate of creatine through the carrier, an activity that may revive energy storage in myocytes and neurons. Tuning creatine uptake by SGK1 and SGK3 might happen due to ubiquitination, IGF-1-mediated pathway, osmolyte regulation, and/or phosphatidylinositol-3-phosphate-5-kinase activation (16), under both physiological and pathophysiological conditions. Kinetic analysis revealed that SGK1 enhanced the maximal current of creatine without significantly altering its affinity; the impact of SGK1 could be mimicked by the constitutively active isoform SGK3 but not by inactive SGK3. In terms of nutrition, 24-h starvation appears to display high levels of SGK1 in IIB fibers from the tibialis anterior (17), with SGK1 required to maintain pharyngeal muscle performance during starvation in *C. elegans* (18). Although CT1 expression and activity were not evaluated in these studies, triggering the serum and glucocorticoid-regulated kinases by food deprivation might be considered as yet another route for controlling the uptake of creatine.

PROTEIN KINASE MTOR AND CT1

The mammalian target of rapamycin (mTOR) is a protein kinase that plays a major role in the regulation of cell growth, proliferation, and autophagy, while sensing cellular nutrient availability and energy levels. The mTOR pathway appears to be the central regulator of mammalian metabolism of tissues including the liver, skeletal muscle, adipose tissue, and brain (19), with the regulation perhaps including creatine metabolism. The group of Florian Lung from the University of Auckland demonstrated that mTOR affects creatine turnover by stimulating CT1 function (20), through mechanisms similar to the serum and glucocorticoid-inducible kinases. The authors found that co-expression of mTOR increased maximal creatine current through CT1 in Xenopus oocytes expressing bovine SLC6A8, while pre-incubation of the oocytes with rapamycin decreased the creatine-induced current and abrogated its stimulation by mTOR. Whether mTOR cross talks with SGK1 in the regulation of CT1 remains currently unknown, yet both kinases might participate in the adjustment of cellular creatine content to nutrient and energy supply (21). For instance, mTOR can be activated by various amino acids (22), time-of-day-dependent caloric restriction (23), or carbohydrate-restricted feeding (24), with diet-driven mTOR activation potentially followed by creatine stream *via* CT1.

HYPERAMMONEMIA ELEVATES CT1 EXPRESSION

Hyperammonemia is a metabolic condition characterized by the elevated levels of blood ammonia (4–150 times normal), leading to alterations in brain energy metabolism, blood–brain barrier

dysfunction, and encephalopathy. Exposure to ammonium chloride (5 mM for 72 h, corresponding to pathophysiological levels observed in the brain in acute liver failure) resulted in a significant increase in mRNA levels of CT1 (1.9-fold increase) in conditionally immortalized mouse brain capillary endothelial cells (25). At the same time, the uptake of radiolabeled ^{14}C-creatine was significantly increased by 18% in cells exposed to ammonia, possibly as a consequence of increased CT1 activity. The authors suggested that the augmented creatine transport across the blood–brain barrier in hyperammonemia could be implicated in neuroprotective mechanisms since creatine can afford significant neuroprotection (26). This is in line with Kosenko et al. (27), who reported that chronic hyperammonemia induced by a 20-day ammonium-containing diet ameliorated the clinical symptoms of acute ammonia intoxication and prevented the associated deficits in energy metabolism. Maintained levels of high-energy phosphates in the brain indicate that diet containing ammonium salts instigates adaptive alterations in energy metabolism that might be due to hyperammonemia-dependent upregulation of CT1. Still, creatine appears to be poorly taken up by immature embryonic brain cells in urea cycle defects that are accompanied by ammonia toxicity (28), suggesting a rather complex interconnection between hyperammonemia and creatine transport.

OTHER CT1 STIMULANTS

Creatine accumulation *via* direct or indirect CT1 stimulation can be achieved by various hormones and hormone analogs (e.g., noradrenaline, isoproterenol, clenbuterol, 3,3′,5-triiodothyronine, amylin, growth hormone, insulin, insulin-like growth factor 1) (29–31). Schlattner et al. (32) reported an upregulation of CT1 after wounding of murine skin and increased abundance of creatine carriers in psoriatic human skin, leading to the accumulation of intracellular creatine. Pre-treatment with calyculin, a protein phosphatase 1a/2a inhibitor, abrogates the doxorubicin-induced creatine transport decrease (33), suggesting that CT1 stimulation is mediated by phosphorylation or a yet to be identified signal (34). CT1 expression and creatine uptake increase after adenoviral overexpression of peroxisome proliferator-activated receptor-γ coactivators 1a and 1b *via* estrogen-related receptor alpha (35), possibly identifying a new therapeutic gene target to increase intracellular creatine and tackle cellular energy homeostasis. A mechanistic nexus between diet and above CT1 excitants that might be involved in CT1 upregulation remains to be discovered.

POSSIBLE RISKS OF CT1 OVEREXPRESSION

Reduced levels of intracellular creatine critically imperil cellular bioenergetics, fostering CT1 upregulation and expedited creatine uptake. However, the cell appears to have an upper limit of creatine accumulation as well, implying a delicate balance between creatine levels and CT1 modulation on both sides of the coin. For instance, long-term creatine ingestion downregulates CT1 in order to prevent the excessive (and potentially harmful) intramuscular accrual of creatine (8). Wallis et al. (36) nicely demonstrated that the overexpression of CT1 in transgenic mice induces an excessive accumulation of creatine inside the myocytes, with an abnormally high intracellular creatine pool (66 ± 6 nmol/mg protein in wild-type controls vs. 133 ± 52 nmol/mg protein in CT1-overexpressing transgenic mice), accompanied by left ventricular dysfunction, myocardial hypertrophy, and heart failure. Likewise, mice overexpressing the myocardial CT1 experienced chronically increased levels of myocardial creatine and developed age-specific progressive hypertrophy and heart failure (37). Supra-normal myocardial creatine and phosphocreatine concentrations thus might lead to energetic impairment, probably due to the fact that the myocardium is incapable of keeping the augmented creatine pool adequately phosphorylated. On the other hand, Santacruz et al. (38) found no cardiac damage in mice with supraphysiological cardiac creatine levels. Adult transgenic animals showed an increase of 5.7-fold in the content of myocardial creatine, yet cardiac morphometry, echocardiography, and pressure–volume loop analyses demonstrated mild hypertrophy but normal function. Another trial suggested that mice overexpressing the creatine transporter in the heart (accompanied by the elevation of myocardial creatine by 20–100%) actually experienced a reduced myocardial stunning and ischemia/reperfusion injury (39), implying that increasing myocardial creatine for up to 100% was not detrimental but beneficial. Having this in mind, the magnitude of CT1 upregulation turns out to be of crucial importance for cell survival, since the maximum CT1 activity that can be attained without adverse metabolic effects is unknown at the moment. A risk-free ceiling for transporter function (along with maximal creatine levels) may vary from one cell type to another, requiring additional CT1 kinetics studies that address salient features encountered in creatine conveyance.

DIET AND CT1 UPREGULATION: WAITING IN THE WINGS

Only a small number of *in vivo* studies reported the effects of controlled dietary regimens on CT1 upregulation, including a 4-day starvation test in male rats (7), a 6-month creatine-free diet in mice (5), and a 7-week creatine depletion feeding in rats (40). All regimens elicited a significant increase in creatine uptake and CT1 activity in the heart and skeletal muscle of experimental animals, likely due to an increased transporter protein expression mediated by low creatine concentrations (41). Those pilot studies were not followed by a torrent of pre-clinical studies and human trials probably due to the somewhat challenging quantification of CT1 expression, activity, and density in target cells (42). An interesting small-scale study observed lower muscle creatine levels and increased capacity to load creatine in seven vegetarian men (four vegans and three lacto-ovo vegetarians) who consumed a vegetarian diet for at least 6 months before the experiment (43). Muscle CT1 mRNA levels tended to be higher in vegetarians against non-vegetarian controls, which could partially explain an increased capacity to accumulate

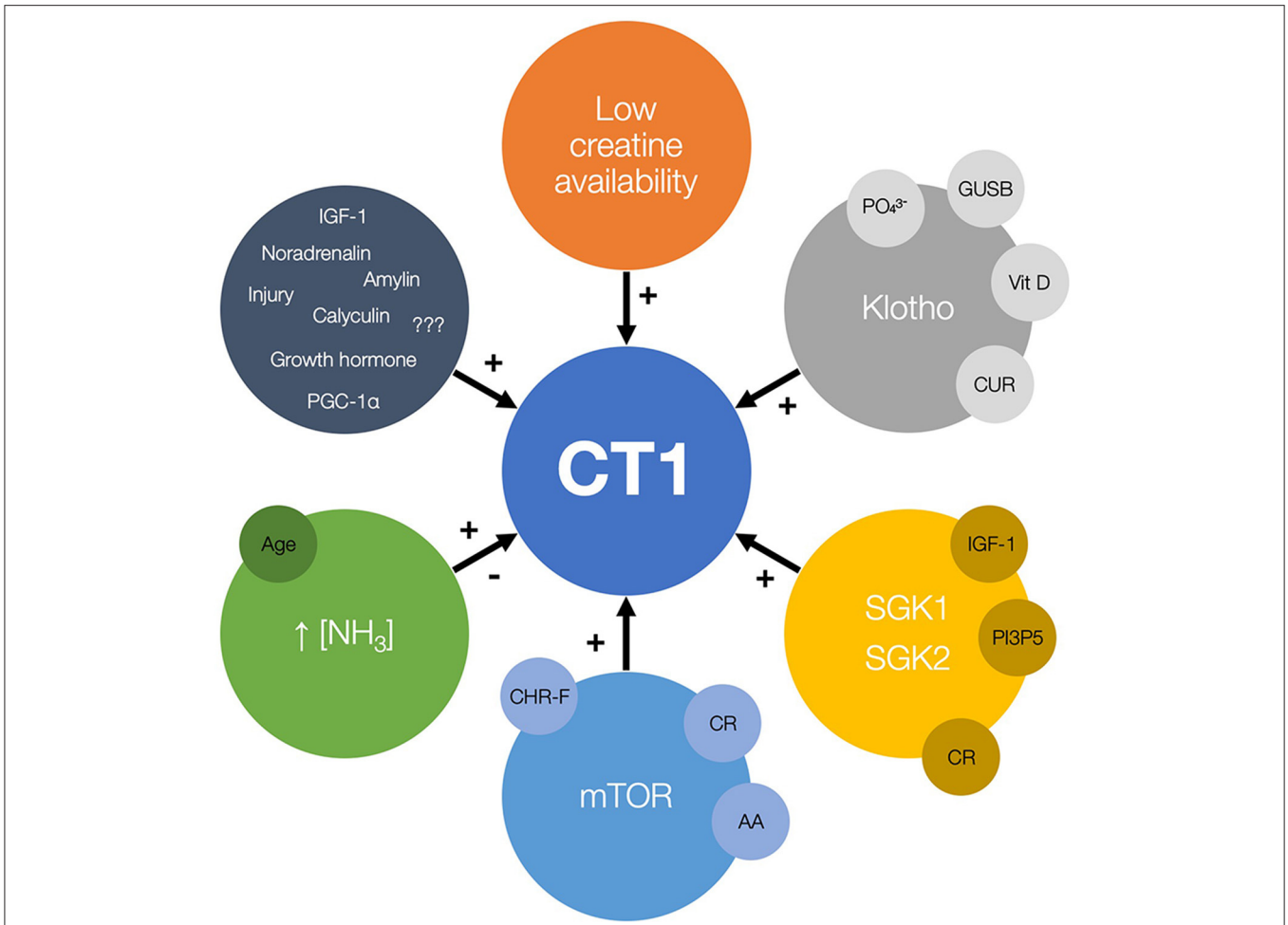

FIGURE 1 | The candidate regulators of creatine transporter (CT1) function and expression, with possible nutrition-related cofactors and modulators (small circles). The plus sign (+) indicates the stimulation of CT1 activity while the minus sign (–) indicates possible inhibition of CT1 function. GUSB, β-glucuronidase; Vit D, vitamin D; CUR, curcumin; SGK, serum and glucocorticoid regulated kinases; IGF-1, insulin-like growth factor 1; PI3P5, phosphatidylinositol-3-phosphate-5-kinase; CR, calorie restriction; mTOR, mammalian target of rapamycin; AA, amino acids; CHR-F, carbohydrate-restricted feeding; PGC-1a, peroxisome proliferator-activated receptor-γ coactivators 1a.

creatine in vegetarians subjected to creatine loading. Better control for diet composition in this pilot trial (i.e., the amount of creatine in vegan and lacto-ovo vegetarian nutrition has not been calculated) along with the inclusion of more participants would possibly reveal a more significant effect of creatine-free diet on CT1 upregulation. However, other possible mechanisms that rule out CT1 expression and density might be involved as well, including an accelerated maximal velocity of CT1, a reduced creatine efflux from the cell, or other unknown channels. An upregulation of CT1 gene expression and creatine deposition has been described in pigs and broilers who were supplemented with guanidinoacetic acid (GAA), a natural precursor of creatine (44, 45), yet the mechanism of GAA-driven CT1 stimulation remains unaddressed. Another nutritional study reported an elevated gene expression of CT1 in mice exposed to a 10-week high-fat diet and treated with nitrite (46), perhaps due to mechanisms that are both dependent and independent of proton-gradient uncoupling. Those exploratory studies lay the first stone of a possible role for diet in CT1 upregulation. This presumably complex tie-in urgently requires auxiliary research, including time-dependent changes in CT1 upregulation driven by a specific dietary regimen (e.g., acute vs. chronic effects of creatine-free diet), a food-driven CT1 triggering in various organs, stages of the life cycle and pathologies, and a possible synergism (or antagonism) of two or more food components to produce a combined effect on CT1 activity, to name just a few.

CONCLUSION

Several vehicles are identified to upregulate or modulate CT1 function and uplift creatine allocation in a handful of *in vitro* and *in vivo* studies (**Figure 1**). Those include carrier modulation by low substrate availability, protein kinases, and hyperammonemia. Importantly, upregulation of CT1 also appears to be triggered by caloric restriction, creatine-free diet

and exposure to ammonium-containing food. Upregulating CT1 could be therefore perceived as an up-and-coming target in nutritional sciences, yet its clinical efficacy, safety, and feasibility require a rather careful scrutinization in the forthcoming years.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: SO serves as a member of the Scientific Advisory Board on creatine in health and medicine (AlzChem LLC). SO owns patent "Sports Supplements Based on Liquid Creatine" at European Patent Office (WO2019150323 A1), and active patent application "Synergistic Creatine" at UK Intellectual Property Office (GB2012773.4). SO has served as a speaker at Abbott Nutrition, a consultant of Allied Beverages Adriatic and IMLEK, and an advisory board member for the University of Novi Sad School of Medicine, and has received research funding related to creatine from the Serbian Ministry of Education, Science, and Technological Development, Provincial Secretariat for Higher Education and Scientific Research, AlzChem GmbH, KW Pfannenschmidt GmbH, ThermoLife International LLC, and Monster Company. SO is an employee of the University of Novi Sad and does not own stocks and shares in any organization. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Effects of Daytime Dry Fasting on Hydration, Glucose Metabolism and Circadian Phase: A Prospective Exploratory Cohort Study in Bahá'í Volunteers

Daniela A. Koppold-Liebscher[1,2], Caroline Klatte[1], Sarah Demmrich[3], Julia Schwarz[4], Farid I. Kandil[1,5], Nico Steckhan[1,6], Raphaela Ring[1], Christian S. Kessler[1,2], Michael Jeitler[1,2], Barbara Koller[7], Bharath Ananthasubramaniam[8], Clemens Eisenmann[9], Anja Mähler[10,11], Michael Boschmann[10], Achim Kramer[7] and Andreas Michalsen[1,2]*

[1] Institute of Social Medicine, Epidemiology and Health Economics, Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany, [2] Department of Internal and Integrative Medicine, Immanuel Hospital Berlin, Berlin, Germany, [3] Department of Sociology, Cluster of Excellence Religion and Politics, University of Münster, Münster, Germany, [4] Department of Oecotrophology, Hochschule Niederrhein, University of Applied Science, Mönchengladbach, Germany, [5] Department of Neurology, Campus Benjamin Franklin, Charité Universitätsmedizin Berlin, Berlin, Germany, [6] Connected Healthcare, Hasso Plattner Institute, University of Potsdam, Potsdam, Germany, [7] Laboratory of Chronobiology, Institute of Medical Immunology, Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany, [8] Institute for Theoretical Biology, Charité Universitätsmedizin Berlin, Berlin, Germany, [9] Department of Sociology, University of Konstanz, Konstanz, Germany, [10] Experimental and Clinical Research Center—a Joint Cooperation Between Charité-Universitätsmedizin Berlin and Max Delbrueck Center for Molecular Medicine, Berlin, Germany, [11] NeuroCure Clinical Research Center, Charité-Universitätsmedizin Berlin, Berlin, Germany

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Anthony Hiu King Tsang,
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***Correspondence:**
Daniela A. Koppold-Liebscher
daniela.liebscher@charite.de

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Background: Religiously motivated Bahá'í fasting (BF) is a form of intermittent dry fasting celebrated by abstaining from food and drinks during daylight hours every year in March for 19 consecutive days.

Aim: To test the safety and effects of BF on hydration, metabolism, and the circadian clock.

Methods: Thirty-four healthy Bahá'í volunteers (15 women) participated in this prospective, exploratory cohort study. Laboratory examinations were carried out in four study visits: before fasting (V0), in the third week of fasting (V1) as well as 3 weeks (V3) and 3 months (V4) after fasting. Data collection included blood and urine samples, anthropometric measurements and bioelectrical impedance analysis. At V0 and V1, 24- and 12-hour urine and serum osmolality were measured. At V0–V2, alterations in the circadian clock phase were monitored in 16 participants. Our study was augmented by an additional survey with 144 healthy Bahá'í volunteers filling out questionnaires and with subgroups attending metabolic measurements ($n = 11$) and qualitative interviews ($n = 13$), the results of which will be published separately.

Results: Exploratory data analysis revealed that serum osmolality ($n = 34$, $p < 0.001$) and 24-hour urine osmolality (n = 34, $p = 0.003$) decreased during daytime fasting but remained largely within the physiological range and returned to pre-fasting levels during

night hours. BMI (body mass index), total body fat mass, and resting metabolic rate decreased during fasting (n = 34, $p < 0.001$), while body cell mass and body water appeared unchanged. The circadian phase estimated by transcript biomarkers of blood monocytes advanced by 1.1 h ($n = 16$, $p < 0.005$) during fasting and returned to pre-fasting values 3 weeks after fasting. Most observed changes were not detectable anymore 3 months after fasting.

Conclusions: Results indicate that BF (Bahá'í fasting) is safe, has no negative effects on hydration, can improve fat metabolism and can cause transient phase shifts of circadian rhythms.

Trial Registration: https://www.clinicaltrials.gov/, identifier: NCT03443739.

Keywords: hydration, religious, intermittent fasting, chronobiology, water deprivation, time-restricted eating, fasting, diurnal fasting

INTRODUCTION

Fasting with its preventive and therapeutic effects has, in general, been explored more and more over the past decade (1–10). However, dry fasting (DF) has not received as much attention in the scientific community. Most studies in this regard have focused on the Ramadan fast (11, 12), not specifically exploring DF as such, while serious consequences of a dysregulated body fluid balance are clear for every clinician (13), making studies on DF difficult to conduct.

However, Ramadan fasting is a complex model for DF, as the daily duration of fasting varies greatly over the years (from 11 to 22 hours a day, depending on the season and geographic location) (14). Apart from studies on Ramadan fasting, only a few studies have focused on DF itself (15–20). From these, only Papagiannopoulos et al. and Papagiannopoulos-Vatopaidinos et al. (15, 20) have focused on the physiology of prolonged DF, examining 10 participants each for 5 consecutive days of water and food deprivation.

The Bahá'í religion is monotheistic and was founded by Bahá'u'lláh (1817–1892) (21). Fasting is seen by Bahá'ís as one of the most significant spiritual duties of a healthy individual (22). The sick, the elderly, as well as children under 15 years of age and pregnant, menstruating, or nursing women are exempt from the religious duty to fast (22). Worldwide, the followers of the Bahá'í religion fast every year in March, abstaining from food and drink from sunrise to sunset for 19 consecutive days (22). This practice could best be described as an intermittent dry fast. No additional dietary regulations exist for believers during the fasting period or in general, except that consumption of alcoholic beverages is prohibited throughout the year. In contrast to Ramadan fasting, Bahá'í fasting (BF) has a fixed date in the year, is of shorter duration (i.e. 19 days) and, since it takes place in March, fasting time varies only between 10–13 hours daily. At the study site in Berlin/Germany, the exact duration is 10.90 hours at the beginning and 12.12 hours at the end of the fasting period. This makes it a good model for exploring the effects of intermittent dry fasting (IDF) on healthy adults, as no relevant changes occur in climate or timing when comparing data from different years or places. Since research has suggested that Ramadan fasting causes alterations of normal circadian rhythms (23), we also explored the effects of BF on the circadian phase (chronotype).

Thus, in this exploratory study, fluid balance in healthy adults during a religiously motivated IDF was assessed. Additionally, it was explored, whether known physiological mechanisms of fasting are also activated through this kind of fasting, which is shorter in duration than other fasting intervals of, for example, time-restricted eating (1) or intermittent fasting (5). More specifically, the aim was to assess urine and serum osmolality, renal and liver function, blood fatty acids, ketones, blood gas analysis, and anthropometric variables, as well as effects on normal daily patterns of eating and drinking. The collected data should serve to generate hypotheses for future studies in the field of IDF in general as well as assessing the safety and effects of BF.

METHODS

Study Design

This longitudinal, exploratory cohort study focussed on changes in physiological parameters. It was augmented by a mixed-methods study on Bahá'í fasting.

This study represents a quasi-experiment with a longitudinal observational design (24). As fasting time and duration are fixed for all Bahá'ís by religious provisions, there was no possibility to randomize the volunteers into a fasting and a non- (or delayed-) fasting group. Using a control group with a non-religious motivation could lead to a bias, as religiously motivated people may have higher compliance rates to dietary norms as well as engage in different health-related behaviors compared

Abbreviations: BIA, bioelectrical impedance analysis; BGA, blood gas analysis; BMI, body mass index; BF, Bahá'í fasting; BQ, participants of the large-scale study with questionnaires; CB, participants of chronobiological measurements; DC, Department of Chronobiology; DF, dry fasting; DLMO, dim light melatonin onset; ECRC, experimental and clinical research center; HbA1c, glycosylated hemoglobin; IDF, intermittent dry fasting; IM, Department of Integrative Medicine under the Institute of Social Medicine, Epidemiology and Health Economics; PP, participants of laboratory and anthropometric measurements; V0, V1, V2, and V3, study visits with laboratory examinations; VQ1, VQ2, VQ3, and VQ4, study visits with questionnaires.

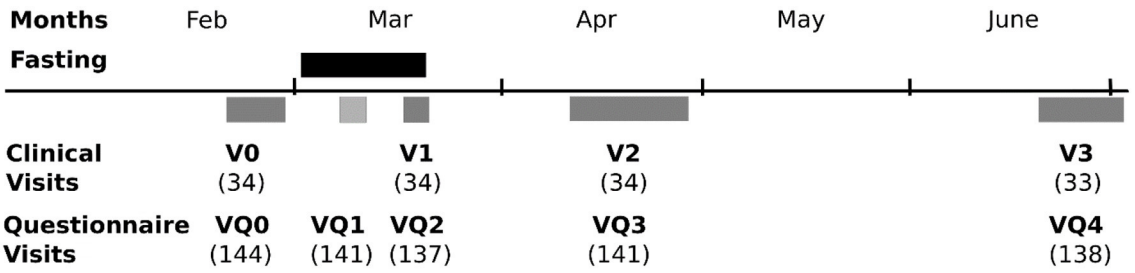

FIGURE 1 | A flow chart of visits with participant numbers.

with non-adherents (25). The external laboratory personnel, as well as the statistician, were blinded. All other study personnel were involved directly with the study participants during visits. Therefore, no blinding was possible in this case.

The study protocol was approved by the institutional review board of Charité Universitätsmedizin Berlin (Charitéplatz 1, 10117 Berlin) in January 2018 (ID: EA4/216/17), was registered with ClinicalTrials.gov (ID: NCT03443739), and carried out according to the standards of the Declaration of Helsinki. Written informed consent was obtained from all the participants prior to study entry.

Setting

Three departments of the same university (Charité Universitätsmedizin Berlin) cooperated for this study: the Department of Integrative Medicine at the Institute of Social Medicine, Epidemiology and Health Economics (IM), the Experimental and Clinical Research Center (ECRC), and the Department of Chronobiology (DC). The two latter departments conducted sub-studies. The main study site was at the Department of Integrative Medicine. Physiological data comprising urine and blood samples, as well as anthropometric measurements, were acquired at the four in-house visits in the Department of Integrative Medicine at the Institute of Social Medicine, Epidemiology and Health Economics (IM) or, for a subgroup, at the Experimental and Clinical Research Center (ECRC). The visits were conducted in the 2 weeks before the beginning of the fasting period (V0), in the last week of the fast (V1), 3 weeks after the end of the fast (V2), and finally, 3 months after the end of the fasting period (V3). **Figure 1** shows these visits along with the additional other visits for the psychometric questionnaires of the companion study and the other sub-studies.

Participants

Original first recruitment was conducted *via* the National Spiritual Assembly of the Bahá'ís of Germany (23), who spread the information via email to all Bahá'ís in Germany. For the aims of this study, 172 healthy volunteers were screened, and 144 were considered eligible (see below) for the questionnaire survey and were enclosed between January and February 2018. All eligible individuals filled out electronic questionnaires on subjective physical and psychological effects of Bahá'í fasting (group "BQ").

Of these, all the participants living in the wider region of Berlin were invited to participate in the additional laboratory tests reported upon here. Thirty-four subjects could be enclosed in this sub-study with its tests for physiological parameters (group "PP"), giving their informed written consent. Again, 17 of these 34 participants agreed to donate additional blood samples for chronobiological measurements and to answer additional questionnaires (group "CB"). The results of the psychometric questionnaires, as well as those of individual in-depth and focus group interviews and another subgroup concentrating on metabolic responses, will be reported separately.

Eligibility Criteria

The participants were screened at the IM and then referred to all sub-studies.

Inclusion criteria were registered membership in the Bahá'í community, planned adherence to fasting in the upcoming fasting period and age between 18 and 69 years (children and elderly are exempt from the religious duty to fast, and, because of the necessity for informed consent, no youth between 15 and 17 years were recruited for the study).

Exclusion criteria were: scheduled interruption of the fast for more than 5 days (for e.g., due to planned travel), pregnancy or breastfeeding, serious physical, or psychological illness, known eating disorder, participation in another study, no email address (because of electronic questionnaires). Individuals who worked in shifts and planned long-distance travel shortly before and during the study period were also excluded from eligibility for the chronobiological measurements.

Outcomes/Variables

Primary outcome measures were the hydration status of fasting individuals and changes in serum osmolality and urine osmolality in 12- and 24-hour-urine samples. Secondary outcome measures were anthropometric parameters, such as body weight, BMI, body composition via bioelectrical impedance analysis (BIA, measured by the octapolar BIACORPUS RX 4004M®) and the waist-to-hip ratio. The resting metabolic rate was calculated by an algorithm implemented in the BIA software provided by the manufacturer. Blood pressure, heart rate, and standard blood count were assessed alongside metabolic parameters, such as

ketone bodies in capillary blood (on the spot, with ACCU-CHEK® device) and urine, blood glucose levels, glycosylated hemoglobin (HbA1c), fructosamine, C-reactive protein, liver enzymes, and serum cholesterol. Additionally, the fluid balance was assessed by kidney parameters, 12- and 24-hour urine creatinine clearance, serum electrolytes, acid-based balance measured by blood gas analysis (on the spot, with the ABL80 FLEX® blood gas analyser), 24-hour and spontaneous urine osmolality, 24-hour urine specific gravity, and cystatin C. All laboratory parameters were measured at Labor Berlin, unless otherwise indicated. As most laboratory measurements had to be carried out in a fasting state, they were carried out between 7:30 a.m. and 11:00 a.m. outside of the fasting period, while, during BF, as most subjects would have breakfast before sunrise, the measurements were done between 4:30 p.m. and 6:30 p.m., if not otherwise indicated.

In group CB (17 subjects), an additional blood sample was taken between ∼8 a.m. and ∼10 a.m. at V0, V1, and V2 to assess the circadian phase (chronotype) (**Supplementary Table 1**) (26). Note that the determination of the circadian phase with the BodyTime assay is independent of the time of sample collection (26). Briefly, monocytes were sorted from whole blood, using magnetic cell sorting, total RNA was prepared, and the expression of 24 biomarker genes was analyzed, using NanoString technology. Based on these data, the body time algorithm allowed the prediction of the circadian phase. In this subgroup, the validated Munich Chronotype questionnaire (27) was also used.

Timing of the measurements constituted a challenge. Due to capacity constraints of the study centers, measurements could not all be done at the same time. Therefore, the measurements had to be spread over the last week of BF and start 2 hours before breaking the fast for dinner so that the mean fasting time for our measurements was 17 fasting days and 11 hours of daily fasting duration.

For a better overview of the whole study setting, we also mention the following measurements, the results of which will be published separately: Body composition was measured by air-displacement plethysmography, parameters of systemic energy metabolism by indirect calorimetry, and parameters of adipose tissue and skeletal muscle metabolism by microdialysis in two subgroups. Furthermore, validated questionnaires on quality of life, mood, mindfulness, and spirituality were used in electronic form for the BQ group. Additionally, the focus group and individual interviews were carried out by a trained sociologist.

Bias

Recruitment for both this study and the accompanying ones was based on voluntary participation and may thus have introduced a sampling bias in favor of motivated and health-conscious subjects. Apart from that, the implementation of an intervention-group-only design is, in so far, justified as the aim of the study was to explore the safety and general health effects of BF, using pre- and post-fasting observations to explore the strength and duration of the fasting effects.

Study Size

The study was planned as an exploratory study to help generate hypotheses for future studies. All p-values lower than 0.10 are regarded as potentially interesting and those below 0.05 as strongly interesting for future studies. The number of the participants ($n = 34$) allows to detect all high-sized and medium-sized effects of $f \geq 0.16$, corresponding to a Cohen's $d \geq 0.32$ (28) under the assumption of standard parameters [alpha = 0.05, beta = 0.20 (power of 80%)] and a higher correlation between measures of 0.7 (based on previous in-house data).

Statistical Methods

As blood parameters are typically not normally distributed, all analyses were conducted, using non-parametric tests. The Friedman test, a counterpart to a one-factorial repeated measures ANOVA, was used to compare the physiological parameters across the four (three) clinical visits, while pairwise *post-hoc* analyses were applied, using the Wilcoxon's signed-rank test. As usual, in exploratory studies, resulting p-values are presented as such and not compared against any adjusted alpha value.

Missing Data

Some physiological data were missing during the laboratory failures and one subject not attending the last visit. The Little test indicated that the missing data were completely at random ("MCAR") (29) and were replaced using multiple imputation methods ("multiple imputer") implemented in the Python's (v. 3.7) SciPy library.

RESULTS

Participants

Thirty-four volunteers (15 w, 19 m, age: 41.09 ± 14.54 years) practicing BF and living in or near Berlin participated in the visits and tests of physiological parameters (group "PP"). They had a mean BMI of 25.74 ± 5.13 kg/m², corresponding well to the average BMI of 26 kg/m² in Germany (30). Further sociodemographic data and baseline characteristics of these participants are shown in **Tables 1**, **2**, respectively. Confidence intervals of the baseline laboratory parameters are clinically unremarkable.

Of these 34 volunteers, 17 subjects (the "CB" subgroup) donated additional blood samples for chronobiological measurements. One participant was not able to attend the last follow-up visit in person. While part of the visit could be conducted via telephone (questionnaires), no physiological samples could be obtained. These, as well as missing laboratory data from some subjects for some visits due to a laboratory failure, were filled in by multiple imputations (see Methods).

Outcomes

Primary Outcome: Hydration Markers

Plasma osmolality changed during fasting [$\chi^2(3) = 19.61$, $P < 0.001$]. It decreased from V0 to V1 and increased to baseline (V0) or even higher levels from V1 to V2, returning to baseline levels by V3 (**Table 3, Figure 2A**). However, all these changes were within the physiological range.

Osmolality of the 24-hour urine (**Figure 2B**) changed similarly [$\chi^2(3) = 13.91$, $P < 0.001$], with a decrease from V0 to

TABLE 1 | Sociodemographic characteristics of the participants in the laboratory measurements.

		Option 1		Option 2		Option 3		Option 4		Option 5	
	n	Answer	*n* (%)	Answer	*n* (%)	Answer	*n* (%)	Answer	*n* (%)	Answer	*n* (%)
Gender	34	Female	15 (44.1)	Male	19 (55.9)						
Graduation	33	A-level	10 (30.3)	University	22 (66.7)	Other	1 (3)				
Gross income per year [kEUR]	33	<20	18 (54.5)	20–40	4 (12.1)	40–60	5 (15.2)	60–80	2 (6.1)	>80	4 (12.1)
Job	33	Self-Employed	7 (21.2)	Employee	11 (33.3)	Worker	2 (6.1)	Student	10 (30.3)	Other	3 (9.1)

TABLE 2 | Characteristics of participants at baseline (V0).

	Original data				Multiply imputed data			
	N	Mean ± SD	Median (IQR)	95% CI	N	Mean ± SD	Median (IQR)	95% CI
Age	34	41.09 ± 14.54	39.5 (29)	36.02; 46.16				
Hight, cm	34	173.49 ± 9.12	175.5 (15.1)	170.31; 176.67				
Weight, kg	34	77.97 ± 19.19	75.3 (19.4)	71.28; 84.67				
BMI, kg/m^2	34	25.74 ± 5.13	24.52 (6.36)	23.95; 27.53				
WHR[a]	34	0.90 ± 0.091	0.90 (0.13)	0.87; 0.93				
Blood pressure sys[b], mmHg	33	123.94 ± 18.32	120 (25)	117.44; 130.43	34	124.25 ± 18.13	120 (25)	117.93; 130.58
Blood pressure dia[c], mmHg	33	79.09 ± 10.71	80 (10)	79.09; 82.89	34	79.4 ± 10.70	80 (10)	75.66; 83.13
ß-Hydroxybutyrate, mmol/l	32	0.17 ± 0.19	0.15 (0.3)	0.10; 0.24	34	0.18 ± 0.20	0.18 (0.3)	0.11; 0.25
Glucose, mg/dl	33	83.48 ± 8.16	84 (13)	80.59; 86.38	34	83.07 ± 8.4	84 (13)	80.1; 86
HbA1c, mmol/mol Hb	34	33.78 ± 3.80	33.3 (4.9)	32.45; 35.10				
Fructosamine, µmol/l	34	239.56 ± 20.49	243.5 (24)	232.41; 246.71				
Body fat, kg	34	22.968 ± 10.81	19.1 (14.3)	19.20; 26.74				
Body water, l	34	39.84 ± 9.56	38.7 (13.5)	36.51; 43.18				
Body cell mass, kg	34	28.83 ± 7.89	29.8 (10.25)	26.08; 31.59				
Resting metabolic rate, kcal	34	1,655.94 ± 323.87	1,626 (447)	1,542.94; 1,768.95				
Haematocrit, l/l	34	0.412 ± 0.033	0.42 (0.04)	0.40; 0.42				
Creatinine, mg/dl	33	0.79 ± 0.13	0.77 (0.21)	0.74; 0.83	34	0.79 ± 0.12	0.77 (0.21)	0.74; 0.83
Cystatin C, mg/l	33	0.911 ± 0.132	0.87 (0.18)	0.86; 0.96	34	0.92 ± 0.13	0.88 (0.2)	0.87; 0.96
GFR (CKD-EPI)	33	89.94 ± 3.58	91 (0)	88.67; 91.21	34	89.89 ± 3.53	91 (0)	88.66; 91.53
HDL, mg/dl	33	59.58 ± 15.68	58 (25)	54.02; 65.13	34	59.61 ± 14.44	58.5 (25)	54.22; 65.00
LDL, mg/dl	33	117.24 ± 27.90	119 (44)	107.35; 127.13	34	117.49 ± 27.51	119 (43)	107.89; 127.09
Sodium (plasma), mmol/l	33	141.33 ± 1.74	141 (32)	140.71; 141.95	34	141.81 ± 1.89	141 (32)	140.81; 142.13
Potassium (plasma), mmol/l	33	4.00 ± 0.29	4 (0.4)	3.90; 4.11	34	3.99 ± 0.31	4 (0.4)	3.88; 4.09
Sodium (bga), mmol/l	31	142.06 ± 1.57	142 (32)	141.49; 142.64	34	144.79 ± 20.03	142 (32)	137.8; 151.78
Potassium (bga), mmol/l	31	3.95 ± 0.23	4 (0.3)	3.86; 4.03	34	3.94 ± 2.08	4 (0.3)	3.22; 4.67
Osmolality plasma, mosmol/kg	33	289.76 ± 4	290 (5)	288.34; 291.18	34	289.61 ± 4.03	289.5 (4)	288.21; 291.02
Osmolality urine 24 h, mosmol/kg	33	471.36 ± 158.36	449 (235)	415.21; 527.52	34	532.16 ± 387.26	454 (257)	397.03; 667.28
Osmolality urine 12-h (6 a.m.–6 p.m.), mosm/kg	31	411 ± 161.98	389 (156)	351.59; 470.41	34	429.88 ± 206.54	399.5 (177)	357.82; 501.94
Osmolality urine 12-h (6 p.m.–6 a.m.), mosm/kg	33	562.73 ± 219.63	550 (347)	484.85; 640.60	34	566.33 ± 217.29	560 (336)	490.51; 642.14

[a]*WHR, waist-to-hip ratio;* [b]*sys, systolic;* [c]*dia, diastolic.*

V1 and a subsequent increase from V1 to V2 to baseline levels. In-depth analysis of the samples, however, revealed that this initial decrease was mainly due to a distinct decrease in nocturnal urine osmolality (sampled from 6 p.m. to 6 a.m.), whereas changes in diurnal urine were less marked. Overall, these changes were largely within the physiological range and, therefore, clinically not relevant.

Kidney Values and Electrolytes

The glomerular filtration rate, as estimated by the equation of the Chronic Kidney Disease Epidemiology Collaboration (GFR via CKD-EPI), creatinine, and cystatin C did not change relevantly during fasting (GFR: $P = 0.584$, creatinine: $p = 0.060$, and cystatin C: $p = 0.073$). Creatinine showed a tendency to increase slightly during fasting (V0–V1: 0.03 ± 0.09 mg/dl) and remained

TABLE 3 | Anthropometric, blood, and urine parameters before, during, and after 19 days of Bahá'í fasting (BF).

	Reference range	V0	V1	V2	V3	Friedmann main analysis		V1 vs. V0		V1 vs. V2		V2 vs. V0		V3 vs. V0		V1 vs. V3		V2 vs. V3	
						χ^2 (3)	*p*	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*
Weight, kg		75.3 (19.4)	72.75 (21.8)	74.2 (20.9)	75.8 (18.8)	18.027	<0.001	4.180	<0.001	−2.114	0.207	2.067	0.233	1.644	0.601	−2.536	0.067	−0.423	1.000
BMI, kg/m^2		24.52 (6.18)	24.24 (6.23)	24.52 (5.71)	24.43 (5.3)	17.74	<0.001	3.983	<0.001	2.359	<0.001	2.342	0.019	2.069	0.039	1.428	0.153	0.239	0.811
WHR		0.895 (0.13)	0.92 (0.13)	0.88 (0.09)	0.87 (0.14)	30.526	<0.001	0.892	1.000	−2.771	0.034	1.879	0.362	4.086	<0.001	−4.978	<0.001	−2.207	0.164
Body fat, kg		19.1 (13.48)	19.05 (12.7)	19.25 (12.98)	18.25 (13.55)	31.83	<0.001	4.36	<0.001	2.248	0.025	3.359	0.001	3.915	<0.001	0.53	0.596	1.103	0.27
Body water, l		38.7 (13.5)	38.95 (15.8)	38.5 (13.1)	39 (13.4)	9.107	0.028	0.282	1.000	−0.845	1.000	1.127	1.000	2.724	0.039	−2.442	0.088	−1.597	0.662
BCM, kg		29.8 (10.25)	29.7 (12.17)	29.83 (10.92)	29.7 (10)	13.823	0.006	1.409	0.953	−2.667	0.045	1.268	1.000	2.020	0.261	−3.429	0.004	−0.751	1.000
BMR, kcal/d		1,626 (422.25)	1,612 (410.0)	1,617 (404.75)	1,604 (383.25)	23.01	<0.001	4.496	<0.001	3.411	0.001	1.958	0.05	2.043	0.041	1.983	0.047	0.128	0.898
SBP, mmHg		120 (25)	120 (21)	115 (20)	120 (20)	12.229	0.007	1.503	0.797	−1.409	0.953	2.912	0.022	0.094	1.000	−1.409	0.953	−2.818	0.029
DBP, mmHg		80 (10)	70 (10)	70 (10)	75 (10)	13.246	0.004	2.959	0.019	0.000	1.000	2.959	0.019	2.160	0.184	−0.798	1.000	−0.798	1.000
Hematocrit	0.395–0.505	0.416 (0.036)	0.40 (0.05)	0.41 (0.03)	0.408 (0.042)	11.620	0.009	3.006	0.016	−2.818	0.029	0.188	1.000	1.127	1.000	−1.879	0.362	−0.939	1.000
Glucose, mg/dl	60–110	84.0 (12.5)	72.5 (7.75)	84.0 (9.75)	84.71 (13.25)	43.45	<0.001	4.59	<0.001	4.915	<0.001	0.658	0.51	1.325	0.185	4.958	<0.001	0.932	0.351
HbA1c, mmol/mol Hb	<42.0	33.3 (4.4)	32.75 (5.23)	31.65 (5.4)	33.16 (5.12)	48.92	<0.001	1.222	0.222	4.838	<0.001	4.471	<0.001	3.368	0.001	3.069	0.002	4.428	<0.001
Fructosamine, µmol/l	205–285	243.5 (24)	253 (32)	248 (22)	248 (17)	32.056	<0.001	5.401	<0.001	−2.630	0.051	2.771	0.034	4.039	<0.001	−1.362	1.000	−1.268	1.000
3-OH-B, mmol/l		0.18 (0.3)	0.15 (0.3)	0.1 (0.2)	0 (0.2)	5.000	0.172												
HDL, mg/dl	>45	58.5 (25)	58.5 (19)	58 (23)	56 (17)	10.495	0.015	0.094	1.000	−0.047	1.000	0.141	1.000	2.489	0.077	−2.583	0.059	−2.630	0.051
LDL, mg/dl	<130	119 (43)	115.5 (47)	117 (45)	119 (33)	2.063	0.559												
Creatinine, mg/dl	0.70–1.20	0.765 (0.21)	0.80 (0.19)	0.81 (0.24)	0.80 (0.21)	7.410	0.060												
Cystatin C, mg/l	0.47–1.09	0.88 (0.2)	0.89 (0.16)	0.9 (0.14)	0.92 (0.21)	6.960	0.073												
GFR (CKD-EPI)		91 (0)	91 (0)	91 (1)	91 (36)	1.943	0.584												
pNa$^+$, mmol/l	136–145	141 (36)	142 (36)	142 (36)	141 (3)	3.884	0.274												
pK$^+$, mmol/l	3.4–4.5	4.0 (0.4)	3.8 (0.4)	4.1 (0.3)	4.0 (0.4)	16.111	0.001	1.315	1.000	−3.851	0.001	2.536	0.067	0.282	1.000	−1.597	0.662	−2.254	0.145
cNa$^+$, mmol/l	136–145	142 (36)	142 (36)	141.5 (3)	141 (3)	6.683	0.083												
cK$^+$, mmol/l	3.3–5.1	4.0 (0.3)	3.8 (0.4)	4.1 (0.3)	4.0 (0.2)	10.867	0.012	1.221	1.000	−2.771	0.034	1.550	0.727	1.362	1.000	−2.583	0.059	−0.188	1.000

(Continued)

TABLE 3 | Continued

	Reference range	V0	V1	V2	V3	Friedmann main analysis		V1 vs. V0		V1 vs. V2		V2 vs. V0		V3 vs. V0		V1 vs. V3		V2 vs. V3	
						χ^2 (3)	*p*	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*
pOsmolality, mosmol/kg	280–300	289.5 (4.0)	284.0 (11.0)	291.5 (4.75)	289.0 (7.5)	18.6	<0.001	3.112	0.002	4.035	<0.001	2.06	0.039	0.402	0.688	2.821	0.005	2.000	0.045
uOsmolality, 24 h, mosmol/kg	50–1,400	454.0 (237.0)	380.0 (190.0)	448.06 (173.26)		13.91	<0.001	2.419	0.016	2.376	0.017	0.282	0.778						
uOsmolality, d−12 h, mosmol/kg	50–1,400	413.0 (195.5)	352.5 (162.75)	417.5 (196.36)		6.18	0.0456	1.658	0.097	2.676	0.007	1.265	0.206						
uOsmolality, *n*−12 h, mosmol/kg	50–1,400	560.0 (353.75)	406.0 (292.25)	524.5 (294.25)		14.51	<0.001	2.753	0.006	3.009	0.003	0.342	0.732						

Data are given as the median and interquartile range (IQR). n = 34 for V0, V1, V2, V3; BMI, body mass index; BCM, body cell mass; RMR, resting metabolic rate; SBP/DBP, systolic/diastolic blood pressure; 3-OH-B, 3-Hydroxy-Butyrate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; GFR, glomerular filtration rate; pNa^+/pK^+, plasma Na^+/K^+; cNa^+/pK^+, capillary Na^+/K^+; pOsmolality, plasma osmolality; uOsmolality, urine osmolality; d−12 h, urine sample 6:00 a.m.–6:00 p.m.; n−12 h, 6:00 p.m.–6:00 a.m.; V0: before BF; V1: during third week of BF; V2: 3 weeks after BF; V3: 3 months after BF.

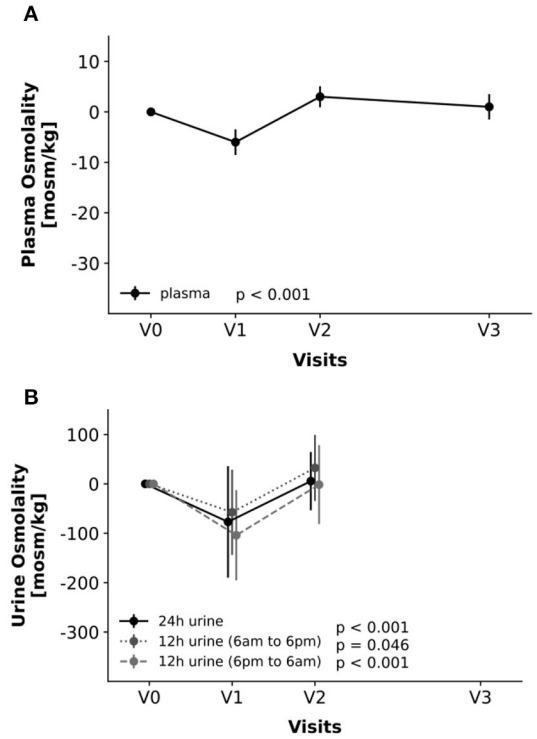

FIGURE 2 | Changes in parameters of hydration, i.e., plasma osmolality **(A)** and urine osmolality in 24-h, 12-h from 6 a.m.−6 p.m. and 12-h from 6 p.m.−6 a.m. samples **(B)**. Changes relative to V0-values. Line graphs and error bars represent means and 95%-confidence intervals accordingly.

on this level after fasting when compared with baseline (V0–V2: 0.04 ± 0.08 mg/dl).

Sodium and potassium were measured in the plasma and by blood gas analysis (BGA). Sodium measures did not change relevantly, neither in plasma nor in the BGA. Potassium showed a slight decrease in both plasma [$\chi^2(3) = 16.111$, $p = 0.001$] and BGA measurements [$\chi^2(3) = 10.867$, $p = 0.012$]. *Post-hoc* testing shows that this result is mainly based on the increase between V1 and V2 to a possibly over-compensational level at V2 for both laboratory values.

Anthropometry

Body weight [$\chi^2(3) = 18.027$, $p < 0.001$], BMI [$\chi^2(3) = 18{,}027$, $p < 0.001$], WHR [$\chi^2(3) = 30.526$, $p < 0.001$], body fat [$\chi^2(3) = 30.154$, $p < 0.001$], body cell mass [$\chi^2(3) = 13.823$, $p = 0.006$], and the resting metabolic rate [$\chi^2(3) = 21.931$, $p < 0.001$] show marked effects during fasting (**Figure 3**). *Post-hoc* analyses revealed that this was mainly due to a decrease between V0 and V1, sometimes extending to V2, rebounding thereafter again to baseline levels until V3.

Glucose Metabolism

Marked changes were seen in plasma glucose [$\chi^2(3) = 43.135$, $P < 0.001$], HbA1c [$\chi^2(3) = 48.921$, $P < 0.001$] and fructosamine [$\chi^2(3) = 32.056$, $P < 0.001$]. During BF, plasma glucose was

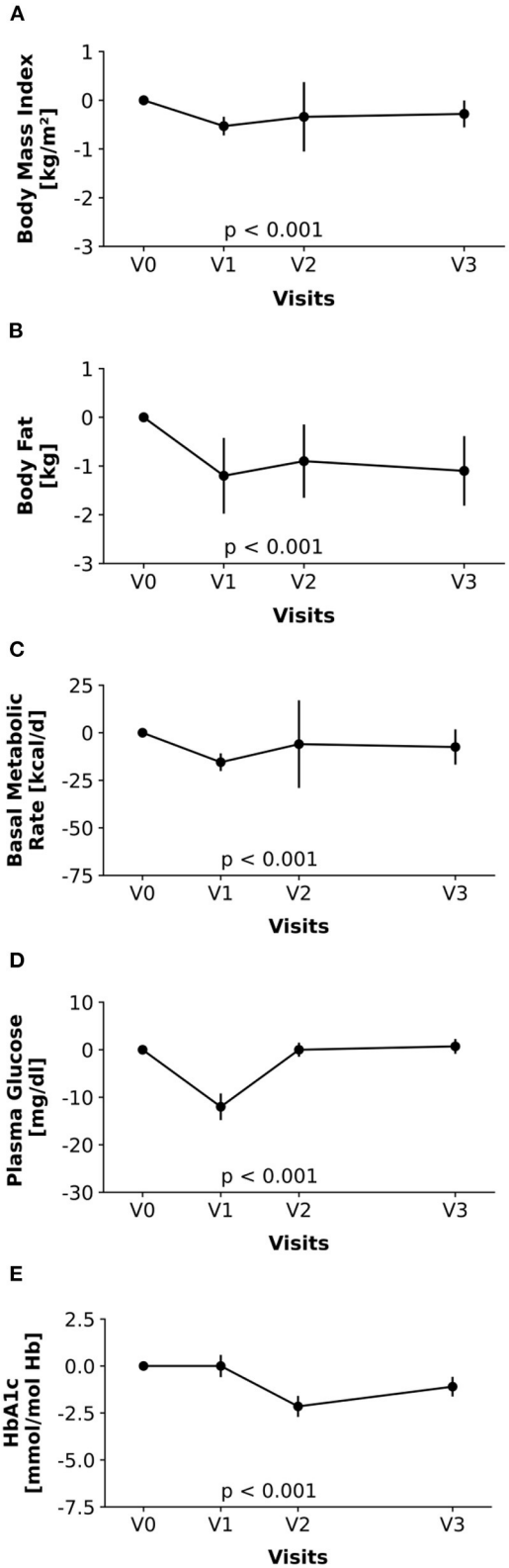

FIGURE 3 | Changes in metabolic parameters over the study period (**A:** BMI, **B:** body fat mass, **C:** resting metabolic rate, **D:** glucose, and **E:** HbA1c). Changes relative to V0-values. Line graphs and error bars represent means and 95%-confidence intervals accordingly.

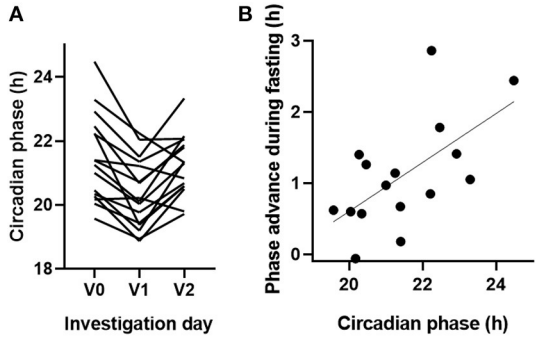

FIGURE 4 | Bahá'í fasting advanced the circadian phase in particular of late chronotypes. **(A)** The circadian phase of 16 study participants was assessed, using the blood monocyte-based BodyTime assay (26) before (V0), during (V1), and 3 weeks after BF (V2). The circadian phase corresponds to the BodyTime-predicted DLMO, which usually occurs about 2 hours before habitual bedtime. **(B)** The extent of advancing the circadian phase during BF correlated with chronotype (i.e., the circadian phase before BF).

considerably lower compared with visits before and after the fast. Interestingly, HbA1c was decreased with a temporal delay to the fast, with a smaller decrease between V0 and V1, reaching the minimum only at V2. Here, even values at V3 were lower when compared with baseline values.

Similarly, fructosamine was elevated during and after the fast (V1, V2, and V3 vs. V0), indicating only slow recovery.

Chronobiology

The circadian phase (chronotype) of a subject, given by the predicted dim-light melatonin onset (DLMO) assessed, using the blood monocyte-based assay BodyTime (26), varied markedly between the days of investigation [$\chi^2(2) = 13.5$, $P = 0.001$]. Chronotype during fasting (V1) was notably earlier than in V0 but returned to pre-fasting levels within the next 3 weeks (V2). The extent of advancing the circadian phase correlated distinctly with the initial chronotype, i.e., late chronotypes advanced more than early chronotypes (slope = 0.34, $P = 0.012$, $r = 0.61$, **Figure 4**). No correlations with other clinical outcomes were found after adjustment.

Sex Effects

Repeated-measures ANOVA was used to examine the changes over time in the interaction between sex and visits for BMI, body fat mass, resting metabolic rate, glucose, HbA1c, plasma, and urine osmolality (including 24-hour, diurnal and nocturnal measurements), as well as for the chronobiological data. In this explorative analysis, we could not detect any relevant differences.

DISCUSSION

This study aimed to generate hypotheses about the health effects and safety of religiously motivated diurnal IDF. Particular emphasis was placed on changes of hydration status, as this aspect is the least scientifically explored until now. Our results showed that hydration status varied slightly

between fasting and non-fasting times in this study sample. Although the mean changes in osmolality were within the physiological range, it could, on scrutiny, be observed that some dynamics seem to be present during nighttime, while diurnal samples remain almost unchanged compared with non-fasting samples.

In summary, our data indicate the safety of BF regarding hydration and renal function. Even a slight diluting effect on urine and plasma was observed. Despite a lower resting metabolic rate, the anthropometric indices, as well as glucose metabolism, seemed to profit from BF, without a rebound being witnessed even 3 months later. The phase of circadian rhythms was changed during BF, an effect being caused either by fasting itself or by concomitant behavioral changes.

To our knowledge, this study is the first worldwide to assess physiological and psychological changes during the religious fast of the Bahá'ís. Different methodological approaches were chosen to unravel cross-links between physiological parameters and religious experience and objective and subjective dimensions of this specific fast. This approach was confirmed even in interpreting the laboratory data on the main study focus, which was the hydration status. We showed that, during BF, hydration parameters remained within physiological limits or even indicated a dilution. Both serum and 24-hour-urine osmolality dropped. In the qualitative interviews, some subjects explained they would more consciously drink in the mornings before sunrise (own data, publication pending), which could explain part of the effect. But, as serum osmolality during BF was measured in the afternoon, there might be more effects leading to the slight dilution observed in most subjects. One of these could be activation of the renin–angiotensin–aldosterone system. The RAAS preserves body water resources by retaining sodium and eliminating potassium. According to our data, the latter actually decreased during BF, a finding which is in line with those in Ramadan fasting (31, 32). This would also suit the slight increase in osmolality in the afternoon spot urine samples. In prolonged DF, an increase in RAAS activity has been described (20), as well as an increase in serum osmolality only after 24-hour of DF, while urine volume decreased (15). Our data showed a decrease in both serum and urine osmolality in the 12- and 24-hour samples, while total body water remained unchanged in the bioimpedance analysis. Our findings suggest that, in the population studied, no relevant dehydration occurred during IDF.

Comparing the outcomes of BF on body weight and the metabolic rate with the results from the studies on Ramadan fasting, we see a diverse picture in Ramadan fasting. Some studies report a weight loss, others no change and even others a weight gain during Ramadan fasting (33). Also, it seems that mostly overweight people lose weight in Ramadan, while those with normal weight do not show as much effect (12). As not enough overweight individuals with a BMI $\geq$ 25 kg/m² were included, this study cannot confirm a similar tendency in BF. A first data analysis of our sub-study at the ECRC indicated an increased adipose tissue lipid mobilization (publication pending). Thus, it could be postulated that BF triggers lipid metabolism, leading to a loss of body fat mass and body weight despite a reduction of the resting metabolic rate.

Furthermore, the observed weight loss and the drop of the resting metabolic rate, alongside the measurement of the activity of the clock genes, show that cyclical changes in energy metabolism influence nutrient utilization and that short-term changes in meal frequency and timing have an effect on chronobiology and energy balance. This is in line with the findings on Ramadan fasting (33).

Previous studies showed that intermittent fasting can affect sleep–wake patterns as well as circadian rhythms in animals and humans. Popular readouts for circadian rhythmicity are repeated measures of body temperature and melatonin levels as well as subjective assessment of chronotype, using questionnaires. Here, a recently developed BodyTime assay was used, in which only one blood sample is needed to objectively assess the phase of the circadian clock of an individual (26). Although the BodyTime assay has not been validated in individuals under specific fasting dietary regimens, it is important to note that, in the study, in which we identified the biomarkers (26), the subjects received hourly isocaloric snacks, whereas, in the subsequent validation study, the participants were free to choose the dietary regimen. At least, when comparing these largely different dietary regimens, we found no difference in accuracy in the circadian phase assessment. The results showed that BF markedly advanced the circadian phase of fasting individuals by more than 1 hour, which is reversed to normal levels 3 weeks after fasting (**Figure 4A**). In animals, restricting food intake to a few hours within their inactive phase substantially alters circadian rhythms in peripheral tissues, while the master clock in the hypothalamic suprachiasmatic nucleus remains largely unaffected [for a review, see Manoogian and Panda (34)]. Studies investigating how Ramadan fasting affects circadian rhythms revealed conflicting results, since they often cannot discriminate direct effects of fasting on the circadian system from indirect effects of lifestyle changes [for a review, see Qasrawi et al. (35)]. However, it remains unclear whether BF alters the circadian system *per se* or whether the observed phase advance of circadian rhythms is rather due to altered sleeping times and thus altered light exposure, which would phase shift circadian rhythms. A limitation of our study is that we recorded neither light levels nor sleeping time, which would have probably been helpful to discriminate between these hypotheses. However, the fact that we observed larger phase advances for later chronotypes (**Figure 4B**) supports the second hypothesis, since the participants reported eating and drinking before sunrise (own data, publication pending), which requires relatively earlier wake-up times for late types during BF compared with early types.

A possible bias of the study may originate from the fact that rather healthy, highly educated volunteers participated in this study. This sociodemographic distribution reflects findings from another sample of Bahá'ís in Germany (21). To counteract these biases and to track intraindividual changes during BF, this study was designed as a longitudinal study with a pretest and a longer follow-up than most of the Ramadan studies. As, in Germany, the Bahá'í religion only has ∼6,000 followers in more than 100 localities (21), it was a challenge to get an adequate sample size. Future confirmatory studies should be conducted in areas with more Bahá'ís to allow higher participant numbers. Timing

of samplings was yet another challenge. During BF, fasting measurements were not possible in the mornings, as breakfast was taken at dawn. Changes seen in physiological parameters during BF may thus include time-of-day effects. To meet this challenge, we included longer-term parameters, for example, 24 h-urine samples for osmolality or HbA1c for serum glucose, as well as follow-up measurements. These show longer-lasting changes, so we can exclude these were only due to time-of-day effects.

Studies with higher participant numbers are needed to validate the hypothesis of urine dilution and weight loss during BF, as well as the improvement of glucose and fat metabolism. Future studies should also focus on exploring the physiological background of these clinical changes and their potential impact on long-term health. It should also be considered to study BF in different cultural contexts, as Bahá'ís fast all over the world. Culturally different approaches to fasting as well as the meal composition could influence the outcomes under discussion.

In summary, this study showed that Bahá'í fasting, although being shorter than other intermittent fasting regimes (5), might have a notable impact on fat and glucose metabolism. Refraining from drinking as well as chronobiological considerations might be crucial in this metabolic shift and should be studied further, especially as this study has not found critical effects on hydration or renal parameters in this kind of intermittent dry fasting.

DATA AVAILABILITY STATEMENT

The original data of the chronobiological measurements can be found in **Supplementary Material**. Futher data described in the article will be made available upon request from the corresponding author pending application and approval.

ETHICS STATEMENT

The study protocol was approved by the institutional review board of Charité Universitätsmedizin Berlin (Charitéplatz 1, 10117 Berlin) in January 2018 (ID: EA4/216/17) and was registered with clinical trials (ID: NCT03443739). The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DK-L conceived and designed the study and had primary responsibility for final content. MB and AMä assisted in designing the part of the metabolic measurements. AK designed the chronobiological part of the study. DK-L, CKl, JS, RR, AMä, MB, BK, and MJ conducted the research. DK-L, CKl, FK, NS, JS, AMä, MB, CKe, RR, AMi, BA, and AK analyzed and interpreted data. DK-L, CKl, FK, AK, AMi, MB, and NS wrote the paper. All the authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.662310/full#supplementary-material

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MINI REVIEW
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Transcriptomic Effects of Healthspan-Promoting Dietary Interventions: Current Evidence and Future Directions

Devin Wahl[1,2†] *and Thomas J. LaRocca*[1,2*†]

[1] *Department of Health and Exercise Science, Colorado State University, Fort Collins, CO, United States,* [2] *Center for Healthy Aging, Colorado State University, Fort Collins, CO, United States*

Aging is the greatest risk factor most diseases, including cardiovascular disorders, cancers, diabetes, and neurodegeneration, but select nutritional interventions may profoundly reduce the risk for these conditions. These interventions include calorie restriction, intermittent fasting, protein restriction, and reducing intake of certain amino acids. Certain *ad libitum* diets, including the Mediterranean, Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability, and Okinawan diets also promote healthy aging. Evidence indicates that these dietary strategies influence aging and healthspan by acting on the biological "hallmarks of aging" and especially upstream nutrient sensing pathways. Recent advances in "omics" technologies, including RNA-sequencing (transcriptomics), have increased our understanding of how such nutritional interventions may influence gene expression related to these biological mediators of aging, primarily in pre-clinical studies. However, whether these effects are also reflected in the human transcriptome, which may provide insight on other downstream/related cellular processes with aging, is an emerging topic. Broadly, the investigation of how these nutritional interventions influence the transcriptome may provide novel insight into pathways associated with aging, and potential targets to treat age-associated disease and increase healthspan. Therefore, the purpose of this mini review is to summarize what is known about the transcriptomic effects of key dietary/nutritional interventions in both pre-clinical models and humans, address gaps in the literature, and provide insight into future research directions.

Keywords: aging, RNA-Seq - RNA sequencing, transcriptome, healthspan, nutrition

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University of Palermo, Italy

****Correspondence:***
Thomas J. LaRocca
Tom.LaRocca@colostate.edu

†These authors have contributed equally to this work

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INTRODUCTION

Older age is the major risk factor for cardiovascular disorders, cancers, diabetes, sarcopenia, frailty, and neurodegeneration (1). As a result, the "Geroscience" concept has emerged in an attempt to understand the relationships between aging biology and age-related diseases, with the hope that targeting aging itself will increase healthspan (the number of healthy, disease-free years in older age) (2). The mechanisms that drive aging/age-related diseases, termed the "hallmarks of aging," represent key molecular targets for interventions to improve healthspan (3).

Nutrition has a profound impact on the biology of aging and disease. Several interventions in particular act on most hallmarks of aging to improve cardiometabolic, physical and cognitive

health, and to delay age-related disease and increase lifespan. These interventions include calorie restriction (CR), intermittent fasting (IF) (4), alternate day fasting (ADF), prolonged fasting (PF), time-restricted feeding (TRF), protein restriction, and reduced intake of certain amino acids. Specific eating patterns, including the Mediterranean, Okinawan, and FINGER (Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability) diets also improve health (5). Information on how these interventions impact health and lifespan (largely by influencing the hallmarks of aging) has accumulated over several decades (1). However, recent advances in "omics" technologies, including RNA-sequencing, may provide additional, important understanding of the common or distinct biological mechanisms underlying these interventions and yield novel insight into targetable, genomic/cellular pathways associated with aging and disease.

The effects of healthy aging interventions on the transcriptome are not fully understood, mostly due to a lack of sequencing data. However, evidence to date suggests that key transcriptomic pathways involved in aging including glucose homeostasis/insulin signaling, inflammation, oxidative phosphorylation, immune responses, and circadian signaling are similarly influenced by most healthy aging/nutritional interventions (**Figure 1** and **Table 1**). The purpose of this mini review is to detail how healthy aging interventions affect these pathways (and related genes/modulators), and to outline additional pathways/genes that are distinctly modulated with select healthy dietary interventions. While most existing transcriptomic and genetic studies have used pre-clinical models, we also provide insight into how some of these interventions affect the human transcriptome. Finally, we describe gaps in the research, future directions, and goals in the field of dietary interventions and transcriptomics.

CALORIE RESTRICTION

Perhaps the most powerful anti-aging nutritional intervention, CR is defined as a reduction in 10–50% daily caloric intake *without malnutrition.* CR increases healthspan and lifespan through many mechanisms, including improved glucose homeostasis and mitochondrial health, and by inhibiting all molecular hallmarks of aging (24). The transcriptomic effects of CR in pre-clinical models have been extensively investigated in multiple tissues. Seminal studies showed that long-term CR reverses gene expression changes associated with age-related inflammation and impaired stress responses, DNA-replication/cell cycle defects, oxidative stress, tumorigenesis, and macromolecular damage (11, 25, 26). Additional results indicate that CR largely reduces transcriptomic signatures associated with immune activation, inflammatory signaling, glucose homeostasis, and AMP-activated protein-kinase/insulin-like growth factor 1 (AMPK/IGF-1) signaling (evolutionarily conserved energy sensing pathways). Also, consistent with the observation that glucose homeostasis/insulin signaling are central players in the effects of CR on metabolism, CR changes the expression of key genes affecting energy/metabolism-modulating proteins and insulin secretion (largely reducing the expression of these genes) (6). In addition to glucose homeostasis, other pathways/genes contributing to DNA repair, fatty acid metabolism, and citric acid cycle/oxidative phosphorylation appear to be key transcriptomic mediators of the effects of CR in white adipose tissue and liver in pre-clinical models (27–29).

More recently, the influence of CR on the liver transcriptome in the *Rhesus macaque* (a close relative to humans sharing 93% DNA homology) was investigated. A two-year CR intervention reduced transcriptome signatures of immune activation and inflammation (a main hallmark of aging) and increased

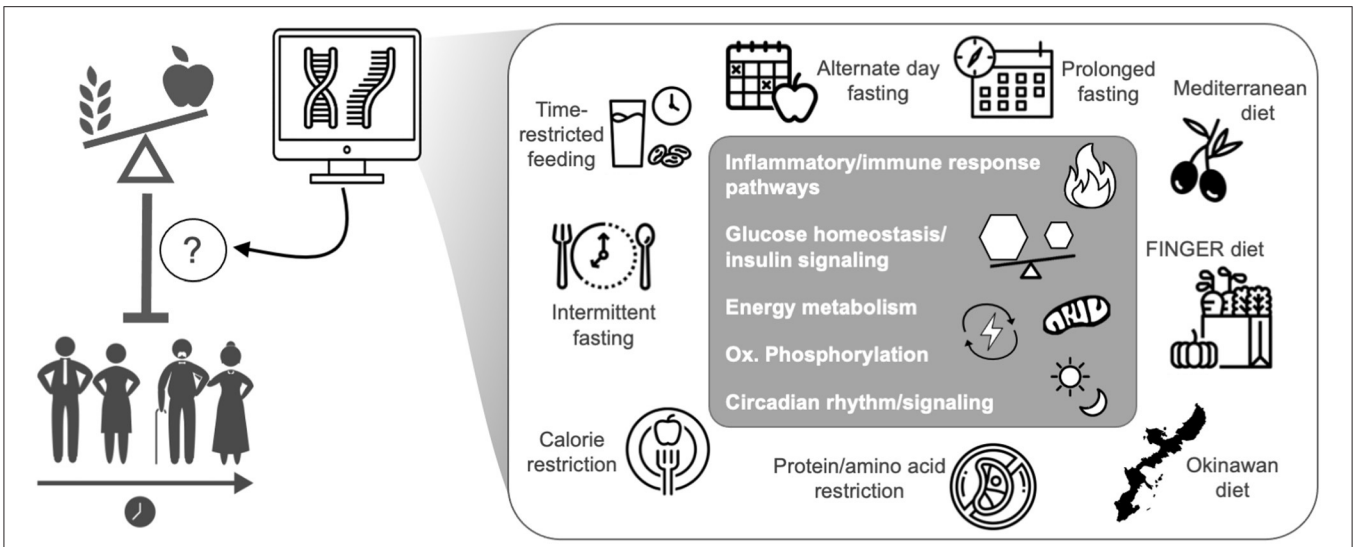

FIGURE 1 | Transcriptomics has increased the understanding of how health-promoting nutritional interventions influence gene expression related to the biological mediators of aging and healthspan, mostly in pre-clinical models. The transcriptomic effects of healthspan-promoting nutritional interventions are still under investigation, but common effects among all interventions are noted in the gray box. Icons from nounproject.com under Creative Commons license.

TABLE 1 | Commonalities among the effects of select healthy dietary interventions on biological hallmarks of aging in transcriptomic evidence.

Dietary intervention	Aging hallmark/transcriptomic pathways influenced by intervention	Species	Tissue(s)	Central signaling mediators	References
Calorie restriction	Glucose dysregulation/insulin signaling ⇓; Inflammation ⇓; Oxidative phosphorylation ⇓; Immune response ⇑⇓; Circadian rhythm ⇑⇓	Rodents, non-human primates, humans	Liver, muscle, brain, white adipose tissue, blood mononuclear cells	*FOXO1, FOX03, IGF-1, GH, MTOR* (insulin signaling) *IL-6, MIF, p53, NF-κB* (inflammation) *AMPK, SIRT1, PPARα, PGC1-α* (oxidative phosphorylation) C4, *C1qa*, *Lzp-s, NF-κB* (immunity) *PER1*, *PER2*, *CRY, ROR-α*, *ROR γ* (circadian rhythm)	(6–13)
Intermittent fasting	Glucose insulin signaling ⇓; Inflammation ⇓; Oxidative phosphorylation ⇓ Immune response ⇑⇓; Circadian rhythm ⇑⇓.	Rodents, humans	Liver, white adipose tissue, brain, muscle, blood mononuclear cells	*SIRT1, SIRT3, IGF-1, FOXO1, GH, MTOR, AMPK* (insulin signaling) *TNF-α, IL1B, IL-6* (inflammation) *PPARα*, *SIRT1, SIRT2, PGC1-α* (oxidative phosphorylation) *APOA2, HPX, NF-κB* (immune response) *PER1*, *PER2*, *CRY* (circadian rhythm)	(6, 14–19)
Alternate day fasting/pro-longed fasting	Glucose insulin signaling ⇓; Inflammation ⇓; Immune response ⇑⇓; Circadian rhythm ⇑⇓.	Rodents, some evidence in humans	Liver, brain, muscle, white adipose tissue	*SIRT1, SIRT3, IGF-1, FOXO1, GH, MTOR, AMPK* (insulin signaling) *TNF-α, IL-6, IL-1β, NF-κB* (inflammation) *NF-κB* (immune response) *PER 1, PER2, BMAL1 and CRY1, CRY2* (circadian rhythm)	(9, 19, 20)
Time restricted feeding	Glucose insulin signaling ⇓; Inflammation ⇓; Oxidative phosphorylation⇓; Immune response ⇑⇓; Circadian rhythm ⇑⇓.	Rodents, some evidence in humans	Liver, muscle, white adipose tissue	*SIRT1 SIRT3, AMPK, IGF-1, GH, MTOR* (insulin signaling) *TNF-α, IL-6, IL-1β* (inflammation) PPAR-α, SIRT1, SIRT3, AMPK (oxidative phosphorylation) *NF-κB* (immune response) *PER 1, PER2, BMAL1 and CRY1, CRY2* (circadian rhythm)	(9, 19–21)
Protein/amino acid restriction	Insulin signaling ⇓ Circadian rhythm ⇑⇓; Immune response ⇓	Rodents, some evidence in humans	Liver, kidney	*MTOR, AMPK, SIRT1* (insulin signaling) *NF-κB* (immune response) IL-6, IL-10, TNF-α (inflammation) *BMAL1, CRY1, NPAS2, REV-ERBα* (circadian rythym)	(10, 22, 23)

Arrows indicate effects on the pathway (increased/decreased). Species, tissues, select central signaling mediators, and references are included.

pathways associated with ribosome activity, branched-chain amino acid metabolism, fatty acid degradation, and RNA transport mechanisms (7). These results suggest that *Rhesus macaques* and mice respond similarly to CR, with main transcriptomic changes in inflammatory pathways, immune function, and fatty acid degradation (related to glucose metabolism) in multiple tissues (30).

In humans, analyses from multiple studies indicates that both short-term (e.g., 4 weeks) and long-term (e.g., 1 year) CR upregulates transcriptome modules associated with stem cell maintenance, blood vessel remodeling, and lipid metabolism in subcutaneous fat tissue (31). Other studies indicate that very long-term (10 years) voluntary CR causes gene expression patterns related to enhanced lipid and glucose metabolism, and downregulation of the insulin/IGF-1 growth pathway (resulting in a younger transcriptional profile) in muscle (a main metabolic tissue), consistent with the pre-clinical mouse and non-human primate data (8).

INTERMITTENT FASTING

IF is an umbrella term for eating patterns (which also include ADF, PF, and TRF) involving an extended time with little or no energy intake (fasting), all of which are associated with improved glucose homeostasis (and reduced insulin) and increased stress resistance (32). It has been suggested that the key benefit of IF comes from fasting periods (rather than reduced total energy intake), which can induce hormesis (responses to moderate stress with downstream protective and anti-aging effects). For example,

11 h/day of restricted feeding (e.g., no calorie intake) reduces liver pathology and extends median lifespan in male mice (33). IF (16 h/day) in mice also results in global transcriptomic changes in the liver (a key metabolic organ) including modulation of pathways contributing to sterol, alcohol, and cholesterol synthesis, and changes in gene regulation modulating circadian rhythm (14). In addition to these effects, IF enhances transcriptional signatures of peroxisome proliferator activator receptor (PPAR) activity, a major mediator of oxidative phosphorylation, highlighting the influence of IF on homeostasis and metabolic function (34). Finally, IF (12–16 h/day) results in global transcriptomic changes in the mouse brain reflecting increased neuroplasticity and reduced inflammation, which parallels the effects of CR on the brain (15).

The transcriptomic effects of IF in humans are still under investigation (studies are underway), but initial results from studies of individual genes indicate that IF (5 days of periodic fasting) increases transcriptomic signatures of nutrient and energy sensing/oxidative phosphorylation gene expression including AMP-activated protein kinase (AMPK), Forkhead box protein 01 (FOXO1), Sirtuin 1 (SIRT1), and Sirtuin 3 (SIRT 3). These genes/proteins are key players in cellular regulation pathways and centrally implicated stress responses, cardiometabolic/systemic health, and longevity (35). Another study (36) confirmed these results with data showing that IF contributes to a marginal increase in SIRT3 gene expression, which was associated with a decrease in plasma insulin levels. While these preliminary results in humans have primarily focused on changes in specific gene expression, the global transcriptomic effects of IF (36-h fast) appear to be similar to CR with changes in gene expression related to fatty acid oxidation, cell cycle and apoptosis pathways, and reductions in key inflammatory genes/pathways (16).

ALTERNATE DAY FASTING AND PROLONGED FASTING

ADF and PF are types of IF involving feeding days (on which food is consumed *ad libitum*), and fasting days on which no calories are consumed. The fasting days can occur every other day or intermittently (e.g., fasting 2 days per week or even up to 21 days once per year, which is more commonly known as PF) (37, 38). Evidence shows that these dietary interventions induce metabolic effects similar to IF, including improving glucose homeostasis and reducing liver triglyceride content in mice (39). In pre-clinical models, these diets flip a "metabolic switch" activating key transcriptional regulators of fatty acid and energy metabolism and inflammation (9, 40), and transcriptional evidence shows that ADF (24-h fast) modulates key processes associated with circadian rhythm, RNA processing, and oxidative metabolism (41).

In humans, a prolonged 10-day fast results in transcriptomic signatures of increased lipolysis/lipid metabolism and reduced activation of pathways related to glycolysis and oxidative phosphorylation. Interestingly, this prolonged fasting period also contributes to the upregulation of inflammatory pathways and macrophage activation in subcutaneous fat tissue (42), reflecting a potential hormesis response and downstream protective mechanisms. Another study showed that short-term fasting is associated with enrichment of transcriptomic pathways involved in fatty acid oxidation, cell cycling and apoptosis, and a decrease in the expression of pro-inflammatory genes in peripheral blood mononuclear cells (16). These apparently divergent results suggest that the protocol, length of fasting, and tissues analyzed are important factors to consider when studying the influence of these dietary interventions on the human transcriptome.

TIME-RESTRICTED FEEDING

TRF is a another type of IF and allows for eating only during a certain time window in the day (e.g., eating *ad libitum* for 8 h or less a day in humans) (37). TRF is different from IF in that the daily eating time frame remains constant, which is thought to influence circadian clock pathways. TRF also promotes cardiometabolic/systemic health by maintaining metabolic homeostasis (e.g., improving glucose tolerance and insulin sensitivity, and reducing blood lipid levels) (43). Additional effects of TRF include enhanced mitochondrial health, DNA-repair, and autophagy, all of which coincide with improved glucose metabolism and potentially reduced risk for most age-related diseases (44). In mice, TRF contributes to transcriptomic changes to circadian clock gene expression (increased expression/rhythmicity) and metabolic/nutrient sensing pathways (changes that parallel improved metabolic health and the prevention of fatty liver) (21), all of which are similar to effects seen with IF, ADF, and PF.

There is limited research on the effects of TRF on the human transcriptome/gene expression, but recent evidence suggests that a daily 8-h eating time frame influences genes responsible for amino acid transport in muscle (45), and reduces gene expression signatures related to growth and metabolism. Research on the effects of fasting has recently focused on peripheral/circadian clock genes, with some studies suggesting no universal effect (17) and others suggesting that that TRF in particular influences several genes implicated in circadian rhythm (46). Such changes in circadian clock gene expression might impact healthspan and lifespan, but these links are poorly understood (47).

PROTEIN AND AMINO ACID RESTRICTION

The restriction of dietary protein and certain amino acids promotes healthspan and lifespan in pre-clinical models (48) and may mimic CR to some extent. This may be an important observation, especially considering that most people may find it difficult to adhere to most calorie deficit interventions, especially in Western societies where there is essentially free access to calorie-dense foods. Long-term reduction of dietary protein intake (through dilution with non-digestible fiber) improves healthspan and lifespan via reduced growth pathway activation and improved mitochondrial function in male and female mice (49), and short-term protein restriction reduces circulating triglycerides via changes in hepatic APOE expression

(50). Similarly, reduced intake of branched-chain amino acids (BCAAs; leucine, isoleucine, and valine) also improves health and lifespan in male and female mice by inhibiting mechanistic target of rapamycin (MTOR) nutrient sensing pathway activation, improving glucose homeostasis, and reducing insulin resistance (22). While the aforementioned studies are not transcriptomic studies, the results suggest that protein and amino acid restriction may partly mimic the gene expression changes and metabolic effects seen with fasting or related nutritional interventions.

Protein restriction is known influence key genes associated with circadian rhythm, metabolic signaling (including insulin signaling), and oxidative phosphorylation, in addition to other transcriptional factors/genes linked with healthspan and lifespan extension (51, 52). On the other hand, BCAA intake is associated with transcriptomic effects including changes in cell cycle/metabolic pathways, apoptosis (programmed cell death), p53 activity (cell division), and NF-κB signaling (innate and adaptive immunity) (22). These results highlight the contribution of these diet-induced transcriptomic effects to changes in metabolic/energy-sensing processes, as well as the modulation of inflammation—all of which are associated with aging and age-related disease (53).

COMMON TRANSCRIPTOMIC PATHWAYS AFFECTED BY MOST WELL-STUDIED HEALTHY DIETARY INTERVENTIONS

The common transcriptomic pathways modulated by most health-promoting dietary interventions involve glucose/insulin signaling, inflammation, oxidative phosphorylation, immune responses, and circadian rhythm (**Table 1**). Based on limited transcriptomic/genetic data, these processes seem to be similarly affected by most interventions, regardless of species or tissue, although specific genes within pathways may be activated/repressed depending on the intervention type (e.g., the duration of CR or fasting period). We also note that some processes like circadian rhythm and immunity are especially complex and may be differentially modulated (e.g., increased or decreased) and highly dependent on length or type of intervention (54). Below, we briefly describe the common biological processes/transcriptomic pathways that are affected by most healthy aging interventions.

Glucose Homeostasis and Insulin Signaling

Metabolic dysfunction is considered a central hallmark of aging, and current transcriptomic data shows that all healthy aging dietary interventions influence pathways involved with glucose homeostasis and insulin signaling. Most evidence suggests that these interventions improve health by suppressing key insulin-related signaling pathways and gene expression. These main genes include Insulin-like growth factor-1 (IGF-1, reduced), Sirtuins (SIRT1 and SIRT3, increased), Growth hormone (GH, increased), Mechanistic target of Rapamycin (MTOR, reduced), and FOXO genes (increased) (6), all of which have key roles in energy sensing, growth and metabolism (5).

Inflammation

Inflammation is considered a key hallmark of aging because it can directly affect all other biological hallmarks of aging (55). Most healthy dietary interventions targeting energy intake reduce transcriptomic evidence of inflammatory activation, and this has been demonstrated in multiple species/tissues. Key inflammatory mediators involved in these pathways include Interluekin-6 (IL-6), Tumor necrosis factor alpha (TNF-α), and Interleukin 1 beta (IL-1β). Importantly, additional health-promoting diets including the FINGER and Mediterranean (described below) also similarly modulate these pro-inflammatory cytokines (and transcriptomic evidence of their activity) in humans (56).

Immune Response

Long periods of calorie restriction or periodic fasting may impair immune system pathways, while short bouts of fasting are emerging as potential interventions for immune system repair and maintenance (10). There is evidence that most healthy aging interventions influence immune responses to a certain degree, mostly by modulating *NF-κB,* a transcriptional factor which plays critical roles in immune response and cytokine production (38). Some evidence suggests that CR reduces additional transcripts (57) involved in immune function including Complement component 4 (*C4*) and Complement C1q (*C1q*), but whether these genes are also influenced by the other interventions remains to be determined.

Oxidative Phosphorylation

All healthy nutritional interventions act on the mitochondria, and consequently, pathways/genes contributing to cellular respiration. Most evidence suggests that these interventions reduce oxidative phosphorylation gene expression (58) consequently attenuating the production of reactive oxygen species (ROS; a key contributor to aging/age-related disease). Increased expression/activity of mitochondrial biogenesis and turnover pathways with healthy nutritional interventions may also contribute to greater mitochondrial efficiency (despite reductions in oxidative phosphorylation). Key genes associated with these pathways are mostly energy sensing in nature and include the Sirtuins, Peroxisome proliferator activated-receptor α (PPAR-α), and AMPK (59).

Circadian Rhythm

Most physiological and metabolic processes are regulated by circadian clock genes that are responsible for the synchronization of biological processes within an organism (60). Recently, it has been suggested that circadian rhythm dictated by peripheral clock genes can be re-synchronized (thus resulting in positive health benefits) under CR or PF (61). Key modulators of circadian rhythm pathways affected by most nutritional interventions include the Period circadian regulators (PER1 and PER2) and the Cryptochromes (CRY) (61).

EXPLORATION OF THE TRANSCRIPTOMIC EFFECTS OF ADDITIONAL HEALTH-PROMOTING DIETS

Most of the well-studied interventions above are calorie or macronutrient restrictive. It is therefore important to understand how other health-promoting *ad libitum* nutritional interventions influence the genome. Indeed, emerging data show that several healthy dietary/nutritional interventions or patterns act on the transcriptome to affect processes associated with aging, age-related disease, and longevity.

The Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER), Mediterranean, and Okinawan diets all improve health in humans. These diets are characterized by ample fruit and vegetable consumption and low-to-moderate protein intake (with limited red meat consumption), low-to-moderate fat consumption (healthy fats including olive oils and avocado), and moderate-to-high carbohydrate consumption (whole grains, potatoes) (62, 63). The FINGER diet also includes cognitive function-enhancing exercises and a weekly physical activity routine (64).

The most well-studied of these diets, in terms of transcriptomic impact, is the Mediterranean diet. The Mediterranean diet elicits a transcriptomic response in humans including reduced inflammatory/inflammation response gene expression (65) (possibly due to a high consumption of monounsaturated fatty acids and olive oils) and enhanced circadian clock transcript levels (66). Other studies show that the Mediterranean diet affects transcriptomic pathways involved with cardiovascular health (e.g., atherosclerosis and hypertension) including lipid and cholesterol metabolism, which appears to be a main benefit of this diet (67). The transcriptomic effects of an Okinawan or FINGER dietary intervention remain to be determined; however, based on the above observations that the Mediterranean diet may have transcriptomic effects similar to those of CR/fasting, profiling the influence of these other diets on the transcriptome could be critical for identifying the ideal, non-fasting dietary pattern to promote healthy aging.

RESEARCH GAPS AND FUTURE DIRECTIONS

As described above and in **Figure 1** and **Table 1**, the available evidence suggests that the common transcriptomic effects of healthspan-promoting nutritional interventions include favorable modulation of gene expression patterns related to inflammatory signaling, immune function, energy sensing/glucose homeostasis, oxidative phosphorylation (including mitochondrial function), and circadian regulation. Importantly, most of this evidence is based on: (1) pre-clinical studies with some limited/emerging clinical data; and (2) various experimental and methodological approaches, including gene expression/qPCR panels, microarrays, and most recently RNA-sequencing (see individual references for details). More research is needed to increase our understanding of human-specific transcriptomic effects of these interventions, and to leverage newer, comprehensive RNA-seq approaches—ideally in "multi-omics" studies (e.g., RNA-seq combined with proteomics) that may provide further mechanistic insight. In addition to these general future directions, below (and in **Figure 2**) we outline several key issues that require further investigation in this area.

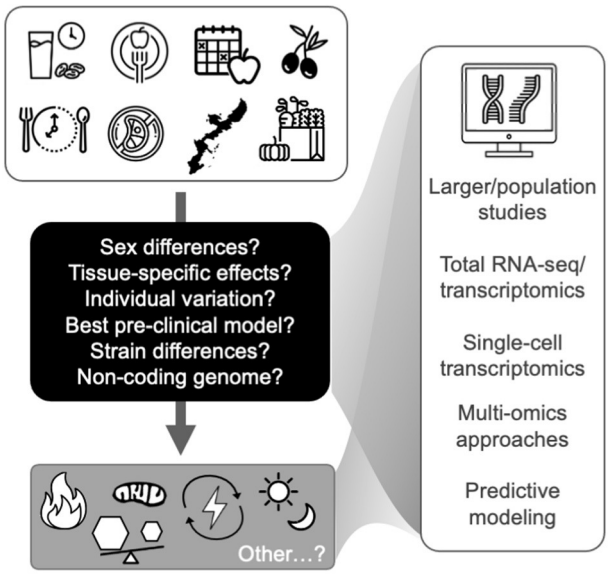

FIGURE 2 | Future studies are needed to identify key transcriptomic modules that relate to the hallmarks of aging (bottom gray box) and represent targets for healthy aging in humans. Future directions and key issues (shown in the black box and at right) in transcriptomic studies of healthspan-promoting nutritional interventions are also noted. Icons from nounproject.com under Creative Commons license.

Suitability of Rodent Models

It is generally accepted that common responses to the most robust healthspan-promoting nutritional interventions are shared among mice and non-human primates, (68). However, it remains to be determined whether mice are a good translational model for studying the transcriptomic impact of nutritional interventions as they relate to humans. With CR (perhaps the most studied and robust nutritional intervention) for example, global plasma biomarker changes with CR in mice mimic those seen with fasting in humans (69), but recent research shows more divergent transcriptome effects of certain healthy nutritional interventions in mice and humans. For instance, an increase in stem cell maintenance and vascularization pathway activation is associated with CR in human subcutaneous white adipose tissue (WAT), but in mouse epididymal WAT these processes are downregulated with CR (31).

In addition to issues related to divergent mouse-human transcriptome effects, some commonly used mouse strains do not respond well to CR (e.g., they exhibit reduced lifespan) and/or other interventions. Most research on nutritional interventions and the transcriptome has utilized C57/B6 mice, a homogenous, inbred strain. However, It appears that CR results in greater lifespan extension in non-inbred mice when

compared to inbred mice and may not be as effective in certain genetic backgrounds/strains (70). The underlying mechanisms are still under investigation, but some evidence shows that certain mouse strains exhibit completely opposite transcriptional responses to CR compared with other strains (e.g., BALBC, DBA), and these effects are tissue-specific (31). As such, when examining the influence of healthy nutritional interventions on the rodent transcriptome, it is important to consider the genetic strain/background and tissue(s) before drawing conclusions. This point parallels the need to consider genetic variability in humans (71) when considering the role and transcriptomic profiles of healthy aging interventions in aging/age-related disease. Indeed, it may be necessary to use transcriptomics and/or other omics approaches to develop "precision" dietary strategies tailored to individual people.

Tissues for Studying the Effects of Nutritional Interventions on the Transcriptome

Most pre-clinical nutritional intervention studies have largely focused on transcriptomic changes in liver and fat, and to a lesser extent, in brain and muscle. Liver, fat, and muscle are key metabolic tissues that are centrally implicated in the transcriptomic and metabolic responses to healthy aging nutritional interventions (72), and it appears that the responses to certain dietary interventions are different depending on type of tissue analyzed. Some transcriptomic pathways, like the insulin/IGF1 signaling response, seem to be equally affected by fasting across multiple tissues in rodents (73). However, the major metabolic tissues/organs of the body have vastly different metabolic rates (74) and may therefore respond to nutritional interventions differently in other gene expression pathways. As an example, AMPK, a key energy sensor and metabolism regulator, is activated by fasting in metabolic tissues like fat, liver, and muscle. However, the activation/response of AMPK (and related downstream mechanisms) to fasting in other tissues (e.g., brain, kidney) may vary (75). Current and future research should aim to determine transcriptomic overlap and differences of healthy nutritional interventions in multiple tissues, as this will broaden the scope of targetable mechanisms in tissue-specific diseases.

Sex-Specific Effects of Healthy Nutritional Interventions on the Aging Transcriptome

Male and female mice respond differently to anti-aging nutritional interventions (76). Key differences between sexes in response to nutritional interventions could be due to amino acid metabolism in the liver (77) or the ability of female mice to use increased energy from fat (through β-oxidation) during food restriction (78). Male and female mice also respond differently to varying intervention regimens (e.g., 20 vs. 40% CR) with different cardiometabolic and lifespan responses (24). Some recent RNA-seq studies have addressed these differences and found sex-specific responses in gene expression patterns with fasting and amino-acid restriction (79). Future research should continue to focus on such similarities/differences in transcriptomic responses to healthy aging nutritional interventions in both male and female mice, which may be key for understanding clinical applications.

The Non-coding Genome and Healthy Aging Nutritional Interventions

The non-coding genome (e.g., non-coding RNAs, microRNAs) is often ignored in transcriptomic studies on aging. However, certain non-coding transcripts are emerging as key players in aging and disease, and could be critical in understanding the transcriptomic effects of nutritional interventions. For example, microRNAs, which negatively regulate gene expression of their targets at post-transcriptional levels, may be intricately involved in aging gene expression pathways and the hallmarks of aging (e.g., genome stability) (80). Some non-coding/microRNAs are influenced by CR to modify chromatin, which may partly explain how this intervention regulates gene expression during aging (81). Even less commonly investigated, non-coding repetitive elements (RE) make up more than 60% of the genome (82), and it has been suggested that some of these RE can become active during aging, perhaps due to decreased chromatin stability (83). Indeed, there is a global increase in RE transcripts in model organisms and humans with aging (84), and some of these RE may be associated with certain hallmarks of aging, including inflammation, oxidative stress, and cellular senescence (85, 86). Interestingly, CR is associated with a global reduction in RE transcripts in liver, which further supports the idea that global RE transcript dysregulation may be an important transcriptional hallmark of aging and healthy nutritional interventions (28). The influence of other nutritional interventions on RE with aging remains to be determined, but could be a promising area of future investigation.

CONCLUSIONS

Nutrition has a profound impact on aging/age-related disease and lifespan. The transcriptomic effects of healthy nutritional interventions are still under investigation, but glucose regulation/insulin sensitivity (growth/metabolic homeostasis), oxidative phosphorylation, inflammatory/immune function, and circadian pathways appear to be key mediators of healthspan that are modulated by these interventions at the transcriptome level in multiple metabolic tissues and species. In order to identify additional genes/transcriptomic modules that may represent targets for promoting healthy aging, future transcriptome/multi-omic studies are needed to address key issues (e.g., differential sex and tissue responses, more comprehensive genomic profiling) and characterize the effects of more diets (e.g., Okinawan)—ideally in human subjects.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Caloric Restriction Mimetics in Nutrition and Clinical Trials

Sebastian J. Hofer[1,2,3]*, *Sergio Davinelli*[4], *Martina Bergmann*[1], *Giovanni Scapagnini*[4] and *Frank Madeo*[1,2,3]

[1] Institute of Molecular Biosciences, NAWI Graz, University of Graz, Graz, Austria, [2] BioTechMed-Graz, Graz, Austria, [3] Field of Excellence BioHealth, University of Graz, Graz, Austria, [4] Department of Medicine and Health Sciences "V. Tiberio", University of Molise, Campobasso, Italy

The human diet and dietary patterns are closely linked to the health status. High-calorie Western-style diets have increasingly come under scrutiny as their caloric load and composition contribute to the development of non-communicable diseases, such as diabetes, cancer, obesity, and cardiovascular disorders. On the other hand, calorie-reduced and health-promoting diets have shown promising results in maintaining health and reducing disease burden throughout aging. More recently, pharmacological Caloric Restriction Mimetics (CRMs) have gained interest of the public and scientific community as promising candidates that mimic some of the myriad of effects induced by caloric restriction. Importantly, many of the CRM candidates activate autophagy, prolong life- and healthspan in model organisms and ameliorate diverse disease symptoms without the need to cut calories. Among others, glycolytic inhibitors (e.g., D-allulose, D-glucosamine), hydroxycitric acid, NAD^+ precursors, polyamines (e.g., spermidine), polyphenols (e.g., resveratrol, dimethoxychalcones, curcumin, EGCG, quercetin) and salicylic acid qualify as CRM candidates, which are naturally available via foods and beverages. However, it is yet unclear how these bioactive substances contribute to the benefits of healthy diets. In this review, we thus discuss dietary sources, availability and intake levels of dietary CRMs. Finally, since translational research on CRMs has entered the clinical stage, we provide a summary of their effects in clinical trials.

Keywords: caloric restriction mimetics, nutrition, spermidine, clinical trials, polyphenols, polyamines, healthy diet

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***Correspondence:**
Sebastian J. Hofer
sebastian.hofer@uni-graz.at

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INTRODUCTION

In addition to genetic, environmental and lifestyle factors, nutrition plays a vital role in shaping health throughout human aging (1, 2). Recently, health was defined as the sum of several hallmarks, including, the ability to react to environmental and cellular stress, integrity of barriers and maintenance of cellular and organismal homeostasis (3), of which many cross-talk with dietary factors. In opposition to health, diseases are more described and defined and nutrition takes a central part in these processes as well, prominently in type 2 diabetes, malnutrition-caused diseases, eating disorders, obesity, chronic inflammation and undernutrition, among others (1).

While a moderate consensus has been reached on what defines an unhealthy diet, the constitution of a healthy diet remains debated and subject to different beliefs (4). In principle, healthy diets should have positive effects on diverse health parameters, while not evoking negative effects (1, 4–6). Different concepts of healthy dietary plans, including the Healthy Eating Index-2010 (HEI-2010), Dietary Approaches to Stop Hypertension (DASH), Alternative Healthy Eating

Index-2010 (AHEI-2010) and the alternate Mediterranean Diet (aMED) have been developed. These indices estimate and rate the intake of 8–12 components (for instance whole grain, nuts, legumes, fruit, vegetable, alcohol, etc.) and good scores are linked to lower cardiovascular disease (CVD) incidence and cancer mortality (2). In comparison to a Western diet, which is high in processed meat, salt, sugar, saturated fat and low in fresh plant-derived ingredients, these health-optimized diets are richer in plant-based food (fruits, vegetables, whole grains, nuts, and legumes), unprocessed meal components and restricted in animal-based foods (focusing on processed and red meat) (1). The famous Mediterranean diet, which comes in different variations, is roughly composed of daily servings of olive oil, vegetables, fruits, cereals, moderate amounts of fish, meat and sweets and represents one form of a healthy diet which is linked to general health promotion (7–9). In agreement, meta analyses suggest that diets preferring non-hydrogenated unsaturated fats, whole grains, lots of vegetables and fruits are efficient measures against coronary heart disease (10). Given the average Western diet, it thus comes unsurprising that half of cardiovascular and type 2 diabetes related deaths are attributed to unhealthy dietary habits in the United States (11).

Accumulating evidence suggests that caloric restriction (CR) and various forms of fasting (intermittent fasting, time restricted eating, periodic fasting), avoiding malnutrition and including an adequate intake of macro- and micronutrients, present yet additional possibilities to promote the health status by reducing CVDs and cancer, among other beneficial effects (12–14).

Recently, the concept of caloric restriction mimetics (CRMs) was developed to describe pharmacologically active substances that mimic some of CR's myriads of effects (15–20). At the core of the CRM definition, we and others argue that potential CR-mimicking compounds should in principle increase life- and/or healthspan and ameliorate age-associated diseases in model organisms, thus often the simultaneous use of the term "anti-aging substances." Additionally, CRMs should be capable of inducing autophagy, a homeostasis-regulating cellular recycling mechanisms that degrades obsolete, damaged or otherwise unneeded proteins, cellular structures or organelles (20, 21), as well as reducing the acetylation status of proteins (e.g., via activation of deacetylases, inhibition of acetylases or depletion of acetyl-CoA) (22–24). The most physiological inducer of autophagy is nutrient and energy deprivation, such as CR and fasting. Genetic and pharmacological induction of autophagy can prolong lifespan in various model organisms, counteract neurodegenerative, cardiovascular diseases, various types of cancer and delay the onset of frailty during aging, among others (21, 25–28). Autophagy naturally declines during aging and diminished autophagic capacity can contribute to progressive age-associated deteriorations and is implicated in neurodegenerative as well as cardiovascular diseases (29–32). Further denominators of CRMs include the capabilities to mimic more general metabolic, physiological, and hormonal alterations induced by CR, activation of stress response pathways and increased stress resilience (17). Different selections of these criteria are used to define CRMs in literature and, due to the rapidly evolving nature of the field and the broad effects attributed to CR, multiple definitions may exist in parallel. Several chemically diverse CRM candidates have been identified and possible sources span multiple different areas and chemical classes, such as glycolysis inhibitors, inhibitors of fat and carbohydrate metabolism, mTOR inhibitors, AMPK activators, sirtuin activators, polyamines and polyphenols, among others.

While CR and fasting are approaching clinical settings, experimental CRM candidates are rare in clinical research. Given the psychologic limitations of CR and fasting applications in humans, these compounds hold promise for medical use. A majority of nutrition research has focused on macronutrient composition, food additives, dietary habits or specific food items, as well as their level of industrial processing. The contribution of single dietary compounds to health outcomes is often elusive and understanding the effects of single dietary compounds on health is crucial for determining optimal diets for individual purposes. Ample reviews have been published on different aspects of the CRM concept [e.g., (15–20, 33–36)]. However, the role of naturally occurring CRM candidates in nutrition has been largely overlooked. Hence, in this review we describe these naturally occurring substances that harbor CR-mimicking and anti-aging properties, focusing on their dietary sources, availability and intake levels (**Table 1**). Several studies suggest that enhanced dietary intake of these substances elicits beneficial effects on human health throughout aging and reduces the incidence of age-associated diseases (**Figure 1**). Thus, we summarize the current status of CRMs in nutritional research and clinical trials.

GLYCOLYSIS INHIBITORS

Early on, CRM candidates were suspected among inhibitors of glycolysis, as an obvious substance class to study for potential CR-mimicking properties. Several compounds have been identified that prolong life/healthspan of model organisms and/or recapitulate other aspects of CR by inhibiting or modulating enzymes of the glycolysis pathway (e.g., hexokinase). Glycolysis inhibitors are comprehensively studied in cancer research, given many cancer types' increased dependence on glycolysis, but are often incomprehensively studied in nutrition and aging research. Generally, a broader research approach into the effects of these substances is needed to evaluate their potential as CRMs.

D-Allulose (also D-psicose), a rare monosaccharide used as a low-calorie sweetener, inhibits glucose metabolism and absorption from the intestinal tract, intracellular glycolysis and starch and disaccharide metabolization in the intestines. This suggested CRM has multiple pre-clinical effects: importantly, nematodes treated with D-allulose have increased lifespan, mediated via AMPK (78), while its effects on autophagy remain elusive. It is mainly studied for its antihyperglycemic and antiobesity effects (79). D-allulose is naturally present in foods, though at very low concentrations, and has been found in wheat, *Itea* plants, and processed cane and beet molasses (80). Interestingly, non-enzymatic reactions during heating of products that contain high levels of sugars, such as seasoning sauces and confectionery items, can yield increased, quantifiable

TABLE 1 | Summary of selected dietarily available compounds with Caloric Restriction Mimetic properties, their estimated intake levels, food sources, and comprehensive literature reviews.

Class	Compound	Estimated dietary intake levels[#+]	Relevant dietary sources[+]	Relevant articles and reviews
Glycolysis inhibitors	Astragalin (glucoside form of kaempferol; also a polyphenol)	Unknown	Various plants, including *Astragalus*, *Cuscuta* (dodder), *Cassia alata*	(17, 37)
	D-Allulose (D-psicose)	Unknown	Wheat, *Itea*, processed cane and beet molasses, high-sugar products (e.g., seasoning sauces, especially after heating)	(17, 38)
	Chrysin (5,7-dihydroxyflavone; also a polyphenol)	Unknown	Honey, propolis, passion flowers, mushrooms	(39, 40)
	Genistein (4′,5,7-trihydroxyisoflavone; also a polyphenol)	2–50 mg/day (total isoflavones of which genistein is a major type)	Various foods, soy-based items, legumes, fruits, nuts, vegetables	(41–44)
	D-Glucosamine	Unknown	Shellfish shells, cartilage, fungi	(17, 45–47)
	Mannoheptulose	Unknown	Unripe avocados	(17, 48, 49)
Di/Polyamines	Putrescine, spermidine, spermine	3–18 mg/day	Various plant and animal-based foods, soy beans, cheese, nuts, seeds, wheat germs	(50–55)
Polyphenols	Total polyphenols	1 g/day	Various	(56–65)
	3,4′-dimethoxychalcone	Unknown	*Unknown	
	4,4′-dimethoxychalcone	Unknown	*Angelica keiskei (ashitaba)	
	Curcumin	29.4 mg/day	Curcuma longa	
	Flavan-3-ols (e.g., epicatechin, EGCG)	23–384 mg/day	Green tea, apples, pears, berries, cocoa, broad beans	
	Gallic acid	25 mg/day	Berries, citrus fruits, leaf vegetables, soy products, tea	
	Isobacachalcone	Unknown	*Angelica keiskei (ashitaba), Artocarpus sp. (breadfruit), Erythrina fusca (purple coraltree), Morus alba (white mulberry), Piper longum (long pepper)	
	Quercetin	13.5–29.4 mg/day	Onions, apples, berries	
	Resveratrol	0.1–8 mg/day	Wines, grapes, lingonberry and >100 other plants	
Others	Hydroxycitric acid (HCA)	Unknown	Garcinia and Hibiscus	(66–70)
	Salicylic acid	1.3–4.4 mg/day	Berries, (citrus) fruits, fruit juices, wines, vegetables (asparagus, onions)	(71–74)
	NAD^+ precursors	21.9–41 niacin equivalents (mg)/day	Various plant and animal-based foods, peanuts, nuts, tuna, fish, pork, beef, soy beans, cheese, wheat germs	(75–77)

**Likely present in a variety of polyphenol-rich food items. [#]Estimated dietary intake levels are subject to profound variations, including different methods of assessment, different dietary habits of study cohorts, large differences of nutritional information in underlying food databases, regional and seasonal variations and diverse food processing techniques. [+]See main text for more details.*

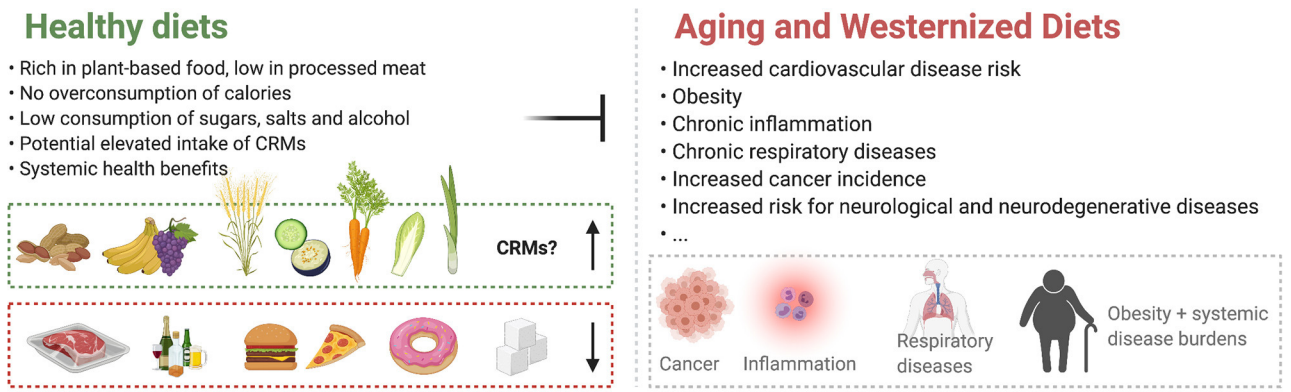

FIGURE 1 | Healthy diet plans stand opposite to Westernized Diets and counteract age-associated deteriorations. The contribution of Caloric Restriction Mimetics (CRMs) to the effects of healthy diets is currently largely undetermined.

levels of D-allulose (e.g., 0.5 mg/100 g in coffee, 130.6 mg/100 g in Worcester sauce) (81).

D-Glucosamine is an amino sugar that serves as a precursor for glycosylated proteins and lipids and acts on glycolysis through hexokinase-1 inhibition. This amino monosaccharide is a CRM candidate due to its lifespan-prolonging effects in nematodes and aging mice (45, 82) and its *in vivo* and *in vitro* autophagy-activating properties (82–84). In aging mice it was also shown to induce mitochondrial biogenesis, to lower blood glucose levels (45), and to counteract high-fat diet induced metabolic changes in rats (85), thus mimicking several effects of CR. D-glucosamine is naturally occurring, but mainly present in cartilage and shells of shellfish (e.g., shrimp, lobster, crab) where it is present as chitin (a polysaccharide built from N-acetylglucuosamine), which are commercially used for the production of glucosamine dietary supplements. It is also found in fungal cell walls at relatively high levels (86). Similar to D-allulose, the rare occurrence and low levels in commonly used food items prevent estimations of intake levels from normal dietary habits without supplements.

Other glycolysis inhibitors exerting some CR-mimicking effects, which naturally occur in plants and other food items, include, for instance, astragalin, chrysin, genistein, mannoheptulose, and resveratrol. **Astragalin** is a glucoside form of kaempferol, a bioactive flavonoid, and present in a wide range of plants. Notable plant sources include *Astragalus* (roots) which has been in medical use in Asia for more than 4,000 years, and *Cuscuta* (dodder) seeds which are also traditionally used in Asian folk medicine and *Cassia alata*, among many other plants [reviewed in (37)]. Similarly, **chrysin** (5,7-dihydroxyflavone) is found in various (medicinal) plants, herbs and fruits and products thereof, including honey [up to 5.3 mg/kg in forest honey, (87)], propolis [up to 28 g/L, (88)], passion flowers (89) and mushrooms at varying levels below 0.5 mg/kg (90, 91), among other sources [reviewed in (40)]. Like other polyphenols, it exerts a wide range of biological activities, but its intake levels from nutrition, stability in food items and bioavailability are poorly understood. **Genistein** (4′,5,7-trihydroxyisoflavone), another phenolic glycolysis inhibitor, belongs to the class of isoflavones and is readily available from diverse food sources, such as soy-based items (mature soy beans contain 5.6 to 276 mg/100 g) (92), legumes (0.2–0.6 mg/100 g), fruits, nuts, and vegetables (41). Germination and fermentation of soy beans have been reported to increase genistein content (93, 94). Intake of isoflavones (of which genistein is a major type) is estimated to range from 25 to 50 mg/day in Asian countries, while Western countries have much lower intake levels (∼2 mg/day) (95, 96) (see also chapter on polyphenols). **Resveratrol**, which is discussed later in the review in more detail, also shows anti-glycolytic activities, as it shows inhibitory effects on hexokinase in cell lines (97). Apart from these phenolic, plant-based compounds, **mannoheptulose**, a rare sugar, also inhibits hexokinases and was suggested as a CRM which is prominently present in unripe avocados (98), but has produced mixed results in preclinical work (17).

Noteworthy, as an example and prototype for glycolytic inhibitors, **2-Deoxy-D-glucose** (2DG) is a well-established and one of the best-known glycolysis inhibitors, acting via its first two enzymatic steps. It was considered one of the first CRM candidates as it lowers body temperature and insulin levels of rats fed a 2DG-containing diet (99), acts cardioprotective, reduces heart rate and blood pressure (100), increases autophagic flux (101), acts as an effective anti-cancer agent (102) and prolongs lifespan, at least in the worm *C. elegans* (103). However, chronic ingestion has been shown to elicit problematic (cardio)toxic effects in rodents, including increased mortality, and has slowed the transition of 2DG into clinical research (17, 101), presenting general challenges for the field. 2DG does not naturally appear in food items and is thus not present in nutritional, epidemiological studies.

Glycolysis Inhibitors in Epidemiological and Clinical Studies

While several inhibitors of glycolysis are widely present in various food items, their effectiveness in humans, especially via dietary intake, is largely elusive. For most of these substances, clinical studies are absent or insufficient to discuss important topics such as bioavailability, toxicity, metabolization, clinical effects

and recommended dosages. Nevertheless, for some glycolysis inhibitors data from clinical trials are available.

Upon consumption, **D-allulose** remains largely unmetabolized and gets secreted to a large extent (104), but seems to reduce glucose uptake from the gut lumen. Few clinical trials (6 interventional trials and 1 meta-analysis registered on clinicaltrials.gov) have investigated the effects and tolerability of D-allulose in humans. One study found decreased glucose levels upon an oral glucose tolerance test (105), matching preclinical reports. This single dose did not change blood glucose levels *per se*. Likewise, postprandial glucose levels were reduced after pre-meal consumption of 5 g D-allulose (106, 107) and metabolism was shifted toward higher fatty acid oxidation and lower carbohydrate utilization over a day's period (107). A similar study confirmed the notion that the glucose response is dampened upon D-allulose consumption, although the effects did not reach statistical significance (108), while the results on postprandial insulin levels are mixed at the moment. In type-2-diabetes patients, increasing doses of D-allulose also slightly lowered early glucose and insulin levels after an oral sucrose load (109), which is in line with previous reports (110). One randomized clinical trial that lasted for 3 months found favorable reductions in abdominal and subcutaneous fat depots, but no changes in various markers of liver and kidney function, glucose, lipids or insulin (111). However, dosing seems to be crucial for this glycolysis inhibitor, as several side effects, including flatulence, diarrhea and general abdominal discomfort have been reported (38, 112). Hayashi and colleagues, however, reported no adverse events or clinical problems in a trial studying the effects of 5 g D-allulose, taken three times a day for 3 months (106). Single doses of up to 0.4 g/kg bodyweight and daily consumptions below total 0.9 g/kg bodyweight seem to be well-tolerable however (112).

Among the discussed glycolysis inhibitors, **glucosamine** is one of the most extensively studied compounds in clinical trials. It is commonly used to treat osteoarthritis, as it is a precursor for glycosaminoglycans in cartilage and is widely available as a dietary supplement. An estimated 7.4% of the US population between 57 and 85 years of age regularly use glucosamine (113) and early prospective studies reported significantly decreased mortality upon regular usage (114, 115). This observation is supported by several recent studies in the US (46, 116) and the UK (47), which found reduced mortality due to all-causes, CVDs, cancer, respiratory and/or digestive diseases. Besides its potentially mortality-reducing effects in humans, glucosamine has been studied for various reasons in clinical trials, including its anti-inflammatory properties. A 4-weeks long RCT using 1.5 g/day in combination with 1.2 g/day chondroitin sulfate (a sulfated type of glucosamine and important structural component of cartilage, which is often sold in combination in supplements) found reductions in CRP (117), which is supported by several pre-clinical studies (118) and epidemiological data (119). Interestingly, regarding its primary reason for application, osteoarthritis, several meta-analyses have been conducted, producing mixed results on its effects for treating symptoms and pain (120–125). Nevertheless, in combination with strong pre-clinical evidence and its good safety profile, ample clinical data speaks for D-glucosamine as a prime CRM candidate with inhibitory functions on glycolysis.

Other glycolytic inhibitors that have been studied in a few clinical trials, include astragalin, chrysin, and genistein. **Astragalin**, as an isolated compound, is largely absent in clinical literature. However, its non-glucoside form kaempferol has been weakly associated with anti-diabetic and anti-cancer effects (126–128). Likewise, administration of astragalus roots which have high astragalin content (129) has shown anti-diabetic effects, lowering fasting glucose and insulin levels, postprandial glucose levels and insulin resistance (130), although the authors of this meta-analysis conclude that some underlying studies lack quality and more rigorous studies of astragalus administration are needed. **Chrysin** has shown promising results on pre-clinical models of metabolic disorder and cancer (39, 131). It is available as a dietary supplement but shows poor oral bioavailability (132), while not evoking problematic toxic effects at the doses studied (single-dose of 400–500 mg) (132, 133). However, its effects as a potential CRM and glycolysis inhibitor, either from diet or as supplement, remain unknown in humans. Similarly, **genistein** has been studied for its anti-cancer properties. It has a bitter taste and is poorly soluble in water with a low bioavailability when consumed orally (41), which might be overcome by encapsulation or using genistin, its glycoside form (134, 135). Evidence of genistein's effects in humans is weak, mainly derived from epidemiological studies and smaller interventional trials that often do not differentiate between multiple isoflavones and use mixtures of several compounds (41). Searching clinicaltrials.gov for genistein reveals 72 registered trials and more can be found in literature databases. Several meta-analyses of clinical trials have been conducted for various purposes. For instance, genistein supplementation at 54 mg/day is associated with beneficial effects on bone mineral density in postmenopausal women (136), longer durations of supplementation (>6 months) may be associated with reduced blood pressure in patients with metabolic syndrome (42) and increased intake with lower type-2-diabetes (137–139) and reduced breast cancer risk (138). Thus, ample data of its effects in pre-clinical models and humans (either via nutritional assessments or interventional supplementations), suggest this isoflavone as an interesting CRM candidate with inhibitory effects on glycolysis, thus warranting more research and larger RCTs into its potential CR-mimicking properties.

HYDROXYCITRIC ACID

Hydroxycitric acid (HCA), a derivative of the TCA-cycle metabolite citric acid, is a phytochemical compound that qualifies as a CRM due to its autophagy-stimulating properties (24). Mechanistically, HCA is a competitive inhibitor of ATP-citrate lyase which is involved in lipogenesis. To date it has been reported in two plant species: *Garcinia* and *Hibiscus*. More specifically, HCA can be extracted from the fruit rind of *Garcinia gummi-gutta*, also known as *Garcinia cambogia* or Malabar Tamarind, *Garcinia indica,* and *Garcinia atroviridis*. Garcinia trees are native to India, as well as Africa, Sri Lanka and Malaysia

(66). The tree produces little green fruits, rich in numerous bioactive phytochemicals, of which HCA is believed to be the major ingredient (67). Garcinia extracts have been mainly studied for anti-inflammatory, -cancer and -obesity effects (66).

Besides Garcinia, HCA is present in *Hibiscus sabdariffa* (Roselle) and *Hibiscus rosa-sinensis* (140). Hibiscus plants are endemic in Africa and South-East-Asia. Like Garcinia, Hibiscus plants are used in multiple ways, as food colorings, jams, cold drinks, teas and nearly all parts of the plant (leaves, stems, fruits, flowers) are used for cooking (68).

While numerous HCA-containing garcinia-derived extracts with varying HCA concentrations are sold over-the-counter mainly for weight loss promotion, no information can be found about dietary intake levels of HCA in regions home to Garcinia or Hibiscus.

Hydroxycitric Acid in Epidemiological and Clinical Studies

Pre-clinical studies of HCA have shown promising results for obesity management, including appetite suppression properties, which is why it is commonly taken for weight management (141), although the effectiveness is questionable. Different doses have been used in human trials, ranging from 5 to 250 mg/kg, or up to 4.7 g, daily HCA supplementation, usually divided into smaller sub-doses taken throughout the day (67, 142). Bioavailability is fairly fast after HCA intake and the compound can be detected in human plasma for several hours after acute intake (143). While there are yet no general recommendations for HCA intake, it has been found safe at daily doses up to 3 g for 30 days, administered in capsules or tablets (141, 144–147). Potential adverse events include mild gastrointestinal problems, diarrhea, nausea and flatulence. This warrants further research into side effects of HCA and HCA-containing extracts, focusing on long-term use (67, 148).

Several randomized clinical trials (RCTs) were conducted with different HCA-containing formulations which reported inconsistent outcomes on energy intake, weight gain, fat oxidation and appetite reduction (142). This could be partly due to the wide range of concentrations and different study designs used in these studies. Also, HCA occurs in different chemical forms, with the lactone form being a less potent inhibitor of ATP-citrate lyase than the open form (149), which might explain some inconsistencies when using different formulations in clinical trials.

Some clinical trials have shown that it can reduce obesity-related visceral fat accumulation (150). However, as summarized and discussed in Onakpoya et al., meta-analyses of RCTs using *Garcinia* extracts for weight loss show only small effects on short term weight loss (69) and the effects of HCA administration in humans remains controversial (142), especially regarding longer term effects. Different types of bowel disorders were treated with the fruit rind of Garcinia (66, 151) and pre-clinical work has shown anti-inflammatory properties of HCA (152).

In summary, HCA has shown promising effects in pre-clinical and encouraging, yet little, evidence for its effectiveness in humans. Its contribution to healthy diets remains elusive and its possible application in clinical settings is yet to be studied with more rigor, particularly in the long term.

NAD^+ PRECURSORS

Nicotinic acid (NA, also named niacin or Vitamin B3), nicotinamide (NAM), nicotinamide riboside (NR), nicotinamide mononucleotide (NMN) and tryptophan are all dietarily available precursors of NAD^+ (nicotinamide adenine dinucleotide) with similar biological activities (75, 76, 153). The universal coenzyme NAD+/NADH and its phosphorylated derivatives NADP/NADPH serve oxidoreductases, dehydrogenases, sirtuins and are central to metabolic pathways (e.g., glycolysis, TCA cycle) and cell signaling (153). Numerous pre-clinical studies have shown the CRM-like properties of these precursors upon supplementation, which can prolong life- and healthspan, promote mitochondrial function, induce autophagy and act cardioprotective and neuroprotective, among others (77, 153–157). NAD^+ concentrations decline with age (156, 158) and replenishing these levels harbors therapeutic potential in humans (157, 159–164).

NAD^+ precursors are abundantly present in foods of animal and plant origins and NAD^+ levels can be increased by dietary habits, as well as physical activity/exercise (75, 165). Taking into account *de novo* synthesis from tryptophan (it is commonly estimated that 60 mg of dietary tryptophan can yield 1 mg niacin in the body, although large interindividual variability exists), dietary supply of NAD^+ precursors is calculated as niacin equivalents (NE) (166). To avoid hypovitaminosis, recommendations for daily NE intake are 14 to 16 mg (166) and niacin is rapidly absorbed from the gastrointestinal tract (167).

Interestingly, in the Bruneck study situated in northern Italy, recent analysis found relative high dietary NE intake of 28.9 mg (23.5 to 35.0) in men and 26.9 mg (21.9 to 33.0) in women per day (154), which is corroborated by similar observations made in the US (28 and 18 mg niacin/day for men and women, respectively) and Canada (41 and 28 mg niacin/day for men and women, respectively) (76).

The highest concentrations of NE can be found in nuts, especially peanuts, (20,833 μg NE/100 g), tuna (14,383 μg NE/100 g), poultry (12,534 μg NE/100 g), beef (9,235 μg NE/100 g) pork, lamb, and fish like trouts and salmons (all >5,000 μg NE/100 g). Other foods rich in NE are curd and cheese (2,800 and 5,226 μg NE/100 g, respectively), along other dairy products, fruits and vegetables, with wheat germs (10,020 μg NE/100 g), mushrooms (5,220 μg NE/100 g), green peas (3,621 μg NE/100 g), garlic (2,300 μg NE/100 g), dried prunes (1,730 μg NE/100 g) and bananas (1,033 μg NE/100 g) ranking among the NE richest items. Potatoes, rice and carbohydrate-based foods, like bread and noodles are also relatively NE-rich (>1,000 μg NE/100 g) (154). NMN was also found to be abundantly present in foods like tomatoes (260–300 μg/100 g), broccoli (250–1,120 μg/100 g), mushrooms (up to 1,010 μg/100 g), and raw beef (60–420 μg/100 g) (168).

Interestingly, pellagra, a niacin- and tryptophan-deficiency caused disease common to rural, southern areas of the US

a century ago, was cured by substituting mainly corn-based diets with milk, eggs and meat (169). Of note, niacin in corn and mature grain is mainly present in bound forms that are poorly bioavailable. Thus, nixtamalization (soaking and cooking in alkaline solution) is often applied to render hemicellulose-bound niacin bioavailable from these sources, a practice that was already used by Native American populations (75, 170).

NAD^+ Precursors in Epidemiological and Clinical Studies

Due to mounting pre-clinical evidence on the beneficial effects of NAD^+ precursor supplementation and NAD^+ depletion as a possible contributor to (age-associated) human diseases, research into the clinical feasibility of these substances beyond the treatment of hypovitaminosis has gained traction (77). Querying "niacin," "NAD+," and "nicotinamide" in clinicaltrials.gov results in hundreds of registered trials in diverse clinical settings and cohorts, many of them with dietary supplements.

Toxicity is low and tolerability high in rodents (161) and several academic sponsors and companies are currently running clinical trials on NAD^+ precursors [for a comprehensive list of completed trials see (77)]. NR, NAM, and other NAD^+ precursors are being tested in clinical trials at doses up to 2 g/day, which overall seem well-tolerable, orally bioavailable and increase blood NAD^+ levels (77, 171–175). One study found reduced circulating inflammatory markers and elevated muscle NAD^+ metabolites upon 3 weeks of daily 1 g NR supplementation (176). The same dose, however, failed to elicit effects on insulin parameters and glucose tolerance after 3 months in non-diabetic obese men (177). Daily supplementation of 500 mg NR with a detectable increase in NAD^+ serum levels did not cause serious adverse effects after 8 weeks (173). This was corroborated by a 6 week long study supplementing NR, via a commercially available supplement, which also found reduced systolic/diastolic blood pressure and arterial stiffness (178).

Niacin has been used in doses >1 g to treat hypercholesterolemia, lowering LDL while raising HDL levels (179). Of note, NAM alone at 1 g/day also evoked similar changes in the LDL/HDL levels (180). A recent study found increased intramuscular NAD+, muscle strength and mitochondrial biogenesis in patients with mitochondrial myopathy after 10 months of up to 1 g/day niacin supplementation (181). This was accompanied by a shift in the muscular metabolomes toward those of controls. A case study found amelioration of movement disorders in a patient with Parkinson Disease (PD) upon 1 g/day niacin supplementation (182). However, double the dose eventually led to nightmares and skin rashes, which stopped upon niacin discontinuing, also reinstating the initial severity of movement disorders. Another case report also found improved motor, cognitive and sleep measures after 0.25 g/day niacin treatment for 1.5 months in a PD patient (183). Interestingly, German PD patients have reportedly lower dietary niacin consumption (184).

As summarized by Katsyuba et al. the sum of clinical trials with NAD^+ precursors supports the general safety of the compounds at the doses indicated. However, effects on different outcomes vary greatly between the studies (77). As outlined before, NAD^+ precursors are important dietary components and widely spread in various foods. Analysis of dietary habits from the Bruneck study have shown lower all-cause and cardiovascular mortality risk, alongside lower systolic blood pressure, associated with diets rich in NAD^+ precursor (154).

POLYAMINES

The naturally occurring, ubiquitously found polyamines spermidine and spermine have been attributed diverse health-promoting effects in model organisms and humans [reviewed in detail in (50, 185)]. Polyamines are available to our bodies via the diet, microbial production in the gut, and endogenous biosynthesis. They serve multiple biological roles, from growth, translation, ion channels and autophagy regulation to binding of nucleic acids and other molecules (186). Externally supplied dietary spermidine evokes cardioprotective and neuroprotective effects in mice, activates autophagy and prolongs life- and healthspan (187–191). Together with precursors (ornithine, arginine, methionine, among others) and the diamine putrescine, these bioactive substances are an unavoidable part of human diets. Additionally, they are synthesized by the gut microbiome, providing an additional polyamine source, and are easily taken up from the gut lumen (51). Several studies have estimated the average intake levels of these compounds across different countries, while variations in microbiota-derived polyamine levels are elusive.

Generally, putrescine seems to make up the greatest share of dietary di/polyamines, both in weight and μmol. At the lower end of estimated intake levels stands Turkey with 8 mg putrescine, 5 mg spermidine, and 3 mg spermine per day (192). Asian countries are estimated to have daily intake levels of 9, 13, and 8.5 mg for putrescine, spermidine and spermine, respectively (193). Countries in the European Union consume 18 mg putrescine, 12.6 mg spermidine, and 11 mg spermine daily on average (52), while the USA report roughly one third lower polyamine consumption levels (194). Due to different dietary habits, great regional variations exist. For instance, while spermidine intake levels in Spain are estimated to be around 15 mg/day, those of Sweden are only 10 mg/day (52). A population-based study in northern Italy, that rigorously assessed the dietary habits via FFQs, came to an estimated intake of 13.4 mg putrescine, 10.1 mg spermidine, and 6.3 mg spermine (195). Interestingly, the same study found a significant trend toward declining spermidine intake levels with age and generally higher dietary consumption in women.

As mentioned, polyamines are ubiquitously present in food items of plant and animal origins. Within food categories, however, wide ranges of concentrations are found, with plant-based food ranking higher on average (52). Thus, it can be speculated that healthy diets as outlined above likely contain elevated polyamine levels. This is corroborated by positive correlations between food items typically consumed in higher quantities in Mediterranean countries and polyamine content (7). A comprehensive summary of polyamine content in various

food items can be found in Atiya Ali et al. (53). Putrescine is found in high quantities in fruits (500–550 μmol/kg), while vegetables and bread contain roughly a tenth of those levels. In contrast, spermidine is more abundant in, particularly aged, cheese (600–700 μmol/kg) and vegetables (200–300 μmol/kg), than in fruits (100–200 μmol/kg), while it's especially low in meat (<50 μmol/kg). Spermine is found in comparable amounts in meat, vegetables and cheese (100–200 μmol/kg), while bread, potatoes and fruits contain <50 μmol/kg (53). Specific food items rich in polyamines are rice bran, wheat germs, nuts, seeds, green pepper, broccoli and its sprouts, fish sauce, oranges, mangos, chicken liver, beef intestines, some shellfish, select mushrooms, and soybeans (196). Natto, which is based on fermented soy beans, is especially rich in spermidine and has led to polyamine-enriched variants being studied in clinical studies (197). Taking portion sizes and intake frequencies into account, within the Bruneck study, the greatest contributors to spermidine intake were whole-grain, apples, pears, salads and vegetable sprouts (195).

Measured or estimated polyamine content varies greatly between different reports. Thus, epidemiological, food-database dependent data are obviously prone to various confounding factors, including the often unknown influences of regional/seasonal variability or preparation techniques, stability, manufacturing, and storage methods in different food items, just to name a few. This applies as well to the other dietary compounds discussed in this review. Reviewing existing literature revealed substantial knowledge gaps on the influence of the named factors on polyamine content (50). No consistent tendencies are present across different reports. However, literature suggests that while spermidine and total polyamines seem rather stable upon boiling/cooking in most foods, polyamines might get lost into excess cooking liquids and fermentation in principle might favor polyamine abundance (50, 54).

Polyamines in Epidemiological and Clinical Studies

Polyamines have been studied in moderate extent in clinical or epidemiological trials. The "Bruneck study," named after the hospital's location in northern Italy where the study visits were conducted, is a prospective population-based study that rigorously assessed dietary habits and health status, including numerous physiological examinations (198). Polyamine intake data were calculated via dietitian-administered food frequency questionnaires (FFQs) and food databases to correlate intake levels to various health parameters. In this cohort it was observed that cardiovascular diseases (188), cognitive impairment (190), and overall mortality (including cancer and vascular deaths) (195) negatively correlated with higher polyamine intake. These associations were robust to withstand corrections for possibly confounding factors including social status, age, BMI, calorie intake, education, alcohol or nicotine consumption, activity and healthy eating, and were more prominently pronounced for spermidine than spermine (both are enzymatically interconvertible), while putrescine intake levels did not show significant correlations. The inverse correlation of spermidine intake and overall mortality was consequently corroborated by the SAPHIR study (195), while the negative correlation with CVD incidence was confirmed by another epidemiological study (199).

Although polyamines show promising effects in pre-clinical studies and epidemiological data point toward benefits of increased dietary intake, only few interventional clinical trials have been conducted so far. One of them, designed as a pilot trial, supplemented elderly people with low doses of polyamines via a wheat-germ extract (1.2 mg spermidine, 0.6 mg/spermine, 0.2 mg putrescine per day) for 3 months and found a positive impact on memory performance (200). The same extract was previously found to be safe in mice and older humans, while not provoking changes in vital signs in the latter after 3 months (201). Another study supplemented spermidine via wheat-germ containing bread rolls (3.3 mg spermidine/piece, ∼23 pieces/month) for 3 months to older adults living in nursing homes and found subtle improvements in cognitive function of patients with mild dementia (202).

Recently, spermidine- and spermine-enriched natto was tested in a 1-year-long intervention study, reaching a daily intake increase of roughly 14.5 mg spermidine and 4.5 mg spermine (197). Interestingly, only spermine blood levels rose by 12% at study end, suggesting either metabolic adaptations in the polyamine pathway or ready tissue uptake and/or metabolization of dietary polyamines. The study showed decreased levels of lymphocyte function-associated antigen 1 (LFA-1) upon elevated polyamine intake (197), suggesting potential anti-inflammatory effects of polyamine supplementation in humans. Interestingly, polyamine modulation cannot only be achieved by direct increase of intake levels, but also via modulation of the polyamine-producing intestinal microbiota. One study administered a yogurt preparation with *Bifidobacterium animalis subsp. lactis* and arginine (precursor of polyamine synthesis) for 3 months and found higher serum putrescine and spermidine levels, decreased heart rate, as well as improved endothelial function in the intervention group compared to the placebo (normal yogurt) (203). Other in-group significant changes included slightly reduced triglycerides, total cholesterol and platelet counts, while HDL-cholesterol increased (changes not significant in comparison to those in the placebo group).

Due to the increased need for polyamines of cancer cells, there was some concern regarding potential cancer-increasing risk of elevated polyamine intake. While one study found increased risk for colorectal adenoma at above-median intake levels (204), the same group found an inverse relationship for colorectal cancer in a different cohort (205), highlighting the need for multiple observational or direct interventional studies. Additionally, multiple other epidemiological studies, as outlined above, did not observe cancer-increasing effects of elevated polyamine intake, rather the opposite. Other interesting avenues of polyamine supplementation in humans include the potentially supporting effects on hair growth (206, 207).

Ongoing or yet-to-be-published trials registered at clinicaltrials.gov, which use dietary spermidine supplementation (4–6 mg/day), include explorative hypothesis-generating

studies against depression and for sleep quality improvement (NCT04823806), one against hypertension (NCT04405388) and one against cognitive decline in elderly subjects (NCT03094546).

POLYPHENOLS

Plant compounds belonging to the polyphenol family may represent promising sources of potential CRMs (15). Polyphenols are ubiquitous phytochemicals characterized by great chemical diversity. They represent one of the largest groups of secondary metabolites in plants with over 8,000 structural variants (208). Polyphenols fulfill multiple ecological roles in the plant kingdom, from defense against biotic and abiotic stressors to inter- and intra-kingdom communication. The most common classification used in the literature implies their subdivision in two main groups: flavonoids (e.g., anthocyanins, flavan-3-ols, flavanones, flavonols, flavonones, and isoflavones) and non-flavonoids (e.g., phenolic acids, stilbenes, and lignans) (209). Like polyamines and NAD^+ precursors, these compounds are an unavoidable component in the human diet.

About 800 different polyphenols have been identified in a wide range of plant foods and beverages, including berries, whole-grain cereals, cacao, coffee, and tea (210, 211). Some food and beverages may be particularly rich in a specific polyphenol class; for example, stilbenes in red wine, phenolic acids in coffee, flavanones in citrus fruits, flavanols in cocoa, and isoflavones in soy products (56). It is important to note that polyphenol content is markedly influenced by plant variety, agricultural practices, and food processing methods. All these factors account for the high variability in the polyphenol profile of plant foods and beverages (212). Although it has often been criticized, the translation of food composition into intakes of specific polyphenols is usually achieved using food composition databases, such as Phenol-Explorer or the database of the United States Department of Agriculture (USDA) for flavonoids (211, 213). Depending on the type diet, gender and other socio-demographic factors, the average polyphenol intake in the human diet is approximately 1 g/day (57, 214). Estimated intake levels for specific polyphenols from different reports need to be treated especially carefully, as the underlying databases and methods of calculation may vary significantly.

A few prominent examples of polyphenols that may mimic CR in humans include resveratrol, curcumin, epicatechin, epigallocatechin-3-gallate (EGCG), gallic acid, and quercetin.

The main representative of stilbenes in the human diet is **resveratrol**. It has been detected in 100 plant species from 35 taxonomic families (215). Estimations of daily resveratrol intake range from 100 to 933 μg in a Spanish study (combined resveratrol and piceid, a glucoside derivative) (216) to 6-8 mg (217), mainly coming from wines and grape products (216). According to Phenol-Explorer, lingonberry (*Vaccinium vitis-idaea*) was found to have the highest content of resveratrol [3.00 mg/100 g fresh weight [FW)] (218). However, the fresh skin of red grapes is also particularly rich in resveratrol, which contributes to its relatively high concentration (3.02 mg/100 ml) in red wine from Muscadine grape (219).

Curcumin is a well-known polyphenolic compound isolated from the rhizomes of *Curcuma longa* (turmeric). The plant is often cultivated to harvest rhizomes and use turmeric powder as a spice and food coloring agent. The average Indian diet provides roughly 60–100 mg per day (58). The contents of curcumin in turmeric rhizomes vary often with varieties, locations, and cultivation conditions. However, by aggregating data from 14 different samples from 3 publications, the average content of curcumin in dried turmeric is 2,213.57 mg/100 g FW (220–222).

Epicatechin and **EGCG** belong to the flavan-3-ol subclass of flavonoids. Dietary intake levels of total flavanols were estimated to be 386 mg/day in Germany (223), 192 mg/day in the US (224), and 23 mg/day in the Netherlands (225), highlighting a high discrepancy in the published literature and problems with differences in the underlying food databases and intake estimations. Of the individual flavan-3-ols, epicatechin, and catechin seem to make up most of the dietary intake (68 and 84 mg/day, respectively), in the US (224). Recently, it has been proposed that the estimated intake of flavan-3-ols can only be interpreted as a marker of specific dietary patterns, but not as the actual intake amount (59). Epicatechin is found abundantly in different fruits and legumes, such as apples, pears, berries, cocoa, and broad beans. Likewise, EGCG is the most biologically active and most abundant flavan-3-ol in green tea. Quantitative data on flavan-3-ol contents of foods are largely debated. This is due to the limitations of self-reporting dietary data (e.g., food-frequency questionnaires) and the inability of currently used methods to accurately estimate the high variability of food composition. Rothwell et al. reported that the values of flavan-3-ols ranged from 3 to 544 mg/100 g in apples, chocolate (dark), and green tea (60).

The flavonol **quercetin** is one of the most extensively studied polyphenols for its anticancer, antiaging, and anti-inflammatory activities. It is mainly found in onions, apples, and berries. Estimated intake levels of quercetin are 29.4 mg/day in the United Kingdom (226), 20 mg/day in the Chinese population (227) and 13.5 mg/day in the US (224). Another example of potential CRMs is **gallic acid**, which is a well-known polyphenol belonging to the class of phenolic acids. A polish study estimated a daily intake of 25 mg gallic acid (228), which can be found in berries, citrus fruits, leaf vegetables, and soy products and it is known mainly for its antioxidant effect (61). However, tea is also an important source of gallic acid. Data reported in Phenol-Explorer indicate that the mean content of gallic acid in black tea infusion is 4.63 mg/100 ml (60).

Recently, **chalcones** have emerged as another specific sub-class of polyphenols that might qualify as CRMs. 3,4-dimethoxychalcone and 4,4'-dimethoxychalcone, among other chalcones, were identified in screens of (plant) metabolites to induce autophagy *in vivo* and prolong health- and/or lifespan of yeast, worms and flies (229–232). 4,4′-dimethoxychalcone was later also shown to ameliorate Parkinson's Disease phenotypes in mice when delivered to neuronal tissue via targeted nanoparticles (233), exemplifying one interesting way of overcoming the *in vivo* limitations of such small molecules. Isobacachalcone has also been shown to induce autophagy and enhance chemotherapy in mice (234). Chalcones are present in a wide range of plants and

plant-derived extracts and are thus dietarily available to humans and have been used in traditional medicines across continents.

However, their concentrations in the identified plants are often unknown and no dietary intake levels can be estimated. For instance, isobacachalcone was found in the edible or partly edible plants *Angelica keiskei* (ashitaba), *Artocarpus sp.* (breadfruit), *Erythrina fusca* (purple coraltree), *Morus alba* (white mulberry), and *Piper longum* (long pepper), among others, and is attributed multiple health-promoting properties [summarized in (234)]. Of note, 4,4'-dimethoxychalcone was also identified in the chalcone-rich ashitaba plant (229). Although no specific information can be found about the presence of these chalcones in other food items, chalcones are generally widely present in plant-based food, such as tomatoes, apples and legumes (62).

Polyphenols in Epidemiological and Clinical Studies

The consumption of polyphenols has been epidemiologically associated with the beneficial modulation of a wide number of health-related variables, including mortality risk (235, 236). However, health benefits and CR-like effects of polyphenols are difficult to demonstrate in humans due to the wide variability of chemical structures, biological actions, and complexity of estimating their content in foods and cooked dishes. Bioavailability is another crucial aspect when the effects of polyphenols are evaluated in humans. It has been estimated that circulating concentrations of both native and metabolic forms of polyphenols are in the nanomolar to low micromolar range and, therefore, only a small percentage is detected in urine and plasma samples (57, 63). Also, many clinical studies concentrate on polyphenol-rich extracts, juices, or diet plans rather than pure compounds, often with unknown exact compositions. Effects often vary significantly between studies, which can likely be attributed to small cohort sizes, big variations in study design, different doses and cohorts and underlying confounding factors (like pre-study dietary intake).

Although **resveratrol** mimics some aspects of CR in humans, current clinical trials with resveratrol supplementation and epidemiological studies report promising but mixed findings. The amount of available data would overstrain the purpose of this review and is more comprehensively reviewed elsewhere (64, 217).

Tolerability of supplemented doses up to 1 g seems fairly good (217). The effects of resveratrol supplementation on BW and/or waist circumference (WC) were investigated by 4 studies (237–240), of which three found a reduction of WC and two studies detected reduced BW after resveratrol supplementation. Two reports found a reduction of cholesterol levels, while six others did not (237, 241–247). Likewise, 1 study showed that resveratrol can improve triglyceride (TG) in diabetic patients (247).

While three meta-analyses observed no effect on glucose levels after treatment with resveratrol (238, 244, 245), three studies reported that resveratrol could decrease blood glucose (237, 242, 248). Four publications also analyzed glucose-related parameters, such as insulin levels and glycated hemoglobin (HbA1c) (238, 242, 243, 248). The authors of 3 meta-analyses evaluating HbA1c reported that patients may benefit from resveratrol treatment.

During aging, chronic, sterile, low-grade inflammation, called inflammaging, contributes to the onset of age-related diseases (249–252). Overall, meta-analyses found reduced levels of C-reactive protein (CRP) and tumor necrosis factor (TNF) in resveratrol-supplemented individuals but no influence on interleukin 6 (IL-6) (242, 245, 253–256). In an intervention trial with patients suffering from type 2 diabetes (T2D), CR-like properties were shown by resveratrol treatment, with activation of AMPK and SIRT1 in the muscle biopsies (257). However, a larger trial demonstrated that resveratrol supplementation does not influence putative molecular targets of CR in postmenopausal women (258).

Epidemiological and clinical data on the benefits of **curcumin** are also growing. Curcumin appears well-tolerated and safe. Its poor bioavailability can be significantly increased by several dietary agents, such as piperine (a component from black pepper). Recently, a number of clinical trials and meta-analyses have aimed at summarizing the CR-like effects of curcumin on humans. Based on data from 8 RCTs, Hariri and Haghighatdoost systematically evaluated the evidence of the effects of curcumin supplementation on anthropometric measures, such as BMI, BW, WC, and fat mass. They found that curcumin, with a long duration of intervention, may reduce total body fat and visceral fat, but it was not enough to decrease BW and BMI significantly (259). Conversely, Akbari et al. pooled results from 21 clinical studies that comprised a total of 1,604 individuals and demonstrated that curcumin intake significantly decreased BMI, BW, and WC (260).

Although the lipid-lowering effects of curcumin remain inconclusive at this time, a meta-analysis of 7 randomized trials found a beneficial effect on total cholesterol and low-density lipoprotein cholesterol (LDL-C) in patients at risk of CVD. However, no significant effect was found with respect to serum high-density lipoprotein cholesterol (HDL-C) (261).

Of interest, curcumin could lower blood glucose concentrations of individuals with dysglycemia. A curcumin supplementation intervention in a pre-diabetic population improved overall function of β-cells and reduced the number of individuals who developed T2D (262). Likewise, it was observed that curcuminoid supplementation (i.e., curcumin, desmethoxycurcumin, and bisdemethoxycurcumin) decreased HbA1c and the homeostasis model assessment index for insulin resistance (HOMA-IR) in diabetic patients (263). These results were only confirmed for HbA1c in a meta-analysis of 11 studies (264). Curcumin has been also subject of intensive research because of its well-known anti-inflammatory properties. Intriguingly, it was observed that supplementation with curcumin reduces circulating concentrations of pro-inflammatory biomarkers and increases anti-inflammatory mediators irrespective of health status. Indeed, pooled from 32 trials showed a reduction in CRP, TNF-α, IL-6, and an increase in IL-10 (265).

Flavan-3-ols, such as **epicatechin** and **EGCG** (also called catechins), have been extensively investigated for their role in human health and nutrition. The beneficial effect of flavan-3-ols

is evident on cardiometabolic outcomes. Results from a meta-analysis of 156 RCTs suggest that flavan-3-ol intake has a positive effect on acute/chronic flow-mediated dilation (FMD), systolic (SBP) and diastolic blood pressure (DBP), total cholesterol, LDL-C, HDL-C, TG, HbA1c, and HOMA-IR (266). Moreover, from the available meta-analyses, it was also reported that catechins have the propensity of reducing BMI, BW and WC, increasing metabolic rate even at low dose (ca. 300 mg per day) (267–269). However, current clinical data, recently meta-analyzed by Haghighatdoost and Hariri, do not suggest benefits of catechins on inflammatory mediators, such as CRP, TNF-α, and IL-6 (270).

Quercetin is one of the most abundantly researched polyphenols. Several clinical trials evaluating the impact of quercetin supplementation on the prevention and treatment of chronic diseases have been completed. We retrieved 4 meta-analyses that covered data on lipid profile after quercetin supplementation (271–274). Although these analyses reported conflicting results on indices of lipid profile after quercetin treatment, it appears that changes in plasma lipids, in particular HDL-C and TG, are associated with quercetin dose (above 50 mg/day) and duration of supplementation (about 8 weeks). The current clinical evidence also suggests that quercetin intake does not affect BMI, BW, and WC (275). Conversely, the results of 4 meta-analysis showed a clear effect of quercetin supplementation in the reduction of BP and management of glucose-related parameters (272, 276, 277). No relevant overall effects on inflammatory mediators were reported, except CRP in individuals with diagnosed diseases (274, 278).

As far as we know, there are no currently running or completed clinical trials evaluating the effects of the herein mentioned **chalcones** (4,4′-dimethoxychalcone, 3,4-dimethoxychalcone, isobacachalcone). However, given the high interest in polyphenol-rich extracts and diets, it is likely that these compounds are present in some of the formulations tested in clinical studies.

SALICYLIC ACID

Salicylic and acetylsalicylic acid (also known as trademark Aspirin™) have been in medical use for more than a century and qualify as CRMs, as they can induce autophagy and prolong lifespan of model organisms (279, 280). Of note, acetylsalicylic acid is rapidly converted to the more active form salicylate by blood and tissue hydrolases (281, 282). As a non-steroid, anti-inflammatory, antimicrobial, antipyretic and analgesic drug, it possesses a high therapeutic potential. Many centuries before the synthetic production of aspirin was available, people made use of these properties by using willow bark as a natural source for salicylic acid. Since salicylic acids are central in plants as protective agents against various pathogens, it is constituent in various foods such as fruits, vegetables, spices, and herbs. Additionally, it is also used as a food preservative.

Daily intake varies greatly depending on different dietary habits (71). Major food sources include fruits, fruit juices, wines and vegetables. For instance, black- and blueberries contain roughly 0.8 and 0.6 mg/kg, respectively, while nectarines contain more than 3 mg/kg. Among vegetables, asparagus is rich in salicylates with up to 1.3 mg/kg, as well as white onions with 0.8 mg/kg (72). Notably, foods containing a lot of spices show relevantly higher salicylate acid levels that can reach the amount of low dose Aspirin medication (283) if consumed in high amounts (for comparison: one standard tablet of Aspirin contains 75 mg acetylsalicylic acid, a more tolerable derivative). For instance, cumin, paprika, thyme and mint contain 20–50 mg/kg salicylate (72). Thus, it is suggested that diets rich in spices, such as south Indian menus, can contain daily levels of 12–13 mg (71). Large variations in the reported levels are present, as exemplified by salicylate levels in orange juice ranging from 0.47 to 3.02 mg per liter (72). A systematic review of salicylates in foods of the Scottish population revealed an estimated intake of salicylates of 4.42 and 3.16 mg/day for men and women, respectively (72). Another study calculated daily intake levels of 1.41 mg (men) and 1.34 mg (women) per day in a southern German cohort, with the major food sources being citrus fruits (30%) and berries (24%) (284).

Salicylic Acid and Derivatives in Epidemiological and Clinical Studies

Salicylic acid and derivatives (e.g., acetylsalicylic acid in Aspirin) in various commercial formulations have been in broadscale medical use for several decades, primarily for their anti-inflammatory and analgesic properties. Aspirin inactivates cyclooxygenase-1 and−2, leading to inhibition of prostaglandin synthesis. Accompanied by reduced platelet aggregation, this can also prevent and treat cardiovascular diseases. Released salicylic acid has a wide range of additional biological activities, including anti-inflammatory, -oxidant, and -proliferative properties.

More recently, long-term low- to middle-doses of Aspirin have gained attention as preventive strategies to promote health. Several clinical trials and meta-analyses thereof have been conducted. Regular Aspirin consumption has been associated with cardiovascular benefits and lower risk for cancers, especially of colorectal type (285–289). Evidence for the anti-cancer effects of aspirin and salicylates comes from both interventional, epidemiological and pre-clinical studies (290). Regarding prophylactic chemopreventive and cardioprotective actions, the cost-benefit profile of low-dose (75–325 mg/day) Aspirin consumption for at least 3 years seems to be largely in favor of Aspirin, although the potential gastrointestinal side-effects must not be neglected (291, 292). At odds with several studies in younger cohorts, a recent large scale Australian and US study gave 100 mg Aspirin to people over 70 and found no difference in overall cancer incidence after 4.7 years, while the risk of incident for late-stage and metastasized cancers was significantly elevated in the Aspirin group (293). This warrants caution for older age groups.

It has been suggested that the chemopreventive effects of aspirin consumption come from the salicylic acid formed in the body and that dietary salicylates could act similarly (290). In line with the higher amount of salicylates in plant-based foods, small-scale studies found that vegetarians have higher serum and urinary excretion levels than non-vegetarians, while average serum levels in vegetarians were only 11% of patients taking daily aspirin (294, 295). The authors found wide ranges and overlaps

in the serum concentrations between vegetarians and aspirin-treated patients, suggesting that it is possible to raise circulating salicylic acid levels by dietary means in some cases. Salicylate tissue levels could respond differently to dietary intake and it is yet unclear what role they play in the ascribed effects. Of note, similar to regular Aspirin consumption, vegetarianism and low-meat diets have been associated with lowered cancer risk several times (296–298). However, studies by Janssen et al. suggest that the amount of acetylsalicylic acids in diets is probably too low to affect disease risk (73, 299). Thus, whether dietary salicylate consumption is sufficient to elicit disease-protecting activities remains debated.

Most trials indicating protective effects of aspirin against various diseases, use doses that likely exceed dietary intake levels by a magnitude of at least 10 and the required trials with doses achievable via the diet (<15 mg/day) are currently absent. Hence, the accumulated effects of long-term and low-level dietary salicylate consumption remain elusive. However, it must be noted, that daily consumption of doses as low as 10 mg have been reported to cause gastrointestinal complications, especially bleeding and ulcers, when consumed for more than a month (300, 301), highlighting the need for rigorous long-term, low-dose interventional studies that take into account dietary intake levels of salicylates.

CONCLUSION AND PERSPECTIVE

CR and different types of fasting are slowly approaching clinical applications, not only as weight management options (12, 302). These developments are accompanied by growing clinical interesting in the potential of naturally occurring and synthetic CRMs to ameliorate and treat diseases or support existing treatments, such as chemotherapy (303). Especially age-associated diseases and those with underlying autophagic disturbances will likely be priority targets. Natural CRM candidates are widely present in foods and, in most cases, inevitably consumed by humans. Given their prominent occurrence in plant-based foods (especially polyphenols and polyamines), it is conceivable that these compounds contribute to the beneficial effects of healthy diets. Nevertheless, to date, specific dietary recommendations must be read with caution as too many uncertainties remain regarding bioavailability, concentration in food, stability and optimal intake levels. Furthermore, estimations of CRM levels in healthy diet plans, such as the DASH, HEI-2010, AHEI-2010, or aMED, are largely elusive and should be evaluated in future studies, as they could add to or be responsible for some of the beneficial effects of these diets. Side by side with the herein discussed naturally occurring CRMs, other non-dietary substances also possess CR-mimicking properties. These prominently include rapamycin, metformin and synthetic sirtuin activators, among others, and are discussed elsewhere (20).

Overall, the promising and emerging field of dietary CRM candidates needs to be considered with scientific rigor, as large parts of evidence on their effects in humans come from epidemiological and/or small-scale studies, often conducted with plant-based extracts that contain numerous bioactive substances. Problems may also arise when translating pre-clinical and epidemiological evidence of dietary and body-endogenous substances to clinical studies. For many of the herein discussed substances important data yet need to be collected: oral bioavailability, stability throughout the intestinal tract, metabolization, cellular uptake, distribution throughout the body, organ-specific effects, interaction with body-endogenous biosynthesis pathways and bioactive levels, just to name a few. More importantly, epidemiological data on dietary components can only be as good as the underlying food databases. Unfortunately, regionally varying food compositions, quality, the influence of meal preparation techniques and storage conditions are sometimes insufficiently studied or documented. Hence, deepened research into these questions is needed for the evolving field of dietary CRMs (and other dietary components). For dietary CRMs, different baseline intake levels likely influence outcomes of different dosing schemes. As an example, daily average spermidine intake levels are estimated to vary greatly between different countries (50), correlating with gross domestic product (193, 304), which might interfere with the effectiveness of doses near baseline dietary intake.

Finally, due to accumulating pre-clinical and clinical evidence, CRMs emerge as a prosperous future field of research that should be tackled in detail by clinical and nutrition researchers alike. Larger interventional studies are needed to validate first promising data from epidemiological and small-scale clinical trials. In terms of dietary CRMs, a detailed evaluation of existing food databases is warranted, and clinical trials should carefully take into account the dietary habits and food compositions of study cohorts. It will be interesting to see how the herein discussed compounds contribute to the beneficial effects of well-characterized healthy diets. Eventually, existing and newly developed healthy diet plans could be optimized with regards to levels of dietary CRM candidates.

AUTHOR CONTRIBUTIONS

SH and SD conceptualized the review. SH, SD, and MB wrote the manuscript. All authors provided critical feedback, edited, proof-read, and helped shape the review.

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Caloric Restriction May Help Delay the Onset of Frailty and Support Frailty Management

Pan Liu[1], *Yun Li*[1] *and Lina Ma*[1,2]*

[1] Department of Geriatrics, Xuanwu Hospital, Capital Medical University, Beijing, China, [2] National Clinical Research Center for Geriatric Medicine, Beijing, China

Frailty is an age-related clinical syndrome that may increase the risk of falls, disability, hospitalization, and death in older adults. Delaying the progression of frailty helps improve the quality of life in older adults. Caloric restriction (CR) may extend lifespan and reduce the risk of age-related diseases. However, few studies have explored the relationship between CR and frailty. In this review, we focused on the impact of CR on frailty and aimed to identify potential associated mechanisms. Although CR may help prevent frailty, further studies are required to determine the underlying mechanisms and specific CR regimens suitable for use in humans.

Keywords: caloric restriction, frailty, older adults, sarcopenia, lifespan

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***Correspondence:**
Lina Ma
malina0883@126.com

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HIGHLIGHTS

- Caloric restriction (CR) has antiaging effects and great significance in delaying frailty and sarcopenia.
- Some suggestions about CR for frailty are proposed.
- Further study is needed to determine the mechanisms and detailed CR interventions appropriate for humans.

INTRODUCTION

Frailty is an age-related clinical geriatric syndrome associated with the decline of multiple physiological systems and increased risk of adverse health outcomes, such as falls, hospitalization, disability, and premature mortality in older adults (1, 2). Frailty is receiving increasing research and clinical attention due to the rapid population aging. The prevalence of frailty is estimated in the range of 4–59% (3). The Fried phenotype (1) and the frailty index (FI) (4) are two widely used methods for frailty assessment. The frailty phenotype includes features, such as unintentional weight loss, poor muscle strength, exhaustion, reduced physical activity, and slow walking speed (1). Interventions that prevent or delay the onset of frailty are required to improve the quality of life among older adults. The previous studies have examined frailty in rodents (5–8) and humans (9).

High-calorie diets are risk factors for obesity and metabolic diseases (10). Caloric restriction (CR) is defined as a reduction in energy intake (typically by 20–40% of *ad libitum* consumption) without malnutrition (11). CR has been reported to considerably extend a healthy lifespan and prevent age-related diseases in both animals and humans (11–14). However, the previous studies have mainly focused on the association between CR and aging, and few studies have explored the relationship between CR and frailty. This review aimed to summarize the evidence on the impact of CR on frailty and to explore candidate underlying mechanisms.

CR AND FRAILTY

In the clinical setting, the FI may be a lifespan biomarker, helping in predicting age-related mortality (15). A previous study has shown that 30% CR may enhance strength in both old- and middle-aged male mice and improve balance and motor coordination in both old- and middle-aged female mice; these outcomes are closely associated with a delay in the onset of age-related frailty (16). In addition, a separate study has shown that both middle-aged and old male mice with the CR of 30% had grip strength greater than that observed in their counterparts (17). Old male C57BL/6 mice that consumed a 40% CR diet over 13 months period, starting from 6 months of age, and that fed an *ad libitum* diet combined with 6 months of resveratrol treatment both improved frailty status compared with their counterparts. However, this difference was not observed in female mice (18). In contrast to the C57BL/6 mice, CR did not delay age-related decline in DBA/2 mice. Male DBA/2 mice on a similar CR diet had a higher risk of frailty than did the matched C57BL/6 mice. There was no difference in frailty assessment by FI among both sexes of CR mice (18). The impact of CR regimens on frailty, activity, and memory in male Wister rats was stratified by CR starting point and duration. A CR of 40% imposed over 6, 12, or 18 months, starting at 6 months of age, improved the general locomotor activity and spatial memory and decreased the age-related frailty. However, the benefits of CR started in late adulthood were unclear; for example, a CR of 3 months starting at the age of 15 and 21 months increased the risk of frailty in old rats (19). Most studies on CR have been conducted in male animals. Further studies in female mice and rats or other species are required.

A 4-year treatment involving 30% CR beginning in adulthood (3.2 ± 0.1 years of age) may extend lifespan by 50% and reduce the risk of age-related diseases in male gray mouse lemurs, without affecting motor and cognitive performance (20). Meanwhile, 30% CR may extend the health span in rhesus monkeys (21). In the same species, Yamada et al. have shown that long-term 30% CR started in adulthood may reduce the incidence of frailty by improving weakness, endurance, slowness, and physical activity and extend healthy lifespan in both the sexes (22).

An interleukin-10 knockout (IL-$10^{-/-}$) mouse model is the genetic model of frailty (8). However, few studies on CR have used this model. Rapamycin, an inhibitor of mammalian target of rapamycin (mTOR), may improve muscle function and prevent frailty in IL-$10^{-/-}$ mice (23). Cu/Zn superoxide dismutase knockout mouse (Sod$1^{-/-}$) is another model of frailty, with characteristics similar to those observed in humans with frailty, such as weight loss, weakness, reduced physical activity, and exhaustion (7). The studies have shown that 40% CR may attenuate age-related loss of muscle mass of Sod$1^{-/-}$ mice by improving mitochondrial function, reducing oxidative stress damage and cellular senescence, and decreasing IL-6 levels (24, 25). Upregulation of SIRT3 and mitochondrial antioxidant manganese superoxide dismutase expression in CR Sod$1^{-/-}$ mice may help protect against muscle damage (24).

CR and Frailty in Humans

The Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy (CALERIE) trial has shown that 6 months of 25% CR reduced the levels of fasting insulin and body temperature in overweight adults (26). Further studies have shown that extending CR for 2 years may improve chronic inflammation markers, blood pressure, the levels of glucose, and blood lipids, alongside other cardiovascular metabolic indicators in young and middle-aged healthy adults (27), while improving cognitive function in non-obese healthy adults (28). Dora et al. found that this CR regimen reduced oxidative stress in male and female adults, as indicated by the urinary concentration of F2-isoprostane (29). Another study indicated that 12 weeks of CR improved cardiometabolic health in sedentary adults with obesity and aged ≥65 years (14). Age-related loss of skeletal muscle quantity and quality is associated with reduced gait speed and overall strength and a high risk of fall and frailty. A previous study has shown that 15–25% CR may prevent age-related muscle atrophy in humans (30), potentially improving the frailty. Other studies have shown that time-restricted feeding improved the walking speed and quality of life in overweight sedentary older adults (aged ≥65 years) (31). However, the generalizability of these findings requires further research.

CR and Sarcopenia

Sarcopenia is an age-related syndrome of muscle strength and functional decline that is closely associated with frailty; in fact, it may contribute to physical frailty. CR exerts a protective effect against sarcopenia in both rodents and non-human primates (32–35). A CR of 30% over 10 weeks may improve skeletal muscle function in male C57BL/6 mice (33). Lifelong 8% CR prevents age-related disruption of the myofiber membrane environment in male Fischer-344 rats (32). The effects of different durations (2.5, 8.5, and 18.5 months) of 40% CR on skeletal muscle may depend on animal strain, sex, and age (36). Vastus lateralis biopsies collected at 6, 9, and 12 years after the treatment that included a 30% CR diet have shown that CR may prevent the shift in fiber type distribution and delay cellular atrophy in male rhesus monkeys (34).

POSSIBLE MECHANISM OF CR EFFECTS ON FRAILTY

The mechanisms of CR impact on frailty remain unclear; several target pathways involved in antiaging may be affected, such as the inhibition of insulin-like growth factor-1 (IGF-1) and mTOR signaling, activation of adenosine 5′-monophosphate-activated protein kinase (AMPK) and sirtuins, and promotion of autophagy (**Figure 1**) (12). Sirtuins are a conserved family of nicotinamide adenine dinucleotide (NAD)-dependent proteins. Silent mating-type information regulation 2 homolog 1 (SIRT1) and other sirtuins may mediate the protective effects of CR (37). SIRT1 activation may extend lifespan through the activation of AMPK, which further inhibits mTOR, promotes lipid catabolism and gluconeogenesis (38). CR may delay cognitive decline in

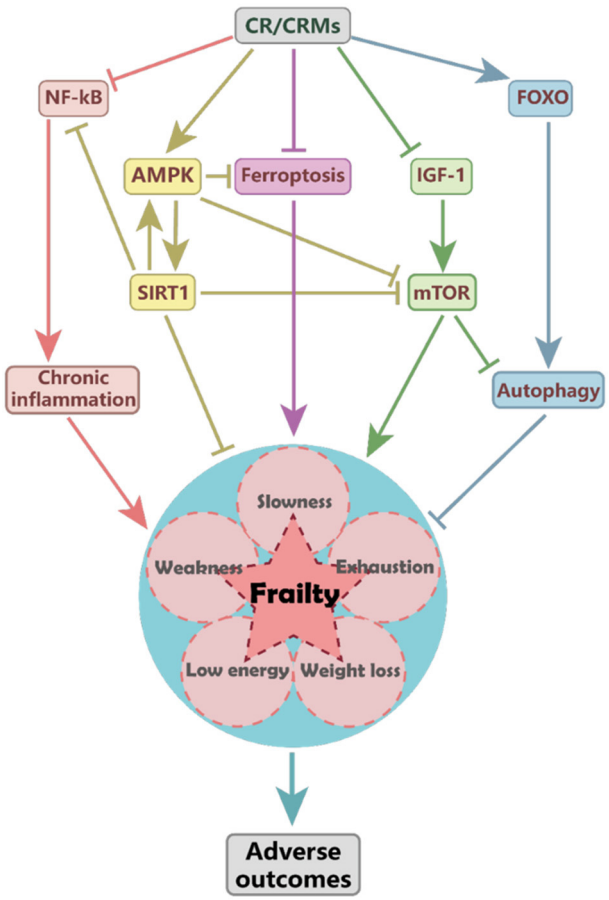

FIGURE 1 | The proposed mechanism of caloric restriction (CR) impacts frailty. CR may reduce the risk of frailty and associated adverse outcomes by activating the AMPK and SIRT1 pathways, inhibiting the IGF-1 and mTOR signaling and ferroptosis, and reducing the inflammation mediated by NF-KB pathways. CR-induced SIRT1 activation may upregulate AMPK and suppress NF-κB and mTOR activity. CR and metformin may attenuate ferroptosis by activating the AMPK pathway and improving frailty. AMPK, adenosine 5′-monophosphate (AMP)-activated protein kinase; CR, caloric restriction; CRMs, caloric restriction mimetics; FOXO, forkhead box O; IGF-1, insulin-like growth factor-1; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; SIRT1, silent mating-type information regulation 2 homolog 1.

mice by modulating the SIRT1/mTOR signaling pathway and by activating SIRT1 and suppressing mTOR signaling (39).

Lower sirtuin levels are independently associated with frailty, regardless of age, sex, and comorbidities. Lower circulating levels of SIRT1 and SIRT3 may indicate frailty (40), and frail older adults are more likely than their counterparts to have lower serum-induced SIRT1 expression levels (41). In contrast, the previous study has shown that frail older patients had higher levels of SIRT1 than did their counterparts. Older adults with elevated SIRT1 levels had decreased physical function (42). Nevertheless, serum-induced SIRT1 expression has not been associated with frailty (43). Further studies are required to elucidate the relationship between SIRT1 and frailty and other signaling pathways that may mediate the relationship between CR and frailty.

Cell senescence and chronic inflammation are important characteristics of aging and frailty (44); CR exhibits anti-senoinflammatory effects by suppressing the expression of cytokines and chemokines in the senescence-associated secretory phenotype. The CR mimetics (CRMs) may improve the dysregulated activity of signaling pathway molecules (45). A CR diet may delay the onset of frailty and improve the progression of several chronic diseases by reducing the development of chronic low-grade inflammation (46), associated with elevated levels of C-reactive protein, IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) (47). Moreover, CR exhibits considerable anti-inflammatory activity by modulating the activity of nuclear factor-κB (NF-κB) and forkhead box O (FOXO) (48). Activation of SIRT1 may suppress the NF-κB pathway (49). Immune senescence is a natural consequence of aging that is associated with frailty. CR may attenuate age-related changes of the natural killer cells and T cells to preserve immune function in later life, which is a system-wide effect (50).

Iron dyshomeostasis and ferroptosis may trigger cell and organismal death in *Caenorhabditis elegans* (51). CR and metformin attenuate ferroptosis by activating the AMPK pathway, which has been associated with extended lifespan and health span and improved frailty (51). CR may protect against cognitive function decline by inducing senescence-accelerated prone eight astrocytes protective gene expression and functional rejuvenation *in vitro* (52). In addition, CR may improve insulin sensitivity (11) by mediating the adipose mTOR2 pathway; however, the activity of this pathway is not necessary for the beneficial effects of CR (53).

Age-related apoptosis in skeletal myocytes may lead to sarcopenia, which involves mitochondria- and TNF-α-mediated pathways (54). Interventions targeting myonuclear apoptosis improve sarcopenia and physical frailty symptoms (55). Lifelong 8% CR has been shown to reduce age-related rates of apoptosis and oxidative damage to the skeletal myocyte by regulating autophagy in rats (56). This mechanism may be associated with heat shock protein 27 signaling, which, when insufficient, may contribute to apoptosis and muscle wasting (57). The upregulation of the IGF1-Akt-mTOR-FOXO signaling pathway may accelerate sarcopenia in aged mice (58). CR may help preserve muscle mass in middle-aged rats by downregulating mTOR and ubiquitin-proteasome pathway signaling (59). Further, CR may delay skeletal muscle aging in rhesus monkeys by inducing metabolic changes (60). These findings indicate that CR may delay sarcopenia by reducing oxidative stress damage, inflammation, and iron overload, as well as improving mitochondrial function, enhancing protein homeostasis, and increasing autophagy and apoptosis (61).

TYPES OF CR

Caloric restriction has been reported to extend health span and lifespan and prevent age-related diseases and frailty. However, the optimum timing of CR initiation or duration remains unclear as few previous studies have focused specifically on frailty. Further studies are required to establish regimens most likely to improve the quality of life of older adults. At the time of writing, several types of CR regimens exist. For example, the Mediterranean

CR diet has been shown to decelerate age-related cognitive decline (62) and the progression of aging and prevent frailty (63), making this approach useful for frailty management in the clinical context (64). The clinical impact of CR may depend on the factors, such as compliance; herein, we describe candidate approaches to CR that include intermittent fasting, CRMs, and protein dietary restriction.

Intermittent Fasting

No diet regimen is suitable for everyone. Different from continuous CR, intermittent fasting consists of periods of little or no energy intake and intervening periods of normal food intake (65), which have benefits for weight loss, healthy aging, and chronic disease prevention (66), such as improving cardiometabolic health in overweight and obese individuals (67). In addition, intermittent fasting may play an important role in reducing oxidative stress, improving insulin sensitivity, repairing autophagy, and improving cognitive function (65). Established intermittent fasting regimens determined by the interval length of fasting (66, 68) include time-restricted feeding, alternate-day fasting, alternate-day modified fasting, and the 5:2 diet. For example, the 5:2 diet involved 2 days of fasting with no more than 25% energy intake and 5 days of regular eating patterns per week (67). Time-restricted feeding may help protect cardiometabolic health; in contrast to CR, it may also be associated with satisfactory compliance as time is relatively easy to monitor (69). In later life, intermittent fasting on alternate days may increase renal gasotransmitter hydrogen sulfide production, which may help reduce age-related frailty in male mice (70).

CR Mimetics

Caloric restriction mimetics are compounds that mimic physiological and metabolic CR effects (71), such as resveratrol, rapamycin, metformin, NAD precursors, and senolytics (15). They have positive effects on the rodent lifespan and human health and are used in interventions against aging and age-related cardiovascular, neurodegenerative, and malignant diseases (72). Moreover, these compounds may help prevent age-related frailty, as assessed using the FI in mice (15). Several CRMs have been shown to prevent frailty (**Table 1**); for example, 6 months of resveratrol treatment (100 mg/kg/day) starting at 18 months of age has been shown to prevent frailty in mice (18). In addition, 6-week resveratrol treatment (150 mg/kg/d) has been shown to improve the grip strength and muscle mass in aged rats through the activation of the AMPK/SIRT1 pathway (73). SRT1720, another SIRT1 activator, may extend lifespan and improve the health of mice through SIRT1 activation and NF-κB expression reduction (74). Frailty is associated with SIRT1 activity in older adults (42); targeting this pathway with CRMs, such as resveratrol may affect both robustness and frailty in humans (37); Metformin has been reported to extend the lifespan of older adults with type 2 diabetes by preventing frailty (75). Exposure to any dose or frequency of metformin administration may reduce the risk of frailty in older adults (76). An 18-month intervention involving rapamycin (1.5 mg/kg/d) for IL-10$^{-/-}$ mice has been shown to prevent muscle mass loss and frailty by decreasing myostatin levels (23). Meanwhile, 12-week treatment with low-dose oral rapamycin (0.5, 1, and 2 mg) failed to improve the frailty status in older adults with coronary artery diseases (77). The combination of dasatinib (5 mg/kg) and quercetin (50 mg/kg), as one of the senolytics, may extend health span and alleviate symptoms of frailty in aged mice (78). In addition, a chronic nicotinamide diet, an NAD+ precursor, at doses in the range of 0.5 or 1.0 g/kg, can improve the health span but not the lifespan of adult mice (79). Future studies are required to elucidate the effects of CRMs on frailty.

Protein Diet

Macronutrient balance is important for healthy aging. Higher protein intake has been associated with worse frailty status over time in a relatively healthy population; no similar effect has been identified for either carbohydrates or fats (80). Further, low-protein high-carbohydrate diets may help expand lifespan (81). Protein restriction has been shown to affect the rodent lifespan in a manner similar to that associated with CR (81, 82). Amino acids, particularly branched-chain amino acids (BCAAs), such as leucine, isoleucine, and valine, are associated with improved health and increased lifespan in different organisms (83, 84). Protein restriction may increase the risk of frailty and sarcopenia (85). Intake of a BCAA-enriched balanced amino acid mixture may help preserve muscle fiber quantity, improve motor coordination and endurance, and extend the lifespan of middle-aged mice by modulating the mTOR/eNOS pathway, which affects mitochondrial biogenesis (84). In addition, a BCAA-enriched diet may help prevent disability and extend a healthy lifespan in older adults (86), suggesting that this diet may be suitable for older adults at risk of frailty. In contrast, Richardson et al. suggested that lifelong restriction of dietary BCAAs may extend lifespan and prevent frailty in aged male mice. Nonetheless, the effect of the BCAA diet on frailty remains unclear (87). Further studies are needed to examine these associations in humans. The controversies regarding the effects of BCAA dietary restriction or enrichment may be associated with different factors, such as intervention onset, duration, and species. Further studies are required to elucidate the relationship between protein intake, lifespan, and age-related diseases.

COMBINATION OF CR AND EXERCISE

Diet and exercise are critical components of healthy aging. Protein supplementation alone may not alleviate sarcopenic symptoms (30). Protein supplementation combined with resistance training is recommended to prevent sarcopenia and frailty (64). The previous studies have shown that a combination of resistance training and CR for 6 months may improve maximal strength in menopausal women with obesity (88). Meanwhile, other studies have shown that CR combined with resistance training may prevent CR-induced muscle loss in older adults with obesity (89). A separate study has shown that the interventions involving CR and exercise may improve age-related conditions in adults with type 2 diabetes (90). Thus, exercise may be considered as another type of CRMs, helping prevent frailty and improve healthy aging alone or in combination with CR (91). These effects are likely mediated by antioxidant-related mechanisms (91). However, it should be noted that the combination of CR and aerobic exercise training

TABLE 1 | Caloric restriction mimetics and frailty assessments.

CRMs	Category	Species	Onset	Dose and duration	Frailty assessment	Results
Resveratrol	SIRT1 activator	Male, C57BL/6J mice	18 months	100 mg/kg/d, 6 months	Mouse FI	Reduces FI scores (18)
		Male, SD rats	24 months	150 mg/kg/d, 6 weeks	Physical function	Improves grip strength and muscle mass (73)
SRT1720	SIRT1 activator	Male C57BL/6J mice	7 months	100 mg/kg/d Natural death	-	Extends lifespan and improves health in mice (74)
Metformin	AMPK activator	Adults aged ≥65 years with type 2 diabetes	Receiving metformin in outpatient care	–	FI	Reduces risk of frailty regardless of dose and frequency (76)
Rapamycin	mTOR inhibitor	IL-$10^{-/-}$ mice	6 weeks	1.5 mg/kg/d, 18 weeks	Mouse FI	Decreases levels of myostatin which may prevent muscle mass loss and frailty (23)
Dasatinib and quercetin	Senolytic drugs	Male, C57BL/6J mice	① 20 months ② 24–27 months	A combination of dasatinib (5 mg/kg) and quercetin (50 mg/kg) ① 4 months ② Natural death	Physical function	Alleviates symptoms of frailty and extends healthspan (78)
Nicotinamide	NAD+ precursor	Male, C57BL/6J mice	56 weeks	0.5 and 1.0 g/kg, 62 weeks	–	Improves healthspan but does not extend lifespan (79)

AMPK, adenosine 5′-monophosphate-activated protein kinase; CRMs, caloric restriction mimetics; FI, frailty index; IL-$10^{-/-}$, interleukin-10 knockout; mTOR, mammalian target of rapamycin; NAD, nicotinamide adenine dinucleotide; SD, Sprague-Dawley; SIRT1, silent mating-type information regulation 2 homolog 1.

practiced for 5 months may not affect cognition in sedentary older adults with obesity (92). Thus, further investigations are required to determine lifestyle interventions suitable for older adults and those with frailty or sarcopenia.

POTENTIAL RISKS ASSOCIATED WITH CR

Malnutrition is common in older adults and increases the risk of frailty, sarcopenia, comorbidities, and premature death. CR may delay the onset of frailty and sarcopenia, potentially helping to improve the quality of life of older people. However, extreme CR may lead to adverse events, such as sarcopenia, osteoporosis, and immune deficiencies (93). Aged rats with 3 months of CR had poorer performance and frailty scores than their counterparts (19). This finding was consistent with that of another study showing that 40% CR initiated in mice aged 22–24 months increased mortality rates in male C57BL/6, DBA/2, and B6D2F1 mice (94). Further, CR accelerated the loss of gray matter but preserved the white matter in the brain of aged mouse lemurs; neither effect altered the cognitive performance (20). While chronic food restriction may impair spatial recognition memory in developing mice (an effect mediated by the extent of food restriction and individual tolerability), acute food restriction exerts negative effects on locomotor activity in mice (95). The relationships between CR, genetics, sex, animal strains, as well as regimen duration and extent, are complex. Future studies are required to elucidate the suitable timing, duration, and extent of CR that may help prevent the onset of frailty in older adults.

CONCLUSION

Caloric restriction has shown some benefits in both animal and human studies; however, the factors that determine the impact of CR remain unclear (19). Rodent and non-human primate models of CR are associated with the limitations that may affect study designs. The impact of CR on aging may be mediated by dietary composition, sex, age at onset, feeding regimens, and genetics (96). There is no standard for CR regimens (e.g., timing of initiation and duration, or caloric intake values). In addition, the evidence on the association between CR and frailty in the clinical setting is insufficient. Moreover, the underlying mechanisms are unclear. Consequently, further studies are required to elucidate the caloric intake and nutrient composition optimal for healthy aging in humans.

AUTHOR CONTRIBUTIONS

PL, YL, and LM contributed to the organization of the manuscript. PL drafted the manuscript and composed the outline. YL and LM reviewed and approved the submitted version. All the authors agree to be accountable for the content of the study.

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Intake of Dietary Fiber From Grains and the Risk of Hypertension in Late Midlife Women: Results From the SWAN Study

Peng Du[1†], *Kaifeng Luo*[1†], *Yali Wang*[2], *Qi Xiao*[1], *Jiansheng Xiao*[1], *Yong Li*[1*] and *Xingjian Zhang*[1*]

[1] Department of General Surgery, The First Affiliated Hospital of Nanchang University, Nanchang, China, [2] Department of Pathology, The Affiliated Stomatological Hospital of Nanchang University, Nanchang, China

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***Correspondence:**
Yong Li
lylfy0606@163.com
Xingjian Zhang
lfyzhangbo@163.com

[†] These authors share first authorship

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Background: The possible effects of dietary fiber intake on hypertension have not been clarified fully. The association of dietary fiber intake with hypertension risk in midlife women was analyzed in this study.

Methods: Baseline data were obtained from the Study of Women's Health Across the Nation (SWAN). Smooth curve, linear regression, and logistic regression analyses were performed to investigate the associations of four indices of daily dietary estimate (DDE) of dietary fiber (dietary fiber intake, dietary fiber intake from beans, dietary fiber intake from vegetables/fruit, and dietary fiber intake from grains) with blood pressure in midlife women. For this research purpose, diastolic blood pressure (DBP) ≥90 mmHg was defined as diastolic hypertension, and systolic blood pressure (SBP) ≥140 mmHg was defined as systolic hypertension.

Results: This study included 2,519 participants with an average age of 46. The smooth curve showed approximate negative correlations between three fiber indices (DDE dietary fiber, DDE fiber from vegetables/fruit, and DDE fiber from grains) and blood pressure, including DBP and SBP (all $P < 0.005$). There were also approximate negative correlations between two fiber indices (DDE dietary fiber and DDE fiber from grains) and the risk of diastolic hypertension and systolic hypertension (all $P < 0.05$). Furthermore, multiple linear regression analysis suggested that DDE dietary fiber (Sβ = −0.057, 95% CI −0.194 – −0.012, $P = 0.027$), DDE fiber from vegetables/fruit (Sβ = −0.046, 95% CI −0.263 – −0.007, $P = 0.039$), and DDE fiber from grains (Sβ = −0.073, 95% CI −0.600 – −0.099, $P = 0.006$, Model 4) were still negatively correlated with DBP after adjusting for confounding factors. Only DDE fiber from grains was independently and negatively associated with SBP (Sβ = −0.060, 95% CI −0.846 – −0.093, $P = 0.015$) after these same confounding factors were adjusted for. Importantly, multiple logistic regression analysis suggested that only higher DDE fiber from grains was independently associated with a reduced risk of diastolic hypertension (OR = 0.848, 95% CI 0.770–0.934,

$P = 0.001$, Model 4) and systolic hypertension (OR = 0.906, 95% CI 0.826–0.993, $P = 0.034$, Model 4) after the adjustments were made for confounding factors.

Conclusions: We found that dietary fiber intake, especially DDE fiber from grains, contributes to a lower risk of systolic hypertension and diastolic hypertension in midlife women.

Keywords: dietary fiber, blood pressure, hypertension, midlife women, smooth curve

INTRODUCTION

Patients with hypertension tend to experience headaches, dizziness, and other symptoms for decades, and the damage caused by hypertension to the cardiovascular system is continuous and aggravating (1, 2). High blood pressure that is not well-controlled contributes significantly to an increased risk of serious diseases, such as myocardial infarction, ischemic and hemorrhagic stroke, aortic dissection or aneurysm, chronic kidney disease, and peripheral artery disease (3). There are many risk factors that are known to be associated with hypertension, such as age, heredity, environment, and lifestyle (4). Unhealthy lifestyles, including a high-salt diet, lack of exercise, fatigue, and high pressure from work and other areas of life, are the most important modifiable factors for developing hypertension (5). Using antihypertensive drugs can alleviate damage to target organs by hypertension and prevent adverse cardiovascular events. However, many individuals are either not aware of their blood pressure condition or are not aware of their true blood pressure values due to inadequate or discontinued antihypertensive treatment. Furthermore, although there are vast options for antihypertensive drugs, some patients with hypertension have difficulty controlling their blood pressure within the normal range by using current drugs, motivating further research in this field. Thus, early prevention of the formation of hypertension is still particularly important at present.

Importantly, the positive effects of dietary fiber supplementation on cardiovascular protection have been recognized for a few years (6–11). Some investigations on the role of dietary fiber intake in patients with hypertension have also been reported. For instance, one previous study reported an association of fiber intake with the prevention of cardiovascular disease, including hypertension (6). Oat, a fiber-rich food, has also been found to have beneficial effects on controlling blood pressure among patients with hypertension (7). However, studies on the exact relationship between dietary fiber intake and hypertension risk are few, and it has been studied inadequately (6). Lifestyle and eating habits in the middle-aged population tend to cause people to be at high risk of hypertension.

Accordingly, in this study, data analysis from a subset of the Study of Women's Health Across the Nation (SWAN) was performed to evaluate the relationships of four indices of daily dietary estimate (DDE) of dietary fiber (dietary fiber intake, dietary fiber intake from beans, dietary fiber intake from vegetables/fruit, and dietary fiber intake from grains) with blood pressure in midlife women.

METHODS

Study Design and Participants

To analyze the baseline data from the SWAN study, a longitudinal, multicenter, and population-based study of the natural history of late midlife women was performed. Between 1995 and 1997, a telephone screening interview was used to determine the eligibility of an individual for the study cohort. Participants from community-based samples were collected at seven collection points across the United States using various sampling frames and recruitment strategies. In summary, 16,065 women completed the telephone screening interview, and only 3,302 (20.6%) women were recruited to the SWAN cohort at baseline. Women who met the inclusion criteria were as follows: (1) had a range of ages from 42 to 53 years; (2) had not used reproductive hormones in the previous 3 months; and (3) had an intact uterus and at least one intact ovary. The institutional review boards at all the sites approved the study protocol, and all included individuals at each site gave informed consent. The SWAN study contained detailed information regarding demographic characteristics, lifestyle, self-reported health, health examination, and medical history. For research purposes, 783 women were excluded due to missing information. A total of 2,519 women were included in this study. The exclusion criteria are described in detail in **Figure 1**.

Measurement and Calculation of Blood Pressure

The measurement of blood pressure in all the included subjects was carried out according to a standardized protocol (12). Subjects did not consume caffeinated beverages or smoke for at least 30 min before measuring blood pressure. The measurements were performed with readings taken on the right arm, with the participant seated and feet flat on the floor for at least 5 min before measuring blood pressure (12). A standard mercury sphygmomanometer was used to record systolic blood pressure (SBP) and diastolic blood pressure (DBP) at the first and the fifth phase Korotkoff sounds. The average of two sequential blood pressure values, with a minimum of a 2-min rest period between measures, was recorded. Using the average of these two sequential blood pressure values, the mean SBP and DBP were calculated (13). For our research purposes, DBP $\geq$ 90 mmHg was defined as diastolic hypertension, and SBP $\geq$ 140 mmHg was defined as systolic hypertension (13).

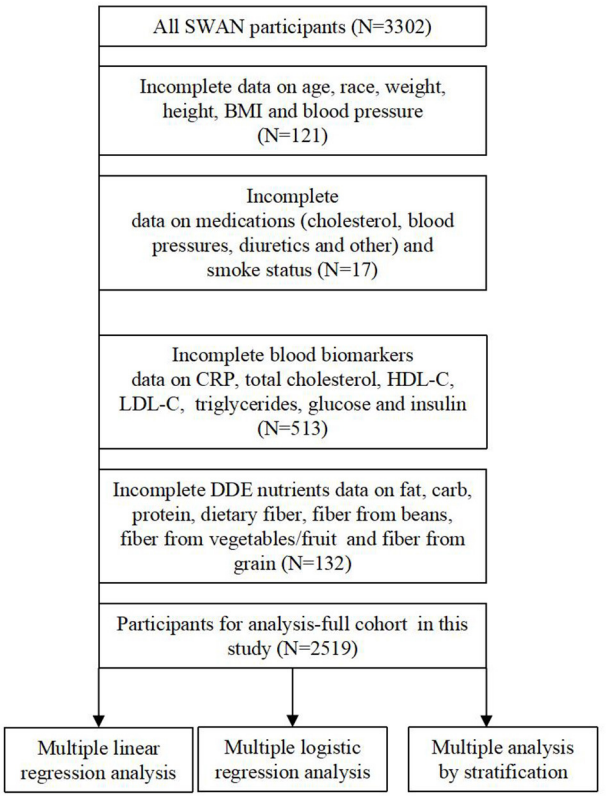

FIGURE 1 | Flow chart of the included participants.

Daily Dietary Estimate

A modified 1995 Block Food Frequency Questionnaire (FFQ) was used to evaluate daily dietary intake with 103 food items (14, 15) based on the Second National Health and Nutrition Examination Survey (NHANES) (14, 16, 17). Four indices of DDE of dietary fiber (dietary fiber intake, dietary fiber intake from beans, dietary fiber intake from vegetables/fruit, and dietary fiber intake from grains) were computed based on a database of food composition from the data of the United States Department of Agriculture (USDA) linked to data of food frequency (18).

Covariates

For research purposes, body mass index (BMI) was calculated as weight (kg) divided by height (meters) squared. Smoking status was divided into "current smoker" and "not current smoker". Medical history (cholesterol medications ever taken, blood pressure medications ever taken, diuretics ever taken, and birth control pills ever taken) was classified as "Yes" and "No". In addition, biochemical parameters including blood C-reactive protein (CRP), total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), glucose, and insulin were detected in the SWAN study.

Statistical Analysis

First, we performed a smooth curve to estimate the associations of four indices of DDE of dietary fiber (dietary fiber intake, dietary fiber intake from beans, dietary fiber intake from vegetables/fruit, and dietary fiber intake from grains) with blood pressure. Then, multiple linear regression models were used to explore the associations between these four fiber indices and SBP and DBP. Furthermore, multiple logistic regression models were also used to explore the relationships between the four DDE fiber indices and systolic hypertension (SBP $\geq$ 140 mm Hg) and diastolic hypertension (DBP $\geq$ 90 mm Hg). In addition, we implemented a stratified analysis to estimate the relationships between the four fiber indices and blood pressure stratified by age, race, BMI, and smoking status. In multivariate analysis, the following five models were used: Crude Model: adjustment for nothing; Model 1: adjustment for age and race; Model 2: adjustment for age, race, DDE fat, DDE carb, and DDE protein; Model 3: adjustment for age, race, DDE fat, carb, protein, smoking status, and BMI; and Model 4: adjustment for age, race, DDE fat, carb, protein, smoking status, BMI, blood CRP, total cholesterol, triglycerides, LDL-C, HDL-C, glucose, and insulin. All of the analyses were performed using EmpowerStats 3.0 (Chinese version). A $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Population Characteristics

A total of 2,519 subjects were included, with an average age of 46. As shown in **Table 1**, the mean values of SBP and DBP were 114 and 74 mmHg, respectively. The mean values of DDE dietary fiber, DDE fiber from beans, DDE fiber from vegetables/fruit, and DDE fiber from grains in the included participants were 11.52, 1.22, 5.36, and 3.91, respectively.

Smooth curves were identified between the four DDE fiber indices and blood pressure. The smooth curve suggested approximate negative correlations between three fiber indices (DDE dietary fiber, DDE fiber from vegetables/fruit, and DDE fiber from grains) and blood pressure, including DBP and SBP (**Figure 2**, all $P < 0.005$). Importantly, there were approximate negative correlations between two fiber indices (DDE dietary fiber and DDE fiber from grains) and the risk of diastolic hypertension and systolic hypertension (**Figure 3**, all $P < 0.05$).

Association Between Dietary Fiber Intake and BP

To explore the correlation between the intake of dietary fiber and blood pressure, a multiple linear regression analysis was performed. As shown in **Table 2**, after adjusting for confounding factors, including age, race, DDE fat, carb, protein, smoking status, BMI, blood CRP, glucose, insulin, LDL-C, HDL-C, triglycerides and total cholesterol, DDE dietary fiber (Sβ = −0.057, 95% CI −0.194 – −0.012, $P = 0.027$), DDE fiber from vegetables/fruit (Sβ = −0.046, 95% CI −0.263 – −0.007, $P = 0.039$), and DDE fiber from grains (Sβ = −0.073, 95% CI −0.600 – −0.099, $P = 0.006$) were still negatively

TABLE 1 | Characteristics of participants ($n = 2519$).

Variables	Mean ± SD or %	Range or N
Age (years)	46 ± 2.69	42–53
Race		
Black/African American (%)	27.79	700
Chinese/Chinese American (%)	7.90	199
Japanese/Japanese American (%)	8.50	214
Caucasian/White Non-Hispanic (%)	48.91	1232
Hispanic	6.91	174
Physical parameters		
Height (cm)	162.40 ± 6.75	140.50–186.20
Weight (kg)	70.20 ± 20.24	37.60–172.10
Waist circumference (cm)	82.5 ± 15.67	59–154.30
Hip circumference (cm)	103.80 ± 14.74	74–173
BMI (kg/m2)	26.47 ± 7.18	14.99–64.83
Smoking status (current smoker), (%)	16.71	421
Average DBP (mmHg)	74.00 ± 10.38	41–144
High DBP (≥90mmHg), (%)	9.83	147
Average SBP (mmHg)	114.00 ±16.88	74–224
High SBP (≥140mmHg), (%)	10.13	253
Medical history		
Cholesterol medications ever taken (%)	0.95	24
Blood pressures medications ever taken (%)	11.23	283
Diuretics ever taken (%)	9.09	229
Birth control pills ever taken (%)	72.89	1836
DDE nutrients		
DDE fat (g/day)	61.75 ± 30.86	12.04–218.67
DDE carb (g/day)	215.92 ± 98.66	18.75–792.56
DDE protein (g/day)	66.74 ± 25.18	17.49–204.49
DDE dietary fiber (g/day)	11.52 ± 5.79	1.66–61.78
DDE fiber from beans (g/day)	1.22 ± 2.46	0–39.62
DDE fiber from vegetables/fruit (g/day)	5.36 ± 3.55	0.60–26.85
DDE fiber from grains (g/day)	3.91 ± 2.16	0.10–16.61
Blood biomarkers		
Total cholesterol (mg/dl)	191 ± 33.75	92–335
HDL-C (mg/dl)	54 ± 14.41	18–166
LDL-C (mg/dl)	114 ± 30.60	25–261
Triglycerides (mg/dl)	89 ± 57.29	31–395
Lipoprotein A-1 (mg/dl)	48 ± 11.42	20–122
Apolipoprotein A-1 (mg/dl)	148 ± 24.95	73–317
Apolipoprotein B (mg/dl)	108 ± 28.41	73–317
CRP (mg/l)	1.50 ± 6.22	0.04–105.60
Glucose (mg/dl)	91 ± 30.45	45–439
Insulin (ulU/ml)	8.50 ± 13.17	2.50–417.20

DBP, diastolic blood pressure; SBP, systolic blood pressure; DDE, daily dietary estimate; BMI, body mass index. CRP, C-reactive protein; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

correlated with DBP in Model 4. However, we observed that only DDE fiber from grains was independently and negatively associated with SBP (Sβ = −0.060, 95% CI −0.846 – −0.093, $P = 0.015$) after these same confounding factors were adjusted.

Association Between the Intake of Dietary Fiber and Hypertension Risk

Multiple logistic regression models were used to further prove the correlation between dietary fiber intake and hypertension risk. As shown in **Table 3**, our results only suggested that higher DDE fiber from grains was independently associated with a reduced risk of diastolic hypertension (OR = 0.848, 95% CI 0.770–0.934, $P = 0.001$, Model 4) after adjustments were made for confounding factors, including age, race, DDE fat, carb, protein, smoking status, BMI, blood CRP, glucose, insulin, LDL-C, HDL-C, triglycerides, and total cholesterol. Similarly, our results also showed that higher DDE fiber from grains was associated with a reduced risk of systolic hypertension (OR = 0.906, 95% CI 0.826–0.993, $P = 0.034$, Model 4) after adjusting for these same confounding factors.

Stratified Analysis of the Association Between DDE Fiber From Grains and Blood Pressure by Age, Race, BMI, and Smoking Status

Multiple linear regression models were used to further explore whether the associations between DDE fiber from grains and blood pressure were affected by sex, race, BMI, and smoking status. As shown in **Table 4**, we observed no significant effect modifiers between DDE fiber from grains and DBP after stratifying the analysis by age (interaction $P = 0.724$), race (interaction $P = 0.125$), BMI (interaction $P = 0.815$), and smoking status (interaction $P = 0.347$). We also observed no significant effect modifiers between DDE fiber from grains and SBP after stratification of the analysis by age (interaction $P = 0.629$), race (interaction $P = 0.114$), BMI (interaction $P = 0.829$), and smoking status (interaction $P = 0.664$).

Multiple logistic regression models were also performed to further investigate whether the associations between DDE fiber from grains and the risk of hypertension were affected by sex, race, BMI, and smoking status (**Table 5**). We observed no significant effect modifiers between DDE fiber from grains and the risk of diastolic hypertension after the stratified analysis by age (interaction $P = 0.898$), race (interaction $P = 0.225$), BMI (interaction $P = 0.716$), or smoking status (interaction $P = 0.450$). We also observed no significant effect modifiers between DDE fiber from grains and the risk of systolic hypertension after the stratified analysis by race (interaction $P = 0.265$), BMI (interaction $P = 0.117$), or smoking status (interaction $P = 0.221$). Importantly, age (<46 and ≥46 years) was an effect modifier of the association between DDE fiber from grains and the risk of systolic hypertension (interaction $P = 0.009$).

DISCUSSION

Our study included baseline data from SWAN including 2,519 subjects to investigate the correlation between dietary fiber intake and hypertension risk. Our results uncovered that DDE fibers from grains were negatively associated with the risk of hypertension after adjusting for confounders. This is the

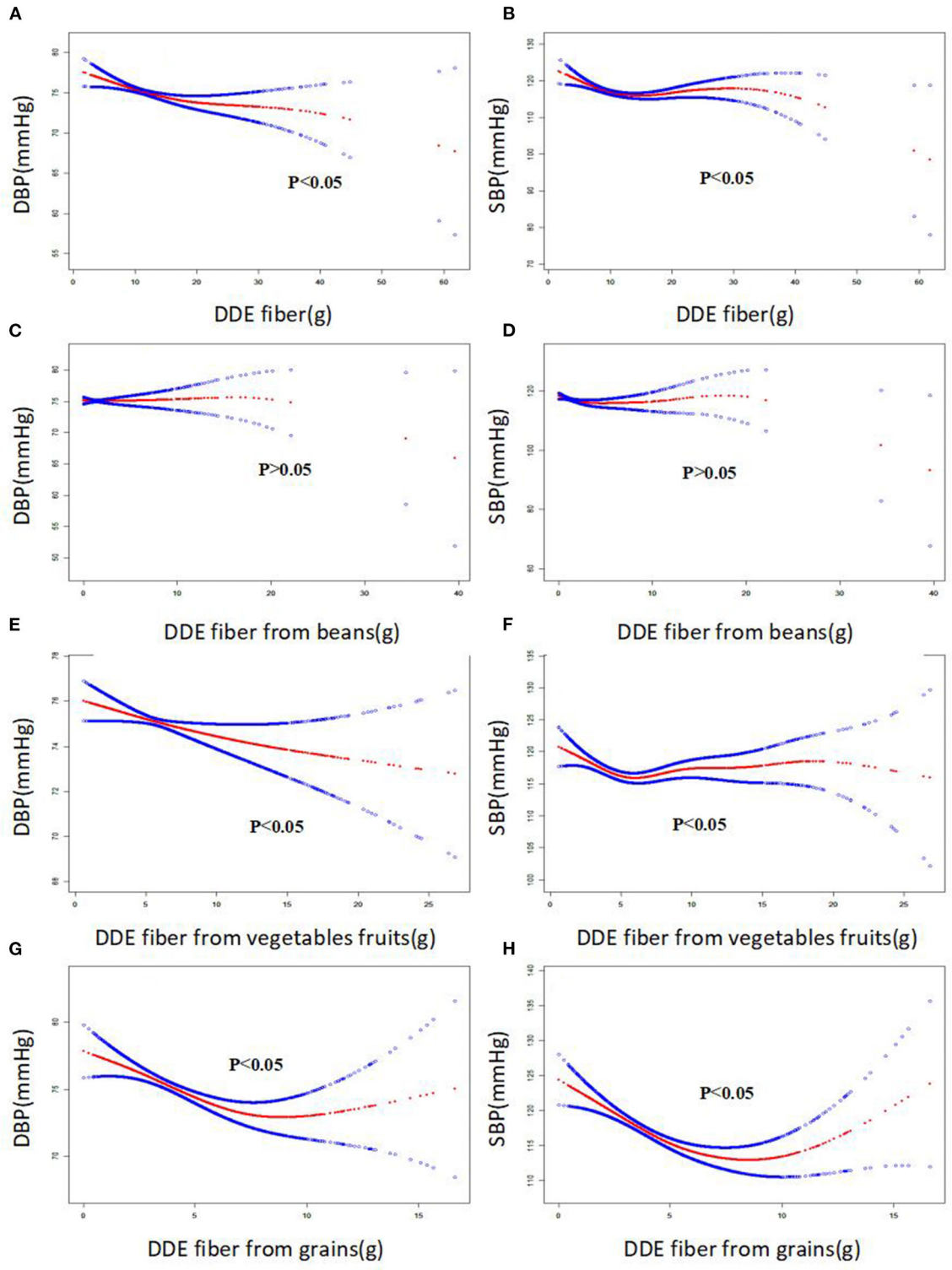

FIGURE 2 | The smooth curve of the associations between dietary fiber intake and systolic blood pressure and diastolic blood pressure (SBP and DBP, respectively).

first study to show that increased intake of DDE fiber from grains may contribute to a reduced risk of hypertension in midlife women.

Dietary fiber intake has great benefits for preventing many chronic diseases, such as cardiovascular diseases and malignant tumors. Studies on the beneficial effect of dietary fiber on

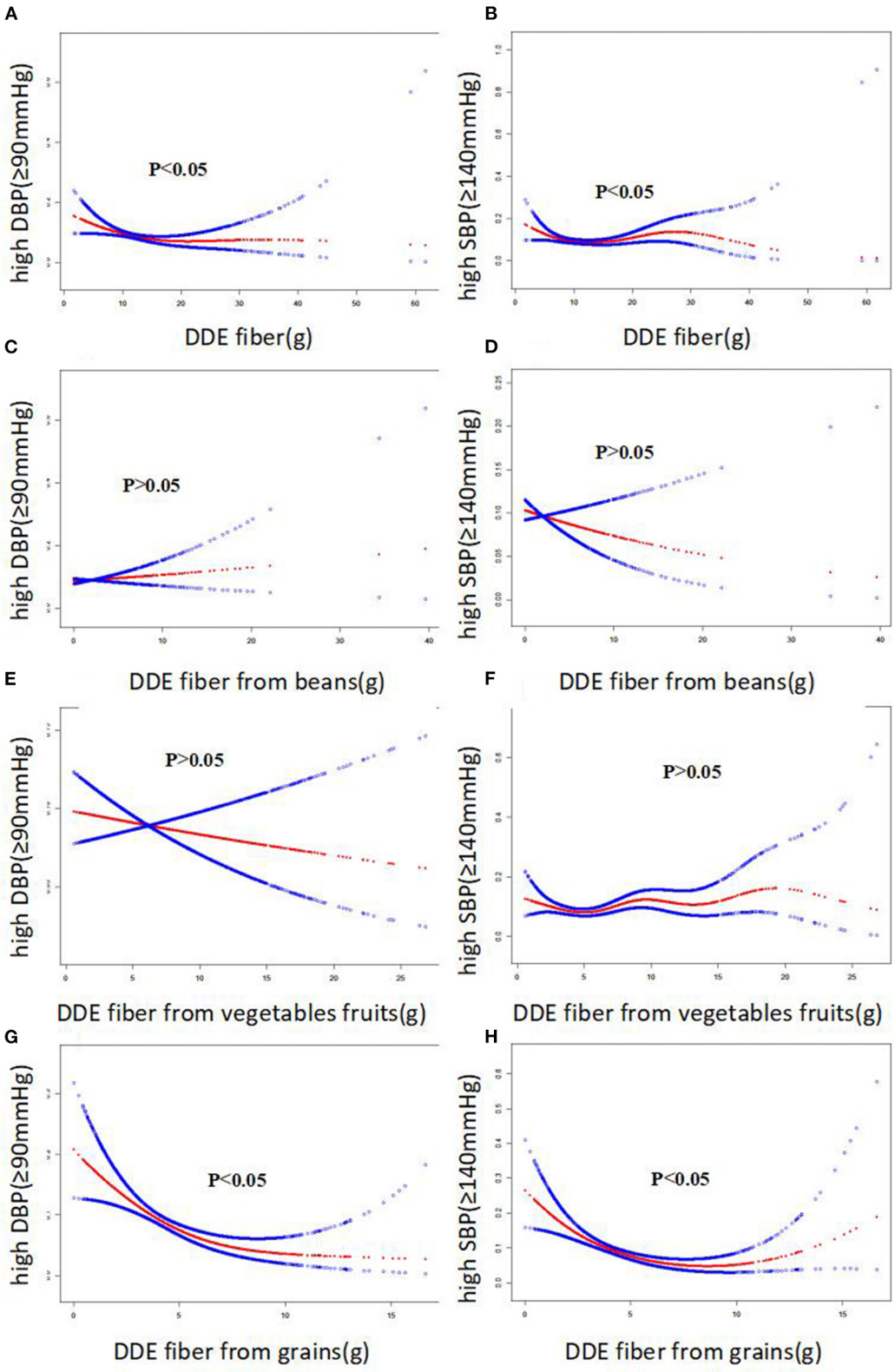

FIGURE 3 | The smooth curve of the associations between dietary fiber intake and risk of systolic and diastolic hypertension, respectively.

TABLE 2 | Multivariate linear regression on association of DDE fiber and blood pressure.

Variables	DBP (mmHg)				SBP (mmHg)			
	B	Sβ	B 95% CI	*P* Value	B	Sβ	B 95% CI	*P* Value
Crude								
DDE dietary fiber	−0.085	−0.047	−0.155, −0.015	0.018	−0.134	−0.046	−0.247, −0.020	0.021
DDE fiber from beans	−0.025	−0.006	−0.190, 0.140	0.766	−0.270	−0.039	−0.537, −0.003	0.048
DDE fiber from vegetables/fruit	−0.117	−0.040	−0.231, −0.003	0.045	−0.060	−0.013	−0.246, 0.125	0.523
DDE fiber from grains	−0.239	−0.050	−0.427, −0.052	0.012	−0.418	−0.054	−0.723, −0.113	0.007
Model 1								
DDE dietary fiber	−0.087	−0.048	−0.156, −0.017	0.015	−0.136	−0.047	−0.246, −0.027	0.015
DDE fiber from beans	0.002	0.000	−0.163, 0.166	0.985	−0.103	−0.015	−0.363, 0.156	0.435
DDE fiber from vegetables/fruit	−0.140	−0.048	−0.254, −0.027	0.016	−0.161	−0.034	−0.340, 0.018	0.077
DDE fiber from grains	−0.227	−0.047	−0.414, −0.041	0.017	−0.380	−0.049	−0.673, −0.086	0.011
Model 2								
DDE dietary fiber	−0.156	−0.087	−0.248, −0.065	0.001	−0.219	−0.075	−0.363, −0.075	0.003
DDE fiber from beans	−0.001	0.000	−0.175, 0.172	0.987	−0.079	−0.012	−0.352, 0.194	0.571
DDE fiber from vegetables/fruit	−0.175	−0.060	−0.305, −0.044	0.009	−0.148	−0.031	−0.353, 0.057	0.157
DDE fiber from grains	−0.491	−0.102	−0.744, −0.238	< 0.001	−0.882	−0.113	−1.279, −0.485	< 0.001
Model 3								
DDE dietary fiber	−0.109	−0.061	−0.200, −0.017	0.020	−0.062	−0.021	−0.200, 0.076	0.376
DDE fiber from beans	0.055	0.013	−0.116, 0.226	0.529	0.092	0.013	−0.166, 0.350	0.486
DDE fiber from vegetables/fruit	−0.137	−0.047	−0.266, −0.008	0.037	−0.023	−0.005	−0.218, 0.171	0.813
DDE fiber from grains	−0.382	−0.080	−0.633, −0.130	0.003	−0.528	−0.068	−0.907, −0.150	0.006
Model 4								
DDE dietary fiber	−0.103	−0.057	−0.194, −0.012	0.027	−0.062	−0.021	−0.199, 0.075	0.372
DDE fiber from beans	0.056	0.013	−0.115, 0.226	0.523	0.083	0.012	−0.174, 0.339	0.527
DDE fiber from vegetables/fruit	−0.135	−0.046	−0.263, −0.007	0.039	−0.035	−0.007	−0.228, 0.158	0.720
DDE fiber from grains	−0.349	−0.073	−0.600, −0.099	0.006	−0.469	−0.060	−0.846, −0.093	0.015

Crude: Adjusted for nothing. Model 1: Adjusted for age and race. Model 2: Adjusted for age, race, DDE fat, DDE carb and DDE protein. Model 3: Adjusted for age, race, DDE fat, carb, protein, smoking status, BMI. Model 4: Adjusted for age, race, DDE fat, carb, protein, smoking status, BMI, blood CRP, total cholesterol, triglycerides, LDL-C, HDL-C, glucose and insulin. DBP, diastolic blood pressure; SBP, systolic blood pressure; DDE, daily dietary estimate; BMI, body mass index. CRP, C-reactive protein; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

blood pressure began to be investigated in experimental animals. For example, previous evidence has suggested that increased intake of certain fibers has beneficial effects on improved blood pressure in rats (19). Obata et al. observed that one type of fiber, the husk of Psyllium seeds, can lower blood pressure in hypertensive rats who were fed a high-salt diet (19). A few years later, Li et al. performed a study on model rats who were fed corn starch, white rice, and rye for 16 weeks. They found that the rye diet lowered SBP significantly after 12 weeks of treatment. This diet also lowered serum levels of triglycerides, LDL cholesterol, and total cholesterol in the animals. They concluded that the intake of dietary fiber could contribute beneficial effects to blood lipids and blood pressure (20). Galisteo et al. also found a similar result in obese rats fed a diet containing 3.5% Plantago ovata for 25 weeks (21). They observed that consumption of a fiber-enriched diet could prevent hypertension, endothelial dysfunction, and obesity in rats. Recently, one study also reported that the short-term intake of cocoa fiber products could lower blood pressure in rats (22).

For the clinical studies, for example, Saltzman and his team investigated the role of oat fiber in hypertension (23). They observed that an oat-rich diet did contribute to a reduction in SBP without changing the levels of DBP (23). In addition, the diet also decreased the levels of blood lipids. Keenan et al. observed that oat cereal supplements could lower the need for improving arterial blood pressure and antihypertensive medication in hypertensive patients (24), and also found beneficial effects of oat fiber in hypertensive patients with grade 1 hypertension. These patients were given soluble oat fiber for 3 months, and this fiber led to a significant decrease in blood pressure (25). Consistent with these previous findings and mechanisms, we found that increased dietary fiber intake, especially DDE fiber from grains, is related to a lower risk of systolic hypertension and diastolic hypertension in midlife women. However, we did not find that increasing the intake of dietary fiber from beans and vegetables/fruit was associated with reduced hypertension risk. Elevated dietary fiber intake from grains contributed to a decreased risk of hypertension. This is a very interesting

TABLE 3 | Multivariate logistic regression on association of DDE fiber and risk of hypertension.

Variables	High DBP (≥90mmHg)			High SBP (≥140mmHg)		
	OR	95% CI	*P* Value	0R	95% CI	*P* Value
Crude						
DDE dietary fiber	0.981	0.956, 1.006	0.132	0.999	0.976, 1.022	0.909
DDE fiber from beans	1.018	0.967, 1.071	0.499	0.963	0.905, 1.026	0.246
DDE fiber from vegetables/fruit	0.980	0.941, 1.020	0.328	1.029	0.994, 1.066	0.108
DDE fiber from grains	0.895	0.834, 0.961	0.002	0.950	0.891, 1.013	0.120
Model 1						
DDE dietary fiber	0.981	0.957, 1.006	0.140	1.001	0.978, 1.025	0.948
DDE fiber from beans	1.031	0.981, 1.084	0.227	0.998	0.939, 1.060	0.942
DDE fiber from vegetables/fruit	0.973	0.934, 1.013	0.186	1.016	0.980, 1.053	0.398
DDE fiber from grains	0.901	0.840, 0.967	0.004	0.956	0.906, 0.996	0.039
Model 2						
DDE dietary fiber	0.971	0.938, 1.005	0.092	0.985	0.953, 1.017	0.341
DDE fiber from beans	1.036	0.983, 1.092	0.185	0.992	0.929, 1,058	0.799
DDE fiber from vegetables/fruit	0.972	0.928, 1.019	0.239	1.010	0.968, 1.053	0.652
DDE fiber from grains	0.823	0.748, 0.906	< 0.001	0.871	0.796, 0.954	0.003
Model 3						
DDE dietary fiber	0.983	0.950, 1.017	0.333	1.003	0.993, 1.037	0.837
DDE fiber from beans	1.051	0.998, 1.108	0.061	1.018	0.955, 1.085	0.585
DDE fiber from vegetables/fruit	0.982	0.937, 1.029	0.436	1.024	0.981, 1.069	0.280
DDE fiber from grains	0.845	0.767, 0.930	0.001	0.906	0.827, 0.992	0.032
Model 4						
DDE dietary fiber	0.984	0.951, 1.018	0.355	1.003	0.970, 1.037	0.859
DDE fiber from beans	1.053	0.999, 1.110	0.057	1.019	0.954, 1.088	0.580
DDE fiber from vegetables/fruit	0.982	0.937, 1.029	0.436	1.023	0.979, 1.068	0.308
DDE fiber from grains	0.848	0.770, 0.934	0.001	0.906	0.826, 0.993	0.034

Crude: Adjusted for nothing. Model 1: Adjusted for age and race. Model 2: Adjusted for age, race, DDE fat, DDE carb and DDE protein. Model 3: Adjusted for age, race, DDE fat, carb, protein, smoking status, BMI. Model 4: Adjusted for age, race, DDE fat, carb, protein, smoking status, BMI, blood CRP, total cholesterol, triglycerides, LDL-C, HDL-C, glucose and insulin. DBP, diastolic blood pressure; SBP, systolic blood pressure; DDE, daily dietary estimate; BMI, body mass index. CRP, C-reactive protein; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

TABLE 4 | Multiple linear regression analysis for relationship between DDE fiber from grains and blood pressure stratified by age, race, BMI and current smoker prespectively.

Subgroup	DBP (mmHg)			SBP (mmHg)		
	B, 95%CI	*P*	Interaction P	B, 95%CI	*P*	Interaction P
Age						
<46	−0.45 (−0.81, −0.10)	0.012	0.724	−0.58 (−1.12, −0.03)	0.037	0.629
≥46	−0.36 (−0.71, −0.01)	0.041		−0.76 (−1.30, −0.23)	0.005	
Race						
White	−0.04 (−0.39, 0.31)	0.817	0.125	0.09 (−0.44, 0.62)	0.736	0.14
No–white	−0.43 (−0.79, −0.07)	0.018		−0.85 (−1.39, −0.31)	0.002	
BMI						
BMI <25 (normal)	−0.41 (−0.82, −0.01)	0.045	0.85	−0.77 (−1.40, −0.14)	0.016	0.829
BMI≥25 (overweight)	−0.35(−0.67, −0.03)	0.030		−0.68 (−1.18, −0.19)	0.006	
Smoking status (current smoker)						
No	−0.32 (−0.59, −0.06)	0.017	0.37	−0.53 (−0.94, −0.13)	0.009	0.64
Yes	−0.56 (−1.03, −0.08)	0.022		−0.70 (−1.42, −0.01)	0.048	

Adjusted for age, race, DDE fat, carb, protein, smoking status, BMI, blood CRP, total cholesterol, triglycerides, LDL-C, HDL-C, glucose and insulin. DBP, diastolic blood pressure; SBP, systolic blood pressure; DDE, daily dietary estimate; BMI, body mass index. CRP, C-reactive protein; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

TABLE 5 | Multiple logistic regression analysis for relationship between DDE fiber from grains and risk of hypertension stratified by age, race, BMI and current smoker prespectively.

Subgroup	High DBP (≥90 mmHg)			HighSBP (≥140 mmHg)		
	OR, 95%CI	*P*	Interaction P	OR, 95%CI	*P*	Interaction P
Age						
<46	0.82 (0.71, 0.96)	0.010	0.898	0.73 (0.62, 0.86)	<0.001	0.009
≥46	0.84 (0.74, 0.95)	0.005		0.95 (0.85, 1.06)	0.334	
Race						
White	0.96 (0.80, 1.15)	0.652	0.225	1.00 (0.84, 1.19)	0.975	0.265
Others	0.84 (0.75, 0.94)	0.003		0.89 (0.80, 0.99)	0.035	
BMI						
BMI <25 normal	0.81 (0.65, 1.00)	0.050	0.716	0.74 (0.58, 0.93)	0.011	0.117
BMI ≥25 overweight	0.84 (0.76, 0.93)	0.001		0.90 (0.82, 0.99)	0.023	
Smoking status (current smoker)						
No	0.86 (0.77, 0.95)	0.005	0.450	0.92 (0.83, 1.01)	0.093	0.221
Yes	0.79 (0.65, 0.96)	0.020		0.81 (0.69, 0.96)	0.017	

Adjusted for age, race, DDE fat, carb, protein, smoking status, BMI, blood CRP, total cholesterol, triglycerides, LDL-C, HDL-C, glucose and insulin. DBP, diastolic blood pressure; SBP, systolic blood pressure; DDE, daily dietary estimate; BMI, body mass index. CRP, C-reactive protein; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

discovery, and more future studies may be necessary to explain the difference.

However, the potential antihypertensive mechanisms of dietary fiber intake have not been elucidated until now. Insulin resistance has been found to be associated with the development of hypertension, and dietary fiber intake might improve blood pressure by modulating insulin metabolism (26, 27). Reduced levels of serum cholesterol are associated with improved endothelial function, which mediates vasodilation and reduces blood pressure (28–30). The weight loss caused by dietary fiber has also been considered the underlying mechanism for the lowering of high blood pressure (31–33). However, these known mechanisms are not enough to explain the protective effect of dietary fiber intake on improving blood pressure. Therefore, more studies are necessary to fully elucidate new mechanisms.

Our study has several notable strengths. First, our study results were from the SWAN study, a multicenter and population-based study of the natural history of late midlife women. The SWAN study has high-quality data, including demographic characteristics, lifestyle, self-reported health, health examination, and medical history, with various sampling frames and recruitment strategies. Second, we comprehensively analyzed the relationship between the main sources of dietary fiber (beans, fruits/vegetables, and grains) and blood pressure. In this cross-sectional study, we are the first to find that increased intake of DDE fiber from grains, rather than the intake of DDE fiber from beans and fruits/vegetables, contributed to a reduced risk of hypertension in midlife women from the United States. Third, enough confounding factors, including demographic characteristics and lifestyle, and biochemical indices, were adjusted by multivariable analysis, which ensures the reliability of our results.

Of course, this study also has several limitations. On the one hand, our data did not distinguish between essential hypertension and secondary hypertension. The diagnosis of hypertension was determined by temporary blood pressure measurement at baseline in the SWAN study. The positive effect of dietary fiber on secondary hypertension caused by other diseases may be insignificant. On the other hand, the results of our study are only applicable to midlife women. The effect of dietary fiber on lowering blood pressure in men and different age groups (minors and the elderly) should be further confirmed in the future. In addition, although 3,302 subjects were included in the SWAN study, only 2,519 subjects were eventually included in our study because approximately 783 individuals were excluded due to missing data.

CONCLUSION

Our results further suggested that fiber intake, especially the DDE fiber from grains, is associated with the risk of hypertension after controlling for potential confounders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The institutional review boards at all sites have approved the study protocol and all included individual at each site gave informed consent.. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Excretion of Heavy Metals and Glyphosate in Urine and Hair Before and After Long-Term Fasting in Humans

Franziska Grundler[1,2], *Gilles-Eric Séralini*[3], *Robin Mesnage*[4], *Vincent Peynet*[5] and *Françoise Wilhelmi de Toledo*[1]*

[1] Buchinger Wilhelmi Clinic, Überlingen, Germany, [2] Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin and Berlin Institute of Health, Berlin, Germany, [3] Department of Biology and Network on Risks, Quality and Sustainable Environment MRSH, University of Caen Normandy, Caen, France, [4] Gene Expression and Therapy Group, Department of Medical and Molecular Genetics, King's College London, Faculty of Life Sciences and Medicine, Tower Wing, Guy's Hospital, London, United Kingdom, [5] Institut de Recherche et d'Expertise Scientifique, Europarc, Strasbourg, France

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Sebastian J. Hofer,
University of Graz, Austria

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Didac Carmona-Gutierrez,
University of Graz, Austria
Maria Virginia Soldovieri,
University of Molise, Italy

***Correspondence:**
Françoise Wilhelmi de Toledo
francoise.wilhelmi@
buchinger-wilhelmi.com

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Background: Dietary exposure to environmental pollutants in humans is an important public health concern. While long-term fasting interrupts the dietary exposure to these substances, fat mobilization as an energy source may also release bioaccumulated substances. This was, to our knowledge, only investigated in obese people decades ago. This study explored the effects of 10-days fasting on the excretion of heavy metals and glyphosate.

Methods: Urinary levels of arsenic, chromium, cobalt, lead, nickel, mercury and glyphosate were measured before and after 10 fasting days in 109 healthy subjects. Additionally, hair analysis was done before and ten weeks after fasting in 22 subjects.

Results: Fasting caused a decrease in body weight, and in urinary arsenic (by 72%) and nickel (by 15%) concentrations. A decrease in lead hair concentrations (by 30%) was documented. Urinary mercury levels were unchanged for chromium, cobalt and glyphosate, which were undetectable in most of the subjects. Additionally, fatigue, sleep disorders, headache and hunger were reduced. Body discomfort symptoms diminished four weeks after food reintroduction.

Conclusions: The results of this study provide the first insights into the changes in heavy metal excretion caused by long-term fasting. Further studies focusing on the kinetics of efflux between different compartments of the body are needed.

Clinical Trial Registration: https://www.drks.de/drks_web/navigate.do?navigationId=trial.HTML&TRIAL_ID=DRKS00016657, identifier: DRKS00016657.

Keywords: Buchinger Wilhelmi fasting, weight loss, arsenic, nickel, lead, glyphosate, biomonitoring

INTRODUCTION

The exposure to heavy metals and pesticides in human populations has increased over the last decades. The main source of contamination by these substances is environmental pollution caused by anthropogenic activities. This includes industries like petroleum refineries, mining, electroplating, painting, or the production and use of synthetic chemicals like pesticides and fertilizers (1). When they are lipophilic, environmental pollutants can also bioaccumulate along the food chain making the diet a significant contributor to human exposure (2).

Heavy metals are defined either according to their high atomic weight or to their high density (above 5 g/cm^3). They are naturally deposited in the ground water and soil (3), and also enter the life cycle and organisms through contaminated food intake (2). Small quantities of metals such as zinc, nickel or cobalt are essential for physiological functions in the human body, e.g., as co-factors of key enzymes or oxidation-reduction reactions (4). However, exposure to higher concentrations through oral ingestion, dermal contact or inhalation can be toxic for human health (3). Some heavy metals accumulate in the body and are stored in various tissues, including the adipose tissue (5). They can also be found in hair (6, 7). Heavy metals are non-biodegradable (3). Toxic levels of arsenic, cadmium, chromium, cobalt, lead, nickel and mercury can trigger DNA damages and structural changes of cellular components either directly through interacting with the organic molecules, or indirectly through production of reactive oxygen species (ROS), leading to various diseases such as cancer, neurological abnormalities, cardiovascular diseases, hormonal diseases and infertility (3). For instance, a recent study showed that urinary arsenic and cadmium associate with vascular brain injury (8). The toxicity mechanisms common to metals and determining their toxicity is the generation of oxidative stress by the production of reactive oxygen and nitrogen species (9).

The exposure to some pesticides has been shown to cause adverse health effects in human populations after acute intoxications (10), repeated occupational exposures (11), or environmental exposures during sensitive periods of the development like pregnancy (12). The toxicity of some pesticides can be amplified by the presence in formulations of other compounds with hazardous properties, as in the case of glyphosate (13, 14). Recent studies also reported that some glyphosate formulations are contaminated by polycyclic aromatic hydrocarbons and heavy metals (1, 15). We focused on glyphosate which is the main declared ingredient used in pesticide formulations worldwide (16). It is generally admitted that approximately 20% of the absorbed glyphosate is excreted in urine after exposure to high concentrations in animal studies. However, recent studies bring contradictory results concerning dietary exposure in humans. It is thus still unclear how the urinary excretion of glyphosate reflects the daily intake (17–19).

The use of dietary supplements in so-called "detox diets" is frequently advocated to mitigate the adverse effects of toxic exposures. Molecular mechanisms of xenobiotics detoxification are well-characterized (20). The detoxification of xenobiotics is a multistep process occurring in different tissues, predominantly in the liver. The different steps consist in a succession of enzymatic reactions which activate (mostly by redox reactions), conjugate and excrete xenobiotics. While some evidence is available to support the detoxifying properties of some plants in laboratory animals (21), limited evidence supports the claim for beneficial effects of detox diets because they are generally not tested with properly designed clinical trials in humans (22). Only one study describes toxic trace element detoxification caused by a switch to an organic plant based diet in a multi-armed randomized clinical trial (23). In addition, some herbal remedies produced with poor-manufacturing practices can also cause liver injuries (24) because they are frequently contaminated with heavy metals (25). The most effective approach to mitigate toxic effects of environmental pollutants is to provide a diet certified free of pollutants. A switch to an organic diet is also sometimes advocated to have health benefits because the consumption of fruits and vegetables with high levels of pesticide residues has been linked to various adverse health outcomes or poor semen quality (26).

Fasting treatments are often associated in the public for their detoxifying properties but only few studies document it. Some of them observed long-term fasting, defined as voluntary interruption of food intake for at least 2 days up to several weeks (27). A general improvement of health in subjects following this fasting program was found (28–31). Moreover, an increase in antioxidant capacity and reduced lipid peroxidation was documented in the cohort we describe in this article (32, 33). However, it was still unclear whether fasting has a measurable detoxifying effect. In this study, we analyzed the presence of heavy metals and glyphosate in urine before and after a 10-days fasting period. Additionally, since metal concentration in hair correlate well with blood levels they are thus known to be a reliable biomonitoring strategy (34). We proceeded to an exploratory hair analysis in a subgroup of subjects.

MATERIALS AND METHODS

Ethics Statement

This prospective, observational study was conducted in accordance with the Declaration of Helsinki. The medical council of Baden-Württemberg, Stuttgart, approved the study protocol on 12 February 2019 (application number F-2018-118) and it was registered on 20 February 2019 in the German Clinical Trials Register (DRKS-ID: DRKS00016657). Subjects were recruited between 15 September 2019 and 18 November 2019. All participants gave their written informed consent before enrolling into the study. After the 10-days fasting period at the clinic, an online follow-up took place 4 weeks after the fasting treatment.

Participants

Participants had to fulfill the following inclusion criteria as described previously (32, 33) in order to be enrolled: subjects had to be between 18 and 70 years old, and underwent a fasting treatment of 10 $\pm$ 3 days. A 10-days fasting period showed in previous studies beneficial health effects (28, 29). A laboratory analysis including blood and urine sampling was done at the start, and a second sampling at the end of the fasting. The intake of

micronutrient supplements was advised to be stopped already 1 week before and during the fast. An exception was made for the mineral magnesium in the form of magnesium citrate (29%) and magnesium oxide (26%), because the clinical routine has shown that the supplementation during fasting minors the risk of muscle cramps that could be provoked by high amounts of liquid intake (3 L per day, see protocol) during fasting. Medical contraindications for fasting led to exclusion as described in the guidelines of fasting therapy (35). Participants had to speak German, English or French, to understand the questionnaires, and not to participate in another study.

The Fasting Protocol

The fasting protocol was precisely and previously described (29). It was conducted under medical supervision and according to peer-reviewed guidelines (35). One-day prior starting the fast, a transition day with a simplified 600 kcal organic diet was completed. The initiation of the fasting period started with the administration of a laxative (20–40 g Na2SO4 in 500 ml water). During fasting subjects received daily 250 ml organic juice at midday, 20 g honey, and 250 ml vegetable soup in the evening, leading to a daily calorie intake of ∼250 kcal. It was recommended to drink at least 2–3 L of water or non-caloric, organic herbal teas. The reintroduction of food occurred stepwise from 800 to 1,600 kcal/day with a vegetarian organic diet. Pesticides were not used in the clinic. The fasting program also included physical exercise and individual physiotherapy (29).

Clinical Data

Before participating in the fasting program, the subjects were thoroughly examined by a medical doctor. Two examinations were performed in the morning during the fasted state. The baseline examination took place before initiating the fasting period, and the second examination was conducted at the end. All clinical data were captured according to the BWC standards. The body height was assessed with seca 285 (Seca, Hamburg, Germany) and the waist circumference was measured with a measuring tape, placed halfway between the lowest rib and the iliac crest. Body weight was measured (with Seca 704/635, Seca, Hamburg, Germany) by trained nurses, while subjects wore light clothing. The medical team documented possible adverse events in a report form.

Blood, Urine and Hair Collections and Analysis

Blood and urine samples were collected in the first morning after arrival, and at the 10 ± 3 fasting day. Routine laboratory blood parameters were measured in the laboratory MVZ Labor Ravensburg as previously described (32, 33). Urine samples were collected from the first urine in the morning. The quantification of the heavy metals arsenic, chromium, cobalt, lead, nickel, and mercury in urine, as well as glyphosate, was done in the Medical laboratory Bremen (Bremen, Germany). Heavy metals were quantified with inductively coupled plasma mass spectrometry (ICP-MS) with a detection limit of 0.1 μg/l as previously described (36). Glyphosate was quantified with gas chromatography (GC) with tandem mass spectrometry (GC-MS-MS) to reach levels of detection of 0.1 μg/l as described in (37). In brief, glyphosate was derivatized with a mixture of trifluoroacetic anhydride and trifluoroethanol before separation using a Agilent Technologies GC system 7890. Quantification was then performed with an Agilent 7000 mass spectrometer (MS-MS) operated in negative ion mode.

Hair samples were collected from a subgroup of subjects who did not have colored hair at the beginning of the fast, and 10 weeks afterwards. The second time point was defined after 10 weeks taking into account the growth rate of hair (1 cm/month), the needed time to grow from the follicle to the scalp (2 weeks) and the approx. required 2 cm hair samples for analysis. For sample collection either a strand of hair was cut as close to the scalp as possible, different spots from the head were possible, or body hair (armpits or pubic hair) was collected. The baseline samples with head hair were collected by trained study staff. Body hair samples as well as the second sampling was done by the participants themselves, using a special collection kit provided by the laboratory. The collected hair samples lengthways into the aluminum foil and the cut ends (ends of the hair nearest the scalp) were placed on the narrow part of the foil. Samples were sent to IRES laboratory (Strasbourg, France) for heavy metal analysis.

Hair samples were cut to keep 3–4 cm proximal segment (closest to the scalp). Hair strands were accurately weighted (ca. 100 mg) in polypropylene tube. Hair were then digested with 70% nitric acid (HNO3 trace metal analysis grade) under oxidative condition with hydrogen peroxide (H2O2, 30%). Digestion of hair was performed in ultrasonic bath for 2 h at 60°C and overnight incubation at 60°C. Extracts were diluted with ultrapure water (Millipore) and analyzed by ICP-MS with a PlasmaQuant MS from Analytik Jena for quantification of arsenic, chromium, Cobalt, lead, nickel. Limits of detection (LOD) in water ranged from 0.21 to 0.53 μg/l, while the limits of quantifications (LOQ) ranged from 0.95 to 2.51 μg/l. Uncertainty calculated within two standard deviations (k2) for these measures was 9.6%. Quantification of mercury was done by Atomic Absorption Spectrometry (AAS) with a RA-4300 Mercury Analyser from Environsciences GmbH. In water, LOD was 0.026 μg/l, and LOQ was 0.126 μg/l. Uncertainty calculated within two standard deviations (k2) for mercury was 15.8%. These corresponded to LOQ in hair of 0.15 ng/mg for arsenic, chromium, cobalt, nickel, lead and 0.015 ng/mg for mercury.

Self-Reported Data

Self-reported data were captured during the stay in a diary to record the symptoms during the fasting period and an additional questionnaire complemented the life style before and 4 weeks after to approach the long-term effects after the fasting period. At baseline, and 4 weeks after fasting, the subjects completed the medical questionnaire (38) to assess their symptoms experienced in the last 4 weeks. All items were rated from 0 (never or almost never have the symptom) to 4 (frequently have it, effect is severe). The overall sum of each item allowed a ranking into three scores: low symptoms (0–14), moderate symptoms (15–49) and high symptoms (>50). The evolution of the intensity for six frequently mentioned symptoms during long-term fasting

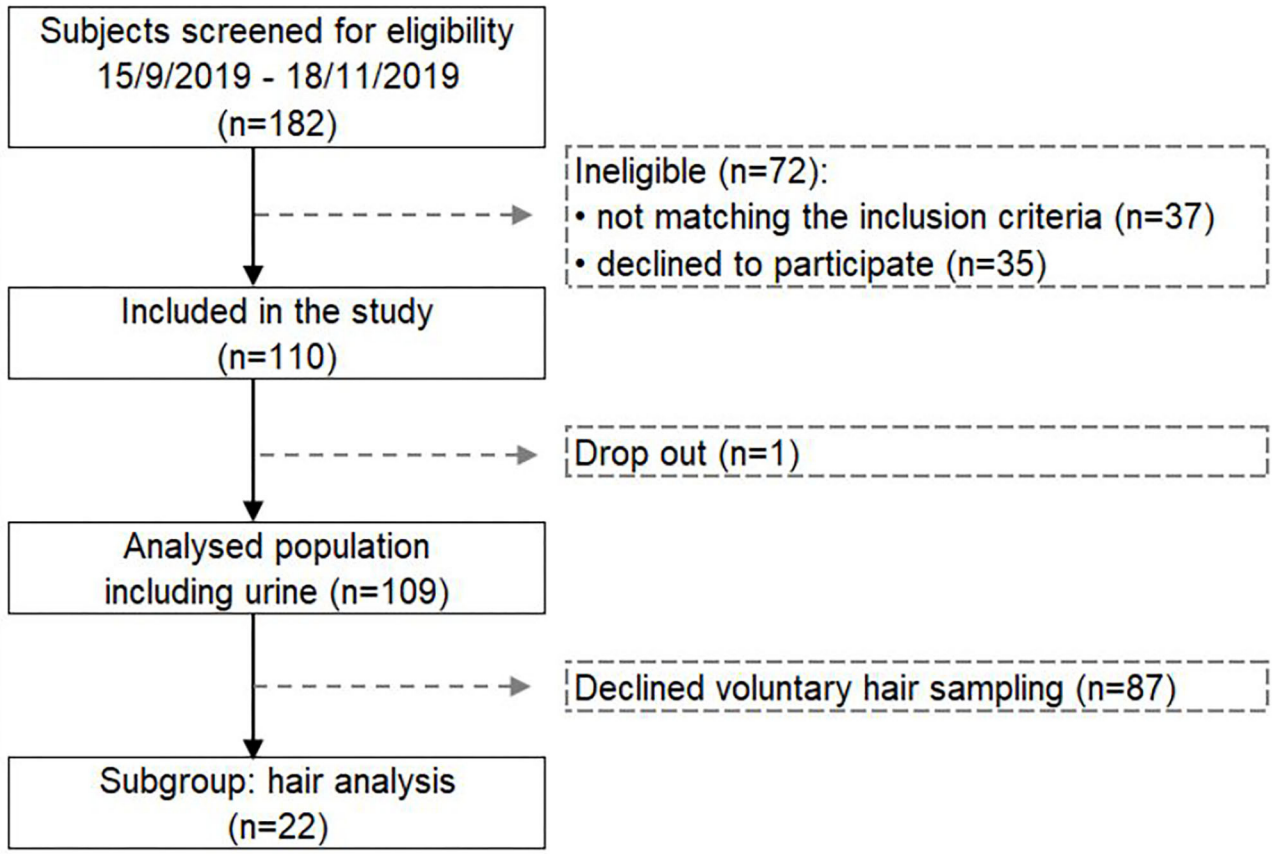

FIGURE 1 | Flow chart presenting the selected study population for measuring the changes in heavy metals and glyphosate excretion during long-term fasting.

(29): fatigue, headache, sleep disorders, hunger, back pain and nausea, was documented daily on numeric self-rating scales from 0 (none) to 10 (very much).

Subjects indicated before and 4 weeks after fasting if they consumed in the last 2 weeks organic food by choosing one of the following categories: no, little, quite often and almost always. The smoking habits were indicated by the subjects before and after fasting and after 4 weeks.

Statistical Analysis

All the statistics were performed with R version 4.0.0. We treated missing values (below threshold of detection) as recommended by the European Human Biomonitoring Initiative (HMB4EU), described in Harel et al. (39). When the proportion of missing values was below 20%, statistical significances were evaluated with the package lmerTest, with linear-mixed models using sex and BMI as a covariate, and the individual identifiers of the subjects as a random effect. For the metabolites for which more than 80% of observations were below the LOQ, we used a mixed effects logistic regression model (R package lme4) with the samples dichotomized as detected/undetected. *P*-values were not calculated when the number of subjects with values above LOQ were below 20%. Summary statistics values were estimated using the maximum likelihood inference for left-censored values when the proportion of missing values was below 80%. The maximum likelihood was estimated using the function cenmle() from R package NADA. When the proportion of samples with missing values were over 80%, the proportion of samples over the limits of detections were indicated. Differences at baseline between the whole cohort and the hair analysis subgroup were evaluated using a *t*-test for continuous variables or a chi-square goodness of fit test for categorical variables. Correlations were calculated using Spearman's rank-order correlation in R.

RESULTS

Out of the 182 screened subjects, 35 subjects declined to participate and 37 subjects did not meet the inclusion criteria, as described previously (32, 33). In total, 41 men and 68 women with an average age of 56.7 ± 10.4 years participated in the study (**Figure 1**). The study population was metabolically healthy before, during and after this long-term fasting study. Only one person had to stop the fasting due to low hemoglobin and sodium levels.

The 10-days fasting period led to a mean weight loss of 4.9 ± 1.9 kg ($p < 0.001$; **Table 1**). Four weeks after fasting the weight

loss was 6.7 ± 11.9 kg ($p < 0.001$). At baseline, 28.4% of the subjects almost always consumed organic food, 35.8% quite often, and 25.7% declared they eat little organic food. 8.3% mentioned not to eat organic products. Four weeks after fasting the number of subjects eating organic food had increased significantly ($p = 0.047$). In total, 40.4% almost always consumed organic food, 33.9% quite often, and 11.9% little. Only 6.4% indicated not to eat organic food (**Table 1**). Nineteen subjects (17.4%) indicated to smoke at baseline (**Table 1**). Out of them only 2 (0.02%) smoked at the end of fasting and 7 after 3 months (0.06%). These results suggest that after voluntary long-term fasting according to this program health consciousness in general has improved.

Heavy Metals and Glyphosate in Urine

Heavy metals and glyphosate excretion was measured in urine before and after long-term fasting in each subject.

At baseline, out of six heavy metals measured, only arsenic was detected in all 109 subjects (**Table 2**). After fasting it was detectable in 104 subjects and the concentration of urinary arsenic levels significantly decreased by 71.5% ($p < 0.0001$). Nickel was present in nearly half of the cohort at baseline and in 28 subjects afterwards. Urinary nickel levels decreased also significantly by 14.6% ($p = 0.004$). In one-third of the cohort ($n = 40$) mercury was over the threshold at baseline, and in 29 subjects after fasting, but the urinary levels remained comparable after fasting (4.3%). Lead was found in one-quarter of the cohort ($n = 26$), and was in only 13 subjects detectable afterwards. The urinary lead levels decreased and were not detectable after fasting. Cobalt and chromium were detected in only a few subjects before and after fasting with urinary levels below quantification.

Nine subjects excreted glyphosate before fasting with levels below quantification. After fasting glyphosate was undetectable in all subjects. A global decrease for most measured compounds in urine is visible in the heatmap (**Figure 2**).

We also examined correlations between heavy metal concentrations in urine and the BMI as previous studies found that environmental pollutant concentrations in serum are proportional to the weight loss. Arsenic levels were correlated to the BMI of the patients (rho = 0.20, $p = 0.0045$). However, the changes in arsenic levels were not correlated to the weight loss (rho = 0.16, $p = 0.11$). This suggested that the excreted arsenic was stored in fat tissue, and that individuals with a high proportion of body fat have higher impregnation by arsenic, but also that the intensity of the weight loss might have limited relationship with the elimination of arsenic. No relationship between weight loss or BMI were found with the other heavy metals measured.

TABLE 1 | Baseline characteristics of the study population as well as weight changes, organic food consumption and smoking habits after fasting.

Fasting effects	Before	After 10 days	After 4 weeks
BMI, kg/m²	28.3 ± 6.0	27.7 ± 5.2***	26.9 ± 5.7***
Weight, kg	82.9 ± 18.8	79.9 ± 16.1***	74.4 ± 14.2***
Estimated organic food consumption, *n* ($p = 0.047$)			
Almost always	31	Organic	44
Quite often	39	during	37
Little	28	food	13
No	9	reintroduction	7
Smoking, n	19	2	7

*After 10 days the subjects stopped fasting, underwent in average a 3-days controlled organic food reintroduction period, and left the clinic. P-values are p < 0.001***.*

Heavy Metals in Hair

Out of the whole cohort, 22 subjects accepted to participate in an exploratory hair analysis. Their mean age of 52.1 ± 12.3 years was lower than that of the whole cohort ($p = 0.023$), less women participated (40.9%; $p = 0.015$) and the subjects had a lower baseline BMI (25.8 ± 4.1 kg/m²; $p = 0.026$) compared with the whole cohort.

Hair samples were collected at the beginning and 10.0 ± 2.7 weeks after fasting in order to wait for the hair growth. Arsenic was not detectable in all hair samples before and after fasting (**Table 3**). Nickel was present in 2 subjects at baseline and in 5 subjects after fasting. At baseline, nickel concentrations in hair were below the quantification level but reached a level of 0.51 ± 0.30 ng/mg 10 weeks after fasting. Mercury could be quantified in all 22 subjects before and after fasting with stable concentrations

TABLE 2 | Environmental pollutants before and after long-term fasting in urine.

All subjects = 109	Before			After			Change
Metabolite, LOQ	***n***	**Mean ± SD**	**Max**	***n***	**Mean ± SD**	**Max**	***p*-value**
Arsenic, ≥1 μg/l	109	28.7 ± 47.5	354.1	104	7.2 ± 8.4	180.7	***
Chromium, ≥1 μg/l	2	ND	7.9	3	ND	13.8	ND
Cobalt, ≥1 μg/l	5	ND	51.7	6	ND	71.7	ND
Lead, ≥1 μg/l	26	1.6 ± 0.4	2.4	13	ND	1.8	ND
Mercury, ≥1 μg/l	40	2.3 ± 1.2	6.2	29	2.4 ± 1.1	7.1	NS
Nickel, ≥1 μg/l	50	2.1 ± 1.0	6.9	28	1.8 ± 0.8	3.8	**
Glyphosate, ≥0.1 μg/l	9	ND	0.9	0	ND	ND	ND

*The heavy metals measured are presented in alphabetical order in μg/l. Glyphosate, as a marker of the main pesticide of the world, is also indicated in μg/l. The total number of subjects is 109; the number of subjects (n) above the level of quantification is given for each parameter. ND, not determined; NS, not significant. P-values < 0.01 are indicated as **; p < 0.001 as ***.*

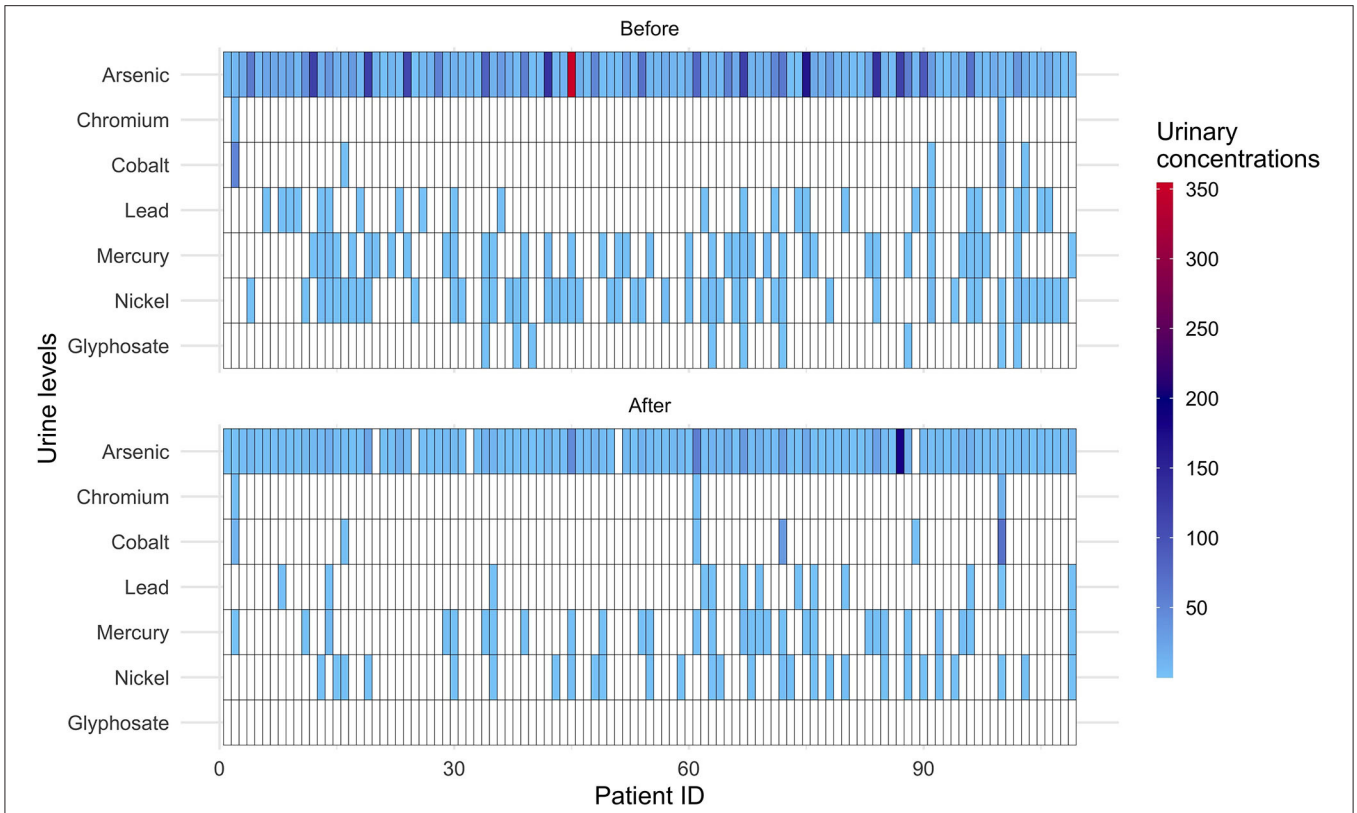

FIGURE 2 | Heatmap displaying the individual excretion of heavy metals and glyphosate before and after 10-days fasting. The color scale shows the urinary levels (μg/l) of heavy metals and glyphosate. Data below detectable levels are indicated in white. Patient ID: individual identification numbers (in total 109).

TABLE 3 | Environmental pollutants in hair before the 10-days fasting period and 10 weeks afterwards.

All subjects = 22	**Before**			**After 10 weeks**			**Change**
Metabolite, LOQ	***n***	**Mean ± SD**	**Max**	***n***	**Mean ± SD**	**Max**	***p*-value**
Arsenic, ≥ 0.15 ng/mg	0	ND	ND	0	ND	ND	ND
Chromium, ≥ 0.15 ng/mg	19	0.35 ± 0.11	0.61	19	0.39 ± 0.12	0.72	NS
Cobalt, ≥ 0.15 ng/mg	1	ND	0.25	2	ND	0.24	ND
Lead, ≥ 0.15 ng/mg	21	1.08 ± 0.98	3.71	20	0.76 ± 0.62	2.60	*
Mercury, ≥ 0.015 ng/mg	22	0.79 ± 0.70	2.90	22	0.81 ± 0.93	3.99	NS
Nickel, ≥ 0.15 ng/mg	2	ND	0.76	5	0.51 ± 0.30	0.97	ND

*The heavy metals measured are presented in alphabetical order in ng/mg. On 22 subjects the number of them (n) above the LOQ is given for each parameter. ND, not determined; NS, not significant. P-values < 0.05 are indicated as *.*

(2.5%). Before fasting lead was detectable in all subjects except one and in 20 subjects after fasting. The lead concentration in hair decreased significantly by 29.6% ($p = 0.036$). Cobalt was only detected in one subject before and two subjects after fasting with concentrations below quantification levels. Chromium was detected in 19 out of the 22 subjects before and after fasting. The chromium concentration remained comparable (11.4%).

Self-Reported Symptoms

The most frequent self-reported symptoms at baseline was fatigue with 4.1 ± 2.8 points (**Figure 3A**; **Table 4**), followed by sleep disorder with 2.5 ± 1.6 points (**Figure 3B**) and headache with 2.0 ± 2.9 points (**Figure 3C**) as well as hunger with 2.0 ± 2.4 points (**Figure 3D**). Fasting decreased all of them significantly: fatigue by 68.4%, sleep disorder by 35.6%, headache by 96.1%, and hunger by 60.6%. Back pain and nausea (**Figures 3E,F**) were less frequent and unchanged during fasting.

The results of the medical symptoms questionnaire indicate, based on 66 items and the specific duration of the symptoms, the symptom profile as high (>50 points) in 23.9%, moderate (15–49 points) in 59.6% and low (<14 points) in 13.8%. Four weeks after fasting high symptoms score were found in only 11.9%, moderate

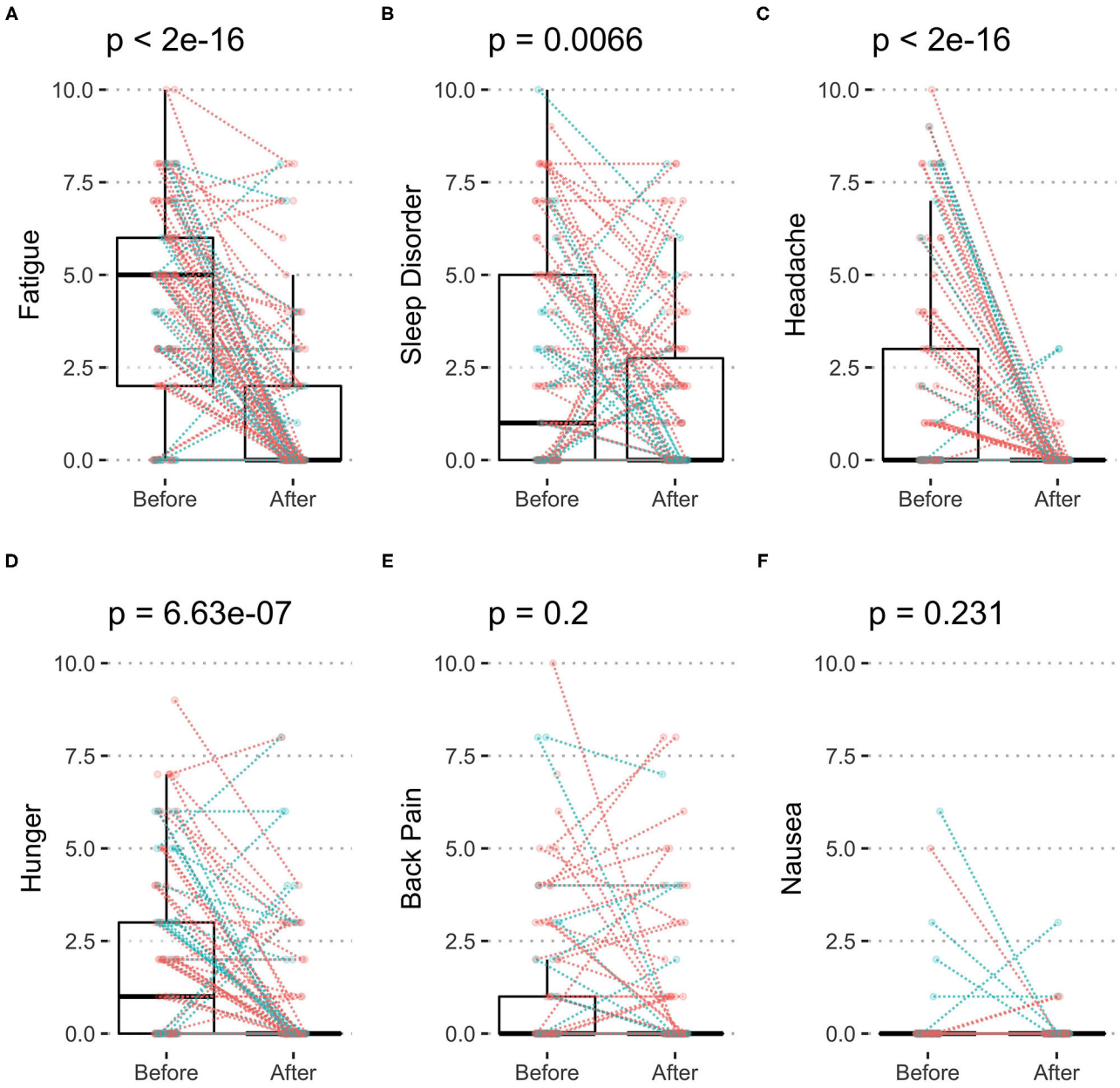

FIGURE 3 | Symptoms of body discomfort before and after 10-days fasting. The individual changes (women in red and men in blue) as well as box plots indicating median and quartiles are presented for fatigue **(A)**, sleep disorder **(B)**, headache **(C)**, hunger **(D)**, back pain **(E)**, and nausea **(F)**.

50.5% and low 30.3%. There was a shift from high and moderate symptoms score to lower score groups, indicating a decrease of these symptoms after fasting ($p = 0.002$; **Table 4**).

DISCUSSION

Chronic exposure to environmental pollutants such as heavy metals and pesticides increasingly influences human health (40). We measured how a 10-day fasting period, where virtually no food was absorbed, influenced the excretion of heavy metals and glyphosate. At baseline, heavy metals were detectable in urine, and their concentration reduced by the fasting intervention. Furthermore, the occurrence of symptoms of chronic body discomfort was improved.

Fasting is defined by the temporary cessation of food intake (27). Consequently, the oral ingestion of possibly contaminated food is stopped. The metabolism switches to the usage of fat and ketones, using endogenous energy reserves (27, 41). Urinary levels of heavy metals or pesticides mainly reflect their exposures in the last few days (42). It is known that arsenic

TABLE 4 | Self-reported symptoms before and after 10 fasting days and self-estimated chronic body discomfort before and after 4 weeks of food reintroduction.

Fasting effects	Before	After 10 days	After 4 weeks
Self-reported symptoms, mean ± SD			
Fatigue	4.1 ± 2.8	1.3 ± 2.1***	ND
Sleep Disorder	2.5 ± 2.9	1.6 ± 2.3**	ND
Headache	2.0 ± 2.9	0.1 ± 0.4***	ND
Hunger	2.0 ± 2.4	0.8 ± 1.7***	ND
Back pain	1.0 ± 2.0	0.8 ± 1.8	ND
Nausea	0.2 ± 0.8	0.1 ± 0.3	ND
Estimated chronic body discomforts, *n* ($p = 0.002$)			
Elevated	26	ND	13
Moderate	65	ND	55
Low	15	ND	33

*ND, not determined. P-values are <0.01** and p < 0.001***.*

and nickel are rapidly absorbed through the gastrointestinal tract (3). Long-term fasting thus interrupts the exposure to dietary pollutants which probably explains at least partly why urinary concentrations of some heavy metals were reduced in our study. Furthermore, the fasting was conducted in BWC, a setting where the exposure to air pollutants from transport or residential exposures to pesticides is very low.

Long-term fasting at BWC can be considered as a health strategy (29) to avoid external contamination with potentially toxic chemicals, because it interrupts the dietary intake of environmental pollutants like heavy metals or glyphosate in this study. However, whether it detoxifies the body in the sense that it decreases pollutant body burden cannot be determined in this study. Some of the heavy metals investigated in this study could have been bioaccumulated from past exposures (2). A recent study demonstrated the presence of nickel, lead, tin, titanium, in 228 adipose tissue samples of humans (43). Mercury was found in half of these same samples. Long-term fasting leads to a significant weight and fat loss, and a reduction in waist circumference, reflecting a decrease in visceral fat tissue (29, 44). It could be hypothesized that pollutants that were accumulated in the fat tissue are slowly released during long-term fasting. In another study, calorie restriction-induced weight loss provoked a release of persistent organic pollutants from adipocytes and increased their levels in the circulation (45). It was reported that the increase of toxic chemicals in serum is proportional to the weight loss (45). A weight loss of 10% was related to an increase of persistent organic pollutants up to 20% in blood over 4–6 months (46, 47). In this study, a weight loss by 3.6% was reached after 10 fasting days and by 10.3% after 4 weeks.

We did not document an enhanced excretion of heavy metals or of glyphosate in urine. The excretion of heavy metals in urine may have been different during the first days of the treatment. This will have to be investigated. By contrast, urinary levels for these compounds were decreased in some cases. It is possible that the decreased dietary intake could have masked the elimination. The high level of water daily ingested may have also contributed to heavy metal elimination since significant proportion of arsenic can be excreted in urine after ingestion (48). Clinical pharmacokinetics includes four components, namely the absorption, distribution, metabolism and excretion (ADME). Our study only evaluated excretion and further studies would be needed to achieve a comprehensive evaluation of chemical pollutant pharmacokinetics during fasting. In comparison with 102 Germans the load of heavy metals in urine seemed to reflect a healthy study population (36). The determination of heavy metal burden directly measured by adipose tissues biopsy could be a suitable way to further investigate changes in bioaccumulation. Our study consisted of a snapshot of urinary pollutants excretion after 10 fasting days. Another option could be to measure pharmacokinetics at different time points to identify the kinetics of elimination. Altogether, little is known about the kinetics of accumulation and washout of these compounds from hair or adipose tissues.

Long-term fasting caused a substantial weight loss in all subjects. On the one hand, weight loss can increase blood levels of bioaccumulated pollutants which are released into the bloodstream as the adipose tissue is used as a source of energy (49). On the other hand, heavy metal body burden can contribute to variations in human weight loss. Body fat percentage correlated with blood levels of lead, cadmium and mercury in a Korean Adult Population (50). Body burdens of lead, cadmium, cobalt, and cesium negatively associated with obesity, while positive association were detected with barium and thallium, in the National Health and Nutrition Examination Survey (NHANES) from 1999–2000 to 2001–2002 (51, 52). A possible explanation is that heavy metals generated metabolic and endocrine disruptions (53). This is also suggested in our study by the positive correlation between arsenic levels and the BMI of the patients. Although it is unclear whether fasting would cause an acceleration of xenobiotics metabolism, leading to a detoxification, our previous studies showed that fasting can enhance protection against cellular damages caused by ROS production (32, 33). In the present cohort, we demonstrated that the total antioxidant capacity significantly increased after 10 fasting days (32, 33).

The decrease in the rate of oxidative damages, and the increase potential for protection against these damages, may explain why we observed that symptoms like fatigue, sleep disorder, headache and hunger, decreased during long-term fasting, indicating that the subjects felt well. The occurrence and intensity of nausea and back pain were at very low levels. As previously described, the well-being of the participants, together with the acceptance and compliance to the protocol, are key elements for a successful fasting procedure (27, 29). Life style changes are also recommended. Studies have demonstrated that long-term fasting is often accompanied by a change toward a healthier life style including an improved eating behavior and an increased physical activity (54). In this study, we also observed changes in life style habits like the enhanced organic food consumption. In general, organic food consumers tend to have a higher awareness toward a healthier life style which explains why large epidemiological studies often found that the consumption of an organic diet correlates with beneficial health

effects (55). The lower intake of food contaminated with residues of pesticides including also heavy metals or antibiotics could participate to this health effect although a direct causal link has not been demonstrated. The consumption of organic food further comprises a predominantly plant based nutrition with a conscious selection of dairy products and meet from organic husbandry (55). The described changes in nutritional habits could contribute to the observed maintenance of the reduced BMI 4 weeks after fasting. The diminished contamination after fasting due to life style changes could contribute to lower self-reported body discomforts. However, the establishment of a causative link between the decreased exposure to pollutants and the reduction of symptoms of chronic body discomfort could only be hypothesized, and would need further investigation.

Urinary glyphosate was found in only 8.3% of the subjects before fasting. This low detection frequency in comparison to other assessments (56, 57) may be explained by the higher specificity of the mass spectrometry used in this study (37) or the general healthy life style of the subjects. Some other published analyses have used immunodetections (i.e., ELISA) which could have led to more artifacts by cross reactions with other compounds since the most commonly used ELISA assay for glyphosate has never been fully validated for human urine. It is also possible that this cohort of health-conscious subjects and their general healthy life style makes them less exposed to glyphosate.

Hair samples can provide more long-term information about the exposure to toxic compounds (58). Reference values for metal content in hair have been reported for healthy individuals in **Supplementary Table 1** (59). These reference levels were comparable to the metal concentrations found in the present study group. Arsenic, cobalt, and nickel were mostly below the detection limit in hair samples in this study, which suggests that these elements are rapidly from the body through urine (3). Hair levels of mercury and chromium were unchanged, whereas lead levels significantly decreased. The decrease in lead concentrations may reflect the lower dietary exposure as food intake was interrupted during fasting. In another study, a calorie reduced diet for 4 weeks in 15 subjects led to a significant decrease of arsenic in hair (23).

Limitations of the study include the high amount of non-detectable values, pointing to the need of more sensitive technologies and a higher number of participants. In addition, a closer monitoring of the kinetics could bring further insides into the excretion of heavy metals and glyphosate, or other pesticides. Nevertheless, this "before–after" analysis lays a good foundation for further studies, which should compare levels of xenobiotics in different compartments like urine, blood, and fat tissue. This will contribute to a better understanding of the exchanges between these compartments during long-term fasting. Moreover, the explorative hair analysis presented in this study should be extended to a higher number of subjects with different time points of sampling. Future studies should also assess more long-term changes in exposure to heavy metals and pesticides, or other pollutants such as plasticisers, brominated and fluorinated compounds. The evaluation of the change in their bioavailability caused by fat mobilization during fasting can help understanding their impact on health.

Altogether, studies about the effects of fasting on the elimination of environmental pollutants are scarce. Our study showed a reduction in the urinary levels of arsenic and nickel, as well as a reduction in hair lead levels during a 10-days fasting period. In parallel, symptoms like fatigue, sleep disorder, headache and hunger were diminished, reflecting the tolerability and benefit of this procedure. Body discomfort symptoms were diminished 4 weeks after food reintroduction. More studies are needed to understand how therapeutic intervention can influence the detoxification capacity of the body for pollutants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study involving human participants was reviewed and approved by Landesärztekammer Baden-Württemberg Ethik-Kommission Liebknechtstr. 33 70565 Stuttgart.

AUTHOR CONTRIBUTIONS

G-ÉS had the idea for this study. G-ÉS, FWT, and FG conceived and conceptualized the study. FG was project manager, coordinated study conduction, data collection and drafted the manuscript. RM performed the bioinformatics and statistical analysis. VP performed parts of the laboratory analyses (hair measurements). G-ÉS and RM contributed to data interpretation and the writing of the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Targeting Cardiovascular Risk Factors Through Dietary Adaptations and Caloric Restriction Mimetics

Julia Voglhuber[1,2], *Senka Ljubojevic-Holzer*[1,2], *Mahmoud Abdellatif*[1,3] *and Simon Sedej*[1,2,4]*

[1] Department of Cardiology, Medical University of Graz, Graz, Austria, [2] BioTechMed Graz, Graz, Austria, [3] Centre de Recherche des Cordeliers, Equipe labellisée par La Ligue Contre le Cancer, Université de Paris, Sorbonne Université, INSERM U1138, Institute Universitaire de France, Paris, France, [4] Faculty of Medicine, Institute of Physiology, University of Maribor, Maribor, Slovenia

The average human life expectancy continues to rise globally and so does the prevalence and absolute burden of cardiovascular disease. Dietary restriction promotes longevity and improves various cardiovascular risk factors, including hypertension, obesity, diabetes mellitus, and metabolic syndrome. However, low adherence to caloric restriction renders this stringent dietary intervention challenging to adopt as a standard practice for cardiovascular disease prevention. Hence, alternative eating patterns and strategies that recapitulate the salutary benefits of caloric restriction are under intense investigation. Here, we first provide an overview of alternative interventions, including intermittent fasting, alternate-day fasting and the Mediterranean diet, along with their cardiometabolic effects in animal models and humans. We then present emerging pharmacological alternatives, including spermidine, NAD^+ precursors, resveratrol, and metformin, as promising caloric restriction mimetics, and briefly touch on the mechanisms underpinning their cardiometabolic and health-promoting effects. We conclude that implementation of feasible dietary approaches holds the promise to attenuate the burden of cardiovascular disease and facilitate healthy aging in humans.

Keywords: cardiovascular risk factors, obesity, hypertension, caloric restriction mimetics, autophagy, dietary regimens, caloric restriction, intermittent fasting

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***Correspondence:**
Simon Sedej
simon.sedej@medunigraz.at

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INTRODUCTION – A BRIEF OVERVIEW OF CARDIOVASCULAR RISK FACTORS

Cardiovascular diseases remain the major cause of morbidity and mortality, accounting for 17.9 million deaths per year or almost one third of all deaths worldwide[1]. Functional decline of the cardiovascular system and increased vulnerability to disease manifestation is accelerated by various risk factors. While some risk factors, such as age, sex, family history and race are unmodifiable, several behavioral and environmental risk factors can be efficiently targeted through lifestyle modifications and/or pharmacological interventions. In this regard, systemic analyses of global cardiovascular disease trends and patterns revealed a cluster of modifiable cardiovascular risk

[1]World Health Organization. Health topics - cardiovascular diseases (2019). Available online at: https://www.who.int/health-topics/cardiovascular-diseases#tab=tab_1 (accessed July 29, 2021).

factors, including high blood pressure, obesity, diabetes mellitus type 2, and hyperlipidemia, which are on the rise due to the global population aging, hypercaloric dietary habits, and sedentary lifestyle (1).

Extensive body of evidence indicates that hypertension is the leading modifiable risk factor for cardiovascular disease and premature mortality (2), accounting for 9.4 million global deaths per year (3). In 2010, around 31.1% of adult population worldwide (or 1.39 billion) were reportedly hypertensive (4). As such, high blood pressure remains an unmet medical need, despite the widespread use of antihypertensive medications. Obesity and unhealthy diets are major behavioral determinants that hamper the long-term control of hypertension (2), contributing to the increased risk for cardiovascular disease (5). Furthermore, obesity-related high levels of low-density lipoprotein-cholesterol (LDL-cholesterol) and triglycerides are causally linked to the progression of atherosclerosis (6), a chronic inflammatory disease associated with increased risk of ischemic cardiomyopathy and myocardial infarctions. Diabetes mellitus type 2 is a global health risk that is often seen concurrently with obesity and obesity-related complications, resulting in a 2-fold increase of cardiovascular disease risk (7). Despite accumulating evidence of the detrimental role of obesity and diabetes mellitus type 2 in the development of cardiovascular disease, their prevalence has reached epidemic dimensions[2]. Importantly, the increasing co-occurrence of multiple morbidities, such as obesity, dyslipidemia, diabetes mellitus type 2, and hypertension, which are referred to as a cluster of conditions also known as metabolic syndrome, typically contributes to an exponential increase in the risk for cardiovascular diseases (8).

In general, patients with cardiovascular disease are commonly affected by more than one risk factor (9). Emerging evidence suggests that most cardiovascular diseases can be prevented using systematic approaches that target behavioral risk factors such as unhealthy diet, obesity, and physical inactivity. Indeed, dietary restrictions or regular exercise have recently attracted much attention for cardiovascular disease prevention as recent estimations suggest that preventive treatments might reduce the development of cardiovascular disease by 80% (3). However, most patients exhibit low adherence to such demanding lifestyle modifications. Therefore, there is a pressing need to identify alternative interventions with better compliance. Various natural and pharmacological supplements or small molecules have emerged as potential candidates to replicate the pleiotropic salutary effects of dietary restriction and, thus, might offer better adherence without reducing calorie intake.

The amount of dietary intake, quality of food and its preparation as well as micronutrient composition together with general eating habits (e.g., meal timing and frequency) significantly contribute to the onset of cardiovascular disease risk factors (3, 10). To this end, many studies have tested various forms of dietary modifications for their efficiency on improving cardiovascular and metabolic health (**Table 1** and **Figure 1**).

[2]World Health Organization. Health topics - diabetes (2019). Available online at: https://www.who.int/health-topics/diabetes#tab=tab_1 (accessed July 29, 2021).

DIETARY APPROACHES FOR IMPROVING CARDIOMETABOLIC HEALTH

Mediterranean Diet

The Mediterranean diet is characterized by high fruit and vegetable intake combined with plenty of fish and unsaturated fatty acids derived mainly from extra-virgin olive oil, with minimal or no consumption of low saturated fat and processed food. Many epidemiological studies and randomized clinical trials report that the traditional Mediterranean diet is associated with lower risk for all-cause and cardiovascular disease mortality, coronary heart disease, metabolic syndrome, and diabetes mellitus type 2 (37, 38). For example, a meta-analysis demonstrated 10% reduction in cardiovascular disease incidence or mortality, and 8% decrease in all-cause mortality (39). In similar vein, a randomized controlled trial (PREDIMED) that included high-risk individuals consuming the Mediterranean diet showed that the cardiovascular disease risk could be lowered by almost 30% (11). In a sub-study derived from the PREDIMED principal trial, the Mediterranean diet was found to improve high-density lipoprotein (HDL) atheroprotective functions (13). Remarkably, similar effects on HDL function were reported in individuals suffering from metabolic syndrome, which were subjected to the Mediterranean diet coupled to exercise for 12 weeks only (12). Increased polyphenol intake from Mediterranean diet is associated with improved levels of LDL-cholesterol, HDL-cholesterol, and systolic and diastolic blood pressures in older participants at high risk for cardiovascular disease. Furthermore, elevated polyphenol consumption reduces circulating inflammatory biomarkers, such as vascular cell adhesion protein-1, interleukin-6, tumor necrosis factor-α, which are linked to atherosclerosis (14). Another sub-study of the PREDIMED trial reported reduced expression of genes involved in vascular inflammation, foam cell formation and thrombosis in a high cardiovascular disease risk population (15).

Growing evidence suggests that the markedly reduced risk for cardiovascular disease by the Mediterranean diet is attributed to its plant-rich nutrient composition with seafood as the predominant source of animal protein. For example, the Women's Health Initiative Observational Study demonstrated that increased consumption of baked or boiled fish, but not fried fish, inversely correlates with heart failure risk in postmenopausal women (17). In agreement with these findings, a 25-year follow-up study suggested that increased intake of long-chain omega-3 polyunsaturated fatty acids (PUFAs) and non-fried fish in early adulthood protects against the development of metabolic syndrome (18). Similarly, the Mediterranean diet enriched with extra-virgin olive oil, but without reduced caloric intake, reduces the risk for diabetes mellitus type 2 in individuals with high cardiovascular risk (16). In sum, the Mediterranean diet is a promising and feasible diet with manifold cardiometabolic benefits. However, since the PREDIMED trial has had its limitations (40), additional randomized clinical studies are warranted to corroborate the efficacy of this most extensively studied dietary regimen.

TABLE 1 | Overview of human trials testing the efficacy of dietary interventions on cardiometabolic risk.

Dietary intervention	Diet characteristics and duration	Follow-up time	Disease/target population	Study design/Number of participants/Sex	Effect	Study outcomes	Reference/Trial title
Mediterranean diet	Until follow-up	4.8 years	High-risk for cardiovascular disease	RCT 7,447 participants 57% women	↓	30% reduced cardiovascular disease risk	(11)
	12 weeks Therapeutic lifestyle changes (diet plus exercise)	-	Metabolic syndrome	Prospective pilot study 25 participants 76% women	↓ ↑	Body weight Body-mass-index Fasting insulin HDL function	(12)
	1-year intervention Enriched with extra-virgin olive oil or nuts	-	High-risk for cardiovascular disease	RCT 296 participants 51% women	↑	HDL atheroprotective functions	(13) PREDIMED
	1-year intervention Enriched with extra-virgin olive oil or nuts	-	High-risk for cardiovascular disease	RCT 1,139 participants 55% women	↓ ↑	LDL-cholesterol Inflammatory biomarkers (VCAM-1, intracellular adhesion molecule, IL-6, TNFα, monocyte chemotactic protein 1) HDL-cholesterol	(14) PREDIMED
	3-month intervention Enriched with extra-virgin olive oil or nuts	-	High-risk for cardiovascular disease	RCT 49 participants 53% women	↓	Pro-atherothrombotic genes	(15) PREDIMED
	Enriched with extra-virgin olive oil or nuts	4.1 years	High-risk for cardiovascular disease	RCT 3541 participants 70% women	↓	Diabetes risk	(16) PREDIMED
	Increased fish consumption (non-fried)	10 years	Healthy post-menopausal women	Observational study 84,493 women	↓	Heart failure risk	(17) WHI-OS
	During early adulthood increased fish (non-fried) and long chain omega-3 PUFAs	25 years	Young adults, free form metabolic syndrome and diabetes	Prospective cohort study 4,356 participants 53% women	↓	Metabolic syndrome incidence	(18) CARDIA
Caloric restriction (CR)	2-year intervention 25% CR	-	Healthy, non-obese	RCT 220 participants 67% women	↓ ↑	Body weight General health	(19) CALERIE 2
	2-year intervention 25% CR	-	Healthy, non-obese	RCT 53 participants (analyzed) 68% women	↓	10-year cardiovascular disease risk by 30% Blood pressure Body weight Subcutaneous and visceral fat Insulin resistance (at 12 months of intervention) LDL-cholesterol Cholesterol Triglycerides	(20) CALERIE 2
	6-month intervention 25% CR plus other groups with exercise and varied % of CR	-	Overweight	RCT 48 participants 57% women	↓	Body weight Fat mass Leptin	(21) CALERIE

(Continued)

TABLE 1 | Continued

Dietary intervention	Diet characteristics and duration	Follow-up time	Disease/target population	Study design/Number of participants/Sex	Effect	Study outcomes	Reference/Trial title
	2-year intervention 25% CR	-	Normal weight to moderately overweight	RCT 218 participants 68% women	↓ ↑	Body weight Blood pressure Insulin resistance Inflammatory biomarkers (triiodothyronine, TNFα) Triglycerides LDL-cholesterol Total cholesterol Energy expenditure HDL-cholesterol	(22) CALERIE
	6-month intervention	-	Metabolic syndrome	Observational study 18 men	↓ ↑	Body weight Insulin levels Fasting glucose Pro-inflammatory cytokines Lipoprotein composition	(23)
	6-month intervention 25% CR, additional subgroup for 2 days/week	-	Healthy, obese or overweight, family history of breast cancer in 54% of participants	RCT 107 women	↓ ↑	Body weight Blood pressure Fasting insulin Insulin resistance Leptin C-reactive Protein LDL-cholesterol Triglycerides IGF-1 BP	(24)
	16-week intervention Calorie reduction of 700 or 500 kcal/day (latter coupled to physical exercise)	-	Diabetes mellitus type 2	RCT 63 participants 51% women	↓ =	Body weight Epicardial fat Total fat mass Cardiometabolomic profile	(25)
	20-week intervention calorie deficit of ∼400 kcal/day	-	Older, heart failure with preserved ejection fraction	RCT 92 participants 80% women	↑	Peak oxygen consumption	(26)
	CR for 6.5 ± 4.6 years	-	Healthy	Cross-sectional 50 participants 19% women	↓ ↑	Blood pressure C-reactive protein TNFα, $TGF\beta_1$ Diastolic function	(27)
Intermittent fasting	2-week intervention ∼17 h fasting cycles	-	Diabetes mellitus type 2 + metformin, obese	Observational study 10 participants 90% women	↓ ↑	Body weight Morning glucose level Postprandial glucose level Physical activity	(28)
	8-week intervention 16 h fasting cycles	-	Healthy men	RCT 34 men	↓ ↑	Fat mass IGF-1 Testosterone Respiratory ratio Adiponectin	(29)

(Continued)

TABLE 1 | Continued

Dietary intervention	Diet characteristics and duration	Follow-up time	Disease/target population	Study design/Number of participants/Sex	Effect	Study outcomes	Reference/Trial title
Alternate-day fasting	8-week intervention Allowed for 25% of energy intake on fasting days	-	Obese	Interventional study 16 participants 75% women	↓	Body weight Body fat percentage Blood pressure Total LDL LDL-cholesterol Triglycerols	(30)
	22-day intervention No control group	-	Non-obese	16 participants 50% women	↓ ↑	Body weight Fasting insulin Respiratory quotient Fat oxidation	(31)
	4-week and 6-month intervention	-	Healthy non-obese	Cohort study with integrated pilot RCT 90 participants for long term ADF 58% women 57 participants in RCT 60% women	↓ ↑	Cardiovascular disease risk Fat-to-lean ratio Inflammatory markers (sICAM-1, triiodothyronine) LDL-cholesterol Ketones PUFAs	(32) InterFast
	8-week intervention High-fat (45%) or low-fat (25%) diet on non-fasting days	-	Obese	RCT 32 women	↓	Coronary heart disease risk Body weight Fat mass LDL-cholesterol Triacylglycerol	(33)
Intermittent fasting *vs.* caloric restriction	12-week intervention Continuous CR (5,000–6,500 kJ/day) or intermittent fasting for 2 days/week	-	Overweight/obese and Diabetes mellitus type 2	RCT 63 participants 52% women	↓	Body weight HbA1c Comparable results between IF and CR	(34)
Alternate-day fasting *vs.* caloric restriction	1-year intervention 25% of energy intake allowed on fasting days or 25% CR continuously	-	Obese	RCT 100 participants 86% women	↓ ↑	Body weight HDL-cholesterol in alternate-day fasting (6 months of intervention)	(35)
	3-week intervention 150 or 200% energy intake on non-fasting days or 25% CR continuously	-	Healthy and lean	RCT 36 participants 58% women	↓ ↑	Body weight (not for 200% energy intake) Body fat (not for 200% energy intake) LDL-cholesterol (only CR) Leptin HDL-cholesterol Adiponectin CR more effectively reduces body weight than alternate-day fasting with 25% reduced energy intake, which confers no additional short-term metabolic or cardiovascular benefits	(36)

We searched the US clinical trial registry (https://www.clinicaltrials.gov/) and PubMed using terms "Mediterranean diet," "Caloric restriction," "Intermittent Fasting," "Alternate-day fasting," and "Cardiovascular risk/disease" for completed, pending or ongoing clinical trials testing the effects of dietary regimes on cardiovascular risk factors.

HDL, High-density lipoprotein; IGF-1, Insulin-like growth factor-1; IGF-1 BP, Insulin-like growth factor-1 binding protein; IL-6, Interleukin-6; LDL, Low-density lipoprotein; PUFA, Polyunsaturated fatty acid; RCT, Randomized clinical trial; sICAM-1, Soluble intercellular adhesion molecule-1; TGFβ_1, Transforming growth factor β_1; TNFα, Tumor necrosis factor α; VCAM-1, Vascular cell adhesion protein-1.

↑ (arrow up) indicates increase or improvement, ↓ (arrow down) indicates decrease or decline, = indicates no change.

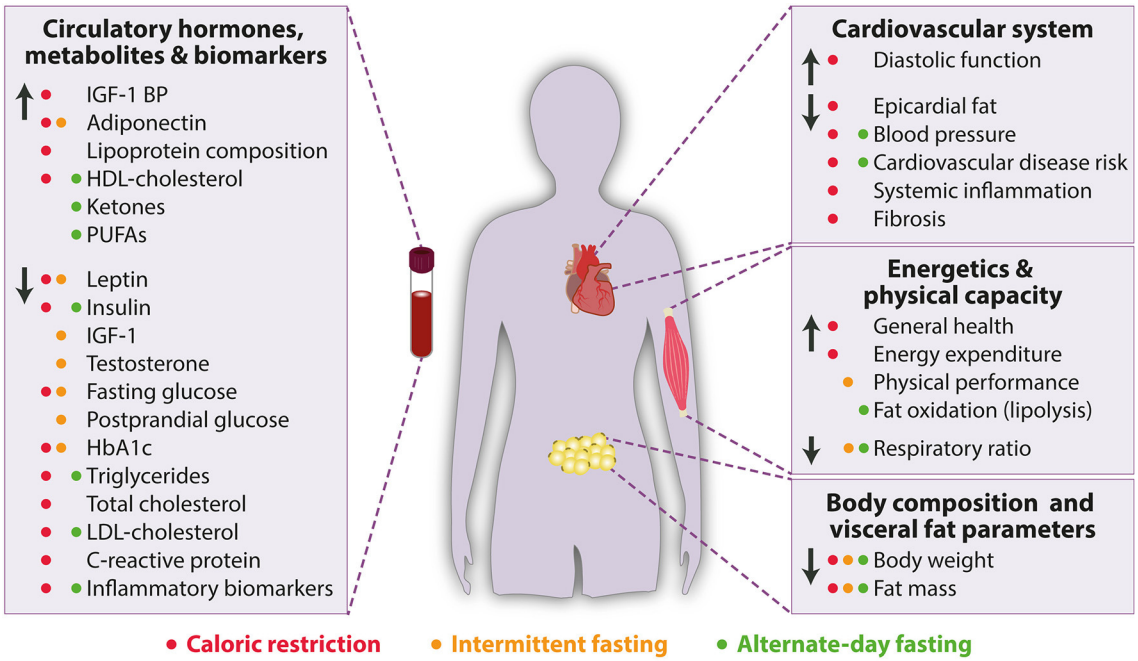

FIGURE 1 | Beneficial effects of caloric restriction (red), intermittent fasting (orange) and alternate-day fasting (green) on cardiometabolic parameters in humans. HbA1c, Glycated hemoglobin; HDL, High-density lipoprotein; IGF-1, Insulin-like growth factor-1, IGF-1 BP, Insulin-like growth factor-1 binding protein; LDL, Low-density lipoprotein; PUFAs, Polyunsaturated fatty acids. Arrow up indicates increase or improvement, arrow down indicates decrease or decline.

Caloric Restriction

Caloric restriction and other forms of stringent eating behaviors, such as intermittent fasting and alternate-day fasting, have recently attracted a lot of attention amongst researchers and have become increasingly popular in the general population to avoid the unhealthy effects of "all-around-the-clock" high caloric diet. Caloric restriction is defined as a chronic reduction of overall calorie consumption without malnutrition. In patients with metabolic syndrome, caloric restriction reduces body weight and exerts beneficial effects on insulin levels, fasting glucose levels, lipoprotein composition and pro-inflammatory cytokines within 6 months of intervention (23). In addition to weight loss in obese or overweight women, caloric restriction reduces leptin, total C-reactive protein (CRP), LDL-cholesterol, triglycerides, blood pressure, fasting insulin and insulin resistance (24). In another study, improved body weight and reduced epicardial fat accumulation were also observed in patients with diabetes mellitus type 2 subjected to caloric restriction. These effects were further augmented by physical activity, while cardiometabolic profiles were apparently unchanged (25).

Caloric restriction was shown to be a safe and well-tolerable intervention in healthy, non-obese individuals (41), leading to body weight loss (19, 20, 42), reduced fat mass and waist circumference (20, 21, 42), and improved general health (19). A long-term clinical trial reported increased energy expenditure without negatively affecting the quality of life in non-obese to moderately overweight cohorts (22). A 20-week long intervention with caloric restriction also potently improved peak oxygen consumption in older and obese patients with heart failure with preserved ejection fraction (26). Beneficial effects of caloric restriction were attributed to reduced blood pressure (20, 22, 24, 42), and lower total cholesterol and LDL-cholesterol concentrations (20) as well as lower leptin levels (21), altogether contributing to reduced 10-year risk for cardiovascular disease by 30% (20). Caloric restriction exerts cardiac-specific effects that ameliorate aging-related decline in diastolic function (27). These salutary effects on heart function might be mediated by the effect of caloric restriction on blood pressure, systemic inflammation, and cardiac fibrosis (43).

Mechanistically, the beneficial effects of caloric restriction are closely linked to autophagy, a cellular recycling process essential for cardiovascular homeostasis (44, 45). Caloric restriction mediates positive effects on the heart also via increased activity of SIRT1 and peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α), leading to reduced amount of reactive oxygen species (ROS), and less fibrosis and inflammation (46). Furthermore, caloric restriction lowers oxidative stress in the heart and vasculature by increasing the expression of endothelial nitric oxide synthase (eNOS), and activating superoxide dismutase (SOD) and NADPH oxidase (47). Importantly, non-cell autonomous mechanisms also contribute to the cardiovascular health benefits of prolonged caloric restriction. Although the mechanisms are still ill-defined, the "metabolic switch" hypothesis may explain, at least in part, improvements in cardiovascular health indicators, such as lower blood pressure in animals and humans (48). In fact, fasting induces the conversion of hepatic fatty acids into ketone bodies (e.g., β-hydroxybutyrate), which act as fuel and potent

signaling molecules, with the capacity to effectively reduce markers of inflammation and control various regulators of systemic metabolism, such as levels of HDL and LDL cholesterol, triglycerides, and glucose (48, 49).

Notably, severe caloric restriction (∼800 kcal per day) induces changes of gut microbiome composition during weight loss (50, 51). However, the consequences of gut microbiome composition alteration for health and disease in response to stringent caloric restriction are only beginning to unveil. A very recent clinical trial, with 80 post-menopausal women who were overweight or obese, revealed that severe calorie restriction imparts a reversible shift in the gut microbiome associated with improved glucose regulation and decreased adiposity, indicating improved metabolic health in dieters (52).

Collectively, caloric restriction exerts clear cardiometabolic benefits in both obese and non-obese individuals. However, caloric restriction might also cause adverse side effects on immunity, fertility and bone density. Hence, further research is warranted to develop more suitable dietary patterns or pharmacological alternatives to reproduce the health benefits of caloric restriction.

Intermittent and Alternate-Day Fasting

In an effort to circumvent the complexity of counting calories and avoid the side effects associated with caloric restriction, other forms of dietary restriction with food intake limited to a daily time window, such as intermittent fasting and alternate-day fasting, have been proposed. Accordingly, different lengths of eating and fasting periods have been tested, with the most common reported of 16/8 h of fasting and eating intervals, respectively (53). Longer fasting periods of 24 h followed by *ad-libitum* food intake for 24 h are also practiced and known as alternate-day fasting. Although intermittent fasting and alternate-day fasting are not as well-studied as caloric restriction, emerging evidence suggests that they are more tolerable and their side effects are less prominent than in caloric restriction and, thus, both dietary interventions could represent promising and more feasible strategies to curtail the hypercaloric pandemic in the Western societies (54).

To this end, a study comparing the efficacy of caloric restriction and intermittent fasting (restricted to 2 days a week) in obese diabetic patients at risk of cardiovascular disease showed that both regimens reduce body weight and HbA1c levels, a measure of long-term blood glucose control (34). Consistently, another small observational study on obese subjects with diabetes mellitus type 2 and receiving metformin reported that short-term intermittent fasting effectively reduces body weight and improves morning glucose levels. Interestingly, 6 out of 10 participants in this study described that intermittent fasting is highly tolerable, and reported readiness to follow intermittent fasting after study completion (28). Of note, intermittent fasting was capable to improve health parameters in healthy, male athletes. Specifically, intermittent fasting reduced body fat mass without worsening body fat-free mass, muscle area and strength. These effects were associated with lower concentrations of insulin-like growth factor-1 (IGF-1) and higher adiponectin levels, while leptin was not found reduced after adjusting for body fat mass (29). By contrast, a recent meta-analysis concluded that the evidence supporting a positive effect of intermittent fasting on glucose remains uncertain, despite the robust body weight-lowering effect (55). Interestingly, the analysis suggested that both intermittent fasting and caloric restriction equally improve cardiometabolic risk factors. Irrespectively, larger studies with long-term follow-up are necessary to clearly determine the effect of either regimen on hard cardiovascular end-points, such as myocardial infarction, heart failure as well as cardiac and all-cause mortality.

With regard to alternate-day fasting, a short-term trial conducted in obese adults, which showed high adherence to alternate-day fasting at least for 8 weeks, revealed manifold cardiometabolic benefits, including reduced body weight, body fat percentage, total and LDL-cholesterol, triglycerides as well as systolic blood pressure (30). It is important to mention that the participants were allowed for 25% energy intake on fasting days. Interestingly, short-term alternate-day fasting effectively reduces body weight, body fat mass and waist circumference despite high-fat dietary intake on non-fasting days. However, although alternate-day fasting improves plasma levels of LDL-cholesterol and triacylglycerol in obese individuals, HDL-cholesterol, blood pressure and heart rate are not altered (33). At variance with short-term studies, a long-term trial reported low adherence to the prescribed amount of energy intake and, accordingly, a high dropout of obese, otherwise metabolically healthy adults subjected to alternate-day fasting within the 1-year follow-up (35). This study also included a caloric restriction group, which exhibited higher compliance rates than the alternate-day fasting group. Although reduction in body weight was evident upon both alternate-day fasting and caloric restriction, none of the fasting regimens improved blood pressure, plasma lipid profile, or markers of glucose control and inflammation. In addition, HDL-cholesterol levels that were higher at 6 months of alternate-day fasting, were not improved after 12 months (35). Recently, a 3-week randomized trial, which is among the first to disentangle the effects of alternate-day fasting and "traditional" daily energy restriction, revealed that alternate-day fasting without energy restriction is not sufficient to reduce body weight in lean individuals. However, although alternate-day fasting with 25% reduced energy intake reduces body mass, the decrease of body fat content is lower compared to a matched traditional daily energy restriction and confers no additional short-term metabolic or cardiovascular benefits (36). Further studies with larger cohorts and longer duration are warranted to examine the fasting-specific effects of alternate-day fasting and intermittent fasting, and directly compare their effects to diets that only reduce daily net calories.

Along similar lines, initial short-term studies in non-obese individuals highlighted the positive impact of alternate-day fasting on body weight loss in absence of clear metabolic changes, but increased fat oxidation. Notably, participants reported difficulty to adhere to alternate-day fasting due to severe hunger on the fasting days (31). By contrast, the InterFast trial showed that alternate-day fasting is capable of improving cardiometabolic markers in healthy non-obese subjects, including reduced body weight, fat-to-lean ratio,

and LDL-cholesterol (32). Furthermore, alternate-day fasting increases ketone bodies (on fasting and non-fasting days), and reduces the inflammatory marker sICAM-1, suggesting that alternate-day fasting is a viable dietary adaptation also for non-obese individuals. Importantly, this 4-week long intervention trial reported no adverse effects on immunity or bone density.

In sum, growing body of evidence indicates potential cardiovascular benefits of intermittent and alternate-day fasting (56). However, it is still not clear whether these nutritional regimens, wherein food intake is limited to a consistent time-restricted interval without changes in nutritional quality or quantity, confer a significantly better adherence than caloric restriction. Also, it remains elusive whether the cardiometabolic benefits of these regimens can be applied to the general healthy population or specific groups with disorders, such as obese individuals with metabolic disease. Hence, larger studies, preferably with long-term follow-up, will be required to address these open issues.

CALORIC RESTRICTION MIMETICS

Recent years have seen an increasing interest in fasting-mimicking diets and caloric restriction, which might offer a more feasible alternative to stringent forms of fasting. For example, a randomized clinical trial was designed to investigate the effects of fasting mimicking diets, which are low in carbohydrates and protein and high in unsaturated fats, on cardiovascular disease and risk factors, including aging and diabetes mellitus type 2 (57). The authors observed that practicing low calorie fasting mimicking diet for only 5 consecutive days per month results in a reduction of body mass index (BMI), arterial blood pressure, fasting glucose, and IGF-1 levels. Generally, subjects who are at greater risk for disease, exhibit a larger benefit than individuals who have no other risk factors, confirming the relevance of fasting mimicking diet for disease prevention. Similarly, caloric restriction mimetics–natural and pharmaceutical compounds with intrinsic pro-autophagic action–might offer superior compliance, and are under intensive investigation as they have been shown to improve cardiovascular health and they might be used for the treatment of cardiovascular disease (58). Therefore, in the following section commonly used and well-studied caloric restriction mimetics will be discussed. Further, we will briefly describe their mode of actions and summarize the current evidence for the cardiovascular and metabolic effects of selected caloric restriction mimetics (**Figure 2**).

Spermidine

Spermidine is a natural polyamine and autophagy inducer that exerts pleiotropic cardioprotective effects by lowering high blood pressure in salt-sensitive *Dahl* rats, while reducing maladaptive hypertrophy and attenuating the decline of diastolic function (59, 60), and arterial elastance in aged mice (61). In addition to its direct cardioprotective effects, accumulating evidence demonstrated the anti-obesity impact of spermidine supplementation in rodents consuming a high-fat diet (HFD). In particular, spermidine counteracts HFD-induced body weight gain and obesity-associated alterations by increasing lipolysis in visceral fat and improving blood glucose control in obese mice (62, 63), and diabetic rats (64). Interestingly, spermidine treatment appears to provide no additional metabolic benefit in young and old mice consuming normal chow (59, 63), suggesting that salutary metabolic effects of spermidine might be limited to hypercaloric and pro-diabetic dietary regimens. Beside the regulation of lipid metabolism, spermidine attenuates inflammatory response in the adipose tissue by decreasing inflammatory cytokine and chemokines expression (65). Spermidine is also capable of reducing circulating TNFα levels during aging, thereby counteracting chronic low-grade inflammation in old mice (59).

The cardiovascular health-promoting effects of spermidine supplementation are predominantly attributed to its cytoprotective autophagy-inducing properties. For example, cardiomyocyte-specific Atg5-deficient mice exhibit no cardiac benefits upon spermidine supplementation (59), while the aortic rings of spermidine-fed mice display no functional advantages over their non-treated controls upon incubation with the autophagy inhibitor chloroquine (61). Autophagy-inducing capacity of spermidine relies on the inhibition of several acetyltransferases, including EP300, one of the major negative regulators of autophagy (66). These autophagy-stimulatory properties are mediated via hypoacetylation of histones (67), and autophagy-related genes, such as *Atg5*, *Atg7,* and *Atg8* (68). In addition, spermidine has been proven to inhibit the mammalian target of rapamycin complex 1 (mTORC1) (66), a key regulator of cell growth and metabolism, and to activate AMP-dependent protein kinase (AMPK) (69). More recently, spermidine was reported to stimulate autophagy through the hypusination of eukaryotic translation initiation factor 5A-1 (eIF5A), which in turn controls the expression of transcription factor EB (TFEB), a master regulator of lysosome biogenesis and autophagy (70). By contrast, age-related decline of spermidine levels and subsequent down-regulation of TFEB may cause reduced autophagic activity in the adaptive immune system, as well as in other tissues. However, although many protective effects of spermidine are autophagy-dependent and associated also with increased mitophagy, a selective form of autophagy that degrades dysfunctional mitochondria (59, 71), a recent study showed that enhanced lipolysis by spermidine was independent of autophagy in adipose tissue (63). Indeed, spermidine effectively stimulated lipolysis in HFD-fed mice with adipose-specific autophagy deficiency. In this regard, further studies are warranted to elucidate, which of the cell type/tissue/organ-specific effects induced by spermidine requires autophagy.

In humans, circulating spermidine levels decline with age (72), and reduced endogenous concentrations of spermidine appear to be associated with age-related deterioration of cellular homeostasis attributed to decreased autophagy (73). The upregulation of endogenous spermidine levels extends lifespan across multiple species, including mice. Spermidine is abundantly found in wheat germ, soybeans, and nuts (73), and reportedly enriched also in the Mediterranean diet (74). While the optimal concentration of spermidine in humans to maintain optimal autophagy levels for healthy aging still needs to be determined, self-reported dietary spermidine intake has been shown to

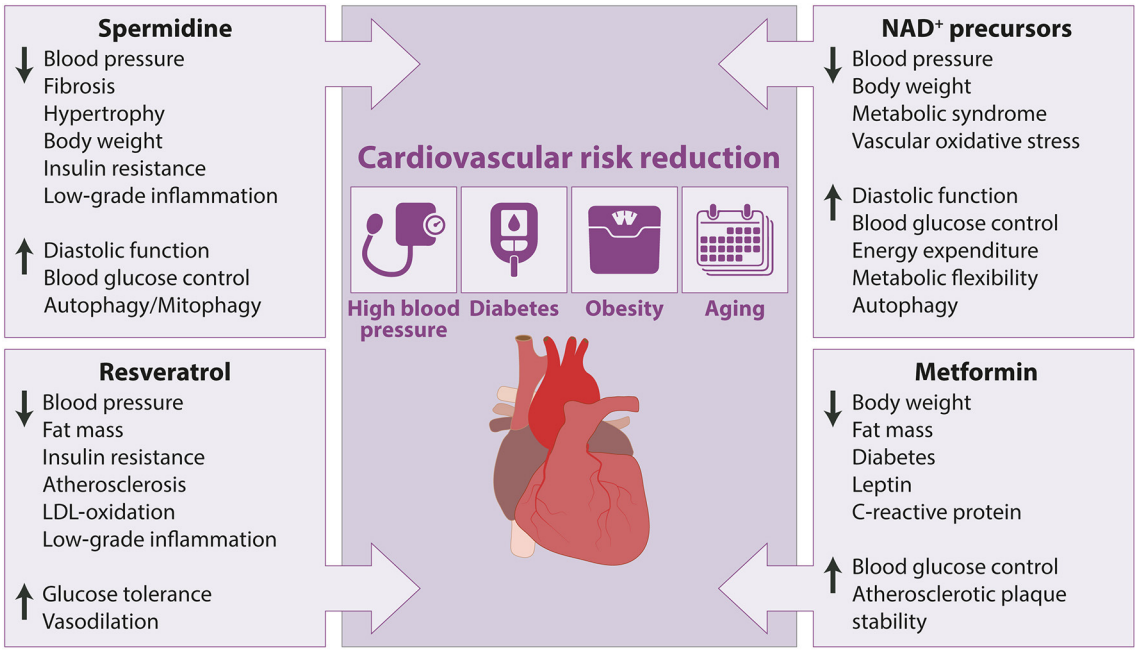

FIGURE 2 | Cardiovascular and metabolic health-promoting effects of caloric restriction mimetics in animal models with cardiovascular risk factors. Arrow up indicates increase or improvement, arrow down indicates decrease or decline.

inversely correlate with arterial blood pressure, risk of both fatal and overt heart failure as also other cardiovascular disease (59), and overall mortality (75).

Taken together preclinical evidence supports the translational potential of spermidine to ameliorate cardiovascular risk factors, including hypertension and HFD-induced obesity. Dietary spermidine supplementation has been proven safe with no adverse effects reported and well-tolerated in healthy volunteers (74, 76), and older adults at risk for dementia (77). Further larger and long-term clinical investigations are needed to elucidate whether cardiovascular risk factors may be counteracted by ingesting polyamine-rich food items, polyamine-enriched plant extracts, synthetic spermidine, or by stimulating polyamine synthesis in the gut microbiome through supplementation of prebiotics or probiotics.

Resveratrol

The polyphenol resveratrol, which is abundantly found in the skin of grapes and red wine, is one of the most extensively studied natural and *bona fide* caloric restriction mimetics. Interest in the cardiovascular health-promoting properties of resveratrol has been greatly influenced by experimental studies, demonstrating that resveratrol protects against metabolic disturbances induced by HFD and, thus, prevent early mortality in obese mice (78). The favorable effects of resveratrol on the cardiovascular system could be, at least in part, explained by its capability to promote vasodilation (79, 80), suppress atherosclerosis (81), improve glucose tolerance and insulin sensitivity (78, 82, 83), inhibit LDL oxidation (84, 85), and decrease plasma triglycerides and cholesterol accumulation (86). In addition to reported protection from the negative consequences of an obesogenic diet, such as insulin resistance (87), resveratrol has been demonstrated to exhibit anti-inflammatory effects (88, 89). The anti-inflammatory properties of resveratrol include down-regulation of genes involved in inflammatory pathways (90), as well as systemically inhibited expression of TNFα, IL-6 (90, 91), IL-1β, ICAM-1, and iNOS (91). Altogether, the anti-inflammatory activity has been postulated to explain a relatively low risk of cardiovascular disease in the French population consuming moderate amounts of resveratrol in red wine, despite high intake of saturated fats (so-called "French Paradox") (92).

Evidence has accumulated indicating that resveratrol, both *in vivo* and at nutritionally relevant concentrations *in vitro*, can activate several interrelated signaling pathways in the cardiovascular system. Many of the beneficial cardiovascular effects of resveratrol are mediated by pathways that require SIRT1 in cardiomyocytes and endothelial cells (93, 94). Although both SIRT1 and AMPK are necessary for resveratrol-induced health promotion (87, 95), there are likely other molecular targets of resveratrol that contribute to its cardioprotective effects. Studies reported that resveratrol inhibits the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) pathway (96), attenuates vascular oxidative stress (97, 98), and upregulates eNOS (99, 100), which is known to improve endothelium-dependent vasodilation through increased nitric oxide bioavailability. Importantly, SIRT1-mediated activation of autophagy is a key process in mediating many beneficial effects of resveratrol (101–103). Very recently, resveratrol was found to promote lysosomal function via endoplasmic reticulum calcium-dependent TFEB activation, which is associated with reduced

intracellular lipid accumulation (104). Importantly, inhibition of mTORC1 activity and presence of Unc-51-like kinase 1 (ULK1) were shown to be required for autophagy induction by resveratrol (105). However, although resveratrol attenuates the activation of mTORC1, low dose resveratrol reportedly induces the expression of Rictor, a component of mTORC2 pathway (106). Overall, despite the large number of molecular targets that have been identified responsible for the promiscuous effects of resveratrol, more research effort is needed before definitive mechanisms can be assigned to its multifaceted cardioprotective benefits. On the basis of available evidence, it can be endorsed that resveratrol-induced cardiovascular protection is controlled by many of the pathways (e.g., NF-kB pathway) and master regulators (e.g., mTORC) involved in cellular stress resistance, redox homeostasis and cellular energetics.

Encouraging results from preclinical research have greatly increased the interest in resveratrol supplementation to mitigate cardiovascular risk factors in humans. A recent meta-analysis of 17 randomized clinical trials validated the blood pressure lowering effect of resveratrol (107). The anti-hypertensive effect of resveratrol that was consistently reproduced only in studies testing doses >300 mg/day was reported mainly in patients with diabetes mellitus type 2 likely due to its favorable effect on insulin sensitivity (108). Of note, lower systolic blood pressure is associated with metabolic changes (90). In this small randomized control trial, 30 days of resveratrol supplementation decreased intrahepatic lipid content, circulating levels of glucose and triglycerides, and inflammation markers, while it stimulated adipose tissue lipolysis in obese men. By contrast, a recent study failed to demonstrate the efficacy of resveratrol against metabolic syndrome (109). In fact, although resveratrol has been shown to modify risk factors in experimental models of obesity and cardiovascular diseases by phenocopying most of the transcriptional aspects and molecular mechanisms of caloric restriction, including the suppression of inflammatory response (91, 110), it is important to note that clinical trials mostly failed to reproduce cardiometabolic improvements likely due to low *in vivo* bioavailability of resveratrol (111). This is particularly relevant because *in vivo* evidence has been viewed increasingly important in endeavors to understand how resveratrol elicits its effects in humans and to ascertain the optimum doses and routes for mitigating cardiovascular risk factors. To this end, other small-molecule activators of SIRT1 have been developed. For instance, SRT1720 has been demonstrated to extend lifespan and improve metabolic syndrome, insulin sensitivity, and endothelial dysfunction in mice, while a related compound, SRT2104, has undergone clinical phase I and II trials, revealing only minor adverse effects (112). Interestingly, rapid metabolism of resveratrol and the composition of the gut microbiome were proposed to control the production of resveratrol metabolites, which are detected at higher levels in humans after intake than their parent compound, with similar biological effects (113). Owing to its capability in modulating the composition of the gut microbiota, resveratrol may affect central energy metabolism and modify concentrations of satiety hormones to produce anti-obesity effects. Similar to resveratrol and spermidine, fasting also induces changes to the gut microbiome and improves immune homeostasis with a sustained beneficial effect on body weight and blood pressure in hypertensive patients with metabolic syndrome (114), suggesting that caloric restriction mimetics and dietary interventions promote cardiovascular health at least in part by regulating the abundance of certain microbes in the gut (115).

NAD^+ Precursors

Recent years have witnessed growing interest in NAD^+ intermediates as molecules that efficiently recapitulate the salutary effects of caloric restriction and exercise by elevating cellular NAD^+ content, which is reduced in aging, obesity and other metabolic disorders (116). Direct supplementation of NAD^+ precursors, in particular nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN), has been shown to alleviate metabolic abnormalities by reducing body weight gain and reinstating blood glucose control in mice consuming HFD (117, 118). Along similar lines, nicotinamide (NAM, also known as vitamin B3) was found to improve glucose homeostasis associated with positive effects on liver metabolism in absence of obesity-lowering effects in aged mice fed HFD (119). Recently, we have also demonstrated that orally administered NAM to male and female ZSF1 obese rats with cardiometabolic syndrome evidently reduces hyperphagia-induced obesity (120). This effect could be partially attributed to increased energy expenditure and improved metabolic flexibility. In addition, NAM moderately lowers high arterial blood pressure, while it improves diastolic dysfunction in ZSF1 obese rats, *Dahl* salt-sensitive rats and aged mice (120). In another study, oral NMN supplementation late in life to aged mice was also found to elicit anti-aging effects on the vasculature by improving aortic stiffness in association with increased arterial SIRT1 activation and reduced vascular oxidative stress, suggesting that NMN delays arterial aging and its pathological sequelae (121).

Mechanistically, increased NAD^+ is required for a sustained SIRT1 deacetylase activity, which regulates autophagy through deacetylation of autophagy-related proteins, such as ATG5, ATG7 and ATG8 (122). In addition, NAD^+ can induce autophagy via AMPK (123). The NAD^+/sirtuin pathway activates mitophagy, which was demonstrated to maintain cardiac function during HFD-induced diabetic cardiomyopathy (124). Moreover, NR supplementation was shown to activate SIRT1 and SIRT3, improve mitochondrial function and protect against HFD-induced obesity in mice (118). It is important to mention, however, that other NAD^+-modulated processes, like inflammation and oxidative stress, which are attenuated by NAD^+, might be involved in the cardiac and more broadly physiological effects of NAD^+ precursors. In fact, health-promoting effects of NAM coincide with reduced inflammation, oxidative stress and adipose tissue infiltration with leukocytes (119, 120).

Ample preclinical evidence has demonstrated that strategies to increase NAD^+ content can mitigate cardiovascular disease in various rodent models. Hence, NAD^+ precursors are increasingly proposed as promising agents to reduce the burden of cardiometabolic diseases in humans. Niacin, which has been typically used in the form of nicotinic acid, is the most extensively studied NAD^+ precursor in humans. The impact of niacin on

lipid control and cardiovascular risk in humans was recently re-examined in a meta-analysis based on a systematic review of 119 clinical trials that included 35,760 patients (125). Collectively, this analysis revealed a marginal benefit of niacin as a monotherapy to elevate HDL-cholesterol levels, but raised doubts about the safety profile of niacin, especially in combination with statins. Despite its poor tolerability, niacin remains in use as an alternative lipid-lowering agent in statin-intolerant patients at cardiovascular risk. First reports on human trials that tested other NAD^+ boosting strategies than niacin have only started to emerge (126), announcing an era of NAD^+ therapeutics. Amongst these, NR and NMN are the main precursors in ongoing or lately completed clinical trials (127). In fact, a recent study in postmenopausal, overweight women with prediabetes, demonstrated that 10 weeks of NMN supplementation increases skeletal muscle insulin signaling, insulin sensitivity, and muscle remodeling (128). These beneficial metabolic effects of NMN supplementation differ from the observations reported from NR trials conducted in obese middle-age and older men and women (129–131), suggesting different biological functions of NMN and NR. Another clinical investigation showed that NR may have the potential for reducing blood pressure and aortic stiffness in healthy middle-aged and older individuals (132). Additionally, NR has been shown to exert anti-inflammatory effects not only in aged healthy individuals, but also in hospitalized patients with heart failure (129, 133). Of note, high doses of oral NAM are safe and have also been shown to reduce non-melanoma skin cancers as well as markers of cardiorenal injury (134), opening a new perspective on the previously understudied therapeutic potential of NAM. In this regard, a diet enriched in NAM and NA is associated with lower blood pressure and a reduced risk of overall and cardiac-specific mortality in humans (120).

Taken together, several challenges need to be overcome before experimental findings on rodent models of cardiovascular risk factors can be translated into clinics. Future clinical trials need to be of longer duration and include a follow-up assessment, involve large numbers of patients, and consider more appropriate conversion of drug doses from rodent studies to human trials (135). In this regard, quantification of potential long-term adverse effects will be instrumental to ensure that NAD^+ precursor administration at higher doses is safe for the use in humans. Head-to-head studies are warranted to answer the outstanding question about the optimal NAD^+ precursor, and determine which of the NAD^+ precursors have superior properties, capable of eliciting a wide range of beneficial effects that may improve cardiovascular risk factors. In addition, several practical hurdles will need to be overcome, such as how to best deliver NAD^+ precursors to achieve the optimal NAD^+ bioavailability, and at what dose and time of the day, as NAD^+ levels are subjected to circadian fluctuations. Future studies should also compare the effects, efficacy and outcomes of pharmacologically increased NAD^+ levels *vs.* physiological means of raising NAD^+ levels, such as regular physical activity and dietary interventions that are designed for older individuals with comorbidities.

Metformin

The biguanide metformin, which originates from the French lilac, is the first-line drug used for the treatment of diabetes mellitus type 2 (136). Although best known for its glucose-lowering effects, a growing body of evidence indicates that metformin extends lifespan and healthspan (137) by mitigating age-associated conditions (138, 139), such as cancer, cognitive decline and cardiovascular diseases (140) across various species (137, 141, 142). Metformin exhibits a plethora of direct effects on the cardiovascular system. For example, it potently protects against hypertrophy in a pressure overload rat model, likely via increased AMPK and eNOS phosphorylation and higher nitric oxide production (143), leading to improved endothelial function and vasodilation (144). Metformin effectively reduces atherosclerotic plaque size in high-cholesterol diet fed rabbits by decreasing high-sensitivity C-reactive protein and inhibiting the NF-kB pathway in the vascular wall (145). In addition, metformin is capable of stabilizing atherosclerotic plaques by activating AMPK in *ApoE*-knock-out mice (146), resulting in better cardiovascular outcomes as calcification of plaques is associated with their instability and serves as a negative predictor of mortality (147, 148). Metformin attenuates inflammatory response in rabbits fed an atherogenic diet by reducing infiltration of macrophages (149), which is known to result in their differentiation to foam cells and atherosclerotic plaque formation (150). Furthermore, metformin suppresses the NLRP3 inflammasome and upregulates autophagy in mice with diabetic cardiomyopathy through the activation of AMPK and inhibition of mTORC (151, 152), both of which regulate aging-related pathways, leading to prolonged lifespan (153). Furthermore, metformin increases the expression and activity of SIRT1, while it attenuates the activation of PGC1α, a central energy metabolism regulator (154).

As most of the research endeavors focused on the glucose-lowering effect of metformin, it is not surprising that the majority of clinical trials were designed to investigate the beneficial role of metformin on diabetes mellitus type 2. However, several human studies assessed the impact of metformin monotherapy on other age-associated comorbidities as well. For example, metformin reduces pro-inflammatory cytokine levels in older diabetic patients, suggesting that metformin has the potential to attenuate age-related low-grade chronic inflammation, reduce the predisposition toward inflammation-related comorbidities, and improve survival of diabetic patients (155). In another clinical investigation, the use of metformin was assessed in the context of cardiovascular outcome in patients with diabetes mellitus type 2 and chronic kidney disease (156). The authors that analyzed data from the TREAT trial (157) demonstrated that metformin reduces the incidence of cardiovascular events as well as cardiovascular death and all-cause mortality. Importantly, metformin was found to be safe for patients with chronic kidney disease, which is in contrast with the previous assertion that metformin commonly induces lactic acidosis (158). In pubertal children with diabetes mellitus type 2 and metabolic syndrome, metformin improves various health parameters, including BMI, leptin levels, fat

mass and liver fat (159). Interestingly, some of these beneficial effects were maintained after completing the 24 months of metformin treatment, suggesting that metformin is well-tolerated and has a potential long-term benefit in adolescents at risk. In the REMOVAL trial, patients with diabetes mellitus type 1 displayed lower LDL-cholesterol levels after 3 years of metformin treatment (160). Recently, a meta-analysis that included 16 studies and nearly 2 million participants revealed that metformin reduces overall cardiovascular risk, including mortality and incidence, in patients with diabetes mellitus type 2 (161). Another comprehensive meta-analysis of 260 studies described a general drop in all-cause mortality and occurrence of cardiovascular disease in diabetic patients upon metformin treatment as compared to diabetic patients receiving other medication and, interestingly, even non-diabetic subjects (139). These observations highlight that metformin could extend lifespan and healthspan by acting as a geroprotective drug. However, studies in healthy or non-diabetic populations are rare and showed conflicting results. For example, the CAMERA study failed to produce the beneficial effects of metformin on cardiovascular disease prevention in non-diabetic patients with high cardiovascular risk (162). By contrast, 6 weeks of metformin treatment reduced body weight, improved insulin secretion, lowered LDL and triglyceride levels in an elderly population exhibiting impaired glucose tolerance but no previous history of diabetes (163).

Of note, the 6-year Targeting Aging with MEtformin (TAME) clinical trial[3], which started in 2016 as a large randomized controlled and multicenter study, including over 3,000 participants (between the ages of 65–79) without diabetes but who are at high risk for the development of chronic diseases of aging, is expected to generate highly valuable new knowledge about the impact of metformin on the primary outcome of death and major age-related chronic disease development, such as cardiovascular disease, cancer, and dementia (164).

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Recent years have seen a growing interest in understanding how dietary interventions shape and interact with the most common cardiovascular risk factors, including hypertension, obesity, metabolic syndrome, and diabetes mellitus type 2. Substantial cardiometabolic improvements have been reported with fasting interventions such as reduction in blood pressure, body weight and fat mass, lower blood glucose, and improvement in insulin sensitivity, both in experimental and clinical studies. Although caloric restriction consistently improves several aspects of health, its application has been hampered by poor compliance and adverse side effects on bone health and immune response, especially in the elderly. To overcome these major hurdles, clinical trials on alternate-day or intermittent fasting, with higher statistical power and follow-up, are strongly needed before they can be implemented as a treatment strategy. Individuals practicing alternate-day or intermittent fasting should consider to include regular physical activity to maintain their energy expenditure. Emerging evidence indicates that the optimal cardioprotective diet is constructed around the traditional Mediterranean eating pattern.

Another interesting aspect that warrants further attention is the effect of caloric restriction mimetics or dietary interventions aimed at weight loss on the gut microbiome changes in obese patients with diabetes mellitus type 2 or metabolic syndrome. Although these interventions propose beneficial clinical outcomes, their effect on the gut microbiome is only beginning to unfold. Interestingly, a combination therapy of resveratrol and spermidine synergistically induces autophagy at doses, which do not trigger effects of the same magnitude if administered alone. At present, however, it remains elusive what is the optimal dose for any of the caloric restriction mimetics that could provide health benefits or protect humans at risk of cardiovascular disease.

Unlike the current drug development approaches that focus on individual diseases in isolation and consider specificity as a desirable outcome in disease prevention and treatment, both caloric restriction mimetics and caloric restriction exhibit a spurious mode of action, intercepting with multiple different targets (165). Such pleiotropic mode of action appears advantageous in targeting the complex process of aging as the greatest risk factor for cardiovascular diseases and associated comorbid conditions. Thus, dietary interventions should aim to maintain optimum health and prevent cardiovascular diseases by attenuating the molecular causes of biological aging directly.

Non-cell autonomous effects of caloric restriction mimetics and caloric restriction itself, such as the anti-inflammatory or immune modulatory functions, are increasingly viewed as relevant as cell autonomous mechanisms. Taking this into account, more research is needed to ascertain how different forms of fasting and caloric restriction mimetics can be the most favorable to further improve cardiometabolic markers in healthy adults and patients living with or at risk of developing cardiovascular disease. Based on the currently available data, harnessing caloric restriction mimetics or dietary interventions, such as intermittent fasting or the Mediterranean diet represent a promising preventive venue, which might reduce cardiovascular risk and the burden of cardiovascular disease.

AUTHOR CONTRIBUTIONS

SS conceptualized the manuscript. JV, MA, and SS contributed to the research for writing the manuscript. JV and SL-H designed the figures and table. All authors

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contributed to the discussion, writing, and review of the manuscript.

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JV is currently trained as a PhD candidate in the Program Molecular Medicine at the Medical University of Graz.

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REVIEW
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Dietary Strategies to Improve Cardiovascular Health: Focus on Increasing High-Density Lipoprotein Functionality

Julia T. Stadler and Gunther Marsche**

Division of Pharmacology, Otto Loewi Research Center, Medical University of Graz, Graz, Austria

Cardiovascular disease is one of the leading causes of morbidity and mortality worldwide, with increasing incidence. A cornerstone of cardiovascular disease prevention is lifestyle modification through dietary changes to influence various risk factors such as obesity, hypertension and diabetes. The effects of diet on cardiovascular health are complex. Some dietary components and metabolites directly affect the composition and structure of high-density lipoproteins (HDL) and increase anti-inflammatory and vasoprotective properties. HDLs are composed of distinct subpopulations of particles of varying size and composition that have several dynamic and context-dependent functions. The identification of potential dietary components that improve HDL functionality is currently an important research goal. One of the best-studied diets for cardiovascular health is the Mediterranean diet, consisting of fish, olive oil, fruits, vegetables, whole grains, legumes/nuts, and moderate consumption of alcohol, most commonly red wine. The Mediterranean diet, especially when supplemented with extra virgin olive oil rich in phenolic compounds, has been shown to markedly improve metrics of HDL functionality and reduce the burden, or even prevent the development of cardiovascular disease. Particularly, the phenolic compounds of extra virgin olive oil seem to exert the significant positive effects on HDL function. Moreover, supplementation of anthocyanins as well as antioxidants such as lycopene or the omega−3 fatty acid eicosapentaenoic acid improve parameters of HDL function. In this review, we aim to highlight recent discoveries on beneficial dietary patterns as well as nutritional components and their effects on cardiovascular health, focusing on HDL function.

Keywords: Mediterranean diet, polyphenols, HDL composition, paraoxonase1, cholesterol efflux capacity

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****Correspondence:***
Julia T. Stadler
julia.stadler@medunigraz.at
Gunther Marsche
gunther.marsche@medunigraz.at

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INTRODUCTION

Cardiovascular disease (CVD) is one of the leading causes of death worldwide and the numbers are on the rise. Data obtained in 2018 indicate that CVD is responsible for more deaths than cancer and chronic lung disease combined (1). Risk factors for CVD comprise age, sex, hypertension, dyslipidemia, and diabetes. However, the likelihood of developing CVD is also increased by various health behaviors such as smoking and tobacco use, physical inactivity, obesity, and most importantly, nutrition. An unhealthy diet, which contributes to disease probability is characterized by increased consumption of processed foods, unhealthy fats, sodium, and added sugars (2–4).

In contrast, results of dietary intervention studies suggest that various foods and healthy dietary patterns, such as the Mediterranean diet, are associated with a markedly lower risk of CVD (5).

Atherosclerosis is an inflammatory disease that underlies a major part of the incidence and mortality of CVD. The inflammatory state promotes the accumulation of extracellular lipids or macrophage foam cells in the vessel wall, leading to atherosclerotic lesions. A poor diet and physical inactivity are risk factors for the disease, but lifestyle changes can prevent the development of atherosclerosis, due to several factors, such as reducing oxidative stress and decreasing the release of pro-inflammatory cytokines (6, 7).

Based on the close relationship between HDL-cholesterol (HDL-C) levels and CVD, efforts have long been made to reduce the risk of the disease by increasing plasma HDL-C levels (8). However, to this point therapeutics to increase HDL-C levels have failed, indicating that simply raising the quantity of HDL-C does not protect from CVD (9, 10). The negative result of HDL-C raising strategies may be partly explained by the recently demonstrated U-shaped association between HDL-C and CVD, with both extreme high- and low HDL-C concentrations associated with increased mortality, indicating that plasma levels of HDL-C do not accurately reflect the atheroprotective potential of HDL (11, 12).

It has to be noted that there is no clear explanation for the "paradoxical" association of very high HDL-C and increased mortality. One hypothesis is that in individuals with extremely high HDL-C, the functional properties of HDL are altered such that HDL no longer functions normally. Given the heterogeneity of HDL particles in terms of structure, size, lipidomic/proteomic composition, and metabolism, steady-state HDL-C levels suffer from the limitations imposed by their mass-based and static measurement. As a snapshot of the steady-state plasma cholesterol levels, HDL-C levels do not provide direct information on the rate of cholesterol efflux from vascular macrophages to the liver, which is influenced by many factors beyond the mass of HDL-C alone. Therefore, circulating HDL-C concentrations do not provide information about the structure and composition of HDL and anti-inflammatory, antioxidant, antithrombotic, and endothelial function-promoting activities of HDL (13–15), although there is increasing evidence of the clinical importance of these pleiotropic functions (16). Therefore, current research strategies focus on improving the atheroprotective functions of HDL.

Recent studies have shown that several dietary strategies and various nutritional components can affect levels of HDL-C and improve/affect some of the atheroprotective functions of HDL. In this review, we summarize established and novel approaches found in literature on the effects of several dietary approaches to influence HDL composition and function, with a particular focus on nutritional phenolic compounds and the Mediterranean diet.

Abbreviations: ABCA1, ATP-binding cassette A1; ABCG1, ATP-binding cassette G1; Apo, apolipoprotein; CETP, cholesteryl-ester transfer protein; CHD, coronary heart disease; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EVOO, extra virgin olive oil; HL, hepatic lipase; LCAT, lecithin-cholesterol acyltransferase; LPS, lipopolysaccharide; NO, nitric oxide; PON1, paraoxonase1; SAA, serum amyloid A; S1P, sphingosine-1-phosphate.

HDL METABOLISM

The first step in the formation of HDL is the production and secretion of the major HDL apolipoprotein, apoA-I, predominantly from the liver and the intestine (**Figure 1**) (17). After secretion, lipid-poor apoA-I interacts with ATP-binding cassette A1 (ABCA1) to acquire cholesterol and phospholipids from cellular lipid pools, which leads to the formation of nascent HDL particles. Cholesterol efflux from peripheral cells results in HDL particles becoming progressively larger and enriched in cholesterol. The acquired cholesterol on the surface of HDL is subsequently converted by the enzyme lecithin-cholesterol acyltransferase (LCAT) into cholesteryl-esters, which form the core of HDL particles (18). ABCA1 preferentially stimulates cholesterol efflux to pre-β HDL and small HDL3 particles, while ATP binding cassette G1 (ABCG1) interacts with large HDL2 particles (19, 20). Further uptake of lipids by HDL occurs via transfer of surface components of triglyceride-rich lipoproteins, during lipolysis by lipoprotein lipase (21).

Clearance of HDL cholesteryl-esters can occur *via* two different routes. First, the cholesterol content of HDL can be taken up selectively by scavenger receptor B1 (SR-BI) from the liver or steroidogenic tissues. Alternatively, cholesteryl-ester clearance can be mediated by cholesteryl-ester transfer protein (CETP), which transfers cholesteryl-ester from HDL to triglyceride-rich lipoproteins, in exchange for triglycerides. The triglyceride-enriched HDL particles are then more susceptible to lipolysis and rapidly catabolized by hepatic or endothelial lipase. Clearance of apoA-I then occurs in the kidney and liver (22). The interplay of the various apolipoproteins, lipid transfer proteins, enzymes, and surface receptors result in HDL particles of distinct sizes and functionality.

HDL STRUCTURE, COMPOSITION, AND FUNCTION

Of particular interest, certain diets and dietary components affect HDL composition, especially lipid components, but also the protein content of HDL can be affected.

HDL particles are very heterogeneous and differ in their size depending on their site of origin, proteomic and lipidomic composition and maturation stage. Approximately 70% of the total protein content of HDL accounts for apoA-I. This apolipoprotein acts as an activator of LCAT, interacts with cellular receptors and exerts several antiatherogenic activities (23). The second major apolipoprotein of HDL is apoA-II with 15–20% of the total protein amount. Other major HDL associated proteins are apoC-II, which serves as an activator of lipoprotein lipase, whereas apoC-III is an inhibitor. ApoE is a key functional apolipoprotein as well (24). Most circulating apoE is associated with triglyceride-rich lipoproteins, where it serves as a ligand for apoE/apoB receptors and facilitates binding of lipoproteins to cell

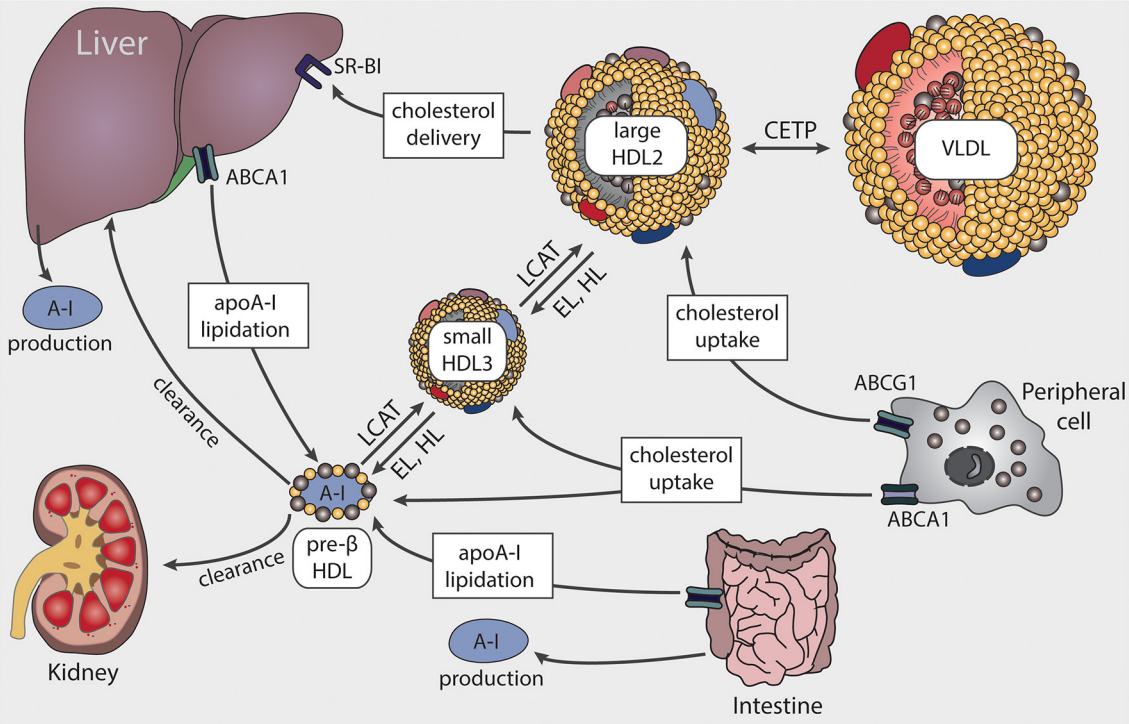

FIGURE 1 | Schematic representation of HDL biosynthesis and maturation. HDL biosynthesis starts with the production and secretion of apolipoprotein A-I (apoA-I) by the liver and intestine. Lipid-poor apoA-I interacts with ATP-binding cassette A1 (ABCA1) to acquire lipids, resulting in pre-β HDL formation. Through lecithin-cholesterol-acyl transferase (LCAT), the ingested free cholesterol on the surface of HDL is esterified to cholesteryl-ester forming larger particles. ABCA1 preferentially interacts with pre-β HDL or small HDL3 particles, while ATP binding cassette G1 (ABCG1) stimulates cholesterol transfer to larger HDL2 particles. Cholesterol is delivered to the liver via scavenger receptor BI (SR-BI) or transferred to very low-density lipoproteins (VLDL) by cholesteryl-ester transfer protein (CETP). HDL-associated triglycerides and phospholipids are mainly hydrolyzed by endothelial lipase (EL) and hepatic lipase (HL).

surfaces. Other minor apolipoprotein components of HDL are apoM, apoA-IV, apoF, apoD, apoJ and apoH, apoO, and apoL-I.

Pre-β particles are the structurally simplest form of HDL. These particles are lipid-poor, monomeric or dimeric apoA-I molecules and account for about 5% of the apoA-I content in the circulation (25). Pre-β particles are discoidal in shape and have a molecular weight of ∼70 kDa. Through their rapid uptake of cholesterol and phospholipids, pre-β particles are transformed into larger HDL subgroups. The small HDL3 particles have a density of 1.125–1.21 g/ml, are rich in proteins and have a molecular weight of ∼175 kDa. The larger size of HDL2 particles is reflected by their increased lipid content. This subclass has a density range of 1.063–1.125 g/ml and a molecular weight of about 350 kDa. In terms of HDL functionality, HDL3 particles have been proposed as the more anti-atherogenic HDL subclass in the general population. The smaller and denser particles display potent cholesterol efflux capacity and possess high antioxidative and anti-inflammatory activities (15, 26). These differences in HDL functionality between the subclasses can be partially explained by their differential proteomic and lipidomic composition. Several proteins are preferentially present on HDL3 particles, such as PON1, apoA-II, and apoM (27). ApoM provides a hydrophobic binding pocket that allows sphingosine-1-phosphate (S1P) to bind (28), which also has shown higher abundance on the HDL3 subclass (29). The apoM/S1P complex exerts several anti-inflammatory and endothelium-protective activities, which seem to account for at least some of the antiatherogenic activities of HDL (30). Recent research further demonstrated that HDL3 produced by the intestine efficiently sequesters lipopolysaccharide (LPS) and thereby protects against liver inflammation (31). Enterically derived HDL3 is enriched in LPS-binding protein and masks LPS from detection by Toll-like receptor 4 (31).

Due to its influence on oxidative stress and inflammation, the activity of the HDL-associated enzyme paraoxonase 1 (PON1) has been investigated in several pathological conditions, including vascular diseases (32, 33), renal disease (34, 35), diabetes (36–39), and cancer (40). Importantly, it has been reported that PON1 activity can be modulated by implementing certain lifestyle habits and dietary patterns, which will be discussed in more detail.

PON1 has a wide range of substrates that can be hydrolyzed. PON1 is mainly expressed and secreted into circulation from the liver, but also to some extent in kidneys and colon (41). Due to its antioxidative capacity, it has been suggested as an important player in atheroprotection (42). PON1 is very unstable, therefore its association with HDL is important to ensure stabilization and to maintain serum enzyme activity (43, 44). PON1 was originally

described to hydrolyze organophosphates such as paraoxon, a metabolite of the pesticide parathion (45). However, more recent studies have demonstrated that PON1 is further able to hydrolyze homocysteine thiolactone, which is a known risk factor for CVD and predictor for CVD mortality (46, 47). Therefore, PON1 is considered to be a protective factor against coronary artery disease (48). Additionally, purified PON1 protects both HDL and LDL from oxidative modifications caused by oxidized lipids (49–51). This ability of PON1, to inactivate the oxidized lipids was attributed to a specific cysteine residue at position 283. PON1-knockout mice show a higher susceptibility to endothelial dysfunction and atherosclerosis (52).

The HDL lipidome is largely composed of phospholipids (40–60%) and cholesteryl-esters (30–40%), while triglycerides (5–12%), and free cholesterol (5–10%) account for smaller proportions. Lipidomic analyses have identified over 200 different lipid species, which, together with the different protein components, are responsible for the high heterogeneity of HDL particles (29).The association of HDL subfractions with HDL function and cardiovascular risk is complex and incompletely understood. In the general population, smaller HDL particles have been shown to be more protective, whereas diameter, cholesterol- and triglyceride- content of very large HDL particles is associated with CAD risk (53). However, several chronic diseases are associated with profound alterations in HDL metabolism and function, caused by increased systemic oxidative stress and inflammation (12, 54). These conditions include obesity (55–57), chronic kidney disease (58, 59), liver disease (60, 61), diabetes (62–64), CVD (65, 66), but also allergic rhinitis (67) and skin diseases (68, 69). Compositional modifications and concomitant changes in parameters of HDL function may lead to development of pro-atherogenic characteristics and enhancement of the inflammatory state. HDL cholesterol efflux capacity is significantly influenced by both the concentration and the functionality of specific HDL particles participating in cell-cholesterol efflux. CAD patients have higher than normal preβ-1 concentrations with decreased functionality, and lower than normal large HDL particle concentrations (70). Concentrations of small HDL particles are sometimes even inversely correlated with cholesterol efflux capacity (71). This suggests a block in maturation of small HDL particles in inflammatory disease states and a complex interrelationship between the lipid-binding capacity of apoA-I and the functionality of HDL particles in disease (72).

HDL-Functionality and Cardiovascular Health

HDL particles display several biological activities, which are involved in atheroprotection (**Figure 2**). The best studied activity of HDL is the ability to remove excess cholesterol from arterial wall cells and subsequent delivery to liver and steroidogenic organs. The first step of reverse cholesterol transport is commonly referred to as the cholesterol efflux capacity of HDL. Indeed, it was shown that this function has a strong inverse association with coronary artery disease, independent of HDL-C levels (73). Another important antiatherogenic function of HDL is endothelial protection. Vascular injury or pro-inflammatory cytokines induce the expression of several adhesion molecules on the endothelium, which attract leukocytes and allow transmigration into the intima. HDL reduces cytokine-triggered expression of adhesion molecules on endothelium, thereby inhibiting adhesion of monocytes to endothelial cells and having a protective effect on endothelium (74–76). This anti-inflammatory capacity, which can be measured in a cell based assay, is inversely associated with incidence of cardiovascular events in the general population (77). Furthermore, HDL is capable to reduce the expression of chemokines and chemokine receptors via nuclear factor B and peroxisome proliferator–activated receptor γ modulation (78). Moreover, HDL has been identified as an important mediator of endothelial progenitor cell mediated cell repair (79, 80). Specifically, HDL pre-incubated endothelial progenitor cells showed improved adhesion to human coronary artery endothelial cells and up-regulated β_2-integrins, which play a unique role in endothelial progenitor cell adhesion (80). Moreover, after injection of recombinant HDL into a mouse model with inflammatory de-endothelialization, endothelial progenitor cell—mediated repair of the endothelium was enhanced (81). Furthermore, in patients with coronary artery disease, a correlation between HDL and circulating endothelial progenitor cells was observed (80). The vasodilatory activity of HDL is generally reflected by its ability to induce endothelial nitric oxide (NO) release, but also prostacyclin production (11, 82–85). HDL mediated activation of endothelial NO synthase is dependent on AMPK activation, which is in turn dependent on S1P-receptors and SR-BI (11).

In addition, HDL is thought to act atheroprotective by reducing oxidative stress. HDL protects other lipoproteins from oxidative damage by removing oxidized lipids caused by free radicals. Components of HDL, such as apoA-I or HDL-associated PON1 are involved in the reduction or hydrolyzation of oxidized lipids (86–91). Additional protective activities of HDL include its antithrombotic effects based on several mechanisms, such as reduced susceptibility of platelets to aggregation and reduced activation of the coagulation cascade (92). Platelet activation is prevented by HDL-induced upregulation of endothelial NO and prostacyclin synthesis (93) and downregulation of thromboxane A2 synthesis and platelet activating factor release (85).

DIETARY STRATEGIES AND HDL FUNCTION

Mediterranean Diet

The nutritional strategy known as the Mediterranean diet is becoming increasingly popular. The Mediterranean diet is characterized by high intake of extra virgin olive oil (EVOO), vegetables, nuts, legumes, whole grain products and fish, moderate consumption of alcohol, typically red wine, and low intake of red and processed meat, poultry and dairy products (94). Interest in the diet began in the 1950's when it was noted that heart disease was not as common in Mediterranean countries. Since then, numerous studies have confirmed that the Mediterranean diet helps to prevent heart disease and

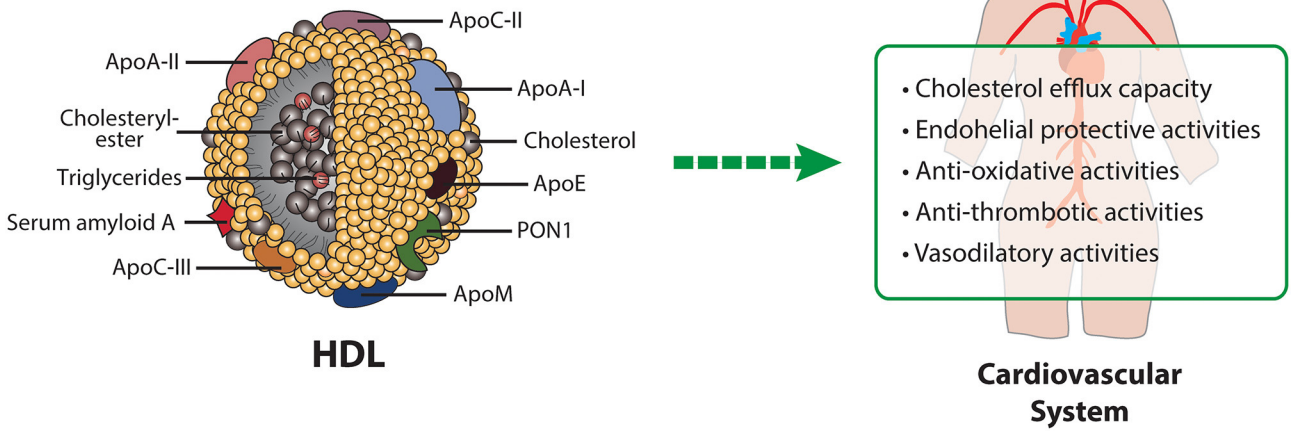

FIGURE 2 | HDL composition and HDL-mediated protective mechanisms in cardiovascular disease. Apo, apolipoprotein; PON1, paraoxonase1.

stroke (95). Notably, the Mediterranean diet is the only dietary pattern, which was shown to markedly improve HDL-functional parameters (**Figure 3**). The PREDIMED trial was one of the largest randomized controlled trials to explore the effects of the Mediterranean diet on cardiovascular disease prevention (5). In this study, 7,447 high-cardiovascular-risk patients were enrolled and assigned to one of three different diets: (1) Mediterranean diet supplemented with nuts, (2) Mediterranean diet supplemented with EVOO, (3) and a control diet with reduced fat intake. Lower incidence of cardiovascular events was observed in both of the groups supplemented with EVOO and nuts (5). In a random subsample of 296 participants of the PREDIMED trial, Hernáez et al. analyzed the effect of this diet on HDL functionality (96). After 1-year intervention, cholesterol efflux capacity of HDL was increased in both Mediterranean diet groups, compared to baseline levels (96). The authors suggested that the improvement in efflux capacity may be explained by increased HDL-related gene expression, changes in HDL-associated lipids and enhanced antioxidative capacity of HDL. Further analyses revealed that in the intervention group supplemented with EVOO, the ability of HDL to esterify cholesterol significantly increased, while activity of CETP decreased relative to baseline levels. LCAT is highly sensitive to oxidative modifications, therefore dietary consumption of antioxidative compounds may protect against oxidative inactivation (97). The arylesterase activity of PON1 did not change after the intervention; however, compared to the low-fat control diet, the activity was increased in the EVOO supplemented group. In addition, the ability of HDL to counteract LDL oxidation increased after EVOO intervention compared to baseline. Concerning compositional parameters of HDL, the authors found a reduced content of triglycerides after both Mediterranean diet interventions, compared to the low-fat control group. Further, the content of HDL surface phospholipids increased in the EVOO group, when compared to baseline and the control group. In this study, the dietary intervention had no effect on apoA-I, apoA-II, and apoC-II content of HDL (96). Further analyses of HDL functional parameters with the consumption of several food groups revealed that the decline in CETP activity was associated with legume and fish consumption (98). Moreover, EVOO intake and whole grain consumption was associated with increased cholesterol efflux capacity, while legume and fish intake was linked to increments of PON1 activity (98). The effect of EVOO on PON1 activity has been confirmed in other studies as well (99, 100). In patients with metabolic syndrome, a 12-week intervention with a Mediterranean diet and additional exercise markedly improved HDL cholesterol efflux capacity (101).

A 3 week intervention with polyphenol-rich olive oil improved cholesterol efflux capacity and increased HDL particle size compared to the control group receiving polyphenol-poor olive oil (102). Olive oil polyphenols increased HDL cholesterol efflux capacity and enhanced the anti-oxidative capacity of HDL through an increase in the olive oil phenolic compounds, such as hydroxytyrosol, glucoronate, and homovanillic acid sulfate. HDL-enrichment with these antioxidative metabolites is expected to provide protection against oxidative modifications (103). Olive oil polyphenols further increased HDL size and promoted a greater HDL stability, reflected by a triglyceride-poor core related to a more stable conformation of apoA-I and PON1 (99, 102–104).

More recently, the effect of EVOO intake on cholesterol efflux capacity of HDL in young and elderly study subjects was investigated. Of particular interest, cholesterol efflux capacity was lower in the elderly group, but returned to normal levels after 12-week of EVOO intervention (105). In addition, HDL subclass analyses showed lower levels of large HDL in the elderly group, which increased again after the intervention. Linear regression analyses showed a strong correlation of large HDL particles with cholesterol efflux. The age-related decrease in cholesterol efflux

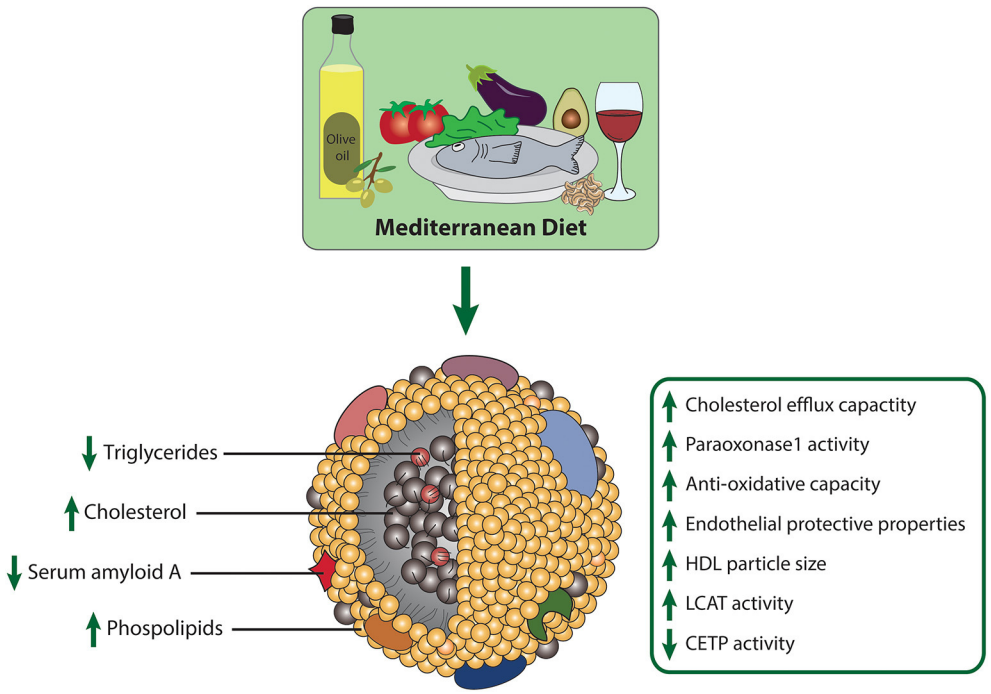

FIGURE 3 | Effects of the Mediterranean diet on composition and metrics of HDL function.

capacity was partly explained by the alteration in the distribution of HDL subclasses, which was modulated after the 12-week EVOO intervention (105).

Tomatoes are readily consumed as part of a Mediterranean diet, and a study of 39,000 women found that ingestion of seven or more servings of tomato-based products per week was associated with a 30% reduction in relative risk of CVD (106). The potential cardiovascular benefits of a tomato-rich diet may be attributed to their high lycopene content, especially as tomatoes account for up to 80% of dietary lycopene intake (107). In one study, HDL functionality was assessed following lycopene supplementation (70 lycopene/week) by monitoring the activities of PON1, CETP, LCAT, and serum amyloid A (SAA) content of HDL (108). After supplementation, lycopene content increased in HDL, and in parallel, PON1 and LCAT activities increased, whereas the content of pro-inflammatory HDL-associated SAA and CETP activity decreased. These results suggest that increased lycopene intake leads to beneficial changes in HDL metabolism, structure and function.

Fish or fish oils, rich in omega-3 fatty acids, are consumed as part of a Mediterranean diet and have been linked to a lower risk of CVD (109). Eicosapentaenoic acid (EPA) is an omega-3 fatty acid that has been shown to reduce levels of pro-atherogenic small dense LDL, remnant lipoprotein particles, and C-reactive protein in metabolic syndrome, presumably due to suppression of hepatic triglyceride production and degradation of CETP after supplementation (110). In patients with dyslipidemia, treatment with EPA (1,800 mg/day) has been shown to improve HDL function, enhancing HDL cholesterol efflux capacity and antioxidant and anti-inflammatory activities (111). EPA-enriched HDL inhibited cytokine-stimulated endothelial VCAM-1 expression and increased production of the anti-inflammatory EPA-derived metabolite resolvin E3 (112). Furthermore, *in vitro* studies revealed that EPA inhibits oxidation of HDL in a dose-dependent manner, which may contribute to the preservation of the antiatherogenic properties of HDL (113). In contrast, the omega-3 fatty acid docosahexaenoic acid (DHA −22:6; n-3) showed an initial antioxidative effect, but this was lost over time. However, comparison studies of EPA and DHA demonstrated that these fatty acids have distinct effects on plasma lipids, with DHA administration being more efficient in raising HDL-C, particularly the HDL2 subfraction and increasing LDL particle size (114–116). A recent study analyzed the effects of 8-week EPA and DHA supplementation on lipoprotein subfractions and HDL proteome in healthy and normolipidemic participants (117). The authors revealed that both fatty acids led to a reduction of VLDL-particle size and VLDL-particle number, suggesting a reduced hepatic VLDL production (118). Both EPA and DHA administration led to a reduction in medium sized HDL-particles and increased large HDL subfraction number. Of particular interest, proteomic analyses showed that supplementation with EPA-rich fish oil increased HDL apoM levels and decreased proteins involved in inflammation (117).

Similar to olive oil, nuts are enriched with mostly monounsaturated and polyunsaturated fatty acids and contain many vitamins and phytosterols (119). Especially walnuts are a rich source of α-linolenic acid and α-linoleic acids and have been shown to improve plasma lipid levels (120). Moreover,

acute consumption of walnuts improves HDL cholesterol efflux capacity, while walnut oil demonstrated beneficial effects on endothelial function (121). In a randomized controlled trial of high-risk CVD patients, the effects of three isocaloric diets were investigated to examine whether the beneficial effects of walnuts on lipid/lipoprotein levels are attributable to their fatty acid content (122). Replacement of saturated fatty acids with unsaturated fats from walnuts or vegetable oils improved lipid/lipoprotein classes, including LDL-cholesterol, non-HDL cholesterol, and total cholesterol but did not affect HDL cholesterol efflux capacity (122).

Another characteristic of the Mediterranean diet is moderate alcohol consumption, usually at mealtimes and in the form of red wine. Consumption of alcoholic beverages is associated with increased plasma levels of HDL-C, phospholipids and apoA-I and a reduction in CETP activity (123–125). Moreover, alcohol is a consistent dietary factor that has shown a positive effect on cholesterol efflux capacity. Moderate intake of alcohol is associated with increased cholesterol efflux (126, 127), but also heavy alcohol intake was shown to enhance cholesterol efflux to HDL2 particles, concomitant with an increase of larger particles (125). The increased cholesterol efflux capacity might be due to the increase of HDL-phospholipids observed in alcohol consumers (128). Interestingly, a study compared the effects of beer, red wine, and spirituous (Dutch gin) consumption on cholesterol efflux and plasma cholesterol esterification-rate. All alcoholic beverages significantly increased cholesterol efflux, without any differences between groups, while plasma esterification rate showed a significant increase after beer and spirituous consumption (126). Of particular interest, 3-week consumption of these beverages also increased PON1 activity, which was strongly correlated to increased HDL-C and apoA-I (129).

Like nuts and olive oil, avocados are a nutrient-rich source of polyphenols and monounsaturated fatty acids (130). In a study comparing different cholesterol-lowering diets, supplementation with 136 g avocado per day for 5 weeks resulted in a reduction of LDL-C and non-HDL-C compared to baseline (131). In addition, the avocado diet reduced LDL particle number, small and dense LDL-C and improved the LDL/HDL ratio (131). The beneficial effect on LDL-C was greater in the avocado diet group than in a diet containing moderate fats and oleic acid oils. These results suggest additional beneficial effects of avocado consumption.

In conclusion, the Mediterranean effectively prevents cardiovascular disease, and the improvement of atheroprotective functions of HDL likely contributes to this. Especially EVOO consumption has been shown to be a potential therapeutic option to promote the cholesterol efflux capacity of HDL. In addition, the antioxidant compounds in EVOO, lycopene, but also fish-derived omega-3 fatty acids may protect HDL from oxidative changes, resulting in more stable and functional particles (103). Moreover, moderate alcohol consumption has a positive effect on HDL cholesterol efflux capacity and appears to increase PON-1 activity.

Caloric Restriction and Intermittent Fasting

Reducing calorie intake, without malnutrition, are commonly implemented lifestyle interventions to lose weight or to improve general health. Of particular interest, caloric restriction, together with intermittent fasting, appear to be an effective dietary intervention to robustly enhance health and reduce age-associated parameters in several organisms (132). Human studies on caloric restriction in non-obese participants reported lower levels of oxidative stress, reduced fasting insulin levels as well as lower circulating levels of tumor necrosis factor alpha (133–135). Furthermore, caloric restriction caused a reduction in body weight as well as an improvement of cardiometabolic health parameters. Effects of caloric restriction on HDL composition and function have been investigated in a few small studies. In a study including 27 diabetic and obese patients, 16-weeks of a low-calorie diet (450 kcal/day) resulted in increased plasma apoA-I levels and markedly decreased plasma CETP concentration, but did not alter cholesterol efflux capacity of HDL (136). Another study evaluated the long-term effects of caloric restriction on risk factors of atherosclerosis (137). Eighteen participants who had been on caloric restriction for an average of 6 years were compared with 18 age-matched individuals who followed a typical American diet. In the caloric restriction group, several risk factors for atherosclerosis were lower, including total cholesterol, LDL-C, triglycerides and blood pressure, whereas HDL-C levels were higher (137). Therefore, long-term caloric restriction appears to be a possible strategy for atherosclerosis prevention. Of particular interest, another study observed that caloric restriction (1,200 kcal/day) for 3 months, combined with physical activity in obese patients with metabolic syndrome led to a surprising decrease of PON1 protein concentration, but increased PON1 activity after weight loss (138). However, in healthy Japanese women, a 2-months intervention of low calorie diet (1,200 kcal/day) was associated with a decrease of PON1 activity (139). It has been hypothesized that this reduction in enzyme activity is an adaptation to reduced LDL-C and HDL-C levels because of a reduced need for antioxidant protection of lipoproteins, but this seems a somewhat far-fetched hypothesis (139). Montefusco et al. studied the effect of a 6-month hypocaloric diet in patients with metabolic syndrome. They demonstrated a reduction in pro-inflammatory cytokine levels and changes in lipoprotein composition, with an increase in triglycerides and apolipoproteins in HDL (140). Interestingly, a positive correlation was observed between CETP levels and cytokine levels, demonstrating a link between lipids and pro-inflammatory cytokines.

Fasting has been practiced for millennia, but only recently studies have shed light on its role in adaptive cellular responses that appear to reduce oxidative damage and inflammation and optimize energy metabolism (141). Intermittent fasting is defined as a period of time, usually from 12 h to 3 weeks, with little or no food intake and abstention from caloric beverages (141).

In a trial of an alternate day fasting regime, with 25% energy intake on fasting days, the intervention reduced body weight, decreased triglyceride levels, and increased LDL particle size,

but did not alter LDL-C and HDL-C levels (142). A short-term intervention of the same fasting regime in obese subjects, showed similar results, with decreases in body weight, systolic blood pressure, triglycerides and LDL-C, while HDL-C remained unchanged (143). A comparison of alternate day fasting with a low-fat diet vs. alternate day fasting with high-fat diet in obese subjects showed a decrease of small LDL particles in both groups, while levels of HDL-C and HDL particle distribution remained unchanged (144). In a study of healthy and non-obese subjects, alternate day fasting for more than 6 months showed improved cholesterol, LDL-C, and VLDL levels but had no effect on HDL-C levels (145). Summarized, intermittent fasting appears to have no direct effect on HDL-C levels, but the possible influences of this diet on HDL functionality remain to be investigated.

IMPACT OF DIETARY INTAKE OF POLYPHENOLS ON HDL FUNCTION

Polyphenols are a large heterogenous family of naturally occurring molecules, which are characterized by the presence of one or more aromatic rings and attached hydroxyl groups (146). More than 8,000 phenolic structures have been reported and most of which are present in plant-based food (146). Dependent on their chemical structure, polyphenols are classified into flavonoids and non-flavonoids (147). Flavonoids are the most numerous of the phenols and are abundant in the entire plant kingdom (148). In recent years, many studies have focused on elucidating the biological activity of polyphenols and polyphenol-enriched foods. While many rodents and *in vitro* studies have been conducted, the available evidence in humans is scarcer. It has been reported that polyphenols exert effects on modulation or prevention of hypertension (149, 150), cardiovascular disease (151, 152), endothelial dysfunction (153), and metabolic syndrome (154). Moreover, recent research has shown that polyphenol intake may also affect HDL composition and functional parameters, such as PON1 activity and cholesterol efflux capacity (155). Therefore, it may be worth considering polyphenols as a dietary supplement to improve HDL functionality. However, further studies are needed to draw firm conclusions.

Anthocyanin

One group of polyphenols belonging to the flavonoid family are anthocyanins, common water-soluble pigments found in flowers and fruits. Structurally, anthocyanins consist of an anthocyanidin (aglycone) and glycosidically bound sugars (156). Studies have reported that these flavonoids possess antioxidative (157) and anti-microbial activities and also improve the lipid profile of healthy adults (158). Several studies have also demonstrated a preventive effect on diseases, such as CVD and diabetes (156).

In recent years, research has also focused on the bioactivity of this flavonoid subclass in the context of HDL composition and function. In a study cohort of dyslipidemic subjects, anthocyanin supplementation (320 mg anthocyanin capsules/day) for 12 weeks led to an increase of HDL-C by 13.7% with a concomitant elevation of cholesterol efflux capacity to serum (159). Furthermore, anthocyanin supplementation resulted in a decrease of plasma CETP mass and activity, which explains the rise in HDL-C (159). Xu et al. showed that anthocyanin supplementation (80–320 mg/day) improved HDL cholesterol efflux capacity and HDL-C levels (160). The increase of HDL-C upon anthocyanin supplementation in hypercholesterolemic patients has been confirmed in other studies, which also reported improved endothelium-dependent vasodilatation (161) and reduced inflammatory response (162). A 24-week consumption period of anthocyanin increased PON1 activity by 17.4% while enhancing antioxidative capacity and reducing HDL-associated lipid hydroperoxides (163). The study further reported an increase of HDL cholesterol efflux capacity (163). Furthermore, anthocyanin supplementation in a cohort of diabetic patients improved dyslipidemia associated with increased HDL-C and antioxidative capacity of plasma (164). Intake of anthocyanine-rich blueberries over a 6-month period resulted in increased HDL-C, as well as HDL particle number and improved vascular function in overweight and obese subjects (165).

Taken together, the results of these studies appear to provide an explanation for the association between anthocyanin intake, increased HDL functionality, and cardioprotection.

Quercetin and Green-Tea Polyphenols

The most frequently occurring compound in the family of flavonols is quercetin, occuring in sources including onions, apples, broccoli, bilberries, grapes and green and black tea (166). Mechanistic *in vitro* studies on this flavonoid mostly focused on PON1 and reported an increase of its activity after treatment of hepatocytes (167). Other *in vitro* studies showed that quercetin increased the expression level of SR-BI in HepG2 cells in a concentration- and time-dependent manner (168) and raises ABCA1 mRNA levels and HDL- and apoA-I-mediated cholesterol efflux (169). In rodents, the induction of PON1 expression induced by quercetin was confirmed (170, 171). Feeding quercetin for 4 weeks increased hepatic expression and serum activity of PON1. In line, the ability of HDL to protect against oxidation of LDL was increased (170). However, studies on healthy adults receiving different doses (six capsules with a total of 50–150 mg/day) of supplementary quercetin for 2 weeks did not show any change in PON1 activity, which was argued to be caused by differences in quercetin metabolism between rodents and humans. The quercetin dosages were selected based on the 5-, 10, and 15-fold estimated daily intake of quercetin in Germany (50, 100, and 150 mg) (172).

The subclass of flavanols is mainly composed of the compounds catechin and epicatechin, which are predominantly found in cocoa, grapes, wine and green tea (173). Administration of green tea through drinking water over a period of 6 weeks in diabetic rodents improved HDL functionality by increasing serum PON1 activity and reducing oxidation of apoB-containing lipoproteins (174). In another study, ApoE-deficient mice received extra virgin olive oil (EVOO) enriched with green tea polyphenols for 2 months. A significantly improved PON1 activity and an increase in HDL cholesterol efflux capacity was observed (175). In patients with end-stage renal disease supplementation with green tea extract improved PON1 activity

and reduced expression of pro-inflammatory cytokines after hemodialysis (176). In a randomized controlled trial with obese subjects comparing the effects of consuming yerba mate, apple tea, or green tea, a significant increase in PON1 activity was found only in the yerba mate group (177). A recent study in hypercholesterolemic rodents demonstrated that long-term administration of matcha green tea (dosage equivalent to 7.5 cups of tea for human individual) led to lower HDL-C, decreased cholesterol efflux capacity as well as reduced cholesteryl-ester transfer to triglyceride-rich particles. Treatment was associated with increased vascular stiffness and greater susceptibility to the development of atherosclerotic lesions (178). Given these controversial results and the lack of literature on human studies, further research is needed to draw firm conclusions.

Resveratrol

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) belongs to polyphenols' stilbenoids group, possessing two phenol rings linked to each other by an ethylene bridge. This natural polyphenol has been detected in more than 70 plant species, especially in grapes' skin and seeds, and was found in discrete amounts in red wines and various human foods. Resveratrol is known for its antioxidant and anti-inflammatory properties and for its ability to upregulate endothelial NO synthase (179–181), but resveratrol also affects the lipid metabolism. Specifically, resveratrol induced a statin-like inhibition of HMG-CoA reductase in a hyperlipidemic rodent model, and lowered cholesterol, triglyceride, apoB, and CETP concentrations, accompanied by an increase in plasma apoA-I levels (182, 183). Resveratrol supplementation in apoE-deficient mice revealed similar results, showing increased levels of HDL-C but also elevated plasma PON1 activity (184). In addition, treated animals showed fewer atherosclerotic lesions and less presence of adhesion molecules in atherosclerotic vessels (184). The upregulation of PON1 expression upon resveratrol treatment was further confirmed *in vitro* (185) and *in vivo* (186). Oxidized LDL is present in atherosclerotic lesions, and disease progression is thought to be decelerated by inhibiting oxidation (187, 188). Of particular interest, resveratrol prevented LDL from peroxidation induced by copper- and γ-radiolysis in a dose dependent manner (189). Resveratrol was suggested to interact with radicals to form stable or non-radical compounds (190). The effect of resveratrol on cholesterol homeostasis has also been demonstrated through its effect on apoA-I-mediated cholesterol efflux by upregulating ABCA1 (189). Interestingly, cholesterol uptake by macrophages or endothelial cells was diminished in the presence of resveratrol. Further experiments showed that resveratrol protected Cu-induced oxidation of human HDL3, which was isolated from healthy volunteers, in a dose-dependent manner and preserved its cholesterol efflux capacity (189). Interestingly, in a recent study in patients with type 2 diabetes, 8 weeks of resveratrol supplementation (1,000 mg/day) resulted in increased PON1 activity and decreased serum levels of asymmetric-dimethylarginine, an inhibitor of endothelial NO synthase (191). There are several randomized controlled trials investigating the lipid-lowering effects of resveratrol in humans, but the results are inconsistent. Some studies reported a positive effect of resveratrol on lipid levels (192–196), while others showed no significant impact (197–201). Due to its poor solubility and bioavailability, application of resveratrol is still a major challenge for pharmaceutical industry. Further studies are needed to definitively determine the effect of resveratrol on metrics of HDL-function.

Curcumin

The polyphenol curcumin, is a well-known and commonly used spice in Middle Eastern and South African cuisine, whose bioactive anti-inflammatory, antioxidant and hepato-protective effects have been investigated in several studies (202–207). Of particular interest is the effect of curcumin on lipid metabolism and the resulting protective effect against atherosclerosis (203, 208). Due to its beneficial properties, curcumin has been suggested as a potential therapeutic to augment HDL functionality (209). *In vitro* experiments examining the effect of curcumin treatment on macrophages revealed a dose-dependent increase in cholesterol efflux through increased expression of ABCA1 and SR-BI mediated by heme oxygenase-1 (210). Interestingly, in a study of hypercholesterolemic rabbits, 6 weeks of curcumin treatment resulted in an increase in HDL-C levels, a decrease in plasma CETP levels, and an increase in antioxidant activity (211).

In a study investigating the potential effect of curcuma on the prevention of atherogenesis in healthy subjects, daily administration of ∼20 mg curcumin for a period of 30 days improved plasma lipid profile (212). Specifically, LDL-C and apoB levels decreased, while levels of HDL-C and apoA-I increased. However, in another study of healthy middle-aged subjects receiving a daily dose of 80 mg curcumin, supplementation had no effect on plasma cholesterol levels but reduced plasma triglyceride levels (213). Interestingly, this study revealed an increase in plasma nitric oxide levels, while levels of the soluble intercellular adhesion molecule were decreased after the intervention. In contrast to numerous animal studies that showed a decrease in myeloperoxidase activity after curcumin administration (214–216), an unexpected increase was observed in the human study (213). A recently published systematic review on the effect of nano-curcumin supplementation revealed an overall increase in HDL-C levels (217). Encapsulation of curcumin in nanoformulations has been shown to prolong circulation time and increase its bioavailability and solubility in several *in vitro* studies (218–221) and already has been used in some clinical trials (222–225). However, in summary the published studies on the potential effects of curcumin on HDL functionality are still inconsistent and further studies are needed to draw firm conclusions.

CONCLUSION

We now understand that the protective effects of HDL are not reflected by the cholesterol content of the particles, so the quality (composition and functionality) of HDL particles must be evaluated. These properties include HDL mediated cholesterol efflux capacity, antioxidant and anti-inflammatory functions, but also immunmodulating and vasoprotective activities.

The benefit of HDL-C elevation is unclear given the conflicting evidence from pharmacological studies on HDL-C elevation, but an examination of the functional properties of HDL deserves attention. Dietary strategies and certain dietary components have been shown to improve HDL functionality. The strongest evidence for modifying parameters of HDL function is available for the Mediterranean diet. This dietary pattern, especially when enriched with EVOO, has been shown to improve HDL cholesterol efflux capacity, to increase PON1 activity, and to augment antioxidant capacity of HDL. Particularly, the phenolic compounds of EVOO seem to exert these effects on HDL function. Supplementation of other polyphenols, such as anthocyanins, but also antioxidants like lycopene and eicosapentaenoic acid appear to improve HDL functionality, highlighting the need for additional research.

AUTHOR CONTRIBUTIONS

JS and GM conceptualized and wrote the manuscript. JS generated the figures. All authors contributed to the article and approved the submitted version.

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The Effect of Polyphenols on Working and Episodic Memory in Non-pathological and Pathological Aging: A Systematic Review and Meta-Analysis

Karin de Vries[1,2†], *Evelyn Medawar*[2,3†], *Aniko Korosi*[1] *and A. Veronica Witte*[2,4*]

[1] *Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, Netherlands,* [2] *Department of Neurology, Max Planck Institute for Human Cognitive and Brain Sciences, Leipzig, Germany,* [3] *Faculty of Philosophy, Berlin School of Mind and Brain, Humboldt-Universität zu Berlin, Berlin, Germany,* [4] *Clinic for Cognitive Neurology, University Medical Center Leipzig, Leipzig, Germany*

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***Correspondence:**
A. Veronica Witte
witte@cbs.mpg.de

[†] *These authors have contributed equally to this work*

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Life expectancy steadily increases, and so do age-associated diseases, leading to a growing population suffering from cognitive decline and dementia. Impairments in working memory (WM) and episodic memory (EM) are associated with an increased risk of developing dementia. While there are no effective pharmacological therapies to preserve or enhance cognition and to slow down the progression from mild memory complaints to dementia so far, plant-based nutrients including polyphenols have been suggested to exert beneficial effects on brain aging. This review studies whether supplementary polyphenols are effective in preserving or enhancing memory in both non-pathological and pathological aging, and whether there are polyphenol efficiency differences between WM and EM. A systematic literature search was conducted and 66 out of 294 randomized clinical trials with 20 participants or more per group, aged 40 years or older were included. These covered a daily intake of 35–1,600 mg polyphenols, e.g., flavonols, flavonoids, isoflovones, anthocyanins, and/or stilbenes, over the course of 2 weeks to 6.5 years duration. In total, around half of the studies reported a significantly improved performance after polyphenol administration compared to control, while three studies reported a worsening of performance, and the remainder did not observe any effects. According to pooled WM and EM meta-analysis of all memory outcomes reported in 49 studies, overall effect size for WM and EM indicated a significant small positive effect on EM and WM with similar estimates ($b \sim 0.24$, $p < 0.001$), with large study heterogeneity and significant Funnel asymmetry tests suggesting a positivity bias. These results remained similar when excluding studies reporting extremely large positive effect sizes from the meta-analyses. While *Ginkgo biloba* and isoflavones did not show benefits in subgroup meta-analyses, those suggested some effects in extracts containing anthocyanins, other flavonoids and resveratrol, again potentially resulting from publication bias. To conclude, a systematic review and meta-analysis indicate that short- to moderate-term polyphenol interventions might improve WM and EM in middle-to older aged adults, however, publication bias in favor of positive results seems likely,

rendering definite conclusions difficult. Future studies with larger, more diverse samples and sensitive monitoring of cardiovascular, metabolic and beginning brain pathologies as well as longer follow-up are needed to better understand the impact of age, (beginning) pathologies, gender, and long-term use on polyphenol action.

Keywords: polyphenol, RCT—randomized controlled trial, aging, episodic memory, working memory

INTRODUCTION

Due to economic, social, and health care developments, the life expectancy of people in all regions of the world is increasing. As a consequence, the proportion of people aged 65 or older is expected to rise from 1 in 11 people in 2019 to 1 in 6 people in 2050 (1). Aging is associated with deteriorating health, including brain health. With aging, for example, pro-inflammatory activity and less efficient anti-oxidative mechanisms lead to higher burden of neuroinflammation and oxidative damage in the brain (2). Moreover, an increase in neurodegeneration (i.e., loss of neurons) and a reduction in neurogenesis (i.e., formation of new neurons) occur with aging and negatively affect the neuronal plasticity of the brain (3, 4). These brain alterations are thought to underly cognitive decline and memory impairment, a key symptom of dementia such as in Alzheimer's disease (4). As the number of people with dementia could likely expand to 132 million people by 2050, causing extreme social and individual costs (5–7), healthy brain aging constitutes a global challenge. However, currently, there are no effective pharmacological therapies to preserve or enhance cognition in older age (8, 9).

While unhealthy lifestyle can accelerate the process of cognitive decline during aging, health-promoting lifestyle factors such as physical activity and nutrition might slow down the trajectory of cognitive decline (10). Therefore, the interest in studying the influence of polyphenols on cognitive functioning is rising. Polyphenols are micronutrients that are found in plant-based foods (4). There are several subclasses of polyphenols, of which flavonoids, stilbenes, and phenolic acids are examples (11). Multiple phenol groups per molecule characterize polyphenols, but the chemical properties of different polyphenols are heterogeneous (12). Studies suggest that polyphenols can cross the blood-brain barrier and affect aging processes due to their anti-inflammatory, antioxidant, and neuroprotective properties (2, 13). In the past ∼15 years, numerous animal experiments and human studies have investigated whether polyphenols enhance cognitive performance or prevent age-related brain pathologies, yet the level of scientific evidence and clinical efficiency in humans still remain unclear [reviewed e.g., in (14–16)].

Therefore, we aimed to perform a systematic review whether polyphenols are effective in preserving or enhancing memory in (non-)pathological human aging. We decided to consider results from randomized-controlled trials (RCT) only, as this study design is the most ideal to demonstrate a causal relationship between an intervention and an effect and is an important step toward evidence-based therapies (17). We focused on the memory system because (1) memory decline may underlie changes in other cognitive functions and (2) memory complaints are reported first in the preclinical trajectory of cognitive decline, sometimes already 16 years before diagnosis (18, 19). Two common types of dementia, namely frontotemporal dementia and Alzheimer's disease, are characterized by early-stage deficits in working memory (WM) and episodic memory (EM) respectively, underlining (partly) independent neuronal underpinnings of the two memory processes (20). WM is required to direct attention and manipulate information that is stored in short-term memory (21, 22) and EM allows to learn, store, and retrieve information about personal experiences (22, 23). In addition, impairments in either WM and EM are associated with an increased risk of developing dementia (24, 25). We therefore studied both WM and EM and asked whether polyphenols are effective in preserving or enhancing memory in (non-)pathological human aging.

METHODS

Literature Search

A literature search was conducted in PubMed in August 2021 (**Figure 1**). We decided to The search term ((((cognitive AND blueberry AND (humans[Filter])) OR (cognitive AND gingko AND (humans[Filter]))) OR (cognitive AND curcumin AND (humans[Filter]))) OR (cognitive AND polyphenol AND (humans[Filter]))) OR (cognitive AND flavonoids AND (humans[Filter])) together with the filter "clinical trial" or "clinical study" or "RCT" resulted in 294 hits. All articles were screened on the following inclusion criteria: (1) RCT study design, (2) administration of polyphenols or polyphenol-rich extracts or supplements, (3) an included measure of working or episodic memory, (4) a sample size of at least 20 participants per group with available follow-up data (i.e., completers), and (5) the participants included in the sample had to be at least 40 years old. Exclusion criteria were non-English articles, single dose trials, severe disease of participants (such as depression, multiple sclerosis, cancer) as well as duplicates or re-analyses of previously published trials. A study outcome measures was identified as memory performance-related and grouped into EM and WM, respectively, based on the author's descriptions and/or by inspecting the description of the used tests in the literature. No self-reported memory measures were evaluated. Note that several neuropsychological tests used in dementia patients, such as the ADAS-cog, usually do not provide raw memory subscale values. These studies were included in the systematic review but excluded from meta-analyses due to the lack of specificity regarding memory functions. A cutoff

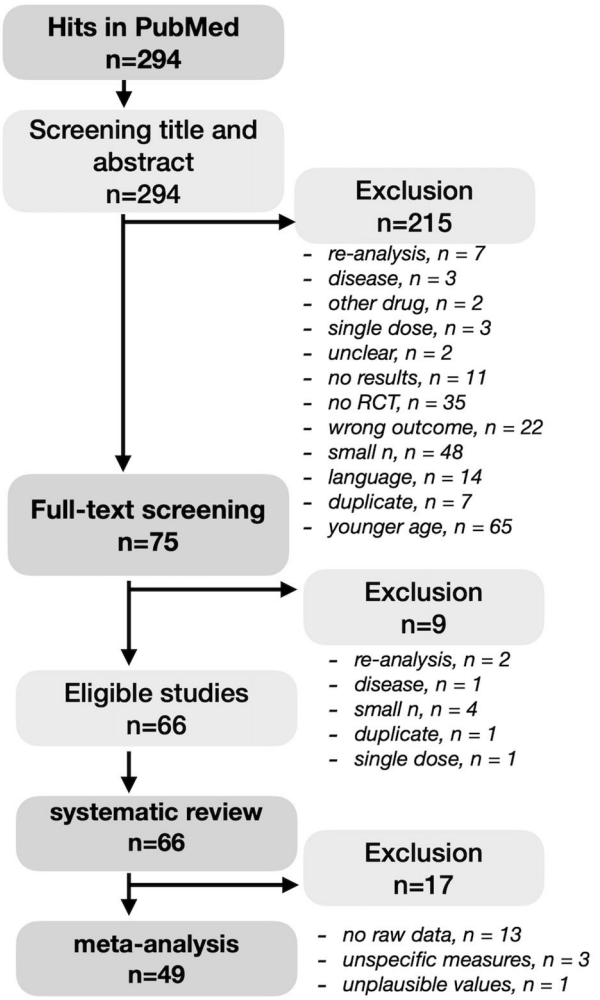

FIGURE 1 | PRISMA diagram of the systematic literature search.

number of minimally 20 participants per group was used to decrease the likelihood of a type II error, which can skew the results toward not finding a (small) effect that is truly there. Considering the age range definition, cognitive decline already starts from young adulthood, but by middle age, from around 40, neuronal volume shrinkage in both white and gray matter becomes more apparent (26, 27). According title and abstract screening, as well as occasionally briefly consulting full-texts, resulted in 75 RCTs with the majority of studies excluded due to wrong population, wrong micronutrient, no memory measure, no RCT, younger age, small sample. During the full-text screening, another nine articles were excluded based on re-analysis ($n = 2$), non-dementia disease ($n = 1$), small sample size ($n = 4$), duplicate ($n = 1$), and single dose study ($n = 1$). Full-text screening eventually resulted in 66 articles that were included in the systematic review. Effect sizes could be derived or calculated from 49 articles and included in a meta-analysis. Due to unavailable raw value information, effect sizes could not be retrieved from 17 studies.

As all included articles provided relatively large sample sizes per group ($>n = 20$) and all were carefully checked for following a randomized trial design being regarded as the gold standard, we refrained from applying additional quality evaluation tools.

Included Polyphenols and Their Effects on Memory

The identified studies investigated either polyphenol-rich plant extracts such as berries, cherries, grapes, pomegranate, green tea, and *Ginkgo biloba*, cocoa flavanols, curcumin, *Pinus radiata* bark, spearmint extract, soy isoflavonol, or rather purified polyphenols such as resveratrol (see **Table 1** for a more detailed overview). In addition, studies included different control conditions, either placebo or no treatment, or a lower dosage of the polyphenol treatment, or an alternative drug, such as rivastigmin or donezepil for treatment of Alzheimer's disease symptoms or alternative substances not containing polyphenols. Results were further reviewed according to these categories and reported WM and EM outcomes, respectively.

Evaluation of Effect Sizes

Effect size d for all studies with available data [mean, standard deviation (SD) or *F*-statistics] was computed in the following way: (1) for studies with available mean and SD for 1 timepoint for 2 groups, we calculated effect sizes using the "Means, Standard Deviations, and Sample Sizes" algorithm available at https://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD1.php, (2) for studies with mean and SD for 2 timepoints for 2 groups (repeated measure design), we calculated effect sizes for mean differences of groups with (un)equal sample size within a pre-post-control design using the algorithm at https://www.psychometrica.de/effect_size.html, and (3) for studies with *F*-statistics only (no mean, SD available), we calculated effect size according to the algorithm at https://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD4.php. If necessary, standard error (SE) was transformed to SD by calculating SD = SE × sqr (n).

Effect size error SE_d was computed according to:

$$SE_d = \sqrt{\frac{n_1 + n_2}{n_1 n_2} + \frac{d^2}{2(n_1 + n_2)}}$$

with effect size (d), intervention sample (n_1) and control sample (n_2). For those memory outcomes, where higher scores relate to lower performance (i.e., reaction time), effect size valence was inversed, to represent memory improvements. At maximum four outcome measures (2 WM, 2 EM) were included in the meta-analysis per study due to feasibility. Effect sizes were calculated for the most sensitive measures (for WM e.g., Stroop inference reaction time and for EM e.g., delayed recall of a 10–15 words list) and in case of more than two groups or timepoints, for the group that had a single formula and/or the highest dose compared to placebo/lowest dose; and for the longest intervention period. A deviation of this rule was made for Wightman et al. (83) reporting effect sizes for (1) *Sideritis scardica* extract and (2) *Ginkgo biloba* compared to placebo.

TABLE 1 | Details of the 66 included studies in the review.

Study no.#	First author	Publication year	Journal	Population	Age range (years)	Name of the examined extract, polyphenol or sublass (referred to as main or effective ingredient by the authors)	Intervention, amount of polyphenols	Control	Duration of intervention/ control period	n	Cognitive tests related to working or episodic memory performance	Any significant outcome (° none, + yes positive; - yes negative)	Significant outcomes in working (WM) or episodic memory (EM), or both	included in metaanalysis	Reference
1	Ahles S	2020	Nutrients	healthy adults (overweight-obese, BMI25-35)	40-60	*anthocyanins*	90 mg Aronia melanocarpa, 150 mg Aronia melanocarpa (18%anthocyanins)/day	maltodextrin	24weeks	102 (97 completed)	Stroop	°		yes	(28)
2	Basaria S	2009	J Endocrinol Invest	perimenopausal women	46-76	*isoflavones*	160 mg of total isoflavones (96 mg aglycones)/day	casein	12 weeks	93 (84 completed, 46+38)	TMTB	°		no (unplausible values)	(29)
3	Beck SM	2016	Hum Psychopharmacol	healthy adults, SMI	50-65	*ginkgo biloba*	240 mg, 22-27% ginkgo flavones/day	placebo (unclear)	8 weeks	75 (61−30,31 completed)	task-set switching, delayed response task, prospective memory	+	WM	yes	(30)
4	Bensalem J	2019	J Gerontol A Biol Sci Med Sci	healthy adults	60-70	*anthocyanins*	600 mg/day	placebo (unclear)	24 weeks	215 (190 completed)	Cambridge Neuropsychological Test Automated Battery (CANTAB): PALTEA, VRMFR, VRMR, SSP, reverse SSP	+	EM	yes	(31)
5	Burns NR	2006	Hum Psychopharmacol	healthy adults	55-79	*ginkgo biloba*	120mg/day	placebo (unclear)	12 weeks	93	test battery including wordlist, Stroop, digit span	+	EM	yes	(32)
6	Carlson JJ	2007	J Am Diet Assoc	healthy adults	65-84	*ginkgo biloba*	3xcapsules, =160 mg ginkgo biloba, 68 mg gotu kola, and 180 mg decosahexaenoic acid, bioflavonoid concentrate (100 mg) and vitamin A (300 IU)/day	identical capsules (gelatin, glycerin, soybean oil, yellow beeswax, lecithin, corn oil, natural caramel color, and maltodextrin)	4 months	90 (78 completed)	benton visual retention, word list learning	-	EM	yes	(33)
7	Casini ML	2006	Fertil Steril	postmenopausal women	>12 months after menses absence	*isoflavones*	600mg/day (60mg/day isoflavones)	identical placebo (unclear)	6 months	78 (76 completed, cross-over)	digit span	+	WM	yes	(34)
8	Cieza A	2003	Fortschr Med Orig	healthy adults	50-65	*ginkgo biloba*	240mg/day	placebo (unclear)	4 weeks	66 (66 completed)	different psychological tests including Stroop, digit span	°		yes	(35)

(Continued)

TABLE 1 | Continued

Study no.#	First author	Publication year	Journal	Population	Age range (years)	Name of the examined extract, polyphenol or sublass (referred to as main or effective ingredient by the authors)	Intervention, amount of polyphenols	Control	Duration of intervention/ control period	n	Cognitive tests related to working or episodic memory performance	Any significant outcome (° none, + yes positive; - yes negative)	Significant outcomes included in working (WM) or episodic memory (EM), or both	metaanalysis	Reference
9	Cox KH	2015	J Psychopharmacol	healthy adults	60–85	*curcuminoids*	400 mg/day Longvida® Optimized Curcumin (containing approximately 80 mg curcumin)	dextrin + yellow food powder	4 weeks	61 (60 completed)	parallel versions of tasks from the Computerised Mental Performance Assessment System	+	WM	yes	(18)
10	Cox KHM	2020	Nutrients	healthy adults	50-80	*curcuminoids*	400mg/day Longvida (80mg/day curcumin)	dextrin + yellow food powder	12 weeks	89 (79 completed)	Serial substractions, Virtual Morris Water Maze (vMWM)	+	WM	yes	(36)
11	Desideri G	2012	Hypertension	MCI	64-82	*cocoa flavanols*	high (HF: ≈990 mg flavanols/day) or intermediate (IF: ≈520 mg flavanols/day	low level (LF: ≈48 mg flavanols/day)	8 weeks	90	Verbal fluency, TMTA, TMTB	+	WM	yes	(37)
12	Dodge HH	2008	Neurology	healthy adults	>85	*ginkgo biloba*	3x80mg, =240mg/day (24% flavone glycosides)	unclear	42 months	134 (118 completed)	Cerad verbal learning	°		no (no raw measures available)	(38)
13	Evans HM	2017	Nutrients	cognitively intact post-menopausal women	45–85	*resveratrol*	150mg/day	placebo (unclear)	14 weeks	80 (72 completed)	Cambridge Semantic Memory Battery; Rey Auditory Verbal Learning Test (RAVLT); Double Span Task	+	EM	yes	(39)
14	Fournier LR	2007	J Nutr Health Aging	postmenopausal women	48-65	*isoflavones*	soy milk or soy supplement (70/72mg isoflavones)	cow milk, placebo supp.	16 weeks	79	Stroop, pattern recognition, benton, etc.	-	WM	no (no raw measures available)	(40)
15	Furlong ON	2020	Eur J Nutr	postmenopausal women	44-63	*isoflavones*	60mg/day or 35mg/day (in 350ml soy drink)	10mg/day	12 weeks	115 (101 analysed)	spatial working memory, spatial span, pattern recognition memory, 5-choice reaction time, and match to sample visual search	°		yes	(41)
16	Gavrilova SI	2014	Int J Geriatr Psychiatry	MCI	>55	*ginkgo biloba*	240mg per day	placebo (unclear)	24 weeks	160(145 analysed)	TMT-B	+	WM	yes	(42)
17	Gleason CE	2015	J Alzheimers Dis	AD	>60	isoflavones	100mg/day	maltodextrin	6 months	65 (59 completed)	verbal and visuospatial memory, Benton retention task, figure recall, TMT, etc)	°		yes	(43)
18	Hartley DE	2004	Nutr Neurosci	postmenopausal women	51-66	*ginkgo biloba*	320mg Gincosan/day (120mg ginkgo + 200mg ginseng)	unclear	12 weeks	70 (57 completed)	verbal and visual memory, CANTAB, delayed matching to sample test	°		yes	(44)

(Continued)

TABLE 1 | Continued

Study no.#	First author	Publication year	Journal	Population	Age range (years)	Name of the examined extract, polyphenol or sublass (referred to as main or effective ingredient by the authors)	Intervention, amount of polyphenols	Control	Duration of intervention/ control period	n	Cognitive tests related to working or episodic memory performance	Any significant outcome (° none, + yes positive; - yes negative)	Significant outcomes included in working (WM) or episodic memory (EM), or both	metaanalysis	Reference
19	Henderson VW	2012	Neurology	perimenopausal women	45–92 years	isoflavones	25 g/day of isoflavone rich soy protein(contained 91 mg aglycone weight isoflavones (154 mg total isoflavone equivalents) composed of genistein (52 mg aglycone equivalents), daidzein (36 mg aglycone equivalents), and gly- citein (3 mg aglycone equivalents))	milk protein placebo	2.5 years	350 (313 completed)	Immediate recall, faces 2 delayed recall	+	EM	yes	(24)
20	Herrlinger KA	2018	J Altern Complement Med	healthy adults	50-70	chlorogenic acid	900mg or 600mg spearmint extract/day	powder with 0 mg/ day spearmint extract	90 days	90 (87 completed)	CDR battery	+	WM	yes	(45)
21	Herrschaft H	2012	J Psychiatr Res	mild-moderate AD	>50	*ginkgo biloba*	240mg/day	unclear	24 weeks	410 (404 analysed)	SKT, CGIC, verbal fluency	+	WM	no (no raw measures available)	(46)
22	Ho SC	2007	Menopause	postmenopausal women	55-76	isoflavones	80 mg/day soy-derived isoflavones	identical appearing placebo	6 months	200 (data analyse van 176)	the Hong Kong List Learning Test= Tests of learning and memory (assesses rate of learning, rate of forgetting, encoding and retrieval deficits, and learning strategies) & Rey- Osterrieth Complex Figure Test and Wechsler Memory Scale-Revised = visuospatial constructional ability and visual memory	°		yes	(47)
23	Huhn S	2018	Neuroimage	healthy adults	60-78	resveratrol	200 mg/day of resveratrol and 320 mg of quercetin	placebo (identical w/o resveratrol)	26 weeks	60 (53 over)	California Verbal Learning Task (CVLT, main outcome), the ModBent task,	°		yes	(48)

(Continued)

TABLE 1 | Continued

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24	Jackson PA	2016	Nutrients	healthy adults, SMI	55-65	*ginkgo biloba*	Ginkgo biloba (240 mg)	2.24 g high oleic acid sunflower oil and 120 mg fish oil (32 mg DHA + EPA)	24 weeks	248 (84 completed)	Cognitive Demand Battery(CDB), Rapid Visual Information Processing(RVIP)	°		yes	(49)
25	Kanowski S	1996	Pharmacopsychiatry	AD or multi-infarct dementia	>55	*ginkgo biloba*	240 mg EGb 761®, 22– 27% Ginkgo flavonoids, 2.8–3.4% ginkgolides A, B, C, 2.6–3.2% bilobalide	placebo (unclear)	24 weeks	216(156 /205 analysed)	CGI, SKT, NAB	+	WM	no (no raw measures available)	(50)
26	Kaschel R	2011	Phytomedicine	healthy adults	45–65	*ginkgo biloba*	240 mg EGb 761®, 22– 27% Ginkgo flavonoids, 2.8–3.4% ginkgolides A, B, C, 2.6–3.2% bilobalide	unclear, placebo tablets	6 weeks	188 (177 for Memory tasks)	standardised free recall paradigm, standardised recognition test	+	EM	yes	(51)
27	Kean RJ	2015	Am J Clin Nutr	healthy adults	60-81	*flavonoids*	549 mg hesperidin/L, 60 mg narirutin/L(250 mL twice per day)	low flavone drink	8 weeks	35-28 (cross-over)	Spatial Working Memory	+	EM	yes	(52)
28	Kent K	2017	Eur J Nutr	mild-moderate AD	>70	*anthocyanins*	200 ml/day of cherry juice	control juice with negligible amounts of anthocyanins	12 weeks	49	battery of seven cognitive tests: RAVLT, SOPT, Boston naming test, TMT(A and B), digit span backwards task, category verbal fluency and letter verbal fluency	+	EM	yes	(13)
29	Kreijkamp-Kaspers S	2004	JAMA	postmenopausal women	60-75	*isoflavones*	25.6 g/day of soy protein (containing 99 mg of isoflavones: 52 mg genistein, 41 mg daidzein, and 6 mg glycetein)	milk protein	12 months	202 (175 completed)	Rey auditory verbal learning test, measures of short-term and long-term verbal and visual memory, Doors test, Digit span test	°		yes	(53)
30	Kritz-Silverstein D	2003	Menopause	healthy adults	55-74	*isoflavones*	110 mg/day total isoflavones	identical placebo	6 months	56 (53 completed)	WMS Logical Memory and Recall	°		yes	(54)
31	Kuszewski JC	2020	J Nutr	healthy adults	50-80	*curcuminoids*	160mg/day curcumin or 160mg/day combined with fish oil supplementation (2000 mg/d DHA + 400 mg/day EPA)	fish oil supplementation (2000 mg/d DHA + 400 mg/day EPA)	16 weeks	152(134 completed, 126 analysed)	neuropsychological test battery	°		no (no raw measures available)	(55)

(Continued)

TABLE 1 | Continued

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32	Le Bars PL	1997	JAMA	dementia	>45	*ginkgo biloba*	120 mg ginkgo	placebo (not specified)	52 weeks	309 (202 completed)	ADAS-cog	+	WM	no (no raw measures available)	(56)
33	Lewis JE	2014	BMC Complement Altern Med	healthy adults	>60	*ginkgo biloba*	120mg ginkgo leaf, 80mg whole gingko +others, +either 700mg choline vs OR anthocyanins mixture, green tea, vitamins etc.	cellulose, lactose, beet powder	6 months	97 (33+31+33)	TMT, Stroop	+	WM	no (no raw measures available)	(57)
34	Li S	2019	J Int Med Res	VaMCI	mean age 65	*ginkgo biloba*	3x19.2mg ginkgo	3x1.8mg pushen (traditional Chinese medicine)	12 weeks	64 (57 completed)	MoCA	°		yes	(58)
35	Mastroiacovo D	2015	Am J Clin Nutr	cognitively intact adults	61-85	*cocoa flavanols*	drink containing 993 mg/ day [high flavanol (HF)], 520 mg [intermediate flavanol (IF)]	48 mg [low flavanol (LF)] cocoa flavanols (CFs)	8 weeks	90	Verbal fluency test	+	WM	yes	(59)
36	Mazza M	2006	Eur J Neurol	mild-moderate AD	50-80	*ginkgo biloba*	daily: Ginkgo biloba: 160 mg	cholinesterase inhibitor: donepezil (5 mg daily dose), placebo	24 weeks	74 (60 completed)	SKT - Syndrome Kurz test (psychometric test battery for assessment of memory and attention), MMSE, CGI	+	WM	no (unspecific outcome)	(60)
37	McCarney R	2008	Int J Geriatr Psychiatry	dementia	>50	*ginkgo biloba*	120 mg daily (60mg twice a day); placebo: lactose-based	lactose-based placebo	6 months	176 (176 analysed)	ADAS-cog	°		no (unspecific outcome)	(61)
38	McNamara RK	2018	Neurobiol Aging	SMI (mild)	62-80	*anthocyanins*	berry powder 25g dry weight, or combined fish oil + blueberry	placebo isocaloric carbohydrates or fish oil	24 weeks	94 (21,24,26,23; 76 analysed)	TMT, verbal learning, fluency	°		no (no raw measures available)	(62)
39	Mix JA	2002	Hum Psychopharmacol	cognitively intact adults	>60	*ginkgo biloba*	180 mg/day of Ginkgo biloba extract	placebo (unclear)	6 weeks	262 (149 analysed)	WMS-R LM	+	EM	yes	(63)
40	Napryeyenko O	2007	Arzneimittelforschung	AD or VaD	>50	*ginkgo biloba*	daily: 2 × 120 mg EGb 761	unclear	22 weeks	395	SKT test battery	+	WM	no (no raw measures available)	(64)
41	Nasab NM	2012	J Pak Med Assoc	mild-moderate AD	50-75	*ginkgo biloba*	120mg ginkgo	4,5mg rivastigmine (cholinesterase inhibitor)	24 weeks	56(51 completed)	7minutes test, MMSE	-	WM	yes	(65)

(Continued)

TABLE 1 | Continued

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42	Nilsson A	2017	PLoS One	healthy adults	50-70	anthocyanins	3x200ml berrymix (1325mg/l=811 mg/d)	placebo (matched to ingredients, pH etc) less fibre in the control drink	5 weeks	46(40 analysed, crossover)	dedicated sentence list - WM test	°		yes	(66)
43	Ochiai R	2019	J Alzheimers Dis	MCI	60-84	*chlorogenic acid*	2x553.6mg CGA/d	placebo (identical wo CDGs)l	12 weeks	34(28 analysed), crossover	ADAScog, TMT	+	WM	yes	(67)
44	Pase MP	2013	J Psychopharmacol	healthy adults	40-65	*cocoa flavanols*	500mg + 250 mg cocoa flavanols	protein, sugar, fat-matched; all had caffeine	30 days	87(77 analysed)	CDR battery	°		yes	(68)
45	Pipingas A	2008	Phytother Res	cognitively intact adults	50-65	*flavonoids*	960 mg of Enzogenol® and 120 mg of vitamin C. Enzogenol® is an aqueous extract from the bark of New Zealand grown Pinus radiata trees containing approximately 80% total proanthocyanins and other water-soluble flavonoids, flavonoid-conjugates and phenolic acids. Rich in proanthocyanins and contain a range of flavonoids including catechin, epicatechin, quercetin, dihydroquercetin, taxifolin and phenolic acids	vitamin c only	5 weeks	42	computer-based cognitive tasks for spatial working memory	+	WM	yes	(69)
46	Rainey-Smith SR	2016	Br J Nutr	healthy adults	40-90	*curcuminoids*	1500 mg/d total (1 ×500mg BCM-95®CG (BiocurcumaxTM) capsule three times a day):	placebo (unclear)	12 months	160	sixteen-item self-report Prospective and Retrospective Memory Questionnaire; Rey Auditory Verbal Learning Test; Subtests of Cogstate battery	°		yes	(70)

(Continued)

TABLE 1 | Continued

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47	Ryan J	2008	J Psychopharmacol	healthy adults	60-85	*flavonoids*	3x50mg PYC	placebo (same size and appearance), unknown	3 months	120(101 completed)	CDR battery	+	WM	yes	(71)
48	Santos RF	2003	Pharmacopsychiatry	healthy men	60-70	*ginkgo biloba*	80mg extract (24%flavonoids)	placebo (same size and appearance), unknown	8 months	48 (all completers)	test battery	+	WM	yes	(72)
49	Schneider LS	2005	Curr Alzheimer Res	mild-moderate AD	>60	*ginkgo biloba*	1x120mg ginkgo or 2x120mg (240mg) ginkgo	placebo	26 weeks	513 (410 completed)	ADAS Cog	°		no (no raw measures available)	(73)
50	Schneider LS	2019	Menopause	perimenopausal women	45-60	*isoflavones*	50mg+100mg/d phytoserm (=isoflavones)	placebo (same size and appearance), unknown	12 weeks	71 (66 completed)	composite measures	°		no (no raw measures available)	(74)
51	Siddarth P	2020	Am J Clin Nutr	normal or MCI	50-75	*anthocyanins*	236,5ml pomegranate/d (368mg punicalagins, ca 100 anthocyanins, etc	placebo (same taste, sugar etc)	12 months	261(200 completed)	Brief Visuospatial Memory Test-Revised (BVMT-R) and Buschke Selective Reminding Test (SRT)	+	EM	yes	(75)
52	Small BJ	2014	Rejuvenation Res	cognitively intact adults	65–85	*anthocyanins*	900 mg NT-020 PlusBiovin / day (containing green tea extract (95% polyphenols), VitaBlue (40% polyphenols, 12.5% anthocyanins from blueberries), grape polyphenols (including 5% resveratrol), carnosine, vitamin D3 (2000 UI/serving) and Biotin 40 mg.	placebo (unclear)	2 months	113	Auditory Verbal Learning Test (AVLT; immediate and delayed recall) voor episodic memory;	°		yes	(76)
53	Snitz BE	2009	JAMA	healthy elderly, MCI	72-96	*ginkgo biloba*	2x120mg ginkgo	placebo (unknown)	6,1 years	3072(1545 +1542 completed)	Stroop, TMT and other	°		yes	(77)
54	Solomon PR	2002	JAMA	healthy adults	60-82	*ginkgo biloba*	3x40mg ginkgo/day	gelatine capsules	6 weeks	230 (203 completed)	Digit span, Stroop and other	°		yes	(78)

(Continued)

TABLE 1 | Continued

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55	Suominen MH	2020	Exp Gerontol	healthy adults	65-75	*cocoa flavanols*	50g dark chocolate (410mg flavanols/day)	50g dark chocolate (86mg flavanols/ day)	8 weeks	104 (100 completed)	TMT	°		yes	(79)
56	Thaung Zaw JJ	2021	Clin Nutr	postmenopausal women	45-85	*resveratrol*	75mg trans-resveratrol	several inert excipients	14 weeks	146 (110 analysed, cross-over)	TMT, list sorting, wordlist, picture memory	+	WM, EM	yes	(80)
57	van Dongen M	2003	J Clin Epidemiol	mild-moderate AD/ VaD or age-associated cognitive impairment	>50	*ginkgo biloba*	240 mg/day ginkgo biloba special extract (high dose) or 160 (usual dose) mg/day	placebo (unclear)	24 weeks	214	NAI-ZN-G; self-perceived health and memory status	°		yes	(81)
58	Whyte AR	2018	Nutrients	healthy adults	65-80	*anthocyanins*	450mg/day blueberry powder (35mg polyphenols, 1.35mg anthocyanins)or 900mg blueberry powder (70mg polyphenols, 2.7mg anthocyanins) or 100mg blueberry extract (50mg polyphenols, 7mg anthocyanins)	maltodextrin	6 months	122 (112 completed)	AVLT, object recognition, serial substractions, Stroop	°		no (no raw measures available)	(82)
59	Wightman EL	2018	Nutrients	healthy adults	50-70	*flavonoids*	475mg or 950 mg/day *Sideritis scardica* (Greek Mountain Tea) or 240mg ginkgo/day	maltodextrin	28 days	155 (140 completed)	own tests, global scores available	+	WM	yes	(83)
60	Witte AV	2014	J Neurosci	healthy overweight adults	50-80	*resveratrol*	200 mg/day of resveratrol and 320 mg of quercetin	placebo (corn oil)	26 weeks	46	Auditory Verbal Learning Test (AVLT)	+	EM	yes	(84)
61	Wong RH	2013	J Hypertens	obese healthy men and postmenopausal women	40-75	*resveratrol*	75mg trans-resveratrol	placebo (identical w/o resveratrol)	6 weeks	28 (cross-over)	Stroop	°		yes	(85)
62	Woo J	2003	Menopause	postmenopausal women	50-65	*isoflavones*	100mg isoflavones	hormone replacement (estrogen/ progesterone) OR no treatment	3 months	127 (completers)	TMT, word list learning	°		yes	(86)

(Continued)

TABLE 1 | Continued

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63	Yakoot M	2013	Clin Interv Aging	MCI	50-80	*ginkgo biloba*	G. biloba leaf extract 120 mg (standardized to contain 24% flavonoid glycosides and 6% terpenoids) and 150 mg of P. ginseng alcohol root extract (containing 40%– 80% ginsenosides).	750 mg of natural lyophilized royal jelly (standardized to at least 6% of 10-hydroxy-2decenoic acid)	4 weeks	66 (60 completed)	MMSE	+	WM	no (unspecific outcome)	(87)
64	Yancheva S	2009	Aging Ment Health	probable AD w neuropsychiatric symptoms	>50	*ginkgo biloba*	2x120mg (240mg/day ginkgo) or combined with donezepil 5-10mg/ day	5-10mg donezepil	22 weeks	96 (88 completed)	SKT	°		no (no raw measures available)	(88)
65	You YX	2021	Nutrients	MCI	60-75	*flavonoids*	2x250mg CC +250maltodextrin	2x500mg maltodextrin	12 weeks	48 (47 completed)	digit span, AVLT	+	WM, EM	yes	(89)
66	Zhang SJ	2012	Asian Pac J Trop Med	cerebral infarction patients/vascular cognitive impairment	60-75	*ginkgo biloba*	3x75mg aspirin/day + 3x40mg ginkgo	3x75mg aspirin/day	3 months	80	MOCA	+	WM, EM	yes	(90)

To analyse overall effect sizes of the included studies and a potential positivity bias in the publication records, we compared effect sizes of WM and EM outcomes, respectively, with forest and funnel plots in the software JASP developed and distributed by JASP Team (91), JASP (Version 0.14.1) [Computer software]. Inference of a random effects meta-analysis per memory category (EM and WM, respectively) was computed with the restricted maximum likelihood methods by pooling all polyphenol interventions and subsequently with sub-analyses in subgroups.

RESULTS

Study Characteristics

The included 66 studies had study durations varying between 4 weeks and 2.5 years and sample sizes between 42 and 350 participants. The investigated polyphenol extracts were administered as tablets, capsules, powder, chocolate bar or drink and comprised a large variety of polyphenol molecules. According to Truzzi et al. (92), polyphenols can be classified in four subclasses according to the number of inert phenolic rings, i.e., flavonoids, phenolic acids, stilbenes and lignans; others that do not fall into those categories are polyphenols such as e.g., curcuminoids. To compare the studies included in this review, we decided to group studies according to the examined extract, polyphenol substance or polyphenol subclass that the authors described as main or most effective ingredient. This resulted in the following subcategories: *Ginkgo biloba* leaf extract (26 studies), soy isoflavones extracts (11 studies), anthocyanins (eight studies, e.g., blueberry extract, pomegranate, cherry juice, etc.), cocoa flavanols (four studies), flavanoid extracts (five studies, e.g., *Sideritis scardica*, *Pinus radiata* bark, etc.), chlorogenic acids (two studies), curcuminoids (four studies), and resveratrol (five studies). Note that all subcategories except chlorogenic acids, curcuminoids, and resveratrol belong to the same subclass of flavonoids (92), and that most extracts contained multiple polyphenols, leading to a certain overlap between study subgroups.

The majority of studies reported to use a placebo tablet, capsule, powder or drink as a control condition for supplementary polyphenols. These placebos comprised for example the intervention formula but only with negligible or no amounts of polyphenols, for example in tablets, capsules or drinks. Some studies incorporated isocaloric maltodextrin or cow milk protein (i.e., for soy isoflavone) as placebo, or studied the effects of polyphenols alone, or added to a certain treatment, e.g., fish oil or vitamins or aspirin, compared to those treatments alone, sometimes including a third arm without any treatment/placebo. Other studies, in some cases including 3–4 study arms, tested effects of polyphenols vs. medications, such as rivastigmin or donezepil in the case of cognitive decline, or hormone replacement therapy in the case of peri/postmenopausal women, or in comparison to lower doses of the polyphenol, for example flavanols. For more information about the study characteristics, (see **Table 1**).

Adverse Events

Most, but not all studies reported on adverse events. Some studies did this extensively, others scantly reported on adverse events. Overall, polyphenols were well-tolerated. Most often, when adverse events were reported, the number and type of adverse events did not differ significantly from the placebo group. One study on *Ginkgo biloba* effects reported the occurrence of stroke in one participant, which could not equivocally related to the treatment (38). Recurring complaints in the reviewed studies were gastrointestinal complaints.

Reported Effects of Polyphenols on Memory

Overall, in this systematic review, 32 out of 66 studies (48.5%) found at least one significant improvement on any of the memory outcomes measured, while 31 (47%) did not report significant performance, and 3 (4.5%) did report decreases in memory in at least one measure (**Table 1**). However, these 66 studies analyzed a multitude of different outcome measures and different polyphenol substances or mixtures. All studies included at least one WM test outcomes, and the majority did also report EM outcomes. However, studies including dementia patients most often included only global assessments with relatively coarse measures of memory performance, such as the ADAS-Cog or SKIT, due to the severity of disease. This somewhat limits the interpretability of possible polyphenol effects on episodic memory in patients suffering more severe cognitive decline.

Tests assessing WM usually ask participants to remember information for a short amount of time and to manipulate this information. Examples of tests that measure WM are the digit span task, where participants have to repeat a series of numbers in the same or reversed order, or the serial subtraction task, where participants are assigned to keep on subtracting the number three or seven from a random number between 800 and 999 (18, 53). Another frequent test was the trail making test (TMT) B, which gives a working memory estimate of mental flexibility. Assessing EM typically entails the visual or verbal presentation of information to a participant and later measures whether this information is remembered (22). An example of an EM test is the Rey Auditory Verbal Learning Test (RAVLT), where participants hear a list of 15 words spoken out loud and are asked to recall the words immediately and after a delay and are asked what words they recognized from a 30-words list comprising old, new and distractor words (53). Forty-five studies included healthy older adults, with some focusing on overweight/obese participants and 12 included only peri/postmenopausal women, one only men. Eleven studies included subjective (SMI) or minor neurocognitive impairment (MCI), while another 14 studies included dementia of the Alzheimer's type (AD) or vascular dementia (VaD), or both. Depending on population, most studies had a relatively broad age range older than 40, 45, or 50, some focused on 65–80 years old only.

Effects of Polyphenols on Working and Episdoic Memory (WM/EM)

Ginkgo biloba

Almost half of the studies included in the review, i.e., 26, investigated the effects of *Ginkgo biloba*, in both healthy and disease samples, two with ginseng added to the treatment. *Ginkgo biloba* leaf extract is comprised of different polyphenols but particularly—similar to soy or certain other plant extracts—rich in flavonoids. The duration of treatment lasted from 4 weeks to up to 3.5 years. Fourteen of those studies reported at least one significant improvement, 11 no changes and two a worsening of functions. Notably, two of the longest studies over 3.5 and 6.5 years, respectively, reported diverging results: the first in 202 completers found a significant beneficial effect of 120 mg/day ginkgo tablets compared to placebo tablets in the ADAS-cog test and the GEFRI test ($p = 0.04$) in dementia patients (56), while the second longer one in a very large group ($n = 3{,}072$ completers) and after a doubled dosage (240 mg *Ginkgo biloba*) could not detect significant differences in healthy older or MCI patients (77). Due to the large sample size and long duration, the latter study is of particular interest, as well as the first one, however we could not include it in the meta-analysis based on missing specific memory outcomes (56). Two shorter studies in mild to moderate AD with doubled dosage over 6 months compared to placebo in around 400 participants also reported benefits (46, 64). However, other large trials with 410/214 completers reported no significant differences for improvements after 6 months in the ADAS cog test (73) (**Table 1**). Some studies also included subjective measures of memory performance, which is by some regarded as biomarker of later cognitive function ("subjective memory impairment"), however a less objective measure of current memory performance and thus not further considered in this review. Notably, two studies reported a worsening of function compared to control condition, one in healthy adults after 160 mg/day in 78 completers (however for EM outcome), and another one in patients with mild to moderate AD when comparing 120 mg/day ginkgo to 4.5 mg/day rivastigmin treatment (a cholinesterase inhibitor).

Soy Isoflavones

Isoflavones show similarities with steroidal estrogens and are suggested to counteract menopause-related estrogen deficiency possibly causing cognitive decline (24, 47). Soy isoflavones were examined in nine studies in peri- or postmenopausal women, one study included also men and another one AD patients. Overall, most studies (72%) could not detect any significant effects of isoflavones (mostly from soy) in dosages between 35 and 160 mg/day over 3–12 months, including a relatively large study with 175 women over the course of 1 year (53). One study in 79 women over three groups reported a worsening of a verbal working memory outcome after 16 weeks of soy isoflavone intake as soymilk, compared to cow milk or isoflavone supplement intervention (40). A cross-over study of 6 months isoflavone tablets compared to placebo ($n = 76$), however, as well as larger ($n = 313$), longer term intervention of soy isoflavones against milk protein-based placebo over 2.5 years, showed improvements in a working memory outcome [TMTB (34)] and in episodic memory (24), respectively. It could be speculated that isoflavones are effective in earlier age only because of their possible action as estrogen-related effects. Some studies indeed showed that effects are mainly found several years before menopause (i.e., perimenopause), when the ovaries start to produce less estrogen, or in young postmenopausal women (93, 94). However, two studies in perimenopausal women included in this review do not support this claim (41).

Berry, Cherry, Grape and Tea Extracts, Curcuminoids, and Related Supplements

Berry, cherry, grape, and green tea extracts are among other polyphenols rich in anthocyanins, evaluated in nine studies. Other extracts included cocoa flavanols (studied in four studies), flavonones (one study), curcuminoids (four studies), and rosmarinic acids (one study). Overall, study results were mixed with nine studies reporting no significant effects and nine studies reporting significant outcomes for WM or EM tasks. One study reported improvements in spatial and WM, but not EM, after intake of rosmarinic acid, containing spearmint extract for 90 days compared to identical placebo supplement (0 mg spearmint), after three months in a group of 87 healthy older adults (45, 69). Considering anthocyanins, for example administered as grape juice or blueberry extracts, most studies including healthy participants could not detect significant changes over 5 weeks to 6 months (four studies). For example, one study performed in 113 healthy elderly did not find any significant improvements on WM or EM outcome measures between the experimental and control group after 2 months (76). Study dosages were partly unclear, as for example, Whyte et al. (82) administered between 35 and 70 mg polyphenols per day and Small et al. (76) reported administering 900 mg of the supplement NT-020 per day, but it was unclear what the amount of which polyphenol was per dosage. In a relatively large sample ($n = 122$) of elderly with subjective self-reported memory complaints (82), the group receiving low dose polyphenols per day, including 7 mg anthocyanin per day, showed a significant improvement compared to placebo on one EM outcome measure after 3 months of supplementation, but not after 6 months. No significant improvements were found on the other five outcome measures of the verbal learning task or on a visual EM task.

In a population of 49 older adults with mild-to-moderate dementia and 138 mg anthocyanins per day intervention, however, Kent et al. (13) found significant improvements on all three measures of EM after 3 months, with moderate to large effect sizes (13). Bensalem et al. (31) reported dosing 258 mg flavonoids per day and showed a significant improvement in one of the three EM outcome measures after 6 months in a sample of 215 healthy elderly (31), and a secondary analysis revealed that polyphenol supplementation significantly improved all three measures of EM in a subgroup with the highest cognitive decline.

The influence of flavanols derived from cocoa was measured in both cognitively intact elderly and elderly with mild neurocognitive disorder (37, 59). In two studies, results showed that participants drinking high and medium amounts of flavanols performed significantly better on a WM task than participants

drinking low amounts of flavanols. In Mastroiacovo et al. (59), the high and medium flavanol groups reduced the time to finish a WM task over 8 weeks with 17 s (21%) and 14 s (18%) respectively, compared to 1 s (1%) in the low flavanol group. However, two studies in 77–100 healthy adults could not detect significant effects after 4–8 weeks intervention with daily drinks or chocolate bars against a protein shake or a low-dose flavanol chocolate, respectively, as control (68, 79). A similar impression comes from four studies on curcuminoids, with two showing no significant results in 134–160 healthy olders after 16 weeks-−12 months intervention (55, 70) and two studies from the same group in 60–79 participants reporting significant improvements in working memory measures after 4 and 12 weeks (18, 36). Here, it seems as if a shorter study duration (i.e., 4 weeks) more likely resulted in positive results, while an intervention period of 1 year did not lead to a difference in composite scores between the placebo and intervention group. On the other hand, the power of the studies with insignificant results was bigger, as the sample size were larger. The dosages of those studies are difficult to compare. Cox et al. (18) for example administered 80 mg curcumin per day. However, Rainey-Smith et al. (70) reported administering 1,320 mg curcuminoids but not mentioning the precise amount of other polyphenols.

In sum, the evidence for a beneficial effect on EM or WM on anthocyanins, flavanols and curcuminoids, is still limited, with a tendency toward more positive effects when cognitive decline is more severe based on two studies only. In line with this, though, the sample of Small et al. (76), showing no significant differences at all, was highly educated and therefore a ceiling effect of the memory task cannot be ruled out. Note also a lack of homogeneity with regard to dosages of different polyphenol subclasses, rendering a direct comparison of study results difficult: some of the flavanol studies for instance varied between 990 and 520 mg flavanol per day, while in the isoflavone studies, lower but still varying doses of 35–160 mg of isoflavones were administered.

Stilbenes

Supplementary doses of isolated polyphenols were rare and often combined with others derivatives or substances, such as in studies examining resveratrol, a polyphenol of the stilbene subclass (five studies). These studies tested postmenopausal women and healthy older adults with again mixed results. For example, Evans et al. (39) administered 150 mg resveratrol per day for over 14 weeks to 80 participants, while Huhn et al. (48) administered 200 mg per day plus 320 mg quercetin for over 26 weeks to 60 participants. Considering WM, although Huhn et al. (48) found no significant improvements, pattern recognition on a spatial WM-related test decreased in the placebo group and did not change in the resveratrol group. This result could suggest that resveratrol helps to preserve cognitive function, while memory otherwise subtly declines with aging. However, this effect was not seen in Evans et al. (39), where the performance in both the placebo and the resveratrol group increased with about the same amount. While quercetin was given to increase bioavailability of resveratrol, it cannot be excluded that a positive result related to the flavonoid-related benefits of quercetin, administered simultaneously in Huhn et al. (48). Considering EM, significant improvements were found in a sample of 46 healthy older adults and in a sample of 80 postmenopausal women (39, 84). Witte et al. (84) administered 200 mg resveratrol and 320 mg quercetin for 26 weeks. In both studies, the positive effect was only found on delayed recall and not on immediate recall of a verbal learning task. According to Evans et al. (39), the effect size was small. The above mentioned study by Huhn et al. (48) researching 60 healthy elderly could not confirm these findings, as no significant improvements in delayed recall, nor on three other outcome measures of a verbal learning task were found after 26 weeks (48). Again, it is imported to highlight that both Witte et al. (84) and Huhn et al. (48) also administered quercetin, to increase the bioavailability of resveratrol, so the results of these studies might be a combined effect of flavonoids and stilbenes.

Related, while one study did not report significant differences after resveratrol in obese older adults (85), others studied an overweight population and found significant improvement in EM performance after resveratrol administration (84). Here, the increased EM performance was associated with improvements in glucose metabolism (i.e., lower HbA1c levels). In addition, ameliorated memory performance was associated with increased functional connectivity between the hippocampus and the medial prefrontal cortex, suggesting a mechanistic link. In addition, a study with cocoa flavanol drink observed significant reductions in blood insulin after treatment, which also explained variance in the memory increases after treatment (55), again pointing to glucose metabolism as a potential mechanism. Positive verbal memory results for 75 mg/day trans-resveratrol intake compared to placebo capsules were also found in postmenopausal women in a very recent study after 14 weeks each in a cross-over design, including 110 women (80).

Evaluation of Effect Size

Considering the difference between the reviewed studies, overall, results appeared to be independent of sample size and study duration. A substantially larger sample size, leading to a greater power to detect an effect that is present, did not always lead to significant results in some samples, similar to smaller samples, suggesting that the effect of polyphenols on memory depends on other factors.

Taken together, available effect sizes of WM outcomes after polyphenol intake interventions appeared in the majority either small or non-existent, with the exception of some studies in both directions and two studies reporting extremely large positive effects on immediate face recognition (24) and TMT-B (59) (**Figure 2**).

When pooled in a meta-analysis, polyphenols showed a small overall positive effect on working memory [Wald test, $b = 0.26$ (95% CI: 0.09; 0.43), $z = 3.07$, $p = 0.002$]. The between-study heterogeneity variance was estimated at $\tau^2 = 0.44$ (95% CI: 0.32–0.7), with an I^2 value of 93% (95% CI: 91–96%), indicating that statistical heterogeneity between studies was highly significantly large [$Q_{(66)} = 710$, $p < 0.001$]. The associated funnel plot indicated a positivity bias in the publication record, according to visual inspection and Egger's regression test ($p = 0.017$) and a trend in the rank correlation test (Kendall's $\tau = 0.15$, $p =$

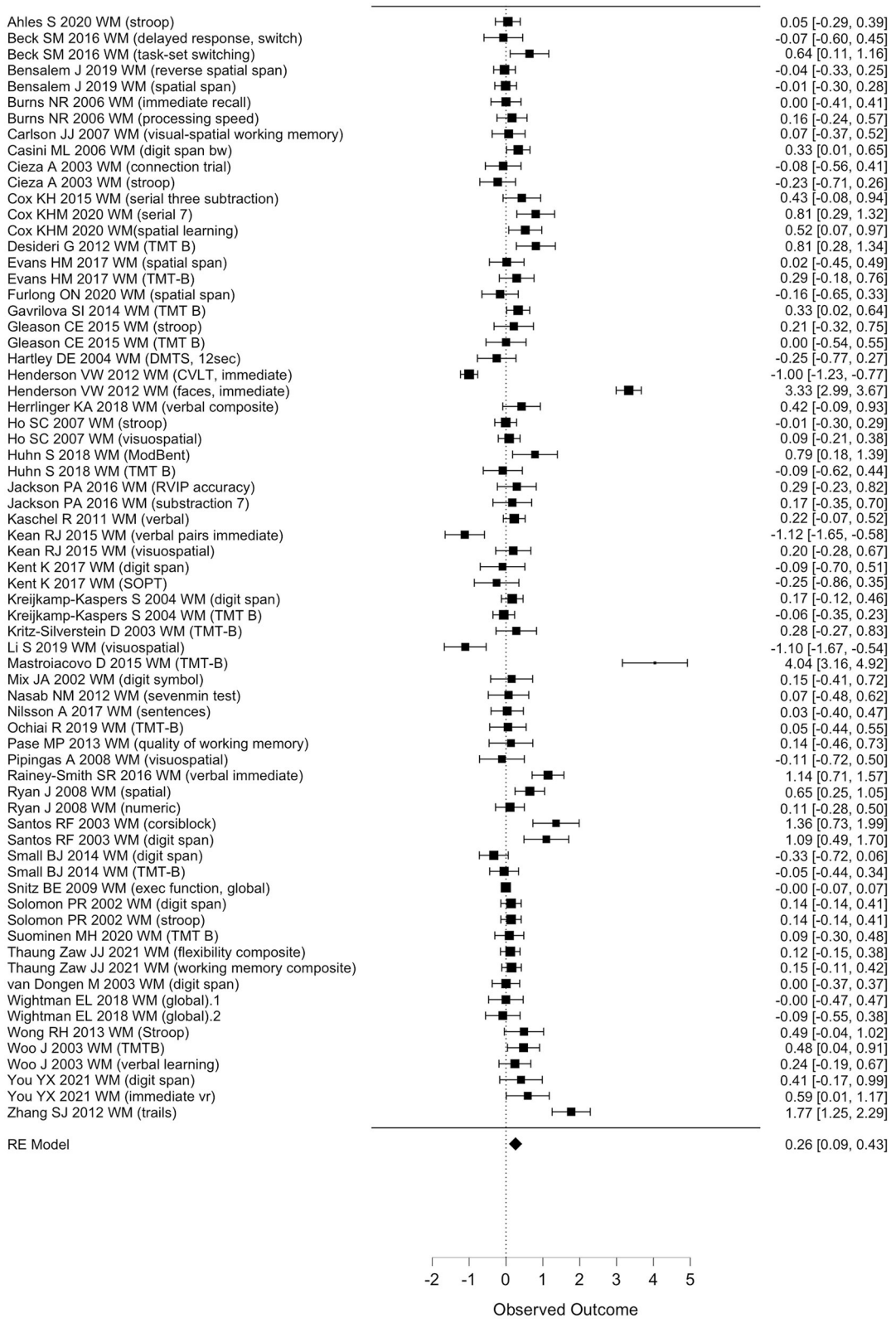

FIGURE 2 | Forest plot for observed effect sizes (d) and standard errors (SE) for available study results for WM. RE, regression estimate.

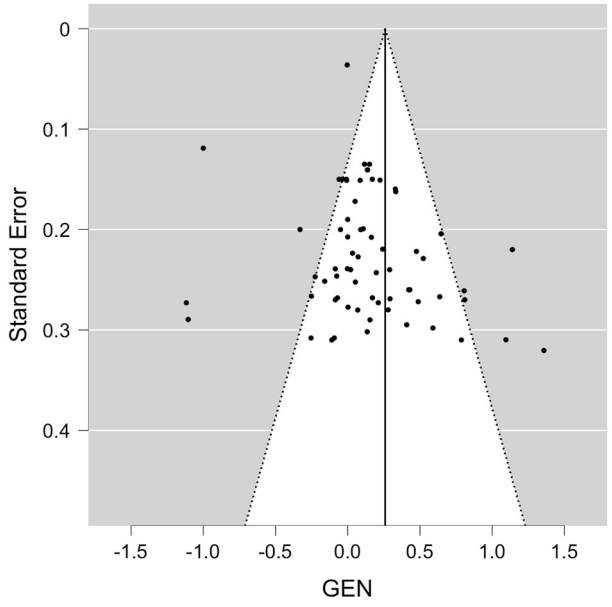

FIGURE 3 | Funnel plot for available study results for WM. GEN, general effect result per study.

0.065) (**Figure 3**). To further evaluate whether the two relatively large RCTs (n = 90–313) that added large positive effect sizes to the meta-analysis had considerable influence on the results, we re-run the meta-analysis excluding these studies (24, 59), however, while the pooled effect size became somewhat smaller, significance level remained unchanged [$b = 0.29$, $z = 3.6$, $p < 0.001$ (95% CI: 0.08–0.28)], with not much lowered between-study heterogeneity [$I^2 = 77\%$, $Q_{(65)} = 205$, $p < 0.001$]. Note that a positivity bias could no longer be observed (Rank test and Egger's test, $p > 0.18$). When focusing on study outcomes that derived from comparisons of placebo formulas without polyphenols or no treatment as control condition (i.e., excluding study outcomes from comparison to lower dosages as control, or from polyphenols compared to fish oil, medications or to alternative extracts), overall results remained stable to the overall meta-analysis (59 outcomes, $b = 0.26$, $p > 0.001$, Rank test $p = 0.045$).

Studies including mainly healthy samples showed similarly a significant effect size on average, yet the likelihood of a positivity bias in those studies was increased (majority of studies, 55 outcomes), $p < 0.032$. In studies including mild cognitive impairment or dementia patients (14 outcomes), though, no positivity bias emerged, and effects were no longer significant ($b = 0.2$, $p = 0.224$). Duration of studies did not affect meta-analysis outcomes that much (<6 months: $b = 0.19$, $p = 0.011$; 6 months or longer: $b = 0.47$, $p = 0.053$), however Funnel asymmetry could not be excluded, and the majority of studies tested <6 months of intervention period. When restricting analyses for studies on ginkgo (23 outcomes, 17 studies, note also a large proportion of patient samples here), meta-analysis on effect sizes did not reach significance for a positive effect ($b = 0.2$, $p = 0.075$) and a positivity bias based on Funnel plot asymmetry was no longer observed ($p > 0.35$). Similarly, meta-analysis for isoflavones (12 outcomes, eight studies; $b = 0.3$, $p = 0.28$, Funnel asymmetry, $p > 0.11$), anthocyanins (eight outcomes, five studies; $b = -0.06$, $p = 0.38$, Funnel asymmetry $p > 0.25$) and flavonoids (eight outcomes, five studies; $b = 0.1$; $p = 0.62$, asymmetry $p > 0.64$) did not show significant effects. However, studies examining other extracts containing resveratrol (seven outcomes, four studies; $b = 0.19$, $p = 0.013$; asymmetry $p > 0.22$) supported a significant positive average effect size. Note that sample size in the subgroup-meta analyses was considerably reduced. No subanalysis was computed in groups with <5 outcomes.

Evaluating EM, the included effect sizes indicated a small positive effect of polyphenol intake on EM outcomes on average (**Figure 4**). Accordingly, polyphenols showed a significant effect on episodic memory when pooled in a meta-analysis [Wald test, $b = 0.24$, $z = 2.88$, $p = 0.004$ (95% CI: 0.08–0.41)]. The between-study heterogeneity variance was moderate to large, estimated at $\tau^2 = 0.31$ (95% CI: 0.21–0.54), with an I^2 value of 91% (95% CI: 88–95%), pointing toward a high statistical heterogeneity between studies [$Q_{(50)} = 430$, $p < 0.001$]. Notably, both the rank correlation test (Kendall's $\tau = 0.34$, $p < 0.001$) and the Egger's regression test ($p = 0.01$) indicated significant asymmetry of the associated funnel plot. The funnel plot did not indicate according to visual inspection more strongly positive effect sizes had been reported in larger studies than could have been expected (**Figure 5**). Three small to large RCTs with small to longest durations added large effect sizes of positive sign (delayed recognition of faces, verbal recall) to the meta-analysis. When excluding these study (13, 24, 90), though, the pooled positive effect size became somewhat smaller yet remained statistically significant [$b = 0.21$, $z = 2.39$, $p = 0.017$ (95% CI: 0.02–0.18)], with a reduced between-study heterogeneity ($I^2 = 51\%$) yet still evidence for positivity bias (asymmetry, $p > 0.021$). When focusing on study outcomes that derived from comparisons of placebo formulas without polyphenols or no treatment as control condition (i.e., excluding study outcomes from comparison to lower dosages as control, or from polyphenols compared to fish oil, medications or to alternative extracts), overall results remained stable to the overall meta-analysis (47 outcomes, $b = 0.23$, $p = 0.009$, Rank test $p < 0.001$).

Note that a meta-analysis of those studies including mainly healthy samples did not reach statistical significance, yet the likelihood of a positivity bias in those studies was decreased majority of studies, 40 outcomes, $b = 0.14$, $p = 0.069$; Funnel asymmetry, $p > 0.052$. In studies including mild cognitive impairment or dementia patients (14 outcomes), effects were again significant, with a smaller estimate ($b = 0.64$, $p = 0.019$) and again positivity bias (Funnel asymmetry, $p < 0.032$). Duration of studies did not affect meta-analysis outcomes that much (<6 months: $b = 0.23$, $p = 0.02$; 6 months or longer: $b = 0.25$, $p = 0.097$), however Funnel asymmetry could not be excluded, and the majority of studies tested again <6 months of intervention period. When restricting analyses for studies on ginkgo (17 outcomes, 13 studies, note also a large proportion of patient samples here), meta-analysis on effect sizes did not reach significance for a positive effect ($b = 0.22$, $p = 0.19$) and

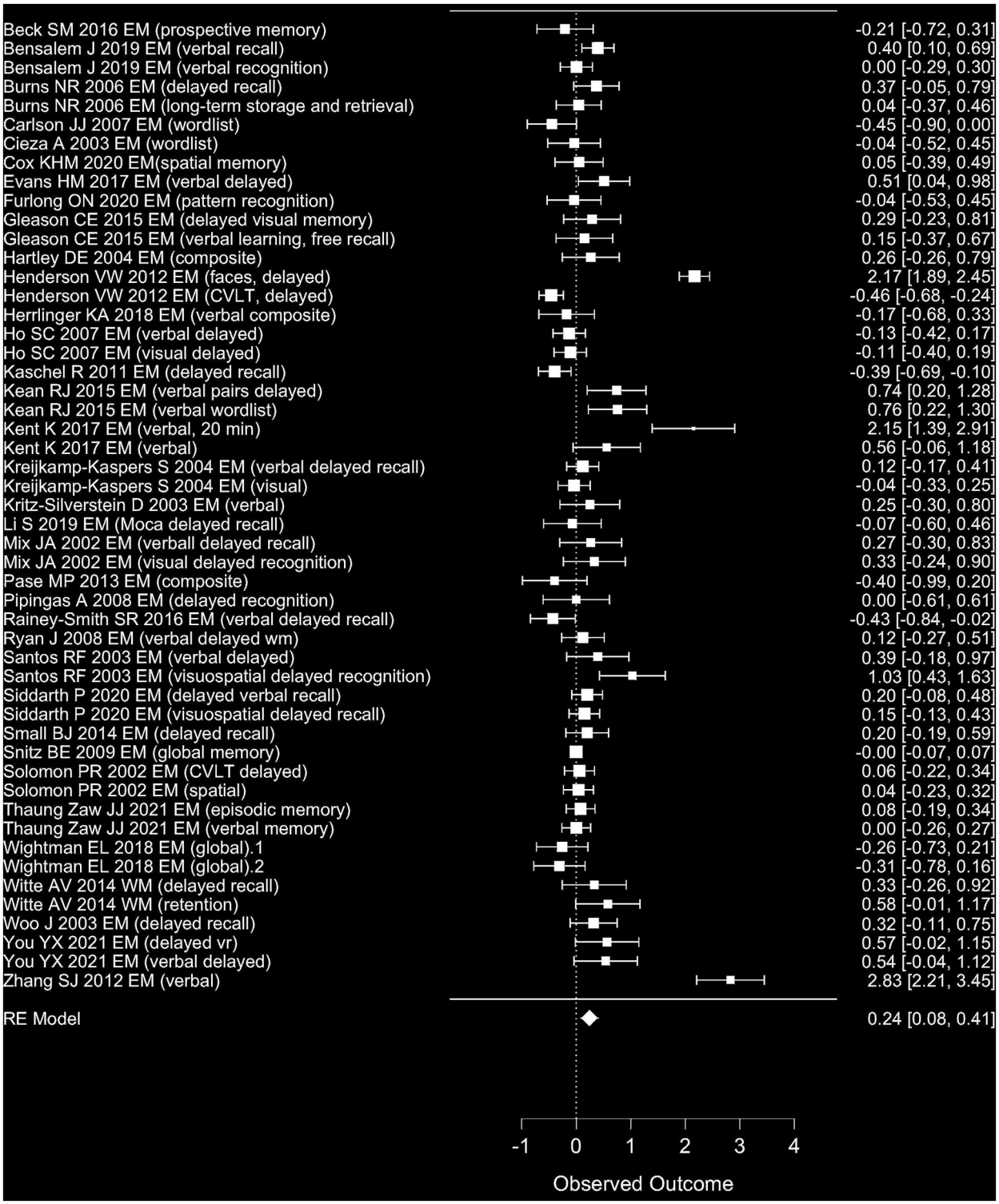

FIGURE 4 | Forest plot for observed effect sizes (d) and standard errors (SE) for available study results for EM. RE, regression estimate.

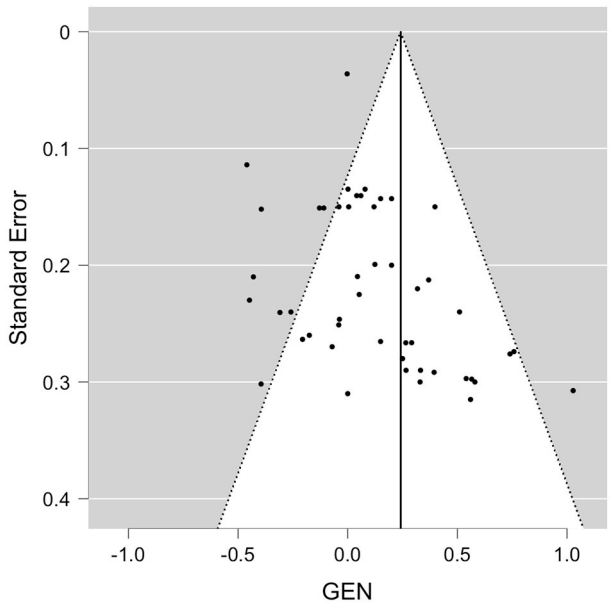

FIGURE 5 | Funnel plot for available study results for EM. GEN, general effect result per study.

a positivity bias based on Funnel plot asymmetry could still not be excluded (Rank test, $p = 0.006$). Similarly, meta-analysis for isoflavones (11 outcomes, seven studies; $b = 0.23$ $p = 0.28$, Funnel asymmetry, $p > 0.24$) did not show significant effects. In contrast, the meta-analysis for anthocyanins (seven outcomes, four studies; $b = 0.45$, $p = 0.05$, Funnel asymmetry Egger's test, $p = 0.001$), for other extracts containing flavonoids (seven outcomes, five studies; $b = 0.34$; $p = 0.029$ Funnel asymmetry $p > 0.3$) and trend-wise for resveratrol (five outcomes, three studies; $b = 0.21$; $p = 0.067$ asymmetry Egger's test, $p = 0.024$) somewhat indicated a positive average effect size, yet both groups raised awareness with regard to positivity bias. Note that sample size in the subgroup meta-analyses was considerably reduced. No subanalysis was computed in groups with <5 outcomes.

DISCUSSION

Based on this systematic review of 66 reported RCTs testing the effects of polyphenols on memory, 33 studies found a significant improvement on at least one memory outcome measure after polyphenol administration, while 30 did not find any significant effects, and three reported a worsening compared to control condition. Reporting was based on a variety of WM and EM outcome measures, of which a selection only were reported as significantly improved after polyphenol consumption compared to placebo with, if reported, small to large effect sizes. Considering the available and calculated effect sizes from available outcomes of 49 studies representing core WM and EM measures, pooled meta-analyses supported a small positive effect on both WM and EM with a mean effect size of 0.26 and 0.24, respectively. However, Funnel plot asymmetry tests detected a significant positivity bias for both WM and EM meta-analysis, questioning the validity of results. When excluding studies with very large positive effects, though, meta-analyses remained significant for a small effect. Our review further indicated a large heterogeneity between studies and outcomes studied, in terms of polyphenol formula and exact memory test used, study duration, sample size and characteristics, as well as considering statistical variance in effects sizes.

Working and Episodic Memory

The evaluated studies can be summarized to show a small positive effect on both WM and EM with similar estimates, however, note that this finding needs to be interpreted with caution due the indicated positivity bias in reporting of polyphenol studies. Still, it has been argued that polyphenols might improve brain function. For example, Brickman et al. (95) showed that specific parts of the hippocampus are activated after polyphenol consumption. When high cocoa-flavanol consumption was compared to low consumption, the middle part (i.e., the body) of the hippocampus was activated. The activation was also associated with higher memory performance. It should be noted though, that this study was performed in a small sample of 37 participants divided into two groups. The hippocampus is necessary for accessing short-term memory representations during WM tasks (20, 96), and hippocampal and parahippocampal areas, parts of the medial temporal lobe (MTL), are critically involved in EM for encoding and retrieval (20, 96), whereas MTL lesions do not lead to extreme WM performance degradation. Later studies however indicated the involvement of the MTL in WM tasks, especially when associations (e.g., location and color) need to be made (96–98).

Future studies involving neuroimaging techniques could help to further understand the neurobiological mechanisms underlying potential benefits of polyphenols on WM and EM.

Possible Sources of Study Heterogeneity or Bias

In this review, we did not observe strong systematic associations between memory improvement and sample size, study duration and mean age. Indeed, significant results were found in studies with long-term follow-up assessment after 2.5 years and in those with follow-up after 6 weeks. Additionally, bigger sample sizes, and thus greater power to detect an effect that is present did not result in more (in)significant findings. Also, the mean age of the samples did not systematically lead to significant or insignificant study results, however, some differences were observed regarding study population and polyphenol subclass.

Polyphenol Subclasses and Dosage

Overall, results of the polyphenol subclasses were mixed. The percentage of significant memory outcome measures for *Ginkgo biloba*, other polyphenol-rich extracts, flavonoids, phenolic acids, and stilbenes were relatively similarly distributed, while subgroup meta-analyses in polyphenol subclasses that could include five outcomes or more failed to show significant benefits for *Ginkgo biloba*, flavanol and soy isoflavones. Here, probability of positivity bias was markedly reduced, indicating a higher

confidence in the results (though note the considerably smaller number of studies included in the sub-analyses). Some support for the expected significant positive effects were found for flavonoids, anthocyanins and resveratrol. In addition, within the polyphenol subclasses, efficiency was sometimes dependent on study dosage. For example, it can be speculated that the dosages in the studies administering *Ginkgo biloba* extract, namely between 38.4 and 57.6 mg flavonoids per day, were too low to affect memory. The polyphenol dosages for *Pinus radiata* bark extract and spearmint extract were higher, namely 768 mg flavonoids per day and 216 mg polyphenols per day, respectively. Also, inter-individual differences in polyphenol absorption, metabolism, and excretion might be other reasons of diverging study results. For instance, Bensalem et al. (31) found that the group with the highest EM decline excreted significantly higher amounts of phenolic metabolites while this group consumed fewer polyphenols than the groups with better performing EM. Possibly, polyphenol supplementation in the group with the highest amount of cognitive decline compensates for the lower intake and higher excretion, leading to improved memory performances in people with more severe memory decline. However, as sample characteristics, exact polyphenol formulas and dosages varied considerably between studies, as well as metabolomics of polyphenols were seldomly reported, results are difficult to harmonize in this regard. A longitudinal observational study in >2,500 older adults also pointed toward the possibility that the effects of polyphenol subclass and molecule may be per se different but also with regard to their impact on cognitive domain: Here, higher intake of e.g., catechins and flavonols were related to both higher verbal memory performance after a period of more than 10 years, while this also correlated with lower performance in tasks on executive function (99). However note that as most extracts in the reviewed studies provided a mixture of polyphenolic molecules, a detailed cause-response investigation with regard to memory function seemed difficult. Future studies should incorporate blood-based biomarkers of individual polyphenol availability and metabolism, to further understand potential differential effects of polyphenol subclasses on cognitive performance.

Impact of Age, Pathologies, and Gender

Mild-to-moderate Alzheimer's disease patients were only studied in a small proportion of RCTs, and presenting on global cognitive test results such as the MMSE instead of WM/EM-focused subtests prevented from adding most of these to the meta-analyses. This underlines the difficulty to differentiate results between non-pathological and pathological aging populations. Considering results of subgroup meta-analyses, however, polyphenols seemed to exert positive effects in MCI and AD or vascular dementia patients on WM, but not significantly on EM, in contrast to meta-analyses in healthy groups. In addition, studies in older women only could not show significant improvements on average, yet, this might be also due to a lack of effects of isoflavonols, as this was the polyphenol used in most of these studies. Another interpretation is that both *Ginkgo biloba* and soy isoflavones had attracted probably the most interest in the last decades as a dietary supplement to combat cognitive decline, potentially due to certain hypes around alternative, nutrition-guided medicine approaches and to the estrogenic action attributed to isoflavones. Thus, these polyphenols were studied most extensively in the literature also with larger, longer-term and high-quality RCTs, leading to a more balanced reporting and resulting (partly) null findings, which would fit to some of the subgroup meta-analyses.

Still, differences in sample age, pathology and gender between studies could have contributed to mixed results. For example, positive effects of *Pinus radiata* bark and spearmint extracts were found in relatively young age, while no effects were seen in samples that were on average 10–25 years older. Moreover, on study that could not demonstrate any positive effect, meaning no objective or subjective effect, of a polyphenol-rich extract on memory, was performed in the oldest sample (81). This was a sample consisting of patients with dementia or age-associated memory impairment. Consequently, it is possible that polyphenols are more effective in relatively young and unaffected samples, but less effective in older patient samples. In addition, menopause in women is associated with an increased risk for developing metabolic syndrome, known for disturbances in glucose metabolism (100). It has been hypothesized that the negative consequences of cardiovascular risk factors on memory performance, for example higher glucose levels, can be compensated by certain polyphenols such as resveratrol administration due to regulation of glucose metabolism and insulin sensitivity (101), rendering a possible efficacy of polyphenols in postmenopausal women likely. In a previous study in older adults, we found that resveratrol lowered glycated hemoglobin A1c in blood, a long-term marker of glucose, which was associated with improved functional connectivity of the hippocampus with the medial prefrontal cortex and eventually memory retention (84). Also, reductions in insulin after cocoa flavonols correlated with increases in memory performance, supporting a potential link between polyphenols and insulin sensitivity as beneficial mechanism (59).

However, samples were often not fully characterized with regard to cardiovascular and metabolic health as well as brain diseases, i.e., (clinical) blood and neuroimaging biomarker analysis has not always been performed. Future studies with larger, more diverse samples and sensitive monitoring of cardiovascular, metabolic and beginning brain pathologies are needed to better understand the impact of age, (beginning) pathologies, and gender on polyphenol action.

Underlying Mechanisms

Several mechanisms have been proposed that might underly the beneficial effects of polyphenols on brain aging. These include anti-oxidative and anti-inflammatory mechanisms and improvements in cardiovascular health such as lower blood pressure and better insulin sensitivity, which are all related to better brain structure and function. *Pinus radiata* bark extract, for example, has been discussed to inhibit oxidation, to reduce systolic blood pressure and to modify signaling in the brain due to the ability of polyphenol metabolites to cross the blood-brain barrier (69). The reduction of oxidation byproducts in the hippocampus has been suggested as a possible mechanism

of spearmint extract (45). Moreover, a recent meta-analysis of carotenoids, known to exert anti-oxidant properties *in vitro* and *in vivo*, provides evidence for a positive effect of carotenoids on improving cognitive performance in middle-aged and older adults, further supporting the hypothesis of a causal role of antioxidant actions in the beneficial effect of plant-derived nutrients on brain health (102). A reduction in pro-inflammatory markers has for instance been observed after anthocyanin-rich supplements, thereby improving hippocampus-dependent memory performance. Kent et al. (13) did however not find altered levels of inflammatory markers in blood, although these alterations might have been undetectable due to disease progression. Yet, significantly lower systolic blood pressure was found by Kent et al. (13) and Whyte et al. (82) after polyphenol administration, which might be a consequence of reduced inflammatory markers and could relate to better memory functioning. For more details on suggested mechanistic pathways linking specific nutrients to cognitive function in aging please see other reviews [e.g., (4, 16, 102–104)].

Limitations

Several limitations of this review should be considered. First, several studies could not be added to our meta-analysis due to lack of raw values, specific information or unplausibility in the given tables, limiting interpretability. Second, only one search base was included (PubMed), due to the limited access to Cochrane and EMBOS library, leading to a risk of omitting published study results. In addition, the results of the different types of polyphenols on WM and EM should be interpreted with care, since the number of studies per polyphenol subclass or extract was still relatively small. Thirdly, a wide variety of memory tests were used, and we cannot exclude that potential arbitrary differences in the classification to WM/EM category per author may have emerged (105). Only a few studies reported test validity and low construct validity might lead to drawing incorrect conclusions from the results. These factors might have contributed to the large statistical heterogeneity observed in the meta-analyses, and reported findings given in the systematic review may not withstand a tight control of type-1 error in the individual studies, in line with the often detected probable positivity bias. In addition, we did not evaluate included studies on study quality e.g., using established tools such as the GRADE or Cochrane risk of bias (106) due to the sheer number of studies screened, which could have revealed for example a lack of blinding or inadequate statistical reporting. However, all studies incorporated a randomized clinical trial design and a sample size of $n \geq 20$ per group, reducing the likelihood of extreme outliers. Also, doses of the different polyphenols were barely comparable and not harmonized at all. All studies reported daily-administered amounts, but the effect of a specific dose of for example isoflavone is hard to compare with the effect of the same amount of flavanol, and compliance measures often relied on self-report or capsule count once at the end of studies.

In general, effect sizes in nutrition sciences and lifestyle interventions are expected to be rather small due to confounding factors in a free-living population. In contrast to other fields, lifestyle interventions have a long tradition of being pre-registered, e.g., on ClinicalTrials.gov and nowadays also on osf.io, which enables to restrict the number of *post-hoc* statistical testing and the possibility to report null or negative findings. Indeed, effect sizes in pre-registered studies were shown to be smaller in pre-registered studies compared to non-pre-registered studies (107). Therefore, the herein presented results of a significant effect for EM and WM are likely to be expected and indeed representative of the field.

Future studies should harmonize control conditions and use memory tests with high construct validity and focus on the quality of the methods. Methodological quality can be increased by for example concealment of allocation, using an intention-to-treat analysis, and measuring compliance as well as implementing memory tests that are insensitive to ceiling or test-retest effects, such as the computerized administration of the Mnemonic Similarity Task (108). Moreover, future studies should include larger samples and longer follow-up to increase power. Yet, the sample sizes and durations of the RCTs included in this review did not seem to affect the results. By including biological parameters, such as urine or blood samples, and functional and structural brain measures using e.g., magnetic resonance imaging, more insights on the mechanisms of polyphenols in improving memory might be gained. Performing longer longitudinal studies in the future could provide insight into whether the consumption of polyphenols decreases progression rates of patients with mild neurocognitive disorder to dementia.

CONCLUSION

Based on reviewing 66 short- to longer-duration daily polyphenol intervention RCTs with small to large sample sizes, a beneficial effect of polyphenols on WM and EM in middle-aged to older adults may be considered small on average, according to qualitative review and a pooled meta-analysis of all available outcomes of 49 studies. The reported outcome measures largely varied and some studies of longer duration and larger sample sizes did not report any significant memory improvement after polyphenol administration. We also noted strong evidence for reporting bias and the statistical heterogeneity was considerably large between studies. Thus interpretation warrants caution and needs to be confirmed by further research. Future studies are encouraged to harmonize polyphenol formulas and doses as well as neuropsychological test methodology, and to increase sample sizes and follow-up periods. Overall, dietary supplementation studies investigating diet-effects on memory of high quality do exist, however, suffer from known limitations in the field and the problem to investigate rather small expected effects. Future studies should aim to address these challenges through rigorously implementing the advantages of open science, including data and code sharing, transparent reporting of neuropsychological methods and null/negative findings, and detailed pre-registration of RCTs, including a detailed statistical analysis plan, to increase reliability and to enable further meta-analyses and replication.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article as meta-data. Further inquiries related to effect sizes per study or other queries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

KV, EM, and AVW: conceptualization and data analysis. KV and AVW: conducted literature search. KV: first draft. EM and AVW: visualization. EM, AVW, and AK: review and correction. All authors contributed to the article and approved the submitted manuscript.

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