Effects of alternate day fasting and exercise on cholesterol metabolism in overweight or obese adults: A pilot randomized controlled trial

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A B S T R A C T

Background and Purpose: The objective of this pilot randomized controlled trial was to investigate the effect of alternate day fasting (ADF) and exercise on serum sterol signatures, which are surrogate markers of cholesterol absorption and biosynthesis.
Methods: We randomly assigned 112 overweight or obese participants to four groups: 1) ADF and exercise (E-ADF); 2) ADF; 3) exercise; and 4) control. We studied 31 completers in this exploratory analysis and measured their serum sterol signatures using gas chromatography–mass spectrometry.
Results: After intervention, most serum sterol signatures that correspond to cholesterol metabolism were significantly different between groups ($p < 0.05$ by analysis of covariance [ANCOVA]). We found no differences in plant sterols, which are markers of cholesterol absorption. In the exercise group, desmosterol, cholesteryl esters, and oxysterols decreased significantly. Furthermore, only changes in physical activity levels negatively correlated with changes in the metabolic ratios of desmosterol and 7-dehydrocholesterol to cholesterol, which reflect cholesterol biosynthesis ($r = -0.411$; $p = 0.030$, and $r = -0.540$; $p = 0.003$, respectively).
Conclusion: These findings suggest that exercise with or without ADF improves cholesterol metabolism as measured by serum sterol signatures, and increased physical activity has a greater effect on cholesterol biosynthesis than weight reduction or calorie restriction.

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1. Introduction

The resulting energy imbalance from increased caloric intake and a decline in physical activity contribute substantially to causing obesity [1]. Obesity is a well-known modifiable risk factor in many metabolic diseases, including dyslipidemia, atherosclerosis, and cardiovascular disease (CVD) [2]. Dietary restrictions like alternate day fasting (ADF) that provide an alternate to traditional calorie restriction are helpful to control weight and have been regarded as therapeutic strategies for dyslipidemia [3,4]. In 3- to 12-week long human trials, ADF effectively reduced body weight by about 3–7%, and decreased low-density lipoprotein (LDL) cholesterol and triglyceride levels in overweight and obese adults [5–7]. However, dietary restriction alone reduces lean mass, and data showing the benefits of physical exercise during calorie restriction to preserve lean mass and maintain weight loss have been presented [8–10]. Exercise and fitness also prevent and regress atherosclerosis by improving lipid metabolism, reverse cholesterol transport (RCT), and antioxidant defense in the arterial wall [11]. The combination of ADF and endurance exercise during a 12-week trial produced remarkable changes in body weight (-6 ± 4 kg), body composition (-5 ± 1 kg for fat mass, 0 ± 1 kg for lean mass), and lipid indicators of heart disease risk ($-12 \pm 5\%$ for LDL cholesterol, $18 \pm 9\%$ for high-density lipoprotein [HDL] cholesterol, 4 ± 1 Å for LDL particle size), when compared to ADF or endurance treatments alone [12]. However, the combined effects of ADF and exercise that is

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composed of both aerobic and resistance training on weight reduction, lean mass preservation, and lipid management have been poorly studied.

Cholesterol, the principal sterol for all animals, is absorbed through diet and also synthesized de novo by cells, particularly hepatocytes. Cholesterol and diet-sourced plant sterols are absorbed into enterocytes via the Niemann-Pick C1-like protein 1 (NPC1L1) transporter, and recycled by a retrograde transport mechanism, depending on the efficiency of intestinal cholesterol absorption. Cellular cholesterol biosynthesis begins with acetyl-coenzyme A and continues through cholesterol precursors to generate cholesterol, via a complicated chain of reactions involving >30 enzymes. Then, excess cholesterol is excreted in as unesterified cholesterols or other metabolic products such as bile acids, passing from hepatocyte to bile and eventually ending in the feces [13]. Unlike cholesterol, which is present in high concentrations in our body, non-cholesterol sterols are detected in very low concentrations in the circulation and tissues. With highly distinct biological activities, non-cholesterol sterols provide biochemical information related to cholesterol metabolic processes, such as cholesterol absorption, biosynthesis, and excretion [14]. Recently, multiple lines of evidence have revealed that sterols are involved in the pathology of atherogenesis, neurodegeneration, and inflammation [15,16]. Therefore, increased understanding of sterol signatures may provide a complementary approach to help elucidate disease-causing mechanisms, such as in atherosclerosis.

Our aim for this pilot study was to investigate the effect of ADF and exercise on serum sterol signatures, body weight, body composition, and metabolic parameters in overweight or obese adults. Furthermore, we examined whether calorie restriction or physical activity had a greater effect on cholesterol metabolism.

2. Methods

2.1. Study Design

This 8-week, randomized, controlled, parallel-arm diet trial was conducted at Yonsei University and Severance Hospital in Seoul, South Korea from April 2014 to March 2016. The study design and experimental protocol were approved by the Institutional Review Board of Severance Hospital (IRB No. 4-2014-0117). This trial was registered at ClinicalTrials.gov (NCT03652532) in August 2018. We obtained written informed consent from each participant before screening and data collection. All methods were carried out in accordance with the approved guidelines and regulations.

2.2. Study Participants

We recruited participants using poster and newspaper advertisements at Yonsei University and Severance Hospital. We screened the volunteers by telephone to assess their eligibility. Eligibility requirements were: age 20–65 years; body mass index (BMI) >23.0 kg/m^2 (overweight or obese for Asian populations, according to the World Health Organization [17]); stable weight for 3 months prior to the study (i.e., weight loss or weight gain <5 kg); no history of bariatric surgery; no secondary obesity, such as hypothyroidism; non-diabetic; aspartate amino-transferase or alanine amino-transferase (ALT) levels <200 mg/dL; serum creatinine level <2.0 mg/dL, no pancreatitis or related disorders; no acute infectious diseases (i.e., pneumonia, acute enteritis, or urinary infection); no chronic inflammatory diseases (i.e., rheumatoid arthritis, or lupus); no history of cardiovascular diseases; no history of cancer; not taking anti-obesity, anti-diabetic, diuretic, central-nervous system, antidepressant, antipsychotic, or steroid medications; no pregnant or lactating women; no overeating behavior; no >30 g of daily alcohol intake; not a night-time or shift-work worker; no chronic malabsorption syndrome or cholestasis; no

other medical conditions that would preclude subjects from participating in exercise and physical test.

2.3. Randomization

We randomly assigned participants in a 1:1:1:1 ratio to four groups: 1) ADF and exercise (E-ADF); 2) ADF; 3) exercise; and 4) control. Block randomization was performed with a computer-generated random number sequence. An independent statistician generated the allocation sequence, and the study coordinator assigned the participants to interventions in chronological order as the participants enrolled. Only outcome assessors were blinded to group allocation.

2.4. Intervention

Each group was instructed to follow their assigned diet or exercise protocol. Control group subjects were required to continue their regular eating and exercise habits. Baseline measurements (body composition, blood samples, questionnaires, and physical fitness test) and post-intervention measurements were performed pre-intervention and after 8 weeks of intervention.

2.4.1. Diet Protocol

Only the E-ADF and ADF groups participated in the dietary intervention. During the 8-week intervention, E-ADF and ADF participants consumed 25% of their daily recommended energy intake (approximately 500 kcal) on each "fast day" (24 h), and consumed food ad libitum on each "feed day" (24 h). The "fast day" and "feed day" were repeated every other day, and the "fast day" occurred 3 days per week. On the "fast day," all participants were instructed to consume one meal between 12 PM and 2 PM to maintain the same fasting times. The nutrient composition of the diet was provided in the daily dietary log by professional nutritionists.

2.4.2. Exercise Protocol

Only the E-ADF and exercise groups participated in the exercise intervention, including resistance training and aerobic exercise. During the 8-week intervention, participants visited the research center gym at least three times per week and received exercise logs. In the first week of intervention, the participants exercised with dedicated instructors, and exercised individually during the remaining 7 weeks.

Each exercise session began with 5 min of warm-up and ended with 5 min of cool-down. Resistance training was performed using weight training machines, barbells, and dumbbells for 40 min. The intensity of resistance training was individualized according to the individual muscle strength and increased periodically each week. Aerobic exercise was performed on motorized treadmills for 20 min. The intensity of aerobic exercise was determined by individual cardiorespiratory fitness, which was measured by maximal oxygen consumption (VO$_2$max).

2.4.3. Diet and Exercise Compliance

During the 8-week intervention, the E-ADF and ADF groups were instructed to report their dietary logs daily, and the E-ADF and exercise groups were instructed to report their exercise log for each visit to the gym. Diet and exercise compliance were assessed by recording attendance. If a "fast day" or an exercise session was missed, the subject was required to make up for the missed day on another day of the week. Participants who missed >8 out of 24 total "fast days" or exercise sessions were considered to have low compliance and excluded from the "per-protocol" analyses, but not the "intention-to-treat" analyses.

2.5. Outcome Measurements

The 24-h dietary recall method was used to collect data concerning food consumption by participants during the previous 24 h. Intake of total calories, protein, carbohydrate, and fat were computed using the

7th revision Standard Food Composition Table produced by the Korea National Rural Resources Development Institute [18]. The individual quantity of physical activity was calculated in metabolic equivalent-minutes per week (MET-min/week), according to the Korean version of the Global Physical Activity Questionnaire [19]. To assess physical fitness, we estimated muscle strength and VO_2 max. Muscle strength was measured using a 10-repetition maximum, while performing chest press, leg press, shoulder press, and lateral pull down. VO_2 max (mL/kg/min), which reflects cardiorespiratory fitness, was estimated during a graded treadmill walk using a modified Bruce protocol [20]. We monitored oxygen concentration, heart rate, and electrocardiogram during all tests.

We measured body weight and height to the nearest 0.1 kg and 0.1 cm, using an automatic extensometer (BSM 330, Biospace, Seoul, Korea) with participants wearing light clothing. BMI was calculated as the ratio of weight (kg) to height^2 (m^2). To assess body composition, we used a bioelectrical impedance analyzer (InBody U20, Biospace, Seoul, Korea) and fat measurement computed tomography (CT) (Tomoscan 350, Philips, Mahwah, NJ, USA). Skeletal muscle mass, fat mass, and fat percentage were assessed by bioelectrical impedance analyzer. Visceral and subcutaneous fat areas were measured using a 10-mm CT slice scan acquired at the level of L4–L5 with subjects in the supine position.

To assess the metabolic parameters, we obtained blood samples from an antecubital vein after a 12-h overnight fast. White blood cell (WBC) counts were quantified using a XN-9000 Hematology Analyzer (Sysmex, IL, USA). Fasting glucose, high-sensitivity C-reactive protein (hs-CRP), total cholesterol, triglycerides, and HDL cholesterol levels were measured with the ADVIA 1650 Clinical Chemistry System (Siemens Medical Solutions, Tarrytown, NY, USA). The LDL cholesterol level was calculated using the Friedewald equation, unless triglycerides were >400 mg/dL, as follows: LDL cholesterol = total cholesterol − HDL cholesterol − triglyceride/5 [21]. Fasting insulin was measured by an electrochemiluminescence immunoassay using an Elecsys 2010 instrument (Roche, Indianapolis, IN, USA). Insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) method, by applying the following formula: [HOMA-IR = fasting insulin (μIU/mL) × fasting glucose (mg/dL)/405] [22].

2.6. Metabolic Signatures of Serum Sterols

The gas chromatography–mass spectrometry (GC–MS) based quantitative profiling of serum sterols, including cholesterol, 3 plant sterols, 4 cholesterol precursors, 2 cholesteryl esters, and 2 oxysterols, were performed using an Agilent 6890 Plus gas chromatograph interfaced with a single-quadruple Agilent 5975C MSD (Agilent Technologies, Palo Alto, CA), as previously described [11,23]. In brief, 20 μL serum samples were spiked with 20 μL of the internal standard mixtures (d6-cholesterol and d6-cholesteryl stearate, 100 μg/mL; d7-7α-hydroxycholesterol, 10 μg/mL) was added to 0.5 mL of methanol. The samples were vortexed, loaded into hybrid solid-phase extraction-precipitation (H-PPT) cartridges, and then eluted three times with 0.5 mL of methanol. The collected eluates were evaporated using an N_2 evaporator at 40 °C and dried in vacuum desiccators over P_2O_5/KOH for at least 30 min. Then, the dried residues were derivatized in 40 μL of N-methyl-N-trifluorotrimethylsilyl acetamide (MSTFA)/ammonium iodide (NH 4 I)/dithioerythritol (DTE) (500:4:2, v/w/w) for 20 min at 60 °C. Finally, 2 μL of the resulting mixture was injected for
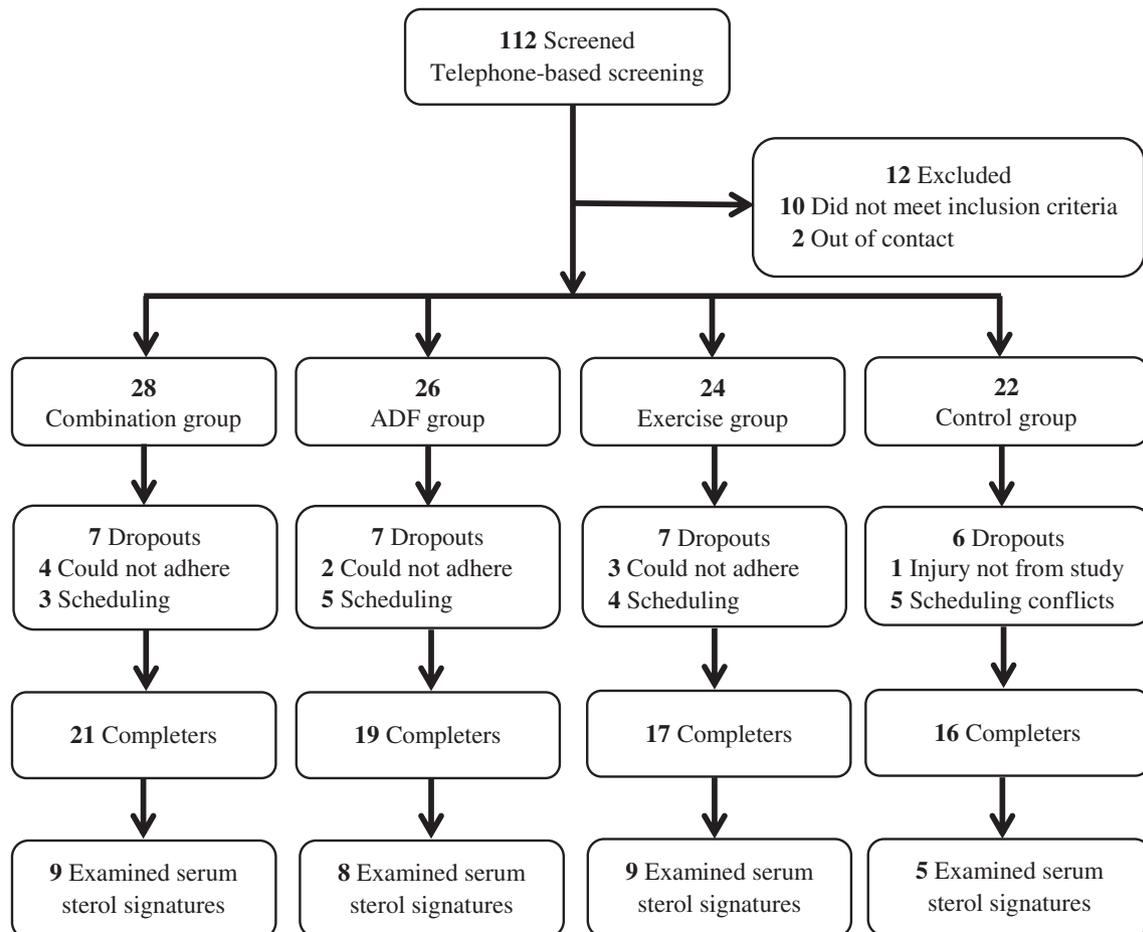

Fig. 1. CONSORT diagram.

GC–MS with the selected-ion monitoring analysis. The devised analytical method has been fully validated with an accuracy (% bias) and precision (% coefficient of variation).

2.7. Sample Size

The sample size calculation was based on a recent 12-week clinical trial that investigated the effect of combining ADF and endurance exercise on body weight [12]. We estimated that 112 participants would need to be enrolled for the trial to obtain a power of 80% and a two-sided significance level of 5% for detecting differences in body weight, assuming 4 kg in E-ADF group, 2 kg in ADF group, 0.67 kg in exercise group, 0 kg in control group, and a standard deviation of 4 kg.

2.8. Statistical Analysis

All data are presented as the mean ± standard deviation (SD), or number (%). We assessed normality with the Kolmogorov-Smirnov test and found no variables to be not normal. Differences in baseline characteristics between the intervention groups were summarized using either one-way analysis of variance for continuous variables or the Chi-square test for categorical variables. Within-group differences after intervention were analyzed by the paired t-test, and differences between groups after intervention were analyzed using analysis of

covariance (ANCOVA), adjusted for age, sex, and baseline body weight. We performed two-way ANCOVA to determine the effects of ADF and exercise, as well as the interaction between ADF and exercise. For these analyses, we used ADF (yes/no) and exercise (yes/no) as fixed factors, and used age, sex, and baseline body weight as covariates. Pearson's partial correlation coefficients were calculated to examine the relationships between changes of weight, calorie intake, physical activity, and changes of cholesterol biosynthesis markers. A p-value <0.05 was considered statistically significant. All analyses were performed using SPSS for Windows (version 20.0; SPSS Inc., Chicago, IL, USA).

3. Results

Among the 112 participants who were randomly assigned to four groups, 73 completed the trial. This pilot study includes 31 completers, who agreed to the examination of their serum sterol signatures for both baseline and post-intervention measurements (Fig. 1).

3.1. Baseline Characteristics

We found no difference between groups for age, sex, body weight, or BMI at baseline. However, LDL cholesterol and total fat intake differed at baseline between groups (Table 1).

Table 1
Baseline characteristics of study participants ($n = 31$).

	E-ADF ($n = 9$)	ADF ($n = 8$)	Exercise ($n = 9$)	Control ($n = 5$)	p-Value[a]
Age (years)	34.5 ± 5.7	33.5 ± 5.0	38.6 ± 8.2	42.6 ± 10.6	0.129
Male sex (%)	5 (55.6)	2 (25.0)	5 (55.6)	3 (60.0)	0.698
Body composition					
Height (cm)	166.4 ± 10.2	163.4 ± 9.0	165.8 ± 7.9	165.4 ± 6.1	0.905
Weight (kg)	78.2 ± 14.5	74.6 ± 13.7	74.2 ± 13.2	71.1 ± 11.7	0.812
BMI (kg/m^2)	28.0 ± 2.6	27.8 ± 3.4	26.9 ± 3.9	25.8 ± 3.4	0.670
Impedance analysis					
Skeletal muscle (kg)	30.0 ± 7.9	26.6 ± 5.8	28.2 ± 6.2	27.5 ± 5.2	0.746
Fat mass (kg)	24.7 ± 3.9	26.5 ± 5.8	23.4 ± 7.9	21.2 ± 4.3	0.460
Fat percentage (%)	32.1 ± 5.6	35.4 ± 4.5	31.3 ± 7.2	29.9 ± 3.4	0.322
WHR	0.90 ± 0.05	0.91 ± 0.06	0.89 ± 0.04	0.91 ± 0.05	0.846
Fat CT					
Visceral fat (cm^2)	79.7 ± 21.5	74.3 ± 21.0	80.6 ± 23.6	82.1 ± 24.1	0.916
SubQ fat (cm^2)	267.2 ± 75.6	237.2 ± 89.4	222.8 ± 59.1	247.1 ± 77.5	0.656
Metabolic parameters					
WBC count (/μL)	6144.4 ± 1196.2	5657.5 ± 1718.9	5624.4 ± 1590.2	5770.0 ± 899.2	0.864
hs-CRP (mg/L)	0.78 ± 1.23	1.20 ± 1.46	1.31 ± 1.63	0.50 ± 0.33	0.672
Fasting glucose (mg/dL)	94.5 ± 13.0	96.2 ± 7.0	90.3 ± 7.2	88.8 ± 12.2	0.480
Insulin (μIU/mL)	9.43 ± 2.24	9.88 ± 5.34	7.37 ± 4.81	4.34 ± 1.47	0.087
HOMA-IR	2.23 ± 0.72	2.35 ± 1.27	1.61 ± 1.04	0.97 ± 0.45	0.063
Total cholesterol (mg/dL)	189.7 ± 25.8	183.0 ± 17.5	172.3 ± 31.8	161.0 ± 37.3	0.279
Triglyceride (mg/dL)	122.0 ± 53.5	117.3 ± 49.5	208.5 ± 154.9	122.6 ± 74.1	0.179
HDL cholesterol (mg/dL)	46.5 ± 10.0	47.9 ± 7.8	41.5 ± 11.5	50.4 ± 14.0	0.447
LDL cholesterol (mg/dL)	118.8 ± 24.4	111.5 ± 14.2	89.1 ± 29.8	86.0 ± 32.4	0.044
Nutrient intake					
Total calorie (kcal/day)	1785.9 ± 311.3	1841.1 ± 359.9	1532.8 ± 410.4	1460.9 ± 448.6	0.182
Protein (g)	73.1 ± 17.0	79.8 ± 11.1	65.1 ± 17.0	66.2 ± 16.6	0.244
Carbohydrate (g)	246.8 ± 43.5	258.4 ± 69.1	220.5 ± 81.2	232.3 ± 77.1	0.692
Total fat (g)	59.8 ± 12.2	61.7 ± 12.0	47.4 ± 19.9	38.6 ± 11.1	0.027
SFA (g)	8.5 ± 6.0	7.0 ± 5.7	4.5 ± 2.4	14.5 ± 13.8	0.110
MUFA (g)	10.1 ± 6.4	8.4 ± 6.6	6.5 ± 3.2	15.0 ± 12.3	0.205
PUFA (g)	8.5 ± 3.8	6.3 ± 4.4	8.8 ± 4.9	10.2 ± 2.5	0.400
Physical activity and fitness					
GPAQ score (MET-min/wk)	1097.7 ± 2352.8	1230.0 ± 1412.2	862.2 ± 910.6	1392.0 ± 2122.3	0.950
Chest press (kg)	38.0 ± 18.6	34.0 ± 20.7	31.6 ± 16.1	35.0 ± 18.3	0.907
Leg press (kg)	101.1 ± 23.6	81.2 ± 22.9	96.6 ± 41.2	74.0 ± 33.6	0.342
Shoulder press (kg)	19.7 ± 13.0	15.6 ± 9.9	18.8 ± 11.7	19.0 ± 13.6	0.905
Lateral pull down (kg)	39.4 ± 14.6	39.3 ± 16.3	34.4 ± 13.3	40.0 ± 17.6	0.867
VO$_2$max (mL/kg/min)	29.0 ± 3.4	28.1 ± 4.5	32.6 ± 7.6	31.8 ± 4.8	0.304

Data are expressed as the mean ± SD or number (%). Abbreviations: E-ADF, exercise and alternate day fasting group; ADF, alternate day fasting group; SD, standard deviation; BMI, body mass index; WHR, waist-hip-ratio; subQ, subcutaneous; WBC, white blood cell; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model of assessment-insulin resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; GPAQ, global physical activity questionnaire; MET, metabolic equivalents.

[a] p-Value calculated by one-way analysis of variance.

3.2. Changes in Body Composition, Metabolic Parameters, Nutrient Intake, and Physical Activity

Body weight, BMI, and fat mass decreased in the three intervention groups after 8 weeks ($p < 0.05$ by paired t-test; Table 2). Body weight and fat mass reduction were significantly greater in the E-ADF group (-3.9 ± 2.1 kg and -3.1 ± 1.9 kg) and ADF group (-3.7 ± 1.9 kg and -2.9 ± 1.4 kg) than in the control group (-0.2 ± 1.5 kg and -0.7 ± 1.8 kg), according to post-hoc analyses ($p < 0.05$ by the Least Significant Difference test). We also observed smaller decreases in body weight and fat mass in the exercise group (-2.0 ± 1.4 kg and -1.8 ± 0.9 kg). There was no significant interaction between ADF and exercise for body weight and fat mass, but ADF significantly decreased body weight ($p = 0.003$) and fat mass ($p = 0.003$) (data not shown).

Among measures of metabolic parameters, we found that only changes in triglyceride were significantly different between the groups ($p = 0.042$ by ANCOVA) (Table 2). Triglyceride levels decreased in the E-ADF group (-43.6 ± 31.0 mg/dL) and increased in the control group (53.2 ± 44.3 mg/dL). Total calorie intake was lower in the E-ADF and ADF groups, and the global physical activity questionnaire (GPAQ) score increased in the E-ADF and exercise groups throughout the trial, as anticipated. Muscle strength, as measured by chest press and lateral pull down, significantly increased in the exercise group (8.6 ± 2.0 kg and 4.7 ± 1.7 kg, respectively). No between-group

differences were observed for specific nutrient intake and VO$_2$ max, which reflects cardiorespiratory fitness.

3.3. Changes in 12 Sterol Signatures

For post-intervention serum sterol signatures, we observed no differences between groups for plant sterols, which are surrogate markers for the efficiency of cholesterol absorption. We found significant differences between groups for most other markers of cholesterol metabolism, except lanosterol and lathosterol, after adjusting for age, sex, and baseline body weight ($p < 0.05$ by ANCOVA; Table 3). Of the cholesterol precursors, desmosterol decreased in the exercise group (-0.05 ± 0.01 ng/mL), whereas 7-dehydrocholesterol (7-DHC) increased in the ADF and control groups (0.12 ± 0.04 ng/mL and 0.29 ± 0.05 ng/mL, respectively) ($p < 0.05$ by paired t-test). Both lanosterol and lathosterol decreased in the E-ADF and exercise groups throughout the course of trial, but there were no significant differences between the groups. All cholesteryl esters and oxysterols significantly decreased in the exercise group ($p < 0.05$ by paired t-test). In the exercise group, cholesteryl myristate and all oxysterols decreased more than the ADF and control groups, while cholesteryl arachidonate decreased more than the control group ($p < 0.05$ by the Least Significant Difference test). The metabolic ratios against cholesterol were similar to those of respective sterols.

Table 2
Changes in body composition, metabolic parameters, nutrient intake, and physical activity after intervention.

	E-ADF ($n = 9$)	ADF ($n = 8$)	Exercise ($n = 9$)	Control ($n = 5$)	p-Value[a]
Body composition					
Weight (kg)	-3.9 ± 0.6[*,¶]	-3.9 ± 0.7[*,¶]	-2.0 ± 0.6[*]	-0.2 ± 0.9[†,‡]	0.019
BMI (kg/m^2)	-1.5 ± 0.3[*,¶]	-1.4 ± 0.3[*,¶]	-0.8 ± 0.3[*]	-0.1 ± 0.4[†,‡]	0.038
Skeletal muscle (kg)	-0.5 ± 0.3[*]	-0.4 ± 0.3	-0.2 ± 0.3	0.0 ± 0.4	0.712
Fat mass (kg)	-3.2 ± 0.5[*,¶]	-3.2 ± 0.6[*,¶]	-1.7 ± 0.5[*]	-0.3 ± 0.8[†,‡]	0.024
Fat percentage (%)	-2.8 ± 0.6[*]	-2.8 ± 0.7[*]	-1.7 ± 0.6[*]	-0.3 ± 0.9	0.131
WHR	-0.03 ± 0.01[*]	-0.02 ± 0.01	-0.02 ± 0.01[*]	-0.03 ± 0.01[*]	0.389
Visceral fat (cm^2)	-2.2 ± 10.9	31.5 ± 12.2	13.0 ± 10.9	4.3 ± 15.5	0.230
SubQ fat (cm^2)	-47.3 ± 33.7	1.3 ± 37.7	-3.9 ± 33.7	-35.7 ± 48.1	0.714
Metabolic parameters					
WBC count (/μL)	-1066.6 ± 387.5[*]	-82.5 ± 433.7	39.2 ± 386.9	759.2 ± 552.7	0.068
hs-CRP (mg/L)	0.26 ± 0.44	-0.35 ± 0.49	-0.14 ± 0.44	0.90 ± 0.62	0.420
Fasting glucose (mg/dL)	-14.1 ± 3.5[*]	-9.7 ± 4.0[*]	-1.3 ± 3.5	-4.0 ± 5.0	0.108
Insulin (μIU/mL)	-3.87 ± 2.78	3.21 ± 3.11	0.04 ± 2.78	5.19 ± 3.97[*]	0.211
HOMA-IR	-1.12 ± 0.68	0.68 ± 0.76	0.01 ± 0.68	1.10 ± 0.97[*]	0.199
Total cholesterol (mg/dL)	15.1 ± 8.7	5.4 ± 9.7	20.3 ± 8.7	33.2 ± 12.4[*]	0.437
Triglyceride (mg/dL)	-43.6 ± 31.0[*]	12.6 ± 34.7[§]	-87.9 ± 31.0[‡,¶]	53.2 ± 44.3[*,§]	0.042
HDL cholesterol (mg/dL)	6.0 ± 2.7	2.9 ± 3.0	11.2 ± 2.7[*]	5.7 ± 3.8[*]	0.237
LDL cholesterol (mg/dL)	17.8 ± 8.9	0.0 ± 9.9	26.7 ± 8.9[*]	16.9 ± 12.6	0.295
Nutrient intake					
Total calorie (kcal/day)	-424.2 ± 175.8[*]	-677.6 ± 196.8[*]	-19.4 ± 175.5	207.8 ± 250.7	0.053
Protein (g)	30.4 ± 26.3	-45.1 ± 29.4[*]	4.9 ± 26.2	18.9 ± 37.4	0.290
Carbohydrate (g)	-32.4 ± 36.0	-93.2 ± 40.3	18.7 ± 35.9	48.6 ± 51.3	0.178
Total fat (g)	-13.0 ± 7.2	-24.0 ± 8.1[*]	-4.6 ± 7.2	8.0 ± 10.3	0.155
SFA (g)	-3.7 ± 2.8	-2.1 ± 3.1	3.4 ± 2.8	-8.1 ± 4.0	0.099
MUFA (g)	-3.7 ± 3.1	-1.1 ± 3.5	2.7 ± 3.1	-8.5 ± 4.4	0.185
PUFA (g)	-3.2 ± 1.5	0.2 ± 1.7	-3.3 ± 1.5	-7.2 ± 2.1	0.101
Physical activity and fitness					
GPAQ score (MET-min/wk)	1241.1 ± 426.7[*]	214.0 ± 477.6	1219.6 ± 426.1[*]	-543.6 ± 608.6	0.051
Chest press (kg)	6.3 ± 2.0[*,‡,¶]	-3.6 ± 2.3[*,†,§]	8.6 ± 2.0[*,‡,¶]	-2.0 ± 2.9[†,§]	0.001
Leg press (kg)	-4.5 ± 5.9	-7.3 ± 6.6	2.7 ± 5.9	-0.2 ± 8.4	0.731
Shoulder press (kg)	2.0 ± 1.9	-0.6 ± 2.2	7.2 ± 2.0[*]	4.7 ± 2.9	0.101
Lateral pull down (kg)	1.4 ± 1.7	-3.0 ± 1.9[§]	4.7 ± 1.7[*,‡,¶]	-4.2 ± 2.4[§]	0.008
VO$_2$max (mL/kg/min)	3.3 ± 1.2[*]	1.4 ± 1.4	1.5 ± 1.2	1.5 ± 1.7	0.677

Changes are mean values $\pm$ SE and are calculated as 8-week value minus baseline value. Abbreviations: E-ADF, exercise and alternate day fasting group; ADF, alternate day fasting group; SE, standard error; BMI, body mass index; WHR, waist-hip-ratio; subQ, subcutaneous; WBC, white blood cell; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model of assessment-insulin resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; GPAQ, global physical activity questionnaire; MET, metabolic equivalents.

[a] p-Value between groups calculated by analysis of covariance (ANCOVA); adjusted for age, sex, and baseline body weight.
[*] p-Value <0.05 by paired t-test.
[†] p-Value <0.05 vs. E-ADF group based on the Least Significant Difference test.
[‡] p-Value <0.05 vs. ADF group based on the Least Significant Difference test.
[§] p-Value <0.05 vs. Exercise group based on the Least Significant Difference test.
[¶] p-Value <0.05 vs. Control group based on the Least Significant Difference test.

Table 3
Changes in 12 sterol signatures measured by gas chromatography–mass spectrometry (GC–MS) after intervention.

	E-ADF ($n = 9$)	ADF ($n = 8$)	Exercise ($n = 9$)	Control ($n = 5$)	p-Value[a]
Cholesterol (μg/mL)	−0.9 ± 12.5	1.5 ± 14.0	2.1 ± 12.5	47.5 ± 17.8	0.156
Plant sterols (μg/mL)					
Sitosterol	0.00 ± 0.08	0.01 ± 0.09	−0.02 ± 0.08	0.14 ± 0.11	0.642
Campesterol	0.00 ± 0.12	0.03 ± 0.13	−0.04 ± 0.12	0.20 ± 0.17	0.679
Stigmasterol	0.093 ± 0.004[*]	0.096 ± 0.005[*]	0.096 ± 0.004[*]	0.098 ± 0.006[*]	0.913
Cholesterol precursors (ng/mL)					
Lanosterol	−0.15 ± 0.05[*]	−0.03 ± 0.06	−0.12 ± 0.05[*]	0.05 ± 0.07	0.113
Lathosterol	−0.57 ± 0.17[*]	−0.06 ± 0.19	−0.44 ± 0.17[*]	−0.03 ± 0.24	0.108
Desmosterol	−0.02 ± 0.01[¶]	0.00 ± 0.02	−0.05 ± 0.01[*,¶]	0.05 ± 0.02[†,§]	0.006
7-DHC	0.06 ± 0.04[¶]	0.12 ± 0.04[*,¶]	0.08 ± 0.04[¶]	0.29 ± 0.05[*,†,‡,§]	0.007
Cholesteryl esters (μg/mL)					
Chol-M	−22.1 ± 4.6[‡,¶]	−7.7 ± 5.1[*,†,§,¶]	−24.7 ± 4.6[‡,¶]	13.5 ± 6.6[†,‡,§]	0.000
Chol-A	−49.6 ± 215	−25.9 ± 24.0	−81.5 ± 21.4[*,¶]	28.3 ± 30.6[§]	0.034
Oxysterols (ng/mL)					
7α-OHC	−0.016 ± 0.004[*,§]	−0.008 ± 0.005[§]	−0.030 ± 0.004[*,†,‡,¶]	−0.005 ± 0.006[§]	0.005
7β-OHC	0.000 ± 0.003[§]	−0.002 ± 0.004[§]	−0.019 ± 0.003[*,†,‡,¶]	0.005 ± 0.005[§]	0.000
Metabolic ratios[b]					
Lano/Chol	−3.08 ± 1.13	−0.82 ± 1.27	−2.61 ± 1.13[*]	0.36 ± 1.62	0.242
Latho/Chol	−10.81 ± 3.67[*]	−1.06 ± 4.11	−9.93 ± 3.66[*]	−3.54 ± 5.23	0.238
Desmo/Chol	−0.37 ± 0.32	0.02 ± 0.36	−0.99 ± 0.32[*,¶]	0.50 ± 0.46[§]	0.047
7-DHC/Chol	1.27 ± 0.86	2.45 ± 0.97[*]	1.83 ± 0.86[*]	5.34 ± 1.23[*]	0.073
Chol-M/Chol	−4.56 ± 0.92[*,‡,¶]	−1.64 ± 1.03[*,†,§]	−5.63 ± 0.92[*,‡,¶]	2.00 ± 1.31[†,§]	0.000
Chol-A/Chol	−0.10 ± 0.04[*]	−0.06 ± 0.05[§]	−0.19 ± 0.04[*,‡,¶]	0.00 ± 0.06[§]	0.041
7α-OHC/Chol	−0.32 ± 0.09[*,§]	−0.18 ± 0.10[§]	−0.68 ± 0.09[*,†,‡,¶]	−0.24 ± 0.13[§]	0.005
7β-OHC/Chol	0.00 ± 0.06[§]	−0.05 ± 0.07[§]	−0.43 ± 0.06[*,†,‡,¶]	0.00 ± 0.09[§]	0.000

Changes are mean values ± SE and are calculated as 8-week value minus baseline value. Abbreviations: E-ADF, exercise and alternate day fasting group; ADF, alternate day fasting group; HTGC-MS, high-temperature gas chromatography–mass spectrometry; SE, standard error; Chol-M, cholesteryl myristate; Chol-A, cholesteryl arachidonate; 7-DHC, 7-dehydrocholesterol; 7α-OHC, 7α-hydroxycholesterol; 7β-OHC, 7β-hydroxycholesterol; Chol, cholesterol; Lano, lanosterol; Latho, lathosterol; Desmo, desmosterol.

[a] p-Value calculated by analysis of covariance (ANCOVA); adjusted for age, sex, and baseline body weight.
[b] The metabolic ratios are based on measured serum concentrations.
[*] p-Value <0.05 by paired t-test.
[†] p-Value <0.05 vs. E-ADF group based on the Least Significant Difference test.
[‡] p-Value <0.05 vs. ADF group based on the Least Significant Difference test.
[§] p-Value <0.05 vs. Exercise group based on the Least Significant Difference test.
[¶] p-Value <0.05 vs. Control group based on the Least Significant Difference test.

We performed two-way ANCOVA to estimate the effects of ADF and exercise, and the interaction between ADF and exercise according to metabolic ratios. We found that exercise significantly decreased metabolic ratios of desmosterol, 7-DHC, cholesteryl arachidonate, and 7α-hydroxycholesterol to cholesterol, with no significant interaction for ADF. There was a significant interaction between ADF and exercise for metabolic ratios of cholesteryl myristate ($p = 0.032$) and 7β-hydroxycholesterol ($p = 0.002$), to cholesterol (data not shown).

3.4. Relationships Between Changes in Weight, Calorie Intake, Physical Activity, and Changes in Cholesterol Biosynthesis Markers

Finally, we found that changes in the metabolic ratios of desmosterol and 7-DHC to cholesterol, which reflect cholesterol biosynthesis, only negatively correlated with changes in physical activity levels as measured by GPAQ score ($r = -0.411$; $p = 0.030$, and $r = -0.540$; $p = 0.003$, respectively) (Fig. 2). These changes were not associated with changes in total calorie intake or body weight.

4. Discussion

Here, we examined the effects of ADF and exercise on body weight, body composition, metabolic parameters, and non-cholesterol sterols in overweight or obese adults. We found that after intervention, 1) after 8 weeks, the E-ADF and ADF groups lost more body weight and fat mass than the control group; 2) among the GC–MS-based serum sterol signatures, desmosterol, all cholesteryl esters, and oxysterols significantly decreased in the exercise group; and 3) changes in metabolic ratios of desmosterol and 7-DHC to cholesterol, which reflect cholesterol biosynthesis, negatively correlated with changes in physical activity, but not with changes in calorie intake or body weight.

Calorie restriction and physical exercise are considered to be key factors that affect weight reduction and the overall risk of CVD. This study is the first to compare the effects of dietary restriction and exercise on metabolic signatures of serum sterols, including cholesterol. Although the traditional lipid profiles are considered to indicate CVD risk, they can only offer limited information about atherosclerosis and cardiovascular pathologies [24]. In contrast, alterations in cholesterol metabolism are known to be powerful predictors of developing cardiovascular events, even in the early stages of atherosclerosis [23]. For example, abnormal cholesterol metabolism, including low intestinal cholesterol absorption and elevated cholesterol biosynthesis, is prevalent in obesity, diabetes, dyslipidemia, and metabolic syndrome [25].

We observed that desmosterol, all cholesteryl esters, and oxysterols significantly decreased in the exercise group. In addition, exercise significantly decreased the metabolic ratios of desmosterol, 7-DHC, cholesteryl esters, and oxysterols to cholesterol, with or without a significant interaction for ADF. Generally, levels of serum cholesterol precursors reflect cholesterol biosynthesis, because their corresponding amounts leak from tissues into circulation, depending on the activity of the cholesterol synthesis pathway [14]. Cholesteryl esters may reflect the enzyme activities of lecithin:cholesterol acyltransferase (LCAT) and acyl CoA:cholesterol acyltransferase (ACAT), and impaired cholesterol metabolism can cause an accumulation of excess cholesteryl ester molecules in the fatty lesions of atherosclerotic plaques [26]. Oxysterols are oxidized derivatives of cholesterol or by-products of its biosynthesis. The first intermediate of classic bile acid synthetic pathway is 7α-hydroxycholesterol (OHC), which is synthesized from the liver by cholesterol 7-hydroxylase (CYP7A1), and acts as regulator of cholesterol metabolism. In contrast, 7β-OHC is a biomarker of oxidative stress with cytotoxic and pro-apoptotic properties that is generated by autoxidation [27]. Therefore, decreased levels of cholesterol precursors, cholesteryl esters, oxysterols, and their ratios to cholesterol suggest
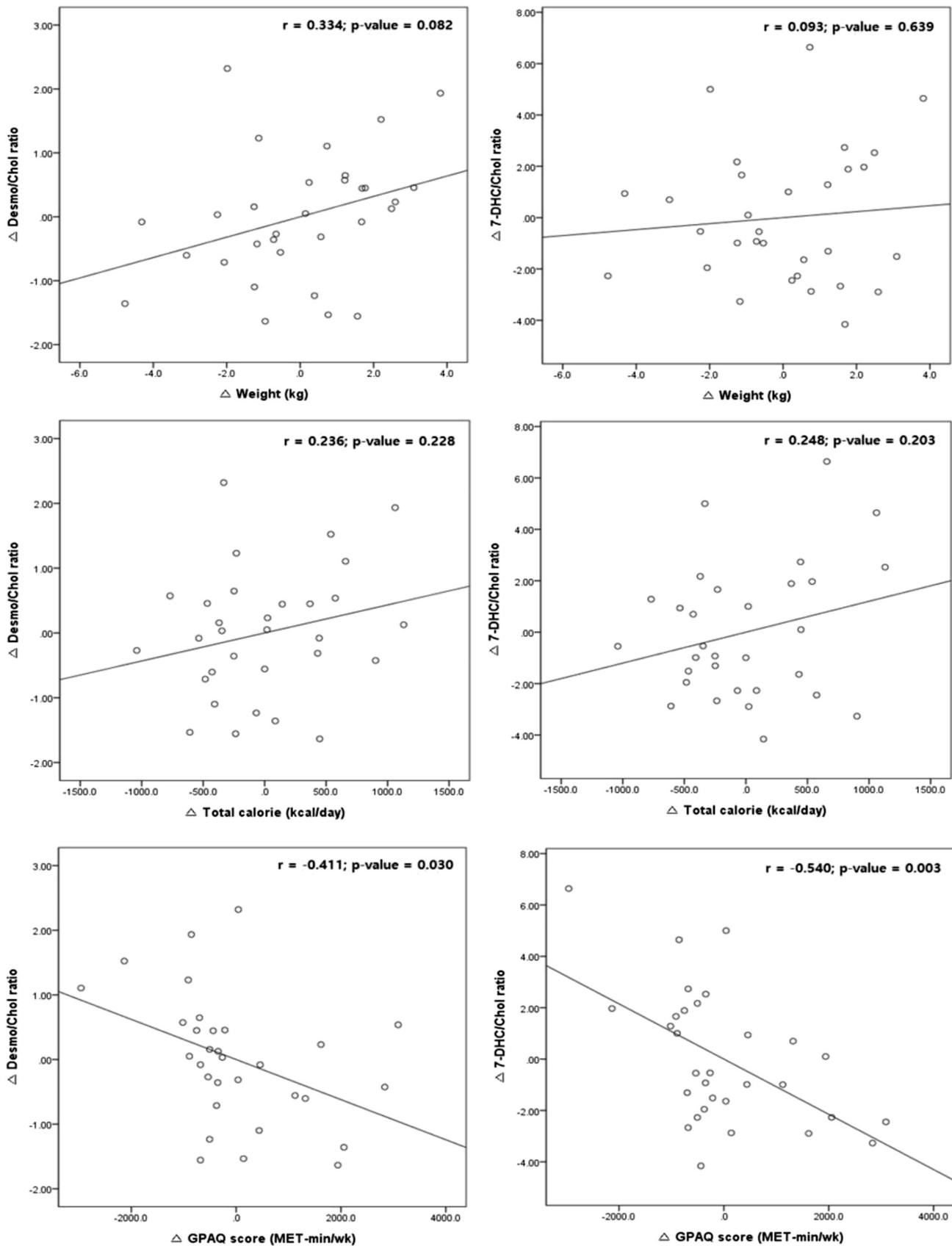
Fig. 2. Relationships between changes in weight, calorie intake, physical activity, and metabolic ratios of desmosterol and 7-dehydrocholesterol to cholesterol. *r*: Pearson's partial correlation coefficient adjusted by age, sex, and initial weight (*r* = 0: no linear relationship, *r* = 1 or −1: perfect linear relationship). x-Axes are based on calculated residuals from regressing changes of weight, total calorie, and GPAQ score. y-Axes are based on calculated residuals from regressing changes of desmosterol/cholesterol and 7-DHC/cholesterol ratios.

that exercise may inhibit excess cholesterol production and oxidative stress more than calorie restriction. This work also suggests that exercise may benefit patients with CVD.

Our finding of correlations between physical activity score and changes in the metabolic ratio of desmosterol and 7-DHC to cholesterol support the conclusion that exercise improves cholesterol metabolism. Although the exact mechanisms that connect exercise to sterol signatures and cholesterol biosynthesis are unknown, our data are consistent with previous observational studies that found that exercise promoted cholesterol efflux via RCT, which is the initial step that releases free cholesterol from cholesteryl esters in peripheral cells [28].

Although the quantification of sterol signatures in obesity and weight change has not been widely utilized, several previous studies evaluated the weight loss effects from dietary restriction and found an accompanying decrease in cholesterol biosynthesis [29] and an increase in cholesterol absorption efficiency [30]. In this study, the changes in levels of cholesterol synthesis markers were smaller in the ADF group than in the exercise group. Among absorption markers, as measured by plant sterols [31], only stigmasterol increased in all groups after 8 weeks, but we found no differences among groups. Inconsistencies with some previous studies might be due to differences in the characteristics of the study population, a different diet control method, or a less-pronounced weight loss in this trial. Large-scale prospective studies with various methods of dietary control and study durations would help reconcile these discrepancies.

Our study has several limitations. First, our 8-week intervention was not long enough to observe significant changes in plasma lipids. Although we found no significant changes in plasma lipids, we observed changes in serum non-cholesterol sterols, indicating their sensitivity to the interventions. Second, this exploratory analysis was limited by a small number of participants. It was probably due to the small sample sizes that baseline LDL cholesterol and total fat intake differed between groups. Third, we collected no information on dietary cholesterol or plant sterols in our 24-h dietary recall. Under caloric restriction conditions with too little plant sterol intake, serum plant sterols do not adequately reflect cholesterol absorption [29]. Future analyses that include the amount of dietary plant sterols could more accurately assess changes in serum plant sterols. Finally, we evaluated cholesterol metabolism using GC–MS based serum sterol signatures, not isotope methods, which directly measure cholesterol absorption, biosynthesis, and excretion [32]. Despite the accuracy of the isotope kinetic and sterol balance methods, these techniques are not suitable for large-scale studies; therefore, GC–MS is a less expensive and simpler method of measuring serum sterol signatures that has been used extensively in recent studies.

In conclusion, we found that exercise, with or without ADF, improved cholesterol metabolism when measured by serum sterol signatures in overweight or obese adults. Moreover, our results indicate that increased physical activity has a greater effect on cholesterol biosynthesis than weight reduction or calorie restriction. These findings provide additional support for the validity of sterol signatures, and suggest that exercise is importance for cholesterol metabolism.

Author Contributions

J.W.L. and M.H.C. conceived and designed the study. A.R.C., S.K., K.Y.A., M.O., and D.H.J. performed the experiments. J.Y.M., J.J., and M.H.C. were involved in data analysis. A.R.C. drafted the manuscript. J.W.L. and M.H.C. edited the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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