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RESEARCH PAPER

Nutrient composition of different energy-restricted diets determines plasma endocannabinoid profiles and adipose tissue DAGL-α expression; a 12-week randomized controlled trial in subjects with abdominal obesity

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Abstract

The endocannabinoid system (ECS) is dysregulated during obesity and metabolic disorders. Weight loss favours the re-establishment of ECS homeostatic conditions, but also the fatty acid composition of the diet can modulate endocannabinoid profiles. However, the combined impact of nutrient quality and energy restriction on the ECS remains unclear. In this 12 weeks randomized controlled trial, men and women (40–70 years) with obesity (BMI: 31.3 ± 3.5 kg/ m^2) followed either a low nutrient quality 25% energy-restricted (ER) diet (n=39) high in saturated fats and fructose, or a high nutrient quality ER diet (n=34) amongst others enriched in n-3 polyunsaturated fatty acids (PUFAs) or kept their habitual diet (controls). Profiles of plasma- and adipose N-acylethanolamines and mono-acyl glycerol esters were quantified using LC-MS/MS. Gene expression of ECS-related enzymes and receptors was determined in adipose tissue. Measurements were performed under fasting conditions before and after 12 weeks. Our results showed that plasma level of the DHA-derived compound docosahexaenoylethanolamide (DHEA) was decreased in the low nutrient quality ER diet (P<0.001) compared with the high nutrient quality ER diet, whereas anandamide (AEA) and arachidonoylglycerol (2-AG) levels were unaltered. However, adipose tissue gene expression of the 2-AG synthesizing enzyme diacylglycerol lipase alpha (DAGL-α) was increased following the low nutrient quality ER diet (P<.009) and differed upon intervention with both other diets.

Concluding, nutrient quality of the diet affects N-acylethanolamine profiles and gene expression of ECS-related enzymes and receptors even under conditions of high energy restriction in abdominally obese humans. ClinicalTrials.gov NCT02194504

Keywords: Endocannabinoid system; Docosahexaenoylethanolamide (DHEA); Metabolic disorders; n-3 PUFA; N-acylethanolamines; CB_1.

1. Introduction

Overweight and obesity have shown to be considerable risk factors for the development of chronic non-communicable diseases, including type 2 diabetes and cardiovascular diseases. Likewise, body weight reduction is one of the most effective means to reduce disease risk [1]. During the past decades, it has been demonstrated that the endocannabinoid system (ECS) plays an important role in both the central and peripheral regulation of food intake and energy homeostasis [2–4]. Stimulation of the cannabinoid receptor 1 (CB_1) promotes amongst others food intake and lipogenesis resulting in increased energy storage. In obesity and its associated metabolic disorders, dysregulation of the ECS in white adipose tissue has been shown to have detrimental

Abbreviations: 2-AG, Arachidonoylglycerol; AA, Arachidonic acid; AEA, Anandamide/*N*-arachidonoylethanolamine; BW, Body weight; CB (-receptor), Cannabinoid receptor; DAGL-α, Diacylglycerol lipase alpha; DAGLA, Diacylglycerol lipase alpha; DEA, Docosatetraenoylethanolamide; DHA, Docosahexaenoic acid; DHAGly, Docosahexaenoylglycine; DHEA, Docosahexaenoylethanolamide; EC(s), Endocannabinoid(s); ECS, Endocannabinoid system; En%, Percent of energy; EPA, Eicosapentaenoic acid; EPEA, Eicosapentaenoylethanolamide; ER, Energy restricted; MQ, Milli-Q; MUFAs, Monounsaturated fatty acids; NAGly, *N*-arachidonoylglycine; OEA, Oleoylethanolamide; OGTT, Oral glucose tolerance test; PEA, Palmitoylethanolamide; PUFAs, Polyunsaturated fatty acids; SAT, Subcutaneous adipose tissue; SEA, Stearoylethanolamide; SFA, Saturated fatty acids; TFA, Trifluoroacetic acid; TG, Triglycerides; VAT, Visceral adipose tissue.

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effects on insulin sensitivity [5]. Besides, levels of the prototypical endocannabinoids, *N*-arachidonoyl ethanolamine (AEA; also known as anandamide) and 2-arachidonoyl glycerol (2-AG) were found to be elevated in obese animals and humans compared to lean [5–7]. Based on this, targeting the CB_1 receptor, its endogenous ligands or enzymes involved in the synthesis or breakdown of these ligands has attracted much attention as potential weight management strategies [4,8]. However, pharmacological reduction of appetite using CB_1 receptor antagonists or inverse agonists has shown to cause severe adverse effects, as was the case for the CB_1 inverse agonist rimonabant [9,10]. An alternative approach could be to modulate an over-activated ECS *via* targeted dietary intervention.

Endocannabinoids (ECs) and related compounds are lipid mediators derived from fatty acids. They form the key elements of the ECS, and their endogenous levels can be influenced by the fatty acid composition of the diet [10,11]. Most of these lipid mediators belong to the class of fatty acid amides (FAAs), with the exception of a number of mono-acyl glycerol esters, including 2-AG. Anandamide and 2-AG are derived from arachidonic acid (AA, C20:4 *n*-6), which mainly originates from dietary animal sources (meat, eggs, dairy) or can be, in limited amounts, synthesized from its precursor, the essential fatty acid linoleic acid (LA, C18:2 *n*-6) [12]. Diets rich in AA could lead to increased levels of AEA and 2-AG in the body [7] resulting in a dysregulated ECS with negative health consequences with respect to metabolic health. Changing the dietary balance from *n*-6 polyunsaturated fatty acid (PUFA)-rich diets to more *n*-3 PUFA-rich ((*e.g.*, eicosapentaenoic acid (EPA; C20:5 *n*-3), docosahexaenoic acid (DHA, C22:6 *n*-3)) diets may decrease AEA and 2-AG levels, and increase levels of the *n*-3 PUFA-derived lipid mediators docosahexaenoyl ethanolamide (DHEA) and eicosapentaenoyl ethanolamide (EPEA) [13–18]. Accumulating evidence in humans and rodents indicates that diets rich in *n*-3 PUFAs have beneficial effects on obesity-related complications by reducing low-grade systemic inflammation and plasma triglycerides (TG) [19–21]. It is assumed that these beneficial effects are at least partly due to changes in the ratios between *n*-6 PUFA-derived- and *n*-3 PUFA-derived lipid mediators [22,23]. We and others suggested that, additionally to an increased formation of *n*-3 PUFA-derived eicosanoids [24] and resolvins [25], changes in EC tone may also play a role here [14,26]. One of the most studied EC-like *n*-3 PUFA-derived lipid mediator is DHEA. This molecule was found to possess anti-inflammatory activity by inhibiting the activity of the cyclooxygenase-2 (COX-2) enzyme [27].

Binding studies show that DHEA and EPEA are weaker ligands of CB_1 than anandamide and 2-AG [28–30]. As a consequence, it is hypothesized that a relative decrease in *n*-6 PUFA-derived endocannabinoids in favor of their *n*-3 PUFA derived congeners will lead to a reduced CB_1 stimulation. Furthermore, other endogenous congeners of AEA and DHEA, like palmitoylethanolamide (PEA), oleoylethanolamide (OEA), stearoylethanolamide (SEA), docosatetraenoylethanolamide (DEA), *N*-arachidonoylglycine (NAGly) and docosahexaenoylglycine (DHAGly), were also shown to have weak or no affinity for CB_1 receptors, while possessing affinity for other receptors such as the peroxisome proliferator-activated receptors PPAR-α or PPAR-γ [31,32]. However, the relevance of these endogenous congeners in obesity is not clear. Their different spectrum of activities and dynamic behavior demands for the combined analysis of the different endocannabinoids and their congeners.

Although it is well established that energy restriction is beneficial for obesity-associated metabolic disorders, it is still unclear whether and to what extent diet composition can further enhance weight loss-induced improvements in metabolic health. Because the ECS, known to regulate important metabolic processes, is affected by diet and dysregulated by obesogenic conditions, it is important to investigate whether nutrient composition during weight loss in obese individuals can beneficially influence the ECS. To achieve an optimal effect, we have designed a high nutrient quality ER diet that includes a combination of high-quality nutrients that specifically target the health of metabolic organs. The high nutrient quality ER diet contains *n*-3 PUFAs, monounsaturated fatty acids (MUFAs), soy protein and fibers [33–35]. Of particular importance for the ECS are the DHA-derived DHEA, EPA-derived EPEA and oleic acid-derived OEA, as they can beneficially affect metabolic health by reducing inflammatory processes, influencing the *n*-3 PUFA/*n*-6 PUFA ethanolamine balance, affect liver health and reduce food intake and restore rewarding, respectively [35]. We particularly aimed to uncover effects of high quality nutrients on ECS regulation and EC levels during 25% energy restriction in humans with abdominal obesity and to compare this with a low nutrient quality 25% ER diet rich in saturated fatty acids (SFAs), monosaccharides such as fructose, and a habitual diet (control). Additionally, we explored how EC levels correlate with observed changes in metabolic parameters. Therefore, we compared effects of high- and low nutrient quality combined with 25% energy restriction on the profiles of 9 different *N*-acyl amides, including DHEA, and 2-AG in plasma and adipose tissue as well as on adipose expressed ECS-related enzymes and receptors from overweight individuals participating in a 12-week randomized controlled trial (RCT) and included a control group that kept its habitual diet [36]. This well-controlled intervention trial is the first investigation exploring such dietary conditions related to the ECS which could be of high relevance for obesity and metabolic dysregulation.

2. Materials and methods

2.1. Subjects

In total, 100 healthy overweight (age 40–70 years) subjects of both sexes with a BMI $>$ 27 kg/m^2 or a waist circumference of $>$102 cm (males) or $>$88 cm (females) were involved in this study (Supplementary Table S1). All subjects were non-smoking, had an omnivore diet and a stable body weight (weight gain or loss $<$ 3 kg in the past three months). Moreover, subjects were not diagnosed with diabetes (oral glucose tolerance test [OGTT]; fasting blood glucose $<$ 7 mmol/L, after 2 h $<$ 11.1 mmol/L) and not using medications known to interfere with glucose or lipid homeostasis. All participants gave written informed consent and the study was approved by an independent medical ethical committee from Wageningen University and registered under ClinicalTrials.gov (ID: NCT02194504; U.S. National Institutes of Health, Bethesda, MD, USA; http://clinicaltrials.gov).

2.2. Study design

The study was a 12-week, randomized controlled, parallel trial in which the 100 participants were assigned to three different dietary groups; a low nutrient quality ER diet group, a high nutrient quality ER diet group, or a control group. Subjects were stratified among the intervention groups based on BMI, age, and gender. The detailed study protocol, dietary compositions, study population and power calculation have been described previously [36]. Prespecified primary outcomes were cardiometabolic risk factors using an array of outcome measures, including whole-body MRI and magnetic resonance spectroscopy (MRS), plasma metabolomics including endocannabinoids, adipose tissue transcriptomics including ECS parameters. Briefly, participants either followed a low nutrient quality ER diet (n=39), high in saturated- as well as unsaturated

fats and fructose, or a high nutrient quality ER diet (n=34), enriched in, MUFAs, PUFAs, soy protein and fiber (see Supplementary Table S2) and [36] together with complex carbohydrates and vegetable-derived proteins or stayed on their habitual diet (n=27). The 2 diets were isocaloric and matched for alcohol, sodium, and total fat. The differences between the two ER diets were that the high nutrient quality ER diet, compared to the low nutrient quality ER diet, was enriched in MUFAs (13.4 En% compared with 9.3 En%, respectively), n-3 PUFAs (7.7 En% compared with 4.1 En%, respectively), fiber (3.1 En% compared with 2.2 En%, respectively), and plant protein (10.3 En% compared with 5.7 En%, respectively) and reduced in SFA (8.3 En% compared with 14.9 En%, respectively) and fructose content (3.4 En% compared with 7.0 En%, respectively). The different fatty acids are precursors for EC-lipid mediators, where OEA is derived from the MUFA oleic acid; DHEA and DHAGly from DHA; EPEA from EPA; AEA, 2-AG and NAGly from AA; PEA and SEA from the SFAs palmitic acid and stearic acid, respectively and DEA from docosatetraenoic acid.

Soy protein has been provided as a healthy sustainable source of protein. The high nutrient quality ER diet had a reported linoleic acid intake of 12.1 g/d, while the low nutrient quality ER diet had a 8.2 g/d reported intake. Recommended doses for linoleic acid are between 12–17 g/d. In the high nutrient quality ER diet, 700 mg of n-3 PUFA was provided each day as capsules. This included 400 mg eicosapentaenoic acid (EPA) and 300 mg docosahexaenoic acid (DHA). Providing capsules enabled the inclusion of participants who did not like fish. Additionally, the high nutrient quality ER diet itself contained approximately 570 mg of n-3 PUFA per day. This, combined with the capsules, resulted in a recommended total daily intake of 1270 mg n-3 PUFAs. In the low nutrient quality ER diet group no additional n-3 PUFA capsules were provided and participants were advised to keep dietary n-3 PUFA intake low (around 33 mg). Individual meal plans were constituted based on the energy composition of the diets, which were communicated to the participants. Participants took part in weekly motivation sessions with a qualified dietician, after which key food and n-3 PUFA capsules products were provided for the next week. Reported intake of energy and nutrients, including that of n-3 PUFA was calculated weekly per participant, using the meal plan, participant's food diary, and reported deviations as a basis. Additional consumed food items were added to the meal plan whereas leftovers from the supplied products were subtracted. The advised and reported nutrient composition, including EPA and DHA is given in Table S2. A reduction of twenty-five percent energy intake was aimed at by allocating participants to an energy group of either 6, 7, 8 or 9 MJ/d based on the estimated energy requirement for each participant. Both ER diets resulted in a 25% reduction of energy intake during the intervention. The control group did not receive any dietary advice and participants were instructed to maintain their habitual diet. Participants from all intervention groups were instructed to maintain their regular physical activity habits and report cases of structural changes in activity regime. On the day prior to each testing day, subjects consumed a standardized low-fat evening meal, refrained from alcohol or strenuous exercise and were not allowed to eat or drink anything except water after 8 PM. The study was conducted at Wageningen University, the Netherlands, from July 21, until October 21, 2014.

2.3. Plasma and adipose tissue collection for EC analysis

Blood and adipose tissue were collected in the morning after an overnight fast, before and after 12 weeks of diet intervention. Subcutaneous abdominal adipose tissue biopsies were collected from the umbilicus under local anesthesia in a subgroup of this study (67/100 participants). Figure 1 shows the flow chart for endocannabinoid analysis of the controlled dietary intervention. Adipose tissue biopsies were subjected to analysis of EC (-related) compounds. To prevent breakdown of the analytes, plasma samples

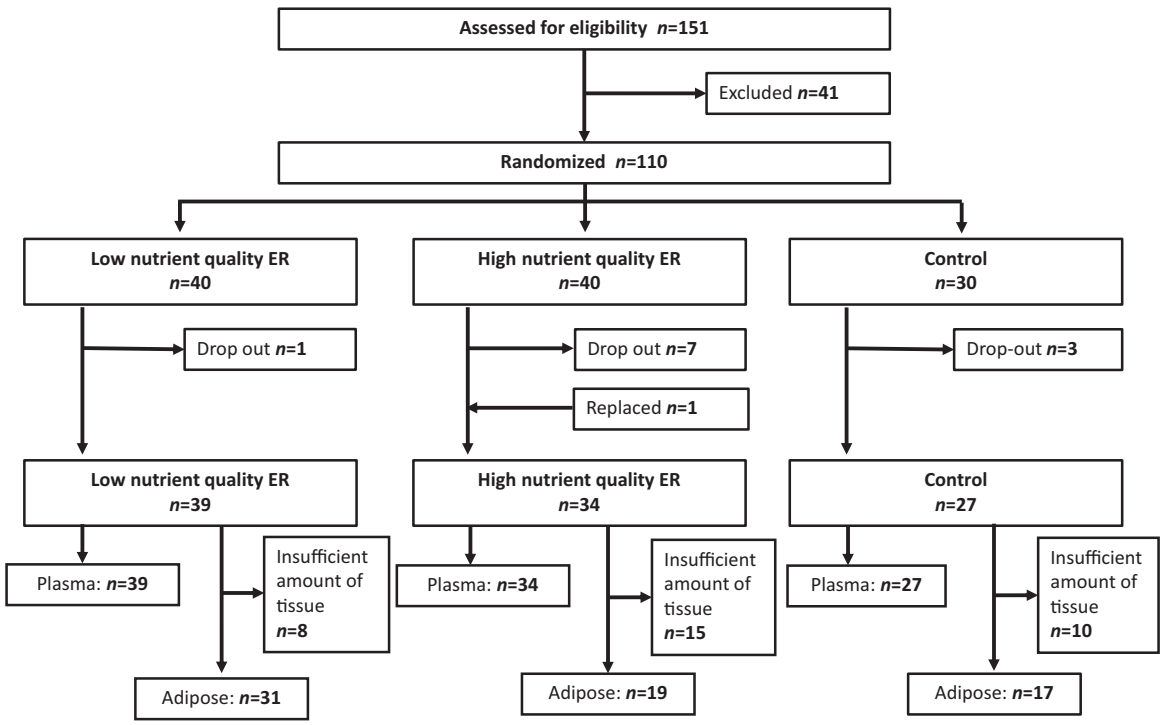

Fig. 1. Flow chart of subjects and number of samples collected for endocannabinoid analysis. One drop-out in the high nutrient quality ER group could be replaced in time with another individual due to the phased entry of participants in the study.

were collected in vials containing phenylmethanesulphonylfluoride (PMSF, final concentration in sample 100 μM) to inhibit fatty acid amide hydrolase (FAAH) activity, and immediately frozen at -80°C. Adipose tissue samples were rinsed with phosphate buffered saline to eliminate blood and immediately frozen in liquid nitrogen and stored at -80°C.

2.4. Analysis of endocannabinoids and related compounds

Sample preparation and analysis of endocannabinoids and related compounds were performed using a modified version of a previously published method [37]. Briefly, plasma (1 mL) was thawed and 4 mL extraction mixture (containing 100 μM PMSF and 10 μL internal standards [AEA-d8 0.03125 μg/mL, 2-AG-d8 0.75 μg/mL and NAGly-d8 0.0625 μg/mL (Cayman, MI, USA)] in acetonitrile ACN) were added while the sample was gently vortexed. After subsequent centrifugation (5 min at 13,000 rpm at RT), 15 mL water containing 0.1% (v/v) trifluoroacetic acid (TFA) was added to the supernatant to dilute the acetonitrile prior to solid phase extraction (SPE) clean-up. SPE was performed using Bond Elute C8 (Varian Incorporated, Lake Forest, CA, USA) by activating the column first with 1 mL methanol followed by a washing step with 1 mL water. The whole diluted extract (20 mL) was subsequently applied on the column and washed with 2 mL 20% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA. Compounds of interest were eluted from the SPE column using 2 mL 80% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA and this lipid-containing fraction was evaporated to dryness using a vacuum concentrator (Thermo Fisher Integrated Speedvac Scientific SPD1010, MA, USA) and stored at −80 °C until further analysis. For adipose tissue, approximately 100 mg "wet" tissue was extracted with 1 mL extraction solution containing internal standards in ACN by sonication [15]. The samples were centrifuged for 5 min at 13,000 rpm at RT, the supernatant was transferred to a clean 2.0 mL Eppendorf tube, and the ACN extraction was repeated once. The 2 mL ACN extract was subsequently evaporated to dryness and stored at −80 °C until further analysis.

Quantification of the endocannabinoids and related compounds AEA, 2-AG, DHEA, PEA, OEA, SEA, DEA, EPEA, NAGly, and DHAGly was achieved by using an Acquity UPLC I-class system coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Etten-Leur, the Netherlands). The dried extracts were reconstituted in 100 μL ACN with 0.1% (v/v) TFA, mixed thoroughly using an ultrasonic bath and vortexer and transferred to an autosampler vial. Finally, 3 μL was injected on an Acquity C8 BEH UPLC column (2.1 × 150 mm, 1.7 μm) and the sample was separated using binary gradient elution with a stable flow of 500 μL/min for 15 min. The gradient started with 100% A (40:40:20 v/v/v of Milli-Q water: methanol: ACN with 0.1% (v/v) FA) which was maintained until 0.35 min, followed by a linear increase to 100% B (7:3 v/v methanol: ACN with 0.1% (v/v) FA) until 8.0 min and was maintained until 11.0 min. At the end of gradient, the column was equilibrated to initial conditions (100% A) for 3.9 min. The column was maintained at 60°C during analysis, and the samples were kept at 10°C. The MS was operated in selective reaction mode using electrospray ionization in positive ion mode, with a capillary voltage of 1.5 kV, a source temperature of 150 °C and a desolvation temperature of 500°C. Cone voltage and collision energy were optimized for each compound individually (see Supplementary Table S3 for precursor and product ion *m/z* values). Peak identification and quantification was performed using MassLynx software version 4.1. In aqueous environments, 2-AG may undergo rapid isomerization into 1-AG, which can be visible as 2 peaks in the 2-AG chromatogram. The quantification of 2-AG was performed using the combined integration of the 2-AG + 1-AG peaks [38]. Calibration curves were run in duplicate from which one regression equation was generated. Concentrations of ECs were calculated based on the regression equations. The two samples (pre- and postintervention taken under fasted conditions) from each individual were extracted and analyzed in the same batch in order to exclude potential batch-to-batch differences. Six quality control (QC) samples at three different concentrations in duplicate were analyzed in every batch to monitor the quality of the analysis and check the accuracy.

2.5. Adipose tissue gene expression

Gene expression of enzymes and receptors related to the ECS were obtained from data of whole genome transcriptome analysis performed on subcutaneous adipose tissue biopsies taken before and after the intervention, under fasted conditions. Adipose tissue biopsies whole genome gene expression was available of 27 subjects in the low nutrient quality ER group, 27 in the high nutrient quality ER group and 18 in the control group. Details on the whole genome transcriptome analysis are described in [36]. In brief, frozen adipose tissue samples were subjected to a Trizol-chloroform extraction (Thermo Fisher Scientific) and subsequently purified by using the Qiagen Mini column kit (Qiagen). Thereafter, with the NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies) RNA yield was quantified. RNA integrity (RIN) was determined on an Agilent 2100 BioAnalyzer with RNA 6000 Nanochips (Agilent Technologies). By using a 1-cycle cDNA labeling kit (MessageAmp II-Biotin Enhanced Kit; Ambion), total RNA of high quality samples was labeled and hybridized to GeneChip Human Gene 2.1 ST arrays (Affymetrix). The affyPLM R package robust Multi-Array Average algorithm [39] was used to normalize the Raw CEL files. Custom annotation was performed based on reorganized oligonucleotide probes combining all individual probes for a gene (MBNI Brainarray CDF file; ENTREZG v21).

2.6. Measurement of metabolic markers and plasma fatty acids

Plasma was collected under fasting conditions before and after the intervention as described in [36]. Briefly, plasma glucose, triglycerides (TG) and insulin were analyzed photometrically (Cobas 8000, Roche Diagnostic Limited, Switzerland) by SHO center for medical diagnostics (Velp, The Netherlands). Subcutaneous (SAT), visceral adipose tissue mass (VAT), SATVAT ratio, and intrahepatic lipids were determined using a 3T whole-body MRI scanner (Siemens) at hospital Gelderse Vallei, Ede, the Netherlands. Subsequent data analysis was performed as described previously [36].

Plasma fatty acid analysis was performed using plasma EDTA samples on a high-throughput NMR metabolomics platform [40] (Nightingale Health Ltd., Vantaa, Finland). Reported fatty acid (FA) concentrations effectively represent those esterified within lipoprotein particles. Capsules containing *n*-3 PUFA were analyzed for fatty acid composition using gas chromatography at our research facility. Analysis showed that the *n*-3 PUFA capsules contained 87% EPA and 97% DHA of the producers claimed content.

2.7. Data analysis

Data are expressed as means ± SD. Statistical analysis was performed on non-transformed data (PASW statistics 18.0.3, IBM). Differences in baseline characteristics between subject groups were analyzed by analysis of variance (ANOVA). Differences in endocannabinoid concentrations within each intervention were analyzed by a paired t-test. Differences in endocannabinoids responses between the different interventions were analyzed by ANOVA on

Table 1
Plasma endocannabinoid profile at baseline and upon 12 weeks intervention (Δ).

Plasma ECs		Baseline	Change (Δ)	*P*-value		
		Mean ± SD	Mean ± SD	Within group	Between group	
					Baseline	After12 wk Δ
AEA (ng/mL)	Control	0.38±0.1	-0.029 ± 0.118	.222	0.562	0.403
	L quality	0.40±0.15	-0.032 ± 0.134	.141		
	H quality	0.40±0.12	0.004 ± 0.107	.836		
DHEA (ng/mL)	Control	0.37±0.12	-0.036 ± 0.101[a,b]	.078	0.966	**0.045**
	L quality	0.36±0.13	-0.076 ± 0.095[b]	**<.001**		
	H quality	0.37±0.12	-0.023 ± 0.085[a]	.126		
PEA (ng/mL)	Control	2.49±0.51	-0.081 ± 0.508	.412	0.356	0.159
	L quality	2.77±0.93	-0.373 ± 0.737	**.003**		
	H quality	2.62±0.63	-0.210 ± 0.518	**.024**		
OEA (ng/mL)	Control	2.4±0.54	-0.159 ± 0.512	.119	0.302	0.480
	L quality	2.63±0.80	-0.235 ± 0.741	**.055**		
	H quality	2.62±0.67	-0.054 ± 0.584	.591		
SEA (ng/mL)	Control	1.55±0.45	-0.059 ± 0.279	.282	0.060	0.175
	L quality	1.87±0.65	-0.248 ± 0.559	**.009**		
	H quality	1.71±0.45	-0.247 ± 0.397	**.001**		
NAGly (ng/mL)	Control	0.26±0.15	-0.012 ± 0.122	.618	0.512	0.536
	L quality	0.26±0.12	0.009 ± 0.050	.256		
	H quality	0.29±0.13	-0.010 ± 0.087	.522		
DHAGly (ng/mL)	Control	0.14±0.08	-0.028 ± 0.060	**.023**	0.925	0.362
	L quality	0.14±0.12	-0.039 ± 0.090	**.009**		
	H quality	0.15±0.08	-0.016 ± 0.046	**.048**		
2-AG (ng/mL)	Control	6.6±5.7	-0.772 ±5.435	.467	0.349	0.178
	L quality	5.3±3.1	0.908 ± 4.928	.257		
	H quality	6.8±4.3	1.835 ± 5.935	.081		

AEA, Anandamide/*N*-arachidonoylethanolamine; DHAGly, Docosahexaenoylglycine; DHEA, Docosahexaenoylethanolamide; PEA, Palmitoylethanolamide; OEA, Oleoylethanolamide; SEA, Stearoylethanolamide; NAGly, *N*-arachidonoylglycine; 2-AG, 2-arachidonoyl glycerol.
Plasma EC concentrations determined before intervention (baseline) and delta changes upon intervention (change) are displayed as means ± SD for each diet group.
Different letters (a, b, c) indicate significant post hoc differences ($P<.05$) between diet groups. Differences in EC concentrations within each diet group were analyzed by a paired t-test. Differences in EC responses between the different interventions were analyzed by ANOVA on delta values (changes from baseline, after-before intervention). A least significant difference (LSD) post-hoc test was performed to identify differences between intervention groups.
P-values<.05 within- and between diet groups are displayed in bold.
Control group (control), $n=27$; low nutrient quality ER diet (L quality), $n=39$; high-nutrient quality ER diet (H quality), $n=34$.

delta values (changes from baseline, after-before intervention). A least significant difference (LSD) post-hoc test was performed to identify differences between intervention groups.

For adipose tissue, differences in gene expression were assessed using Linear Model for Microarray Analysis. For the selected EC(-related) genes, changes (12 weeks − 0 weeks) in fasting gene expression between the 3 different groups (F-test and t-tests), were defined as significantly different at a *P* value <.05. Analysis within an intervention group were performed using paired *t*-test with *P* value<.05. Correlation analyses at baseline were performed on the absolute values and after combining the three intervention groups. Correlations in response to the interventions were performed on delta values (changes from baseline, after-before intervention) for each intervention group. Spearman correlations and hierarchical clustering were performed in R. Data were log2 transformed and correlations were FDR corrected for multiple testing. A *P*-value <.05 was considered to be significant.

3. Results

3.1. Baseline characteristics of the subjects

A total of 100 participants completed the study, from which 39 participants were assigned to the low nutrient quality ER diet, 34 participants to the high nutrient quality ER diet and 27 participants to the control group (Fig. 1). Details of exclusion and drop-out information have been described before [36]. Baseline characteristics of the subjects from the 3 different groups have been described previously and are also displayed in supplemental data, SupplementaryTable S1.

3.2. Anthropometric variables, plasma metabolic markers and exposure markers of participants before and after the intervention

Intervention-induced changes in clinical chemistry and anthropometric variables have been published before [36]. In both ER diet groups, 12 weeks of intervention significantly reduced body weight, BMI, waist circumference, plasma glucose, insulin and TG levels and SAT, VAT, VATSAT ratio and intrahepatic lipids ($P<.05$) compared to the control group. Among these changed metabolic variables, body weight and BMI in the high nutrient quality ER diet were significantly more reduced compared to the low nutrient quality ER diet ($P<.05$) [36].

Plasma fatty acid analysis have been performed on fasted EDTA plasma samples of the participants to designate the main group of metabolites that could reflect dietary intake. Data are given in Supplementary Table S4. Changes in total FA, MUFA, SFA, PUFA and omega-6-fatty acids as well as the ratio of SFA to total FA, the ratio of LA to total FA and the estimated degree of unsaturation were significantly different between groups. Post hoc analyses showed that these effects were caused by a reduction in levels of total FA, MUFA, SFA, PUFA and omega-6-fatty acid in the high nutrient quality ER diet and an increase in estimated degree of unsaturation in the high nutrient quality ER group. However, further explorative analyses showed that the ratio of DHA-, PUFA, omega-6 fatty acids and omega-3 fatty acids each, relative to total fatty acids, were significantly increased within the high nutrient quality ER group.

3.3. Effects on plasma endocannabinoid profile upon intervention

Plasma collection and endocannabinoid analyses were successfully completed for all 100 participants. A total of 8 ECs and related compounds, *i.e.,* AEA, 2-AG, DHEA, PEA, OEA, SEA, NAGly and DHAGly, could be adequately quantified in plasma. Some species, such as DEA and EPEA, were undetectable in the study samples, presumably because their concentrations were below detection limits. Changes in EC-(related) compounds in plasma upon intervention are listed in Table 1. At baseline groups did not differ in plasma EC concentrations. Comparisons between the 3 different diet groups showed that plasma DHEA differed significantly between the diet groups ($P<.05$). Posthoc analysis revealed that DHEA was significantly ($P<.001$) lower in the low nutrient quality ER diet compared to the high nutrient quality ER diet. For the other compounds there were no significant differences between the 3 groups upon intervention. Although not significant between diet groups, a significant reduction with the diet groups was found for PEA ($P=.003$) and SEA ($P=.009$) in the low nutrient quality ER diet, as well as for PEA ($P=.024$) and SEA ($P=.001$) in the high nutrient quality ER diet. Decreases of OEA plasma levels ($P=.055$) in the low nutrient quality ER diet showed a trend.

3.4. Changes in adipose tissue endocannabinoid profiles and gene expression upon intervention

Adipose tissue EC-(related) compounds and expression levels of 20 ECS-related genes were analyzed in a subset of the study population. For whole genome-wide gene expression, biopsies were obtained from 72 subjects. Out of the analyzed genes, expression of diacylglycerol lipase alpha (*DAGLA; P*=.026) was significantly different between the three diet groups (Table 2) which was due to an increase in the low nutrient quality ER diet ($P=.009$) upon intervention, which was significantly different from both other diet groups. To explore possible differences in intervention caused by

Table 2
Expression of EC-related genes in adipose tissue upon 12 weeks intervention.

		EFFECTS BETWEEN DIET GROUPS				EFFECTS WITHIN DIET GROUPS					
		Effects Fasting	**LQD vs. Con**	**HQD vs. Con**	**LQD vs. HQD**	**Control Diet**		**Low Quality Diet**		**High Quality Diet**	
		P-Value				Change SLR	*P-Value*	Change SLR	*P-Value*	Change SLR	*P-Value*
LEP	leptin	0.083				-0.15	0.26	-0.30	**0.004**	-0.51	**0**
MGLL	monoglyceride lipase	0.717				-0.08	0.16	-0.12	**0.018**	-0.15	**0.003**
DAGLA	diacylglycerol lipase alpha	**0.026**	**0.03**	0.991	**0.016**	-0.05	0.51	0.17	**0.009**	-0.05	0.41
LIPE	lipase E, hormone sensitive type	0.898				-0.10	0.10	-0.11	**0.036**	-0.13	**0.009**
ABHD6	abhydrolase domain containing 6	0.946				-0.09	0.22	-0.13	**0.046**	-0.11	0.094
MAPK3	mitogen-activated protein kinase 3	0.182				0.01	0.79	-0.06	0.078	0.03	0.48
ADIPOQ	adiponectin, C1Q and collagen domain containing	0.655				-0.02	0.74	-0.05	0.24	-0.08	0.066
PRKAA1	protein kinase AMP-activated catalytic subunit alpha 1	0.773				0.01	0.86	0.06	0.18	0.04	0.32
FAAH2	fatty acid amide hydrolase 2	0.962				0.09	0.26	0.07	0.29	0.06	0.36
NAAA	N-acylethanolamine acid amidase	0.761				0.08	0.31	0.07	0.32	0.01	0.86
ABHD12	abhydrolase domain containing 12	0.998				0.05	0.43	0.05	0.32	0.05	0.29
NAPEPLD	N-acyl phosphatidylethanolamine phospholipase D	0.949				0.04	0.67	0.05	0.50	0.07	0.32
DAGLB	diacylglycerol lipase beta	0.947				0.06	0.35	0.04	0.53	0.05	0.42
CNR1	cannabinoid receptor 1	0.249				0.05	0.64	0.05	0.58	0.23	**0.008**
PTGS2	prostaglandin-endoperoxide synthase 2	0.67				0.07	0.63	0.04	0.73	-0.08	0.50
CNR2	cannabinoid receptor 2	0.775				0.05	0.69	-0.03	0.78	-0.07	0.52
SREBF1	sterol regulatory element binding transcription factor 1	0.956				0.03	0.82	0.02	0.83	-0.02	0.89
ADGRF1	adhesion G protein-coupled receptor F1	0.439				-0.10	0.36	0.02	0.86	0.08	0.37
MAPK1	mitogen-activated protein kinase 1	0.741				0.01	0.80	0.01	0.89	-0.03	0.45

Fold change (Δ log2; 12 weeks–0 weeks) in gene expression of EC-related genes expressed in adipose tissue biopsies taken before and after intervention for the 3 different diet groups.
Biopsies were taken under fasted conditions from a subset of 72 subjects in the study population. Control group (con), $n=18$; low nutrient quality ER diet (LQD), $n=27$; high nutrient quality ER diet (HQD), $n=27$.
Gene expression was measured by microarray analysis, having significant differences assessed by using Linear Model for Microarray Analysis. Effects between diet groups with *P* values<.05 were defined as significant (F-test and *t* test). Effects within the different diet groups with *P* values <.05 are depicted in bold (paired *t*-test).

Table 3
Adipose tissue endocannabinoid profile at baseline and upon 12 weeks intervention (Δ).

Adipose tissue ECs		Baseline	Change (Δ)	*P*-value		
		Mean ± SD	Mean ± SD	Within group	Between groups	
					Baseline	After12 wk Δ
AEA (ng/mL/g tissue)	Control	1.61 ± 0.72	-0.10 ± 0.59	.493	0.832	0.670
	L quality	1.62 ± 0.69	0.06 ± 0.70	.646		
	H quality	1.73 ± 0.59	0.04 ± 0.46	.681		
DHEA (ng/mL/g tissue)	Control	0.76 ± 0.2	-0.04 ± 0.22	.421	0.941	0.428
	L quality	0.75 ± 0.31	-0.11 ± 0.23	**.013**		
	H quality	0.78 ± 0.31	-0.03 ± 0.24	.564		
EPEA (ng/mL/g tissue)	Control	1.24 ± 0.5	-0.25 ± 0.78	.200	0.490	0.889
	L quality	1.35 ± 0.77	-0.19 ± 0.69	.136		
	H quality	1.51 ± 0.7	-0.14 ± 0.71	.414		
OEA (ng/mL/g tissue)	Control	13.81 ± 2.9	-1.29 ± 3.03	.099	0.854	0.653
	L quality	13.56 ± 3.7	-1.15 ± 4.63	.176		
	H quality	14.08 ± 2.5	-0.26 ± 2.58	.663		
PEA (ng/mLg tissue)	Control	16.14 ± 3.8	-0.42 ± 3.99	.669	0.355	0.795
	L quality	17.26 ± 4.7	-1.10 ± 5.25	.251		
	H quality	18.15 ± 3.4	-0.30 ± 3.54	.780		
SEA (ng/mL/g tissue)	Control	22.2 ± 4.4	-2.38 ± 4.65	.051	0.757	0.835
	L quality	22.5 ± 6.2	-2.96 ± 6.62	**.019**		
	H quality	23.5 ± 5.4	-1.98 ± 4.85	.091		
DEA (ng/mL/g tissue)	Control	0.13 ± 0.11	0.02 ± 0.08	.282	0.491	0.523
	L quality	0.15 ± 0.09	0.00 ± 0.09	.963		
	H quality	0.17 ± 0.08	0.02 ± 0.08	.185		
DHAGly (ng/mL/g tissue)	Control	15 ± 3.1	-1.94 ± 4.07[a]	.067	0.831	0.073
	L quality	15.2 ± 3.6	-1.38 ± 6.11[a]	.218		
	H quality	14.6 ± 3.4	1.99 ± 6.18[b]	.177		
2-AG (ng/mL/g tissue)	Control	150.1 ± 150.3	-4.39 ± 132.82	.893	0.615	0.460
	L quality	187.7 ± 162.7	-41.04 ± 220.26	.308		
	H quality	150.6 ± 142	26.30 ± 166.91	.501		

AEA, Anandamide/*N*-arachidonoylethanolamine; DEA, Docosatetraenoylethanolamide; DHAGly, Docosahexaenoylglycine; DHEA, Docosahexaenoylethanolamide; EPEA, Eicosapentaenoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide; SEA, Stearoylethanolamide; 2-AG, 2-arachidonoyl glycerol.
Adipose tissue EC concentrations determined before intervention (baseline) and delta changes upon intervention (change) are displayed as means ± SD for each diet group.
Different letters (a, b, c) indicate significant, or close to significant, post hoc differences ($P<.05$) between diet groups. Differences in EC concentrations within each diet group were analyzed by a paired t-test. Differences in EC responses between the different interventions were analysed by ANOVA on delta values (changes from baseline, after-before intervention). A least significant difference (LSD) post-hoc test was performed to identify differences between intervention groups.
P-values<.05 within- and between diet groups are displayed in bold. Control group (control), $n=17$; low nutrient quality ER diet (L quality), $n=31$; high nutrient quality ER diet (H quality), $n=19$.

either the diet- or energy restriction, within group analysis was performed. Only within the high nutrient quality ER diet, gene expression of CB1 (*CNR1*) was found to be significantly increased ($P=.008$), while expression of leptin (*LEP*), MAG-lipase (*MGLL*), and lipase E (*LIPE*) were significantly reduced within both ER-groups (Table 2).

In total, 67 participants, of which 31 in the low nutrient quality ER diet, 19 in the high nutrient quality ER diet and 17 in the control group, were included for EC analysis (Fig. 1). No differences in baseline characteristics (listed in Supplementary Table S5) between groups were observed in this subset. From the EC-(related) compounds 9 could be detected in adipose tissue, including AEA, 2-AG, DHEA, PEA, OEA, SEA, DEA, EPEA, and DHAGly. In general, concentrations of EC-(related) compounds were several times higher than in plasma. The effects of the dietary intervention on these compounds are listed in Table 3. No significant differences were found between the three diet groups. A trend was found for DHAGly ($P=.073$) between diet groups and was significantly increased in the high nutrient quality ER diet group compared to the low nutrient quality ER diet- and control group. Although not significant

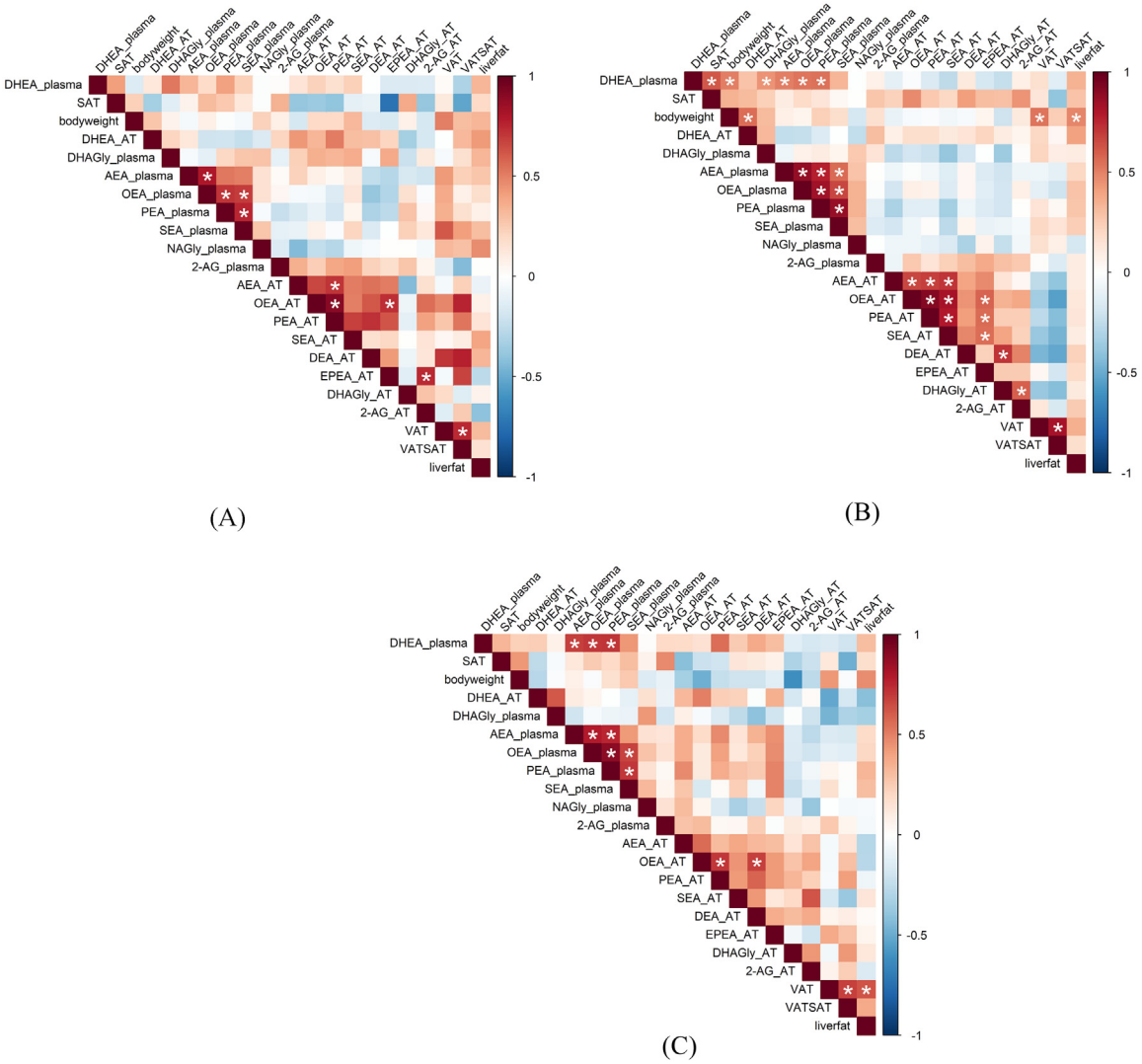

Fig. 2. (A–C) Correlation heat maps for plasma- and adipose tissue endocannabinoids with body composition parameters; body weight, subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), VATSAT ratio and intra hepatic lipids (liver fat), on changes upon intervention for (A) control diet, (B) the low nutrient quality ER diet and (C) the high nutrient quality ER diet. *P* values<.05 are indicated with * and are corrected for false positives using FDR (false discovery rate). Spearman correlation was performed for all heat maps.

between diets, DHEA (*P*=.013) and SEA (*P*=.019) levels in AT were found significantly reduced within the low nutrient quality ER diet group (Table 3).

3.5. Correlation of changes in EC levels with changes in plasma-, liver- and adipose tissue metabolic markers in response to intervention

To explore potential relationships of ECs with metabolic health markers upon the dietary/ER intervention, correlation analyzes were performed between changes in the 8 plasma- and 9 adipose tissue ECs, with changes in the following body composition markers of liver and adipose tissue (AT); body weight (BW), subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), VATSAT ratio and intra hepatic-lipids (liver fat) (Fig. 2A–C). Out of all ECs measured, significant correlations with health measures were only found for DHEA. In the low nutrient quality ER diet group (Fig. 2B), decreases for DHEA in plasma upon intervention were significantly positive correlated with decreases in body weight (*P*=.01; Spearman correlation=0.5), and SAT (*P*=.006; Spearman correlation = 0.6), while in the high nutrient quality ER diet group (Fig. 2C) and the control group (Fig. 2A) no such correlations were found. Likewise, in the low nutrient quality ER diet group (Fig. 2B), decreases in DHEA levels in adipose tissue (*P*=.01; Spearman correlation= 0.55) were significantly positive correlated with decreases in body weight, but not with SAT. There were no significant correlations found between intervention-induced changes in EC plasma and EC AT levels and plasma glucose, triglycerides and insulin (data not shown).

In plasma and AT, changes in levels of AEA, OEA, PEA as well as SEA were highly correlated with each other (*P*< 0.001; Spearman correlations: 0.7-0.9) upon intervention, particularly in the low nutrient quality ER diet (Fig. 2B) but also in the control group (Fig. 2A), while significant correlations for DHEA with the above mentioned ECs were around 0.5. Changes for plasma DHEA correlated with those for DHAGly in the low nutrient quality ER diet group. For the high nutrient quality ER diet in plasma, high correlations for the above mentioned ECs were present and included DHEA but not always SEA. In AT obtained from participants of the high nutrient quality ER diet only PEA and OEA (*P*=.02;

Spearman correlation=0.7) and DEA and OEA (P=.03; Spearman correlation=0.7) were significantly correlated. Changes upon intervention for 2-AG in AT were significantly correlated with AT DHAGly in the low nutrient quality ER diet group and with AT EPEA in the control group. Notably, changes in levels upon ER intervention for ECs in plasma and ECs in AT were not correlated with each other.

By calculating the correlations between the EC profile in plasma with adipose tissue before intervention of all participants, we found that only for AEA (R=0.375, P=.002) and DHEA (R=0.654, P<.001) levels were significantly correlated between plasma and AT, while for the other EC related compounds no such correlations were found.

4. Discussion

Within this randomized controlled intervention trial we investigated whether the nutrient quality of a weight-loss diet affects the ECS in overweight people. Our findings revealed that consumption of a low nutrient quality ER diet with reported intake of 103 (± 226) mg omega-3-fatty acids (DHA and EPA) resulted in significant (P<.001) decreased plasma concentrations of the DHA-derived endocannabinoid-like compound DHEA compared to the high nutrient quality ER diet that contained 1224 (± 55) mg omega-3-fatty acids (DHA and EPA). Although AEA and 2-AG levels did not differ between the 2 ER groups, the gene expression of the 2-AG synthesizing enzyme DAGL-α was significantly increased (P<.009) in adipose tissue in the group following the low nutrient quality ER diet, compared with those of the high nutrient quality ER- and the control diet. Our findings indicate that following a high nutrient quality ER diet for 12-weeks prevents a decline in DHEA plasma and an increase in adipose tissue DAGL-α gene expression, thereby providing additional metabolic health benefits over a low nutrient quality ER diet, in addition to the metabolic improvements already obtained as a result of the 25%ER [36].

4.1. Diet intervention alters DHEA plasma levels and adipose tissue DAGL-α expression in the low nutrient quality ER diet group

In addition to the significant low nutrient quality ER diet-induced reduction in plasma DHEA, DHEA levels were also measured in adipose tissue and although not significant between the diets, within groups analyses also showed a significant reduction in DHEA in adipose tissue upon the low nutrient quality ER diet. The main differences between both ER diets were that the low nutrient quality ER diet (n=39) was high in saturated- as well as unsaturated fats and fructose and the high nutrient quality ER diet (n=34) was enriched in n-3 PUFAs, MUFAs, soy protein and fiber. Previous animal and human studies showed that a diet rich in n-3 PUFA can result in increased DHEA concentrations in several tissues [11,15–17,41–43] and even alter the balance between EC-derived n-3 and n-6 PUFAs by also decreasing circulating AEA and/or 2-AG concentrations [44–46]. As DHEA derives from the n-3 PUFA DHA, we had expected an increase in DHEA levels in the high nutrient quality ER diet. Instead, we observed in the high nutrient quality ER diet no changes in DHEA but in the low nutrient quality ER diet without additional n-3 PUFA we did see a reduction in DHEA levels. It was also only in this low nutrient quality ER diet that a reduction in DHEA was significantly correlated to a reduction in body weight and reductions in subcutaneous adipose tissue.

Fatty acid analysis of plasma fatty acids before and after the intervention shows that the high nutrient quality ER diet resulted in a significant reduction in total plasma fatty acids, SFA, MUFA, PUFA and omega-6-fatty acids and an increase in the estimated degree of unsaturation compared to the control, while no such change was found for DHA and omega-3-fatty acids. Further exploration reveals that the ratio of DHA-, PUFA, omega-6 fatty acids and omega-3 fatty acids each, relative to total fatty acids, only significantly increases within the high nutrient quality ER group. Based on this observation it can be reasoned that the high intake of 1224 (± 55) mg omega-3 fatty acids may have prevented a decrease in omega-3 fatty acids and DHA in the high nutrient quality ER diet along with energy restriction. Other reports using FAME analysis support our findings that plasma total fatty acids and SFA [47,48] as well as MUFA [48] decrease with calorie restriction [47]. Further explorative analysis of our data showed similar results within the low nutrient quality ER group. Interestingly, in contrast to its precursor DHA, DHEA plasma concentrations seemed to better reflect the intake levels of DHA in the different diet groups during energy restriction. It is known that dietary DHA is uniquely accreted in tissue- and cell phospholipid membranes, where it amongst others is a precursor for the endocannabinoid DHEA. It is thought that the production of N-docosahexaenoyl-phosphatidylethanolamine by the N-acylation-phosphodiesterase pathway is the first step in the synthesis of DHEA, which is then further hydrolyzed by predominantly NAPE-PLD to yield DHEA in the second step of the pathway. Therefore, it might be that the reduction in DHEA in the low nutrient quality ER diet is due to use of the body's DHEA or DHA cell phospholipid membrane reserves, while in the high nutrient quality ER diet, the additional provision of DHA may have prevented this. Several studies have shown that DHEA concentrations in tissues like adipose tissue, gut, liver and intestine are influenced by the dietary levels of DHA [11,15–17,41–43]. Fisk et al., indicated that participants with metabolically healthy obesity had less DHEA in their white adipose tissue compared to healthy normal weight participants, while both followed the same n-3 PUFA intervention [42]. Possibly higher levels of AA in the obese group might have led to substrate competition with DHA for the synthesizing enzymes of the corresponding ECs. In our study, calorie restriction might have even further blurred entanglement of underlying mechanisms. Altogether, our findings suggest that n-3 PUFA supplementation in the high nutrient quality ER diet may have prevented a decrease in fasted DHEA concentrations due to 25% ER which was observed in the low nutrient quality ER diet. However, we cannot rule out that other components such as MUFA, soy protein or fiber have been indirectly responsible for this effect.

It is known that the prototypical endocannabinoids AEA and 2-AG act upon CB_1 receptors in brain and peripheral tissues. The physiological role of the CB_1 receptor in food intake, satiety and energy homeostasis has been intensively studied [49]. Interestingly, DHEA and DHEA-derived metabolites were found to have considerably lower affinity for the CB_1 receptor compared to AEA [30,50,51]. Therefore, it may be speculated that a shift in the balance between n-3 PUFA derived-DHEA and n-6 PUFA derived-AEA may lead to a reduced EC tone [52,53]. While we found differences in the concentration of DHEA between diet groups, without concomitant changes in AEA and/or 2-AG, further exploration of our data revealed a significant increase in expression of the CB_1 receptor within the high nutrient quality ER diet. Previous studies in healthy obese subjects also showed that CB_1 expression in abdominal adipose tissue increased, after approximately 10% weight loss, while no changes were observed in AEA levels in the same tissue [54]. While both our ER diet groups had an average weight loss between 6.3% and 8.4% of their body weight, the weight loss per se might not have been large enough to induce a change in adipose tissue CB_1 expression in the low nutrient quality ER diet group. Our data indicate that combining a high nutrient quality diet with ER seems to have additional effects on CB_1 expression in adipose tissue. We suggest that the observed increase in adipose

tissue CB_1 expression reflects a return to basal ECS homeostatic conditions, as also others reported that improvements of metabolic conditions are accompanied with increases in CB_1 expression [22,54,55].

Interestingly, expression of adipose tissue *DAGLA*, coding for DAGL-α, which is responsible for 2-AG synthesis, was significantly increased following the low nutrient quality ER diet ($P<.009$) and differed upon intervention with both other diets. Knocking out *DAGLA* has been shown to generate the same favorable metabolic phenotype as murine KOs of the CB_1 receptor [56]. Besides, *DAGLA* has been reported to be upregulated in adipose tissue of obese human subjects but stayed unaltered after a 2 weeks intervention with a low fat diet [57]. DAGL-α and β are the enzymes responsible for the synthesis of 2-AG, which is derived from cell membrane arachidonic acid (AA) derivatives. The enzyme mono-acylglycerol lipase (MAGL) degrades 2-AG. It is reported that gene expression of *DAGLA* and *DAGLB* as well as *MGLL* is upregulated in the WAT of mice subjected to a high fat diet (HFD) [58]. *DAGLA* already showed highest upregulation after a few days of HFD intervention and matched with the upregulated expression pattern of gene *MGLL* coding for the 2-AG degrading enzyme MAGL. Still, plasma 2-AG levels slightly raised at this timepoint. We found after an intervention of 12 weeks an upregulated expression of *DAGLA* in adipose tissue. Because in our study, the amount of total dietary fat was the same between both ER diets, the type of fatty acids in the diet likely has been of influence on the expression of *DAGLA*. Energy restriction appears to have had no effect, as no changes on *DAGLA* were found in the high quality nutrient ER diet. Further exploration of our data showed a downregulation of *MGLL* within both ER diet groups, thus as a result of 25% ER and unrelated to the nutrient composition of the diet. Notably, although expression of *DAGLA* increased in the low nutrient quality ER diet, fasted levels of 2-AG in adipose tissue and plasma were not changed. A further increase in adipose tissue 2-AG would have been expected by the downregulation of *MGLL*. However, it is reported that MAGL activity is location dependent, being only responsible for 30% of 2-AG degradation in adipose tissue [59,60]. It seems plausible that over the time-course of the 12-weeks intervention *DAGLA* might have showed a different expression (possible higher) in the low nutrient quality ER group, thereby resulting in elevated levels of 2-AG. As we had only 2 time points and subjects had a better metabolic health at the end of the 12 weeks intervention, we might have missed more pronounced changes. Alternatively, *DAGLB* might also play a role in the formation of 2-AG in adipose tissue, which has been indicated to be of influence for elevated plasma 2-AG in HFD mice [58]. It should be stated that our participants had a BMI of around 31, which does not reflect extreme obesity and is perhaps associated with a relatively low degree of disruption of the ECS. Possible, different sensitivities of the techniques and methods used might have resulted in a mismatch on outcomes of *DAGLA* expression- and 2-AG levels in adipose tissue needed to assess subtle changes in the population studied here.

Although our findings showed that reduced plasma DHEA levels were accompanied by increased *DAGLA* gene expression in adipose tissue following a low nutrient quality ER diet for 12-weeks, no direct relationship between DHA-derived DHEA and the 2-AG synthesizing enzyme DAGL-α is known from the literature. However, it seems conceivable that both can influence each other indirectly *via* the enzyme COX-2. DHEA can specifically reduce the formation of AA-derived prostaglandins through COX-2 [27]. Furthermore, both DHEA [61] and 2-AG [62,63] act as a substrate for COX-2. It is assumed that these pathways serve to diminish the bioactivity of the parent compound. COX-2 is upregulated during inflammatory conditions and has been shown to also be functional in adipose tissue during conditions of low-grade chronic inflammation in obesity [64,65]. It is possible that a decrease in DHEA upon intervention with the low nutrient quality ER diet could have resulted in increased oxygenation of 2-AG by COX-2, due to a lack of substrate competition with DHEA. This in turn could have influenced gene expression of *DAGLA*, possibly involving feedback mechanisms. Homeostasis of endocannabinoid tone is the result of a delicate balance between the activity of synthesizing and degrading ECS enzymes and the presence of EC fatty acid precursors, *e.g.,* AA, DHA, in the diet. Understanding how energy restriction interferes with this balance is still in its infancy. Further studies are warranted to provide evidence of whether and how DHEA levels may influence those of 2-AG and it's synthesizing enzyme DAGL-α.

4.2. Levels of AEA and 2-AG stay unaltered with 25%ER

After a period of 12 weeks on 2 different diets providing 25 EN% less, participants from both groups showed a marked decrease in body weight, a reduction in abdominal circumference and an improvement in metabolic parameters compared to the group that maintained its habitual diet. Remarkably, these improvements were not associated with significant reductions in the fasted plasma and adipose tissue concentrations of AEA and 2-AG, the most studied, "prototypical" endocannabinoids. Although this finding is in line with some studies [6,66], other reports do show a reduction in 2-AG upon weight loss [54] or found different levels of AEA and 2-AG between lean and obese or subjects with T2D [6,67]. Differences in BMI, disease state (T2D), hormonal state and amount of weight loss might all have been off influence. Besides, dietary content of linoleic acid, the precursor for *n*-6 PUFAs are never reported.

4.3. Endocannabinoid-congeners PEA, SEA, OEA, NAGly, and DHAGly, respond differently upon 12 weeks intervention

We further explored the data in order to gain new insights into the potential effects of nutrient quality combined with ER on the other studied endocannabinoid-congeners. Although not significant between the dietary groups both ER diets reduced levels of the saturated fatty acid derivatives SEA (precursor stearic acid) and PEA (precursor palmitic acid) within the group. Interestingly, increased levels of saturated fatty acids in the low nutrient quality ER diet could not prevent the reduction of SEA and PEA plasma levels as observed in this diet group. Interestingly, most studies in animal models show that levels of the saturated fatty acid derivatives SEA and PEA are not sensitive to the fatty acid composition of the diet, in contrast to *n*-3 PUFA-derived compounds [15,68]. An often used explanation for this is that their fatty acid precursors are endogenously produced from carbohydrates and are not likely to become limiting. Our human study gives nice indication for similar findings. For the oleic acid-derived OEA effects were less clear as we just observed that OEA was borderline significantly decreased in plasma from the low nutrient quality ER group. The MUFA-derived OEA was found unchanged upon intervention in the high nutrient quality ER diet that was enriched in MUFA (originating from dietary olive oil). OEA is known to be involved in food regulation and satiety by acting locally in the gastrointestinal tract and gained interest due to its reported weight loss-inducing properties. OEA is synthesized in the proximal small intestine from oleic acid and phosphatidylethanolamine by N-acyltransferase (NAT) enzyme in the cellular membrane [32]. OEA activates PPARα, the transient receptor potential vanilloid receptor 1 (TRPV1), and GPR119. Despite its reported effects on weight [69], we did not find any correlation of OEA- plasma or -adipose tissue concentrations with body weight or SAT in the different diet groups. This correlation might however

been glossed up, by the effect of the 25% ER on plasma MUFA levels, which were found to be reduced in the high nutrient quality ER diet and likely have involved oleic acid the precursor of OEA. Alternatively, as the MUFA content in the high quality nutrient ER diet was 1.44 times (En%) higher than in the low nutrient quality ER diet, this difference might by itself not have led to any pronounced effects on weight loss. However, the combination of the higher protein (20.6 En% compared with 16.0 En%, respectively), fiber (3.1 En% compared with 2.2 En%, respectively), MUFA and linoleic acid intake might have had led to a more significant increase in weight loss in the high nutrient quality ER diet compared to the low nutrient quality ER diet as was reported in [36]. Interestingly, recently the NAE of linoleic acid, linoleoylethanolamide (LEA) has been shown to be capable to normalize the metabolic and inflammatory changes induced by a high fat diet in mice [70]. Linoleic acid content was higher in the high nutrient quality ER diet and adjusted to recommended intake levels.

In this study, we also investigated the effects of diets on two glycine conjugates of fatty acids, NAGly and DHAGly. Previous studies reported that NAGly might be an endogenous metabolite of AEA generated *via* both oxidative metabolism of AEA and (or) through the conjugation of glycine to arachidonic acid which is released during hydrolysis of AEA by FAAH [71]. We did not find significant variation in plasma NAGly between groups and treatments. NAGly was only found in human plasma but not in adipose tissue. Interestingly, for fasted DHAGly levels, a trend towards significance was found between the diets in adipose tissue only, which seemed to be caused by increased levels in the high nutrient quality ER diet group compared to a decrease in the low nutrient quality ER diet, however, these were not significant. As the physiological roles of both glycine conjugates have not been fully elucidated, it is difficult to draw a conclusion from the data here. In addition, we observed large individual variations of each compound for all three groups at baseline levels and their response to intervention in both plasma and adipose tissue.

We found much higher concentrations of endocannabinoids and their congeners in adipose tissue than in plasma. This is line with the general viewpoint that ECs are produced locally (*e.g.,* in adipose tissue) with an unknown proportion spilling over into the systemic circulation [15,66]. However the EC profile in plasma does not fully reflect the adipose tissue profile as SEA and particularly 2-AG and DHAGly are relatively much higher in adipose tissue, (2-AG and DHAGly >100 times), while DHEA was relatively similar in adipose tissue. Notably, explorative correlation analyzes of EC profiles in both plasma and adipose tissue of the participants before intervention revealed that only the levels of DHEA were highly correlated between plasma and adipose tissue (R=0.654, $P<.001$).

Next to the numerous physiological roles ascribed to the prototypical endocannabinoids 2-AG and AEA [72,73], the possible roles of their structural analogues, like OEA and PEA, have gained increasing attention [69,74]. Interestingly, this includes the ethanol amine DHEA derived from the long chain *n*-3 PUFA DHA [27,75]. DHEA has shown anti-inflammatory, antihyperalgesic and potential tumor-modulating effects in cell lines, animal models and human trials [18,27,76–78]. Its level is controlled by the enzymes FAAH and NAAA, which play important roles in the ECS [79]. In macrophages, DHEA acts on COX-2, a key enzyme involved in inflammation and pain [27], and displays more potent anti-inflammatory effects than AEA [79,80]. Also, similarly to AEA, DHEA is a ligand for the pain receptor TRPV1 [81]. Furthermore, GPR110 (ADGRF1) was proposed as a functional receptor mediating DHEA-induced neurite growth, synaptogenesis neurogenic differentiation, and immune modulation [82,83]. Together with the known relevance of DHA as essential fatty acid, these findings underline the potential role of *n*-3 PUFA derived N-acylethanolamides as endogenous messengers.

This study includes a number of strengths. Changes in EC profiles and expression of ECS-related genes were assessed in a well-controlled, parallel-designed, randomized dietary intervention study with a relatively high number of participants. The trial resulted in clinically relevant outcomes including weight loss and significant improvements in metabolic parameters such as TG, insulin, glucose, SAT, VAT and intra-hepatic lipids after 12 weeks of ER. In addition, a broad range of endocannabinoids was measured in both plasma and adipose tissue. A limitation of the study is that the included subjects with abdominal obesity were relatively healthy and might not have had largely disturbed EC profiles. In addition, the biological interpretation of our data remains complex given the subtle and predominantly local effects of endocannabinoids that according to the current insights are produced “on demand” in tissues.

In conclusion, here we show that dietary nutrient composition on top of ER influences EC levels and expression of their enzymes and receptors in subjects with obesity. Our data demonstrate that combining ER with a high-quality diet that includes among others 1.2 g *n*-3 PUFAs (per day) is able to prevent a decline in fasted plasma DHEA levels as was found in the low nutrient quality ER diet with very little *n*-3 PUFAs. Although no correlations with metabolic markers of health were found, given the positive effects of DHEA found in previous studies this might in the long run result in more beneficial effects than the low nutrient quality ER diet. Furthermore, while the prototypical endocannabinoids AEA and 2-AG were not significantly altered by intervention, adipose tissue expression of the 2-AG synthesizing enzyme DAGL-α was significantly increased due to the low nutrient quality ER diet. Our data highlight the relevance of diet quality for the ECS especially under conditions of energy restriction.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ya Wang: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Michiel G.J. Balvers:** Methodology, Validation, Writing – review & editing. **Diederik Esser:** Investigation, Formal analysis. **Sophie Schutte:** Visualization, Investigation, Formal analysis. **Jean-Paul Vincken:** Resources, Writing – review & editing. **Lydia A. Afman:** Conceptualization, Supervision, Project administration, Writing – review & editing. **Renger F. Witkamp:** Conceptualization, Writing – review & editing. **Jocelijn Meijerink:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Formal analysis, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2024.109605.

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