Journal of Physiology and Biochemistry
https://doi.org/10.1007/s13105-024-01063-6

RESEARCH

Higher plasma levels of endocannabinoids and analogues are correlated with a worse cardiometabolic profile in middle-aged adults

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Received: 11 September 2024 / Accepted: 14 November 2024

Abstract

The increase in age-related comorbidities, such as cardiometabolic diseases, has become a global health priority. There is a growing need to find new parameters capable of improving the detection of cardiometabolic risk factors, and circulating endocannabinoids (eCBs) are a promising tool in this context. Here, we aimed to investigate the relationship between plasma levels of eCBs and their analogues with body composition and cardiometabolic risk factors in middle-aged adults. Seventy-two individuals (54% women; 53.6 ± 5.1 years old) were included in this study. Plasma levels of eCBs and analogues were determined using liquid chromatography-tandem mass spectrometry. Body composition was measured by dual-energy X-ray absorptiometry. Cardiometabolic risk factors (i.e., glucose and lipid profile, blood pressure, liver and renal parameters, and gonadal hormones) were also assessed. The plasma levels of 1- and 2-arachidonylglycerol (1-AG&2-AG) were positively correlated with adiposity (all $r \geq 0.23$, $P < 0.05$). Interestingly, the plasma levels of 1-AG&2-AG, arachidonoylethanolamide, and palmitoyl-ethanolamide were positively correlated with the homeostatic model assessment index – Insulin Resistance (HOMA-IR) (all $r \geq 0.32$, $P < 0.01$). Our results also showed that high levels of 1-AG&2-AG, arachidonoylethanolamide, linoleoyl ethanolamide, and palmitoleoyl ethanolamide were correlated with poorer liver (all $r \geq 0.27$, $P < 0.05$), kidney (all $r \geq 0.24$, $P < 0.05$), and gonadal function parameters (testosterone: all $r > 0.26$, $P < 0.05$, SHBG: 1-AG&2-AG r=-0.33, $P < 0.01$). The plasma levels of some eCBs and analogues are correlated with a worse cardiometabolic profile in middle-aged adults.

Keywords Cardiometabolic risk factors · Endocannabinoid system · Insulin resistance · Fatty liver · Visceral obesity

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Published online: 05 December 2024

Introduction

As life expectancy increases, the health and social needs of an ageing population have multiplied due to age-related comorbidities, such as obesity and cardiometabolic diseases [1]. Within this framework, cardiometabolic risk factors, such as visceral adiposity, dyslipidemia, and hyperglycemia, represent a cornerstone in the knowledge and treatment of age-related comorbidities [2, 3]. Understanding the underlying mechanisms contributing to the development of cardiometabolic disorders is essential for implementing effective preventive and therapeutic strategies. In this context, there is growing interest in the role that the endocannabinoid system (ECS) may play in the regulation of cardiometabolic health [4, 5].

ECS is a widespread complex cell-signalling system present in central and peripheral tissues that regulates many physiological processes, including energy homeostasis, cognitive function, and immune response [6, 7]. It comprises endogenous lipid messengers named endocannabinoids (eCBs), G-protein-coupled receptors (GPCR), and enzymes responsible for the synthesis and degradation of the eCBs. The main eCBs, i.e., 2-arachidonylglycerol (2-AG) and arachidonoylethanolamide (AEA), act predominantly via the membrane cannabinoid receptors (CB) CB1 and CB2 [8, 9], but mainly CB2 receptors, which are more expressed in peripherical tissues such as adipose tissue, muscle, and pancreas [10, 11]. Meanwhile, eCBs analogues, e.g., palmitoylethanolamide (PEA) and oleoyl-ethanolamide (OEA), bind mainly to nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs) [12].

eCBs play a role in the pathogenesis of some cardiometabolic diseases (e.g., type 2 diabetes, or metabolic syndrome) [13, 14]. Indeed, elevated circulating levels of 2-AG and AEA [15, 16] are associated with insulin resistance and visceral adiposity in individuals with overweight and obesity [17–19]. Moreover, recent research suggests that the ECS fluctuates over the lifetime, affecting the balance of eCBs synthesis, CBs sensitivity, and eCBs degradation [20]. Given the potential role of eCBS in cardiometabolic health, targeting the ECS (e.g., by regulating the circulating levels of eCBs) may offer a viable therapeutic approach to cardiometabolic diseases. Therefore, the aim of this study was to investigate the relationship between plasma levels of eCBs and analogues with body composition and cardiometabolic risk factors in middle-aged adults.

Methods

Study design and participants

The present work is a cross-sectional study conducted as part of the FIT-AGEING study (ClinicalTrials.gov. ID: NCT03334357) [21], involving 72 participants (39 women and 33 men). Inclusion criteria included reporting to be sedentary (i.e., ≤ 20 min of moderate-intensity physical activity on ≤ 3 days/week over the last 3 months), and having a stable weight in the previous 6 months (change ≤ 5 kg). Exclusion criteria encompassed pregnant or lactating women, individuals taking medication, and those with a significant illness that could interfere with or be exacerbated by a training program. Participants were instructed to be well-rested, use public transportation or a car to come to the study ward, abstain from stimulants and/or alcohol on measurement days, and avoid moderate exercise in the 24 h or vigorous exercise in the 48 h preceding the measurements, respectively. The study was approved by the Ethics Committee on Human Research at the University of Granada and "Servicio Andaluz de Salud" (CEI-Granada) [0838-N-2017], and all participants provided informed consent. The study protocol and experimental design adhered to the latest revised ethical guidelines of the Declaration of Helsinki.

Blood collection and determination of plasma levels of endocannabinoids and analogues

Blood samples were collected between 8:00 and 9:00 A.M. following an overnight fast. The collection was performed from the antecubital vein in Vacutainer® Hemogard™ tubes containing K_2EDTA as an anticoagulant. Blood samples were immediately centrifuged (10,000 rpm, 10 min, 4ºC temperature) to obtain plasma. Subsequently, the samples were directly aliquoted and preserved at -80ºC until analysis.

The plasma levels of eCBs and analogues were determined using a validated liquid chromatography-tandem mass spectrometry method described elsewhere [22] (Table S1). Briefly, plasma samples were prepared by liquid-liquid extraction and analysed using a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan) connected to a SCIEX QTRAP 7500 + mass spectrometer (SCIEX, Framingham, MA, USA). In addition to the study samples, quality control samples were analysed to evaluate the data quality and correct for between-batch variations. The relative standard deviations of peak area ratios (i.e., the analyte's peak area divided by the respective internal standard's peak area) were calculated for each analyte in the quality control samples. Metabolites with RSDs ≤ 15% were included in further data analyses. Metabolites showing RSDs higher than 30% on

peak area ratios in QC samples were excluded. Metabolites with 15% ≤ RSDs < 30% were cautiously interpreted (Table S1). The isotopically-labeled internal standards used for the liquid chromatography-tandem mass spectrometry protocol are detailed in Table S2.

The liquid chromatography-tandem mass spectrometry method allowed for the relative quantitation of the eCBs 1-&2-arachidonoylglycerol (1-AG&2-AG) and AEA, as well as the analogues PEA, stearoyl ethanolamide (SEA), linoleoyl ethanolamide (LEA), docosahexaenoyl ethanolamide (DHEA), OEA, PDEA, dihomo-gamma-linolenoyl ethanolamide (DGLEA) and palmitoleoyl ethanolamide (POEA). Acyl glycerols occur naturally in two isomeric forms: 1-AG and 2-AG. The conversion of the 2-isomer to the 1-isomer during the analytical procedure is a known mechanism that cannot be controlled, especially for 2-AG. Therefore, due to this isomerisation, we report 1-AG and 2-AG as one entity and sum their peak areas before calculating the peak area ratio and refer to them collectively as 1-AG&2-AG.

Anthropometric and body composition measurements

Body weight was measured using a model 799 scale and height was measured using a stadiometer (both from Seca, Hamburg, Germany), without shoes and with light clothing. Body mass index (BMI) was calculated from weight and height (kg/m^2). Waist circumference (WC) was measured at the minimum perimeter at the end of a normal expiration, with the arms relaxed at both sides of the body. When the minimum perimeter could not be detected, measurements were taken just above the umbilicus, in a horizontal plane. Hip circumference was measured around the widest portion of the buttocks. WC and hip circumference were determined using a plastic tape measure as the average of two measurements. The waist-hip ratio (WHR) was calculated by dividing the waist and hip measurements, and the waist-height ratio (WHtR) was calculated by dividing the waist and height measurements.

Bone mineral content (BMC), bone mineral density (BMD), fat-free mass (FFM), lean mass, fat mass, and visceral adipose tissue (VAT) mass were measured by dual-energy X-ray absorptiometry using a Discovery Wi device (Hologic Inc., Bedford, MA, USA) equipped with analysis software (APEX version 4.0.2). VAT mass was estimated using an algorithm applied to the abdominal region, but the device does not directly distinguish between visceral and subcutaneous adipose tissue (SAT). Therefore, this measurement refers to estimated VAT throughout the study. Fat-free, lean, and fat mass indices (FFMI, LMI, and FMI, respectively) were expressed as kg/m^2; fat mass was also described as a percentage of body weight. The Sarcopenic Obesity Index (SOI) was calculated using the formula SOI = WC (cm) / SMI (kg/m²). SMI (skeletal muscle index) was determined as FFM (kg) / height² (m²), where FFM represents fat-free mass. Waist circumference (WC) was measured in centimetres. This index allows the assessment of sarcopenic obesity by relating central adiposity to skeletal muscle mass.

Determination of cardiometabolic risk factors and related biomarkers

Traditional cardiometabolic risk factors and hormones, i.e., glucose, insulin, total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides, glutamic pyruvic transaminase (GPT), gamma-glutamyl transferase (GGT), albumin, creatinine, creatine kinase (CK), somatostatin, cortisol, testosterone, free testosterone, sex hormone binding globulin (SHBG), dehydroepiandrosterone, were measured in plasma. Low-density lipoprotein cholesterol (LDL-C) was calculated from the Friedewald formula. Insulin resistance/sensitivity was estimated via the homeostatic model assessment index – Insulin Resistance (HOMA-IR) [23] and the quantitative insulin sensitivity check index (QUICKI), respectively [24]. The fatty liver index was calculated as a validated surrogate marker of non-alcoholic fatty liver disease [25].

Lastly, blood pressure was determined in the right arm after a 30-min rest in a supine position, using an Omrom® HEM 705 CP automatic monitor (OMROM Health-Care Co., Kyoto, Japan), following the recommendations of the European Heart Society [26]. Three measurements were taken 1 min apart, and the mean value was calculated.

Classification of individuals with metabolically healthy overweight-obesity and with metabolically unhealthy overweight-obesity

Participants were retrospectively classified as metabolically healthy overweight-obesity (MHOO) or metabolically unhealthy overweight-obesity (MUOO) following previously proposed criteria (Table S3) [27]. MUOO was defined as having a BMI ≥ 25 kg/m^2 and presenting at least one of the following risk factors: (a) fasting triglyceride levels ≥ 150 mg/dL; (b) fasting HDL-C levels < 40 mg/dL for men and < 50 mg/dL for women, respectively; (c) resting systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg, respectively; and/or (d) fasting glucose levels > 100 mg/dL. MHOO was defined as having a BMI ≥ 25 kg/m^2 and not presenting any of the abovementioned risk factors.

Statistical analyses

This study is based on a secondary analysis using baseline data from the FIT-AGEING project [21]; therefore, no specific power calculation was performed for this analysis.

The normal distribution assumption was tested using the Shapiro-Wilk test, visual histograms, and Q-Q plots. The plasma levels of eCBs and analogues were log_2-transformed, whereas cardiometabolic risk parameters were log_{10}-transformed before further analysis. Since no effect of sex was observed, data from men and women were analysed together.

The baseline characteristics and outcomes of the study participants were expressed as mean ± standard deviation (unless otherwise stated). We conducted Pearson partial correlation analyses to examine the relationship between plasma levels of eCBs and analogues and body composition and cardiometabolic risk parameters adjusting for sex and age. All correlation analyses and plots were designed using the 'corrplot' package in R software (V.3.6.0).

In separate models, forward stepwise regression was performed using HOMA-IR, GPT, and GGT as dependent outcomes. The eCBs and analogues correlated with HOMA-IR (1-AG&2-AG, AEA, and PEA), with GPT (1-AG&2-AG) and GGT (1-AG&2-AG, AEA, LEA, and POEA) together with body composition parameters (BMI, WHR, FFM, FFMI, lean mass, LMI, fat mass (%), FMI, VAT mass) were introduced as predictors using a forward stepwise procedure, which presents the predicting components step-by-step into the model (if $P < 0.05$) according to the strength of their correlation with the dependent outcome.

We performed one-way analyses of covariance (ANCOVA) adjusted for sex and age to assess differences in the plasma levels of eCBs and analogues between participants with MHOO or MUOO.

The forward stepwise regression analyses and the one-way ANCOVAs were performed using the Statistical Package for the Social Sciences (SPSS) v.25.0 (IBM Corporation, Chicago, IL, USA). The significance level was set at $P < 0.05$ for all the analyses.

Results

The characteristics of the 72 participants (54% women; 53.0 ± 5.0 years old, 46% men; 54.4 ± 5.1 years old) included in this study are presented in Table 1.

Plasma 1-AG&2-AG levels are positively correlated with adiposity in middle-aged adults

Plasma levels of 1-AG&2-AG were positively correlated with fat mass percentage, FMI and SOI (all r ≥ 0.23, $P < 0.05$; Fig. 1), whereas they were inversely correlated with FFM, FFMI, and lean mass (all r ≤ -0.23, $P < 0.05$; Fig. 1). Moreover, circulating levels of LEA were negatively correlated with FFMI (r=-0.27, $P < 0.05$; Fig. 1). However, the plasma levels of OEA solely were negatively correlated with BMD (r=-0.25, $P < 0.05$; Fig. 1).

Plasma levels of 1-AG&2-AG, AEA and PEA are positively correlated with insulin resistance in middle-aged adults

We found that the plasma levels of 1-AG&2-AG, AEA, and PEA were positively correlated with insulin and HOMA-IR (all r ≥ 0.3, $P < 0.01$; Fig. 2), whereas they were negatively correlated with QUICKI (all r ≤ -0.28, $P < 0.05$; Fig. 2). Among other hormones involved in pancreatic insulin secretion, the plasma levels of 1-AG&2-AG and POEA presented a negative correlation with somatostatin levels (all r ≤ -0.3, $P = 0.01$; Fig. 2).

Regarding lipid metabolism, our analysis showed that the plasma levels of 1-AG&2-AG were inversely correlated with LDL-C concentration (r=-0.25, $P < 0.05$; Fig. 2), while the plasma levels of PDEA were negatively correlated with TC and LDL-C concentrations (all r ≤ -0.23, $P < 0.05$; Fig. 2).

Based on the significant correlations observed, we performed forward stepwise regression models to investigate whether the plasma levels of 1-AG&2-AG, AEA, and/or PEA could improve the prediction of HOMA-IR compared to a baseline model or without these variables. These analyses showed that PEA levels improved the prediction of HOMA-IR in addition to fat mass percentage (+ 9.7%; $P < 0.001$; Table 2).

Plasma levels of endocannabinoids and analogues are correlated with poorer liver and kidney function parameters

Next, we found that the plasma levels of 1-AG&2-AG eCBs were positively correlated with fatty liver index, GPT, and GGT (all r ≥ 0.27, $P < 0.05$; Fig. 2). Furthermore, AEA, LEA, and POEA circulating levels were also positively correlated with GGT (all r ≥ 0.27, $P < 0.05$; Fig. 2). In this case, the forward stepwise regression models showed that 1-AG&2-AG improved the prediction of GPT in addition to FFM (+ 10.1%; $P < 0.001$; Table 2), and 1-AG&2-AG together with AEA improved the prediction of GGT in addition to

Table 1 Descriptive parameters of the study participants

	Total ($n=72$)	Men ($n=33$)	Women ($n=39$)
Age (years)	53.6±5.1	54.4±5.1	53.0±5.0
BMI (kg/m^2)	26.7±3.8	28.3±3.7	25.3±3.3**
Waist circumference (cm)	94.8±11.7	102.6±8.9	88.2±9.7**
WHR	0.9±0.1	1.0±0.1	0.9±0.1**
WHtR	0.6±0.1	0.6±0.1	0.5±0.1**
BMC (g)	2242.1±448.4	2621.4±303.4	1921.1±259.8**
BMD (g/cm^2)	1.1±0.1	1.2±0.1	1.0±0.1**
FFM (kg)	45.4±12.0	56.5±6.6	36.0±6.0**
FFMI (kg/m^2)	26.8±6.1	32.2±3.4	22.3±3.5**
Lean mass	43.1±11.6	53.8±6.3	34.15.8**
LMI (kg/m^2)	15.2±2.9	17.5±1.9	13.2±1.8**
Fat mass (%)	40.0±9.0	34.6±7.8	44.5±7.4**
FMI (kg/m^2)	10.8±3.1	10.0±3.2	11.4±2.9
VAT mass (g)	786.0±382.6	975.3±383.7	625.8±303.4**
SOI (cm/(kg/m²))	3.7±0.8	3.2±0.4	4.1±0.9**
Fatty liver index	49.5±26.3	66.6±20.0	34.8±21.9**
Glucose (mg/dL)	93.6±11.3	95.0±13.6	92.4±8.8
Insulin (µUI/mL)	8.1±5.6	8.7±6.7	7.6±4.6
HOMA-IR	1.9±1.8	2.2±2.4	1.6±1.1
QUICKI	0.4±0.0	0.4±0.0	0.4±0.0
Total cholesterol (mg/dL)	207.2±33.6	203.2±37.1	210.6±30.5
HDL-C (mg/dL)	59.5±12.6	56.2±12.4	62.3±12.2*
LDL-C (mg/dL)	126.4±29.5	127.5±32.3	125.5±27.2
SBP (mmHg)	127.1±15.3	132.5±15.1	122.7±14.1**
DBP (mmHg)	81.2±11.7	83.5±12.1	79.2±11.2
Triglycerides (mg/dL)	133.1±63.7	141.5±77.5	126.1±49.1
GPT (IU/L)	23.3±12.6	29.2±13.8	18.3±9.0**
GGT (IU/L)	34.4±23.4	41.1±23.7	28.7±21.9**
Albumin (g/dL)	4.40±0.24	4.4±0.2	4.4±0.3
Creatinine (mg/dL)	0.82±0.17	1.0±0.1	0.7±0.1**
Creatine kinase (IU/L)	121.4±56.5	125.6±55.0	118.0±58.8
Somatostatine (nm/L)	1.43±2.55	0.56±1.8	2.2±2.9**
Cortisol (µg/dL)	12.83±4.20	13.4±4.6	12.4±3.9
Testosterone (ng/dL)	181.8±183.9	346.6±128.0	42.4±76.7**
Free testosterone (ng/dL)	3.41±3.52	6.5±2.3	0.8±1.9**
SHBG (nmol/L)	47.2±23.9	35.7±16.6	56.8±25.0**
Dehydroepiandrosterone (µg/dL)	110.2±67.3	136.8±66.8	87.7±59.9**

Data are presented as mean and standard deviation (SD), otherwise stated. Asterisks (*) indicate statistically significant differences between sexes for the respective variables (*$p<0.05$; **$p<0.001$). Statistical differences between groups were assessed using a t-test. *Abbreviations*: BMC, bone mineral content; BMD, Bone mineral density; BMI, body mass index; CDV risk score IDF, Cardiometabolic risk score of the International Diabetes Federation; CK, creatine kinase; DBP, diastolic blood pressure; FFM, fat-free mass; FFMI, fat-free mass index; FMI, fat mass index; GGT, gamma-glutamyl transferase; GPT, glutamic pyruvic transaminase; HDL-C, High-density lipoprotein-cholesterol; HOMA, homeostatic model assessment index – Insulin Resistance; LDL-C, Low-density lipoprotein-cholesterol; LMI, lean mass index; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; SHBG, sex hormone binding globulin; SOI, sarcopenic obesity index; VAT, visceral adipose tissue; WC, waist circumference; WHR, waist-hip ratio; WHtR, waist-height ratio

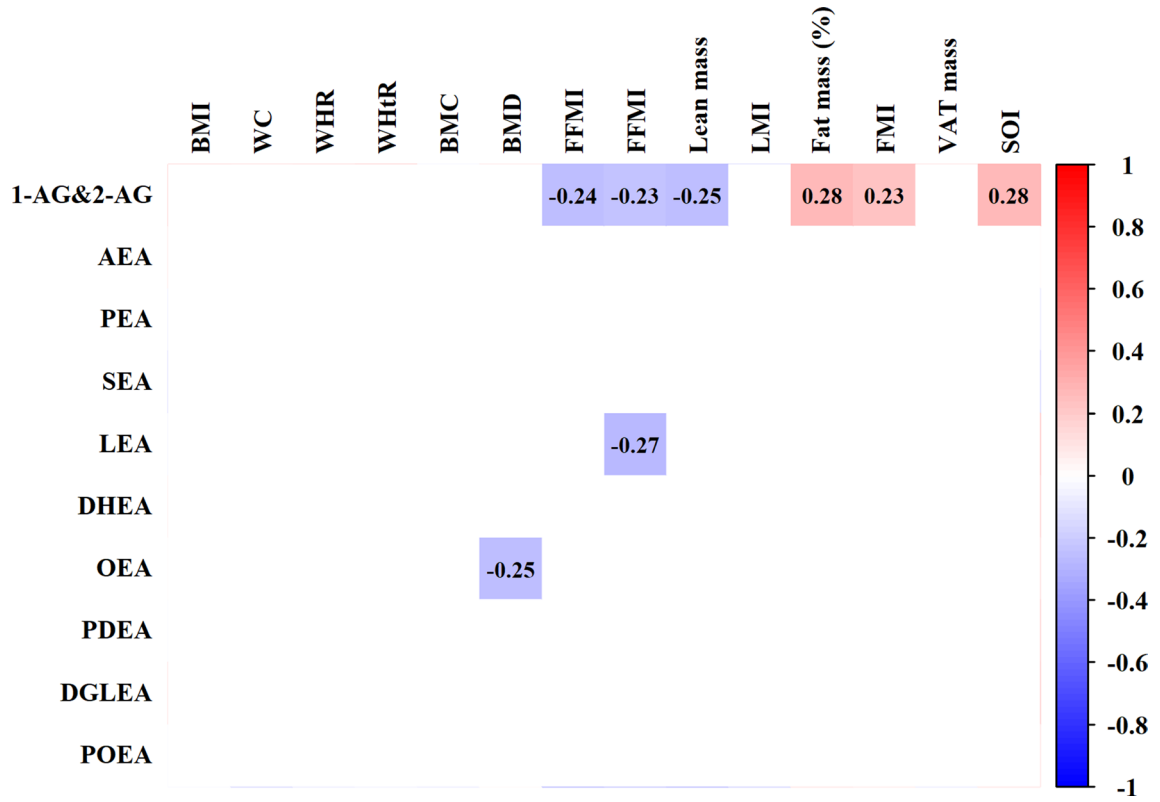

Fig. 1 Pearson correlation analyses between plasma levels of endocannabinoids and analogues with body composition parameters in middle-aged adults adjusted for sex and age. Every box represents a significant correlation coefficient (all $p < 0.05$), whereas empty spaces represent no significant correlations. Red and blue boxes indicate positive and negative correlations, respectively. All endocannabinoids and analogues were log2-transformed before data analysis. *Abbreviations*: 1-AG&2-AG, 1-&2-arachidonoylglycerol; AEA, anandamide; BMC, bone mineral content; BMD, Bone mineral density; BMI, body mass index; DGLEA, dihomo-gamma-linolenoyl ethanolamide; DHEA, docosahexaenoyl ethanolamide; FFM, fat-free mass; FFMI, fat-free mass index; FMI, fat mass index; LEA, linoleoyl ethanolamide; LMI, lean mass index; OEA, oleoyl-ethanolamide; PDEA, pentadecanoyl ethanolamine; PEA, palmitoyl ethanolamide; POEA, palmitoleoyl ethanolamide; SEA, stearoyl ethanolamide; SOI, sarcopenic obesity index; VAT, visceral adipose tissue; WC, waist circumference; WHR, waist-hip ratio; WHtR, waist-height ratio

FFMI (6.8%; $P < 0.001$; Table 2). We also observed that the 1-AG&2-AG, LEA, and DHEA plasma levels were positively correlated with albumin concentration (all r≥0.25, $P < 0.05$; Fig. 2).

Regarding renal function parameters, the levels of LEA and POEA had a positive correlation with CK (all r≥0.24, $P < 0.05$; Fig. 2), while AEA levels had a moderate positive correlation with CK ($r = 0.44$, $P < 0.001$; Fig. 2).

Plasma levels of endocannabinoids and analogues are correlated with altered levels of gonadal steroids

Generally, gonadal hormones levels tend to decline with age and cardiovascular disease [28]. Our results showed that the plasma levels of PEA were positively correlated with testosterone and free testosterone concentrations (all r≥0.26, $P < 0.05$; Fig. 2). However, the plasma levels of 1-AG&2-AG were negatively correlated with SHBG levels (r=-0.33, $P < 0.01$; Fig. 2), while the plasma levels of SEA were positively correlated with SHBG levels ($r = 0.34$, $P < 0.01$; Fig. 2). Furthermore, OEA and DGLEA plasma levels showed an inverse correlation with dehydroepiandrosterone concentration (all r≤-0.29, $P < 0.05$; Fig. 2).

Participants with metabolically healthy overweight or obesity present similar plasma levels of endocannabinoids and analogues compared with their metabolically unhealthy counterparts

Based on the higher cardiometabolic risk of individuals with MUOO than those with MHOO, we divided our cohort into individuals who were MHOO ($n = 14$) or MUOO ($n = 35$). We investigated whether differences in plasma levels of eCBs were present between MHOO and MUOO but observed no differences among plasma levels of eCBs and analogues between both groups (all $P \geq 0.1$; Table S4). Furthermore, we investigated whether differences in plasma

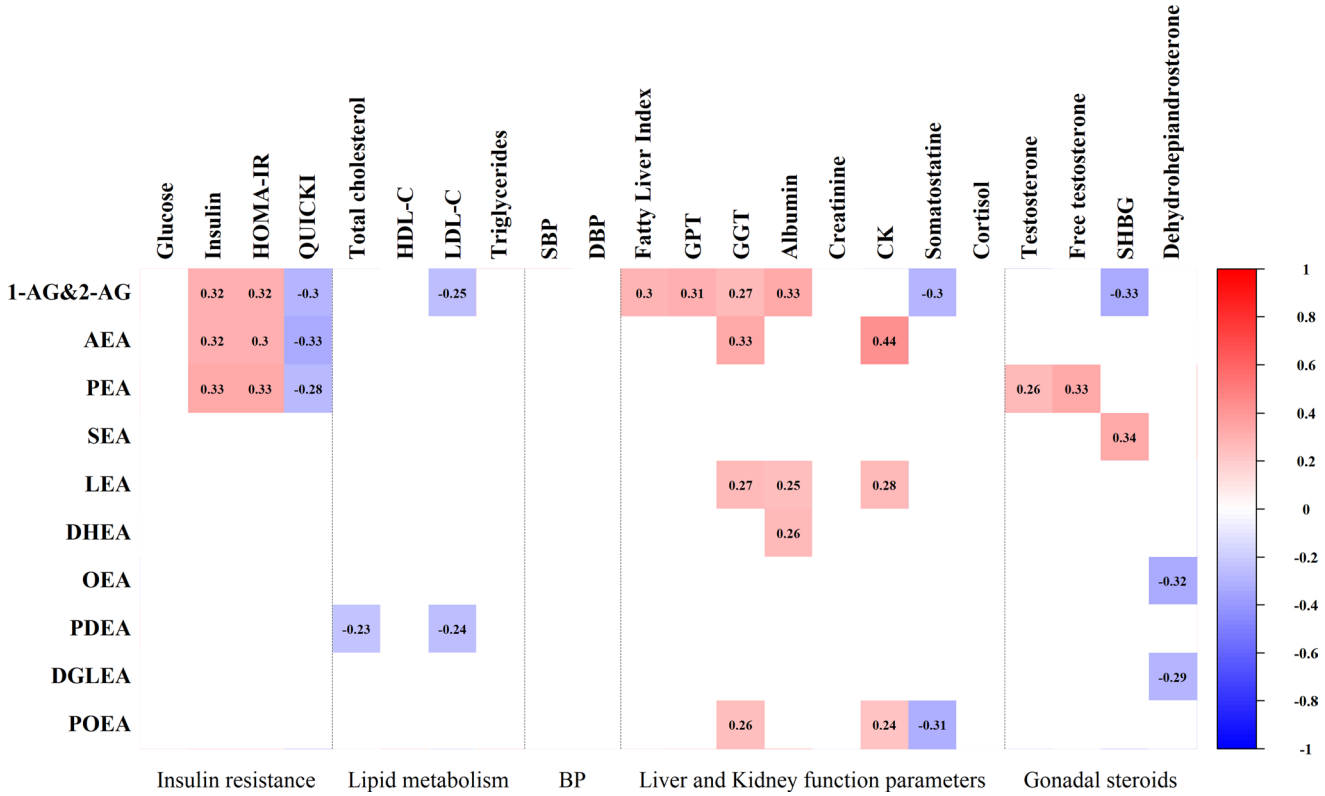

Fig. 2 Pearson correlation analyses between plasma levels of endocannabinoids and analogues with cardiometabolic risk factors in middle-aged adults adjusted for sex and age. Every box represents a significant correlation coefficient (all $p < 0.05$), whereas empty spaces represent no significant correlations. Red and blue boxes indicate positive and negative correlations, respectively. All endocannabinoids and analogues were log2-transformed before data analysis and cardiometabolic risk factors were log10-transformed. Values are displayed with up to two decimal places. For values where the second decimal is zero (e.g. 0.30), only one decimal is shown. *Abbreviations*: 1-AG&2-AG, 1-&2-arachidonoylglycerol; AEA, anandamide; BP, blood pressure; CK, creatine kinase; DBP, diastolic blood pressure; DGLEA, dihomo-gamma-linolenoyl ethanolamide; DHEA, docosahexaenoyl ethanolamide; GGT, gamma-glutamyl transferase; GPT, glutamic pyruvic transaminase; HDL-C, High-density lipoprotein-cholesterol; HOMA-IR, homeostatic model assessment index – Insulin Resistance; LDL-C, Low-density lipoprotein-cholesterol; LEA, linoleoyl ethanolamide; OEA, oleoyl-ethanolamide; PDEA, pentadecanoyl ethanolamine; PEA, palmitoyl ethanolamide; POEA, palmitoleoyl ethanolamide; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; SEA, stearoyl ethanolamide; SHBG, sex hormone binding globulin

levels of eCBs were present between normal-weight, overweight, and individuals with obesity. However, no statistically significant differences were observed among the groups in the levels of eCBs and their analogues (all $P \geq 0.1$; Table S5).

Discussion

In this study, we found that some eCBs and analogues plasma levels are correlated with higher adiposity and insulin resistance markers and worse liver and kidney function parameters in middle-aged adults. Our results may suggest that eCBs (i.e., 1AG&2AG and AEA) and some analogues (i.e., PEA, LEA, and POEA) could be used as potential markers of cardiometabolic risk in middle-aged adults. However, it is essential to note that most of the eCBs and analogues analysed in our cohort do not correlate significantly with cardiometabolic risk parameters, and among those that do correlate, plasma levels of 1-AG&2-AG have the most substantial potential as a predictor of cardiometabolic risk.

Adiposity is associated with a higher cumulative burden of morbidity and a significant increase in all-cause mortality [29, 30]. Concretely, visceral obesity is considered a major cardiometabolic risk factor [31]. Interestingly, we revealed that in middle-aged adults, plasma levels of 1-AG&2-AG were positively correlated with adiposity, which concurs with our previous results showing a positive correlation between plasma levels of 2-AG and adiposity in young adults [32]. However, plasma levels of 1-AG&2-AG were not positively correlated with VAT, which was unexpected [32]. Other studies have observed higher 2-AG plasma levels in individuals with obesity compared to lean counterparts. In those with obesity, the circulating 2-AG concentration was positively correlated with BMI, fat mass, and VAT mass [18, 19, 33–36]. Interestingly, interventions that

Table 2 Forward stepwise regression models assessing the independent correlations of the predictors (selected eCBs and body composition parameters), with HOMA-IR, GPT, and GGT as dependent outcomes in separate models in middle-aged adults

	β	B	95% CI	P	R^2	R^2 change	F Change	P (model)
HOMA-IR								
Step 1					0.119	-	9.476	**0.003**
PEA	0.345	0.444	0.156, 0.732	**0.003**				
Step 2					0.219	0.122	11.052	**<0.001**
PEA	0.417	0.537	0.262, 0.812	**<0.001**				
Fat mass (%)	0.356	0.011	0.004, 0.018	**0.001**				
GPT								
Step 1					0.180	-	15.317	**<0.001**
FFM	0.424	0.000	0.000, 0.000	**<0.001**				
Step 2					0.280	0.101	9.675	**<0.001**
FFM	0.406	0.000	0.000, 0.000	**<0.001**				
1-AG&2-AG	0.318	0.068	0.024, 0.112	**0.003**				
GGT								
Step 1					0.123	-	9.834	**0.003**
FFMI	0.351	0.015	0.005, 0.024	**0.003**				
Step 2					0.214	0.091	8.009	**<0.001**
FFMI	0.334	0.014	0.005, 0.023	**0.003**				
1-AG&2-AG	0.302	0.076	0.022, 0.129	**0.006**				
Step 3					0.282	0.068	6.428	**<0.001**
FFMI	0.344	0.014	0.006, 0.023	**0.001**				
1-AG&2-AG	0.270	0.068	0.016, 0.120	**0.011**				
AEA	0.263	0.162	0.034, 0.289	**0.014**				

Selected endocannabinoids and analogues for HOMA-IR model: 1-AG&2-AG, AEA, PEA. Selected endocannabinoids and analogues for GPT and GGT models: 1-AG&2-AG, AEA, LEA, and POEA. Selected body composition parameters: BMI, WHR, FFM, FFMI, lean mass, LMI, fat mass (%), FMI, VAT mass. β, standardised regression coefficient; B: unstandardised regression coefficient; CI, confidence interval; R^2, adjusted coefficient of determination, expressing the percent variability of the dependent variable explained by each model; R^2 change, additional percent variability explained by the model due to the inclusion of the new outcome. Abbreviations: FFM, fat free mass; FFMI, fat free mass index

aimed to improve body composition, i.e., bariatric surgery, physical activity, and healthy eating, reported a decrease in 2-AG levels associated with reduced fat mass and VAT [37, 38]. Indeed, a higher adherence to a Western dietary pattern leads to significantly higher levels of 2-AG and AEA compared to a healthy dietary pattern (such as Mediterranean Diet) [39]. The effect of eCBs, such as 2-AG and AEA, on adrenergic stimulation of adipose tissue can be explained by their interaction with CB1 receptors. Activation of CB1 receptors in adipocytes inhibits the β-adrenergic pathway, which usually stimulates lipolysis through the action of catecholamines such as norepinephrine. By suppressing lipolysis, eCBs reduce fat breakdown and promote fat storage instead. This results in an autocrine/paracrine negative feedback loop in which eCBs counteract the fat-reducing effects of adrenergic stimulation, thereby favouring fat accumulation and contributing to the development of adiposity [40]. These results suggest a potential link between elevated plasma 2-AG levels and adiposity in middle-aged adults.

Beyond increased adiposity, other detrimental body composition changes, such as lean mass and BMD reductions, raise the risk of cardiometabolic disease [20]. Contrary to the current findings in middle-aged adults, we previously found a positive correlation between 2-AG plasma levels and lean mass in young adults [32]. This result could be due to lean mass decrements during ageing [20]. However, other authors have suggested that 2-AG may be an endogenous repressor of myoblast differentiation via CB1-mediated inhibition of the $K_v7.4$ channel (pro-myogenic effects) [41], a process that would support our correlations with middle-aged adults. We also observed that 1-AG&2-AG plasma levels were positively correlated with SOI. Similarly, in a murine study, sarcopenia was associated with a 32% increase in 2-AG levels, although this increase did not reach statistical significance ($P=0.07$) [42]. Interestingly, we observed that plasma levels of OEA were negatively correlated with BMD in middle-aged adults. BMD is a widely used measurement for assessing bone health and is well known to decrease with age [43]. Moreover, circulating OEA concentrations also showed a negative correlation with dehydroepiandrosterone levels. BMD levels follow the same pattern across age as serum dehydroepiandrosterone levels. Indeed, when BMD levels are relatively high, dehydroepiandrosterone also peaks, and when BMD levels are relatively low, dehydroepiandrosterone levels are also low [44–46]. However, contrary to our results, a murine study observed that OEA was correlated with bone health and locomotor activity in rats [42]. The observed differences

among these studies could be attributed to interspecies variations or other confounding factors such as age and metabolic status. Further studies are needed to confirm or refute these findings and better understand the relationship between OEA and human bone health.

Along with changes in body composition, hyperglycemia is considered a crucial and well-recognized cardiometabolic risk factor [47]. We observed that plasma levels of 1-AG&2-AG, AEA, and PEA were positively correlated with insulin resistance in our cohort of middle-aged adults. Similar to our results, AEA levels have been associated with glucose intolerance [48], and circulating 2-AG has been proposed as a biomarker of insulin resistance and dyslipidemia independent of BMI [49–51]. In this sense, our findings show that plasma levels of PEA improved the prediction of HOMA-IR and fat mass percentage. In contrast, studies have suggested that PEA positively modulates inflammation, hepatic function, and glucose homeostasis [52, 53]. This controversy may be explained by the pleiotropic effects of eCBs depending on the tissue involved and its microenvironment [54]. The observed results might be mediated by disrupting the interaction of eCBs with CBs [55]. Indeed, the subcutaneous adipose tissue CB1 gene expression is increased in people with type 2 diabetes and insulin resistance. At the same time, gene expression levels of enzymes that break down eCBs (such as FAAH) are reduced, leading to high eCB tone and decreased glucose uptake by adipocytes [56]. Several mechanisms have been proposed to explain how eCBs and their analogues may influence insulin resistance. For example, activation of CB1 receptors in adipose tissue has been shown to disrupt insulin signalling pathways such as the Akt pathway, reducing glucose uptake by adipocytes and exacerbating insulin resistance [55]. These mechanisms may partially explain the correlations observed in our study, suggesting that fluctuation of ECS tone in obesity could lead to glucose homeostasis dysregulation in middle-aged adults.

Our data also showed that the plasma levels of 1-AG&2-AG were positively correlated with poor liver function parameters. In this sense, 2-AG and AEA have been implicated in the pathogenesis of non-alcoholic fatty liver disease in both animal models and humans [57–59]. Excessive activation of cannabinoid receptors, particularly CB1, partially contributes to the development and progression of fatty liver by promoting lipogenesis, fatty acid synthesis, and lipid accumulation in the liver [60]. In line with this, eCBs levels are higher in patients with non-alcoholic fatty liver disease than in healthy controls, these levels being positively correlated with the disease severity [61, 62]. Similarly, in patients with chronic hepatitis, the plasma levels of 2-AG and AEA were approximately two-fold higher than in healthy controls [63]. Furthermore, high levels of 2-AG can affect SHBG production by the liver, affecting hormone balance and contributing to metabolic dysfunction [64]. Overall, these results may suggest that high levels of 1-AG&2-AG might be involved in liver dysfunction. We also observed that 1-AG&2-AG levels were inversely correlated with SHBG. However, this correlation was not sustained in association analysis (regression analysis), suggesting that adiposity may play a key role in this correlation.

Finally, we found significant correlations between the plasma levels of 1-AG&2-AG, AEA, LEA, and POEA with renal function parameters. Specifically, we observed positive correlations of AEA, LEA, and POEA levels with CK. In individuals with obesity, elevated AEA levels have been associated with impaired kidney function [65]. Aligned with this, in murine studies, 2-AG and AEA levels have been related to renal dysfunction [66, 67]. Therefore, it seems plausible that eCBs regulation directly affects renal function or reflects a broader physiological state involving renal function and eCB activity [68]. In addition, the moderate correlation between AEA and CK levels may suggest a potential implication for muscle health since elevated CK levels are considered a predictor of poor muscle health, indicating muscle damage, breakdown, or disease [69]. These results could contribute to a better understanding of the relationship between circulating eCBs and renal and muscle function in middle-aged adults.

This is the first study to investigate differences in eCBs between middle-aged adults with MUOO and MHOO. Previously, we described significantly higher levels of plasma eCBs in young adults with MUOO than in those with MHOO [32]. However, these differences disappeared when VAT was included as a confounder in the analysis, suggesting that MUOO young adults might have elevated circulating eCBs levels due to VAT dysfunction. In contrast, we observed no differences in plasma levels of eCBs and analogues in the present study between MUOO middle-aged adults and their metabolically healthy counterparts. The apparent metabolic and hormonal regulation differences between young and middle-aged adults may explain this finding. During ageing, the balance of eCBs may be adjusted to adapt to changing metabolic needs, which may contribute to the absence of differences in plasma levels of eCBs and analogues in middle-aged adults. Overall, further studies are needed to establish a relationship between circulating levels of eCBs and their analogues and metabolically healthy and unhealthy obesity.

Limitations

Our study has some limitations, including its observational nature, which precludes establishing causal relationships, and the sample size, which may affect the generalisability of our findings. Furthermore, only 14 individuals with MHOO

were evaluated against 35 individuals with MUOO. Therefore, it remains uncertain whether activation of the ECS leads to an unfavourable cardiometabolic state or whether cardiometabolic issues such as obesity, insulin resistance, and inflammation trigger the ECS. Additionally, the menopausal status of female participants was not considered, which could potentially influence the levels of gonadal hormones and, consequently, the observed associations with cardiometabolic outcomes. Further research is needed to determine this relationship's direction and understand the underlying mechanisms. A limitation beyond the scope of this study is the complexity of current analytical methods for measuring eCBs, such as LC-MS, which requires specialised equipment and technical expertise. Further research is needed to support the development of more cost-effective and accessible techniques that could facilitate routine clinical implementation.

Conclusion

Altogether, our study posits further evidence of the relationship between plasma levels of certain eCBs (mainly 1-AG and 2-AG) and cardiometabolic risk factors. Although significant correlations were observed between certain eCBs (mainly 1-AG and 2-AG) and markers such as HOMA-IR, GPT, and GGT, the lack of consistent correlations with other eCBs, analogs, and additional cardiometabolic markers suggests that multiple underlying mechanisms may be involved in these interactions. These findings highlight the complexity of the ECS and the need for further research to understand its role in cardiometabolic disease. Therefore, the potential role of eCBs in reflecting cardiometabolic risk should be interpreted with caution until more extensive studies are conducted to validate these correlations.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13105-024-01063-6.

Acknowledgements The authors thank this study's participants for their time and effort.

Author contributions LJF, FJOP, BMT, MJC, and FJAG conceived and designed the study; LJF, IK, FJOP, and FJAG acquired data; LJF performed the data analysis; CRG and LJF drafted, and all the authors revised the manuscript; all authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Funding This study was supported by the Spanish Ministry of Education (FPU18/03357, and PID2022-141442OA-I00), the Redes Temáticas de Investigación Cooperativa RETIC (Red SAMID RD16/0022), the University of Granada Plan Propio de Investigación 2016 -Excellence actions: Unit of Excellence on Exercise and Health (UCEES), the Junta de Andalucía, Consejería de Conocimiento, Investigación y Universidades (ERDF; ref. SOMM17/6107/UGR and DOC 01151). CRG was supported by , Texas A&M AgriLife. LJF was funded by the University of Granada Plan Propio de Investigacion (Programa Perfeccionamiento Doctores). F.J.O-P was supported by a Sara Borrell contract (CD23/00231) from the MCIN/ ISCIII.This project was partially funded by MCIN/AEI/ 10.13039/501100011033 and, as appropriate, by "ESF Investing in your future", by EASO-New Clinical Investigator Award 2024 and by the EFSD-Rising Star 2024. BMT was funded by RYC2022-036473-I.

Data availability Data is provided within the manuscript or supplementary information files.

Declarations

Competing interests The authors declare no competing interests.

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