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Combatting metabolic disease : ethnic aspects, mechanisms and novel treatment strategies

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CHAPTER 3

Gene expression of endocannabinoid system components in skeletal muscle and adipose tissue of South Asians and white Caucasians with overweight

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ABSTRACT

The study aimed to investigate whether markers of endocannabinoid signaling differed between overweight men of South Asian and white Caucasian descent. We included overweight, prediabetic South Asian (n=10) and white Caucasian (n=10) men aged 35-50 years. Plasma samples were analyzed for endocannabinoids, their congeners and lipids. In white adipose tissue (WAT) and skeletal muscle biopsies, mRNA expression of genes involved in the endocannabinoid system (ECS) was assessed using qRT-PCR. Fasting lipid oxidation and glucose oxidation were determined with indirect calorimetry. Compared to white Caucasians, South Asians had higher levels of plasma 2-linoleoyl glycerol ($p<0.01$) and *N*-linoleoylethanolamine ($p<0.05$). Interestingly, in skeletal muscle of South Asians, expression of cannabinoid receptors CB_1 and CB_2 was 10-fold lower ($p<0.001$) and that of the endocannabinoid degradation enzyme FAAH2 was 5-fold lower ($p<0.001$) compared to white Caucasians. Expression of genes involved in the ECS in WAT were not different between the two ethnicities. After pooling of both ethnicities, plasma 2-AG positively correlated with plasma triglycerides ($R=0.77$, $p<0.001$) and lipid oxidation ($R=0.55$, $p<0.05$). Overweight South Asian men have higher plasma 2-linoleoyl glycerol and *N*-linoleoylethanolamine levels and lower expression of CB receptors and the endocannabinoid degradation enzyme FAAH2 in skeletal muscle compared to white Caucasians.

INTRODUCTION

The prevalence of obesity and related diseases, such as type 2 diabetes (T2D), is rapidly increasing worldwide. Particularly in South Asians, constituting nearly one-fourth of the world's population (1), an unfavorable metabolic profile consisting of obesity, dyslipidaemia and T2D, is highly prevalent (2). Moreover, South Asians have a higher risk of developing cardiovascular disease, resulting in high morbidity and mortality (3). It is generally thought that the increased susceptibility to metabolic disease of South Asians might be due to a disturbed energy metabolism (4).

The endocannabinoid system (ECS) is involved in maintaining energy balance by impacting on energy intake and expenditure as well as lipolysis (5). The ECS is present both centrally and in peripheral metabolic tissues, including the liver, pancreas, skeletal muscle, white adipose tissue (WAT) and brown adipose tissue (6). In skeletal muscle, endocannabinoids reduce glucose uptake and oxidative pathways (7, 8), thereby reducing energy expenditure. In WAT, endocannabinoids stimulate energy storage by increasing lipogenesis and adipogenesis (5, 9, 10). Thus endocannabinoid stimulation of skeletal muscle and WAT collectively results in a positive energy balance.

The ECS consists of G-protein-coupled cannabinoid receptors, i.e. type 1 and type 2 (CB_1 and CB_2), the endogenous lipid ligands (endocannabinoids) and the enzymatic machinery for their synthesis and degradation. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the most studied endocannabinoids and are synthesized on demand from their membrane lipids through the action of the biosynthetic enzymes N-acyl-phosphatidyl-ethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipases (DAGL), respectively. Endocannabinoids are degraded by specific enzymes. Fatty acid amide hydrolase (FAAH1 and FAAH2) inactivates AEA and monoglycerol lipase (MAGL) hydrolyzes 2-AG. Arachidonic acid is a precursor molecule and metabolite for endocannabinoids. AEA belongs to a family of bioactive *N*-acylethanolamines, which include *N*-linoleoylethanolamine, *N*-palmitoylethanolamine, *N*-oleoylethanolamine, *N*-stearoylethanolamine and *N*-docosatetraenylethanolamide. These N-acylethanolamines arise from the same biosynthetic pathway as AEA and are capable to indirectly modulate cannabinoid receptor activity by interfering with endocannabinoid metabolism, known as an "entourage effect" (11). Palmitoylethanolamine and, especially, *N*-oleoylethanolamine interact with nuclear receptor peroxisome proliferator-activated receptor α, stimulate lipolysis in the liver and adipocytes and act as a satiety factor (9, 12).

We previously showed that young healthy lean South Asian men have lower resting energy expenditure (13) and higher circulating endocannabinoid levels compared to matched white Caucasians (14). High plasma endocannabinoid levels are associated with obesity (15) and T2D (6) and affect peripheral metabolic organs, including adipose tissue and skeletal muscle (5). In addition, obese individuals have reduced expression

of endocannabinoid degradation enzymes and CB_1 receptor in adipose tissue (16, 17). Elevated circulating endocannabinoid levels might be a result of decreased enzymatic degradation (16). As little is known about ECS in overweight, prediabetic South Asian compared with white Caucasian men, we investigated circulating endocannabinoid levels and gene expression of CB receptors and enzymes involved in endocannabinoid synthesis and degradation in WAT and skeletal muscle of middle-aged men of South Asian and white Caucasian descent. We hypothesized that overweight, prediabetic South Asian men would have elevated plasma endocannabinoid levels and consequently lower expression of CB receptors on peripheral tissues compared to overweight, prediabetic Caucasian men.

MATERIALS AND METHODS

Ethics

Blood and tissue samples were collected as part of a previously conducted cross-over study to investigate the effect of L-arginine on brown adipose tissue activity and resting energy expenditure in overweight, prediabetic South Asian and white Caucasian men (18). This study was approved by the Ethics Committee of Maastricht University Medical Center (MUMC) and the Leiden University Medical Center (LUMC) and undertaken in accordance with the principles of the revised Declaration of Helsinki. All volunteers provided written informed consent. Trial registration number and date: NCT02291458, 14 November 2014.

Participants and study design

Twenty Dutch overweight (BMI 25-35 kg/m^2) prediabetic men of South Asian (n=10) and white Caucasian (n=10) descent (aged 35-55 years) were enrolled. South Asians and white Caucasians were matched for BMI. This study was conducted at the LUMC (The Netherlands) between November 2014 and August 2015 (18). Subjects underwent medical screening including an oral glucose tolerance test to identify individuals with impaired glucose tolerance according to the American Diabetes Association 2010 (19) and/or WHO criteria. In line with the ADA criteria (19), prediabetes was defined as having either fasting plasma glucose levels between 5.6 and 6.9 mmol/L or plasma glucose levels 2 h after an oral glucose tolerance test between 7.8 and 11.1 mmol/L. Exclusion criteria included the presence of chronic disease including T2D, smoking and recent body weight change up to 3 months prior to the start of the study.

Study procedures

The study procedures were described previously (18). In short, subjects were treated for 6 weeks with either L-arginine (9 g/day) (Argimax, Hankintatukku Oy, Karkkila, Finland) or placebo supplements (containing 70% microcrystalline cellulose, 29% maize starch and 1% magnesium stearate) in a randomized double-blind cross-over design, with a 4-week washout period. Each treatment period was followed by two consecutive study days, which consisted of several measurements. During the first study day lipid oxidation and glucose oxidation were determined for 30 minutes with indirect calorimetry using a face-mask (EZcal, IDEE, Maastricht, the Netherlands) and subsequently fasted venous blood samples were collected to quantify plasma triglycerides (using commercially available enzymatic colorimetric kits and an ABX Pentra 400 autoanalyzer) and endocannabinoids (liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)) as described previously (14). The next day, a fasted WAT (abdominal subcutaneous adipose tissue) and skeletal muscle (vastus lateralis) biopsy were collected as described previously (20). The tissues were instantly placed in ice-cold preservation medium (BIOPS, OROBOROS Instruments, Innsbruck, Austria) and stored at -80°C. Thereafter, body composition was determined by means of dual x-ray absorptiometry (DEXA; Discovery A, Hologic, Bedford, MA, USA). From one white Caucasian no DEXA scan data was available due to technical problems. For the purpose of this study only the fasted blood samples and WAT and skeletal muscle biopsies taken after placebo treatment were analyzed. All samples were taken at the same time of the day in all volunteers. Blood samples were obtained during the first study day at 11 A.M. after a 4-hour fast. WAT and skeletal muscle biopsies were obtained during the second study day at 8:00 A.M. after an overnight fast.

Endocannabinoid measurements

Plasma endocannabinoids and their congeners were quantified with LC-MS/MS. From the pool of individual study samples, quality controls were used to generate calibration curves. Additionally, all samples were randomized and each batch included calibration samples and an even distribution of quality control samples and blanks. The sample extraction procedure and method is as described in detail previously (14).

WAT and skeletal muscle gene expression

WAT and skeletal muscle biopsies of South Asians and white Caucasians after placebo treatment were analyzed. RNA was extracted using Tripure RNA isolation reagents (Roche, Molecular Biochemicals, Indianapolis, USA). Total RNA was reverse transcribed using Promega M1705 Reverse transcriptase for RT-PCR according to the instructions by the manufacturer to produce cDNA. Expression levels of enzymes involved in endocannabinoid synthesis (*DAGLA* and *DAGLB, NAPEPLD* and *PLA2G4E*) and degradation (*MGLL,*

FAAH1 and *FAAH2*) and CB receptors (*CNR1* and *CNR2*) were determined in a 96-well PCR plate by real-time PCR (Bio-Rad CFX96), using gene-specific primers (**Table 1**) and IQ SYBR-Green super mix (PromegaA6002). For analysis and quantification, Bio-Rad CFX Manager software version 3.1 was used. Gene expression was normalized by using *18S* (for WAT) or *GAPDH* (for skeletal muscle) mRNA content and expressed as fold difference compared to white Caucasian using the ΔΔCT method.

Table 1. Sequences of forward and reverse primers

Gene	Forward primer	Reverse primer
18S	AGGATCCATTGGAGGGCAAGT	TCCAACTACGAGCTTTTTAACTGCA
CNR1	GATGTACTTGCCCTGACCATA	AACATTCTAGGACTGATTCATCATG
CNR2	AAGATTGGCAGCGTGACTATG	CAGGCAGAGGTATCGGTCAA
DAGLA	CCATCTTCCTCTTTCTCCT	CTCGTGCGGGTTATAGAC
DAGLB	GTCTTCCCAGGGTTCTTC	TGAGGACGATCAAGTAAC
FAAH1	GGGCCGTCAGCTACACTATGC	ATGTTCCATCTGGGCCTCGTC
FAAH2	CGCTAGGCTTTCTCATAGGC	CCGAAAGCAGAAGCAATGGTT
GAPDH	TTGCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG
MGLL	TCTTCCTTCTGGGCCACTCCA	GGATTGGCAAGAACCAGAGG
NAPEPLD	CCCTCTATTCCAAATGTTCT	CATCCATTTCCACCATTAC
PLA2G4E	GAGCCCACAAACGGATGAAG	GTCTGTCTGGCTCAGCATATCA

Statistical analysis

Data were collected and analyzed using IBM SPSS statistics version 23.0. Baseline characteristics and differences in plasma endocannabinoid levels between ethnicities were compared using unpaired student t-tests. Furthermore, linear regression analysis computed by Pearson's correlation was used to determine correlations between plasma endocannabinoid levels and different metabolic parameters. Regression analysis was performed both with and without correction for the effect of ethnicity, by respectively including/excluding ethnicity as a covariate. P values < 0.05 were considered as statistical significant. Data are presented as mean ± SEM. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

RESULTS

Clinical characteristics

The clinical characteristics of the participants were partly described previously (18). In brief, twenty overweight, prediabetic men of South Asian (n=10) and white Caucasian

(n=10) descent were included. Mean age (46.5±2.8 *vs* 47.5±2.0 years) and BMI (30.1±1.1 *vs* 30.7±1.2 kg/m^2) were comparable between South Asians and white Caucasians, respectively, as were body fat percentage (31.0±1.1% *vs* 30.1±1.0%), plasma triglyceride concentration (1.6±0.2 *vs* 1.5±0.2 mmol/L) and fasting glucose level (5.6±0.5 *vs* 5.7±0.7 mmol/L). Resting energy expenditure was lower in South Asians as compared to white Caucasians (4.9±0.2 *vs.* 5.7±0.2 kJ/min; p<0.05), while lipid and glucose oxidation did not differ significantly.

South Asians have higher plasma levels of some endocannabinoids

First, we studied circulating endocannabinoid levels in both ethnicities. Plasma 2-AG and AEA levels were comparable between South Asians and white Caucasians (12.8±1.5 *vs* 10.6±1.2 pmol/mL; **Figure 1A**, and 1.0±0.1 *vs* 0.9±0.1 pmol/mL; **Figure 1B**, respectively). Plasma arachidonic acid tended to be higher in South Asians (18.3±1.1 *vs* 14.6±1.6 nmol/mL, p=0.07; **Figure 1C**). In addition, plasma 2-linoleoyl glycerol (208±23 *vs* 114±17 pmol/mL, p<0.01**; Figure 1D**) and *N*-linoleoylethanolamine (3.0±0.2 *vs* 2.2±0.2 pmol/mL, p<0.05; **Figure 1E**) were higher in South Asians compared to white Caucasians. Plasma 1-linoleoyl glycerol (243±28 *vs* 169±30 pmol/mL, p=0.09; **Table S1**), and *N*-docosatetraenylethanolamide (6.3±0.4 *vs* 5.3±0.4 pmol/mL, p=0.08; **Table S1**) both tended to be higher in South Asians. No significant differences were observed for other *N*-acylethanolamines and mono- and di-acyl glycerols measured.

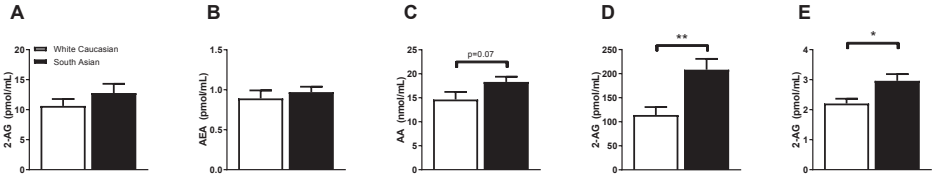

Figure 1. Circulating endocannabinoid levels in South Asian and white Caucasian men. Blood was collected from overweight, prediabetic South Asian (n=10; black bars) and matched white Caucasian (n=10; white bars) men. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used to measure plasma concentrations of 2-AG **(A)**, AEA **(B)**, arachidonic acid (AA) **(C),** 2-linoleoyl glycerol (2-LG) **(D)** and *N*-linoleoylethanolamine (LEA) **(E)** concentrations. Values are mean ± SEM. P-values are based on unpaired t-tests. * p<0.05, ** p<0.01.

Circulating endocannabinoid levels correlate with plasma triglycerides and lipid oxidation

We next investigated whether circulating endocannabinoid levels correlated with metabolic parameters in our study by pooling endocannabinoid levels of the two ethnicities. Plasma 2-AG levels positively correlated with plasma triglyceride levels (R=0.77, p<0.001; **Figure 2A**) and lipid oxidation (R=0.55, p<0.05; **Figure 2B**). In contrast, AEA levels did

not correlate with any of these parameters (data not shown). To test whether the effects could be attributed to ethnicity, we repeated the regression analysis including ethnicity as covariate and found that this did not change the results.

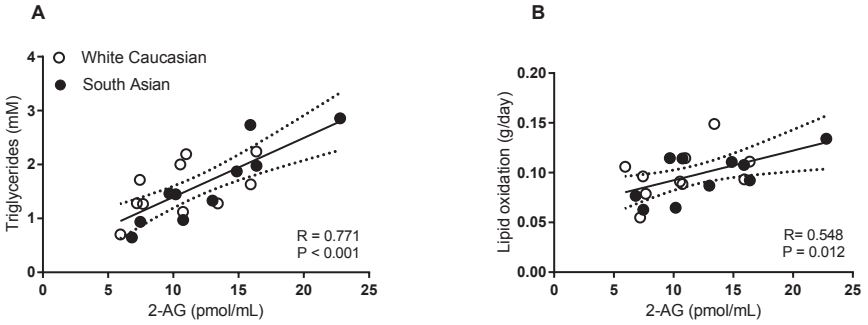

Figure 2. Plasma 2-AG levels positively correlate with plasma triglyceride levels and with lipid oxidation in South Asian and white Caucasian men. Scatterplot of the correlations between 2-AG levels and plasma triglycerides **(A)** or lipid oxidation **(B)**. Correlations are shown for the total group combined (n=20), white circles represent white Caucasian men (n=10) and black circles represent South Asian men (n=10), with 95% confidence limits. Correlations were analyzed using linear regression analysis.

South Asians have lower mRNA expression of CB receptors and endocannabinoid degradation enzymes in skeletal muscle

Since circulating endocannabinoid levels not necessarily reflect tissue-specific signaling, we next assessed mRNA expression of CB receptors and enzymes involved in endocannabinoid synthesis and degradation in WAT and skeletal muscle biopsies of South Asian and white Caucasian men.

In WAT, expression levels of all measured genes were not different between the two ethnicities (**Figure 3A**). However, in skeletal muscle, the relative expression of the degradation enzyme FAAH2 *(FAAH2)* was 5-fold lower in South Asians compared to white Caucasians (-81%, p<0.001; **Figure 3B**). In addition, expression of the other degradation enzymes, MAGL *(MGLL)* and FAAH1 *(FAAH1)* tended to be lower in South Asians (-22%, p=0.05 and -18%, p=0.07, respectively; **Figure 3B**). Moreover, expression of both the CB_1 (*CNR1)* and CB_2 *(CNR2)* receptor was approx. 10-fold lower in South Asians as compared to white Caucasians (-93%, p<0.001 and -90%, p<0.001; **Figure 3B**). Expression of endocannabinoid synthesis enzymes was comparable (**Figure 3B**) between the two ethnicities.

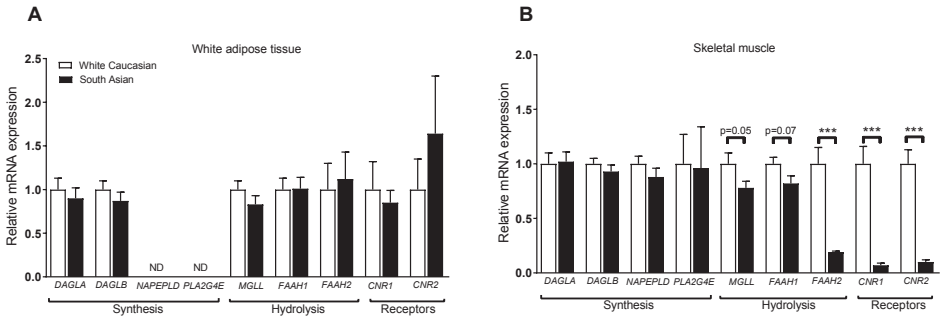

Figure 3. Relative expression of synthesis and degradation enzymes of endocannabinoids and mRNA expression of cannabinoid (CB) receptors in South Asian and white Caucasian men. Abdominal white adipose tissue and skeletal muscle tissue biopsies from the vastus lateralis muscle were taken after an overnight fasting period. Gene expression in white adipose tissue **(A)** and skeletal muscle **(B)** was determined by qPCR. Gene expression was normalized by using *18S* (white adipose tissue) or *GAPDH* (skeletal muscle) mRNA content and expressed as fold change compared to white Caucasians using the ΔΔCT method. ND is not detectable. Values are presented as mean ± SEM. p-values are based on an unpaired student t-test. *** p<0.001.

DISCUSSION

The incidence of obesity and T2D is particularly high in individuals of South Asian descent. In the present study, we investigated different markers of endocannabinoid signaling in overweight, prediabetic South Asian and white Caucasian men. Here we report that, plasma 2-linoleoyl glycerol and *N*-linoleoylethanolamine were higher in South Asians compared to white Caucasians. In addition, gene expression of the enzymes involved in endocannabinoid degradation as well as CB receptors in skeletal muscle was markedly lower in South Asians as compared to white Caucasians. In addition, plasma 2-AG correlated positively with plasma triglycerides and lipid oxidation.

We observed higher levels of the endocannabinoids 2-linoleoylglycerol and *N*-linoleoylethanolamine in overweight, prediabetic South Asian compared to white Caucasian men. In our previous study in healthy lean South Asians and white Caucasians we also observed higher endocannabinoid levels in South Asians, however, in that study other endocannabinoids (*i.e.* 2-AG and AEA) were higher (14). If we compare the effect sizes between the two different cohorts we observe that except for *N*-palmitoylethanolamide, all effects are in the same direction albeit that the effect sizes differ for most endocannabinoids between the two cohorts. Furthermore, there is no clear pattern in the effect size with respect to whether it is larger in either the lean or the overweight cohorts. This is likely due to the different phenotype of the subjects in both cohorts. Of note, circulating endocannabinoid levels in this cohort of overweight, prediabetic men were not significantly higher compared to endocannabinoid levels measured in our previous cohort of healthy lean men. Probably, the difference in BMI between the two cohorts

was insufficiently large (average BMI 22 kg/m^2 in lean versus BMI 30 kg/m^2 in overweight men) to observe differences in circulating endocannabinoid levels between the two cohorts.

We also found a strong correlation between 2-AG and plasma triglyceride levels. This correlation was also present in our previous cohort of healthy lean South Asian and white Caucasian men (14) and is in line with results of Bluher et al. (21). The biological explanation for this correlation is currently unknown. Possibly, it can at least partly be explained by the fact that both 2-AG and triglycerides are lipid molecules with common lipid intermediates as precursors (e.g. diacylglycerol), resulting in production and secretion into blood of 2-AG concomitant with VLDL-triglyceride.

To investigate potential differences in endocannabinoid signaling in metabolically active tissues we examined WAT and skeletal muscle biopsies from these overweight, prediabetic men. We did not observe differences in expression of genes involved in endocannabinoid signaling in WAT between the two ethnicities. Interestingly, in skeletal muscle, we found that mRNA expression of the endocannabinoid degradation enzymes and CB receptors was lower in South Asians as compared to white Caucasians. Possibly, low expression of degradation enzymes in skeletal muscle of the South Asian men might have contributed to higher local endocannabinoid levels within skeletal muscle thereby possibly inducing CB receptor downregulation in this tissue (15). Alternatively, the dietary intake between South Asians and white Caucasians may have been different, as South Asian diets often contain low n-3 PUFA which has been shown to modulate the expression of endocannabinoid synthesis and degradation enzymes and CB receptors (22). Although all subjects used a standardized meal the evening before the study day, we cannot exclude that differences in diet might have influenced our results. Based on our study, we can thus only speculate about the underlying mechanisms, which is an interesting subject of future studies. Interestingly, the CB1 receptor regulates metabolic processes including insulin signaling, glucose uptake, and fatty acid oxidation in skeletal muscle (7,8). Moreover, overstimulation of CB receptors in skeletal muscle can disrupt insulin signaling thereby promoting insulin resistance which could eventually lead to development of (pre)diabetes (5). Since South Asians are known to have an increased risk for the development of metabolic disease, including T2D (2), dysregulation of ECS in skeletal muscle might thus contribute to diabetes development in this population.

Our study has several limitations. Firstly, we had a small sample size of ten South Asian and ten white Caucasian men. Secondly, all individuals were already metabolically deregulated as they are overweight and have prediabetes. This may have limited the differences we could observe between the two ethnic groups. In addition, we can only speculate about the mechanisms underlying the differences in ECS signaling between South Asians and white Caucasians. Future studies in larger cohort should investigate if these results also apply to women and lean, non-prediabetic individuals to determine

if these results could be translated to the general population. A strength of our study is that we measured both circulating endocannabinoids and gene expression of the ECS in WAT and skeletal muscle in the same individuals.

Taken together, our data show that, plasma 2-linoleoyl glycerol and *N*-linoleoylethanolamine were higher in overweight, prediabetic South Asians compared to white Caucasians. In addition, South Asian individuals had lower expression of enzymes involved in endocannabinoid degradation and CB receptors in skeletal muscle. Although it remains speculative, high endocannabinoid levels may deteriorate endocannabinoid signaling in metabolic organs, including skeletal muscle and thereby may contribute to the development and/or progression of obesity and possibly even T2D, both of which are highly prevalent in South Asians. Further studies are required to show if therapeutic interventions that target the ECS will improve the metabolic profile of these individuals.

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SUPPLEMENTAL APPENDIX

Supplementary Table S1. MRM (Multiple reaction monitoring) parameters and endogenous concentrations of the target list

S.No	ID	Metabolite Name	HMBD ID	Q1	Q3	DP, CE	Concentration (pmol/mL)		
							White Caucasians	South Asians	p-Value
1	1-1OG (18:1)	1-Oleoyl Glycerol	HMDB11567	357	265	80, 20	2441±353	2372±261	0.876
2	2-OG (18:1)	2-Oleoyl Glycerol	HMDB11537	357	265	80, 20	248±42	250±36	0.978
3	1-LG (18:2)	1-Linoleoyl Glycerol	HMDB11568	355	263	80, 15	169±30	243±28	0.086
4	2-LG (18:2)	2-Linoleoyl Glycerol	HMDB11538	355	263	80, 20	114±17	208±23	0.004
5	2&1-AG (20:4)	2&1-Arachidonoyl Glycerol	HMDB04666	379	287	56.19	11±1	13±2	0.276
6	2AGE (20:4)	2-Arachidonyl Glycerol ether	HMDB13657	365	273	72, 25	ND	ND	
7	PDEA (15:0)	*N*-Pentadecanoyl Ethanolamide	53832-58-9a	286	62	80, 18	0.09±0.01	0.08±0.01	0.440
8	PEA (16:0)	*N*-Palmitoyl Ethanolamide	HMDB02100	300	62	78, 36	9.3±0.7	9.1±0.5	0.847
9	SEA (18:0)	*N*-Stearoyl Ethanolamide	HMDB13078	328	62	72, 31	6.3±0.5	6.1±0.2	0.672
10	POEA (16:1)	*N*-Palmitoleoyl Ethanolamide	HMDB13648	298	62	86, 32	0.53±0.06	0.41±0.04	0.145
11	OEA (18:1)	*N*-Oleoylethanolamide	HMDB02088	326	62	72, 30	5.9±0.7	5.5±0.3	0.602
12	LEA (18:2)	*N*-Linoleoyl Ethanolamide	HMDB12252	324	62	74, 36	2.2±0.2	3.0±0.2	0.014
13	Alpha-LEA (18:2)	*N*-α-Linolenyl Ethanolamide	HMDB13624	322	62	80, 20	0.013±0.001	0.011±0.001	0.339
14	DGLEA (18:3)	Dihomo-γ-Linolenoyl ethanolamide	HMDB13625	350	62	70, 20	0.24±0.03	0.24±0.02	0.904
15	ETAEA (20:3)	Eicosatrienoic Acid Ethanolamide	169232-04-6a	350	62	80, 46	ND	ND	
16	AEA (20:4)	Anandamide	HMDB04080	348	62	70, 42	0.89±0.10	0.97±0.07	0.522

Supplementary Table S1. MRM (Multiple reaction monitoring) parameters and endogenous concentrations of the target list (continued)

S.No	ID	Metabolite Name	HMBD ID	Q1	Q3	DP, CE	Concentration (pmol/mL)		
							White Caucasians	South Asians	p-Value
17	O-AEA (20:4)	O-Arachidonoyl ethanolamine	HMDB13655	348	62	70, 25	ND	ND	
18	EPEA (20:5)	Eicosapentaenoyl Ethanolamide	HMDB13649	346	62	80, 20	0.06±0.01	0.06±0.01	0.910
19	DEA (22:4)	*N*-Docosatetraenoyl Ethanolamide	HMDB13626	376	62	72, 20	0.24±0.03	0.25±0.02	0.843
20	DHEA (22:6)	*N*-Docosahexaenoyl Ethanolamide	HMDB13658	372	62	70, 25	5.3±0.4	6.3±0.4	0.078
21	NADA (28:4)	*N*-Arachidonoyl dopamine	199875-69-9a	440	137	75, 24	ND	ND	
22	Arachidonic Acid (20:4)	5,8,11,14-Eicosatetraenoic acid	HMDB01043	303	259	-80, -18	14639±1578	18300±1076	0.071

The target list includes endocannabinoids and *N*-acylethanolamines (NAEs). The compound ID is the abbreviation of metabolite name along with number of carbon atoms and number of double bonds in the fatty acid chain of the molecule, respectively. All compounds are analyzed in positive mode except arachidonic acid in negative mode. Q1 and Q3 are optimized precursor ion and product ion, expressed as *m/z*. DP and CE are declustering potential (Volts) and collision energy (volts). Endogenous concentrations are denoted as mean ± SEM, n=10 in each group. P-values are based on unpaired t-tests. ND: Not detected in human plasma.