Dysregulation of endocannabinoid concentrations in human subcutaneous adipose tissue in obesity and modulation by omega-3 polyunsaturated fatty acids

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Abbreviations: 14:0, myristic acid; 16:0, palmitic acid; 16:1n-7, palmitoleic acid; 18:0, stearic acid; 18:1n-7, vaccenic acid; 18:1n-9, oleic acid; 18:3n-3, α-linolenic acid; 20:0 arachidic acid; 20:3n-6, 20:4n-3, eicosatetraenoic acid; dihomo-gamma-linolenic acid; DPA, 22:5n-3, docosapentaenoic acid; AA, 20:4n-6, arachidonic acid; AEA, anandamide; 2-AG, 2-arachidonyl glycerol; CPM, counts per million; EPA, 20:5n-3, eicosapentaenoic acid; EPEA, eicosapentaenoyl ethanolamide; DAGLA, diacyglycerol-α lipase; DHA, 22:6n-3,

docosahexaenoic acid; DHEA, docosahexaenoyl ethanolamide; FA, fatty acid; FAAH, fatty acid amide hydrolase; FAME, fatty acid methyl ester; HDL-C, high density lipoprotein cholesterol; LA, linoleic acid, 18:2n-6; LC n-3 PUFA, long chain omega-3 polyunsaturated fatty acid; LDL-C, low density lipoprotein cholesterol; MGLL, monoacylglycerol lipase; NEFA, non-esterified fatty acid; PLA_2, phospholipase A_2; PLA2G2D, phospholipase A_2 Group IID; PLA2G4A, phospholipase A_2 Group IVA; PLA2G7, lipoprotein-associated phospholipase A_2 Group VII; PPAR, peroxisome proliferator-activated receptor; RIN, RNA integrity score; SFA, saturated fatty acid; scWAT, subcutaneous white adipose tissue; SLC27A1, long chain fatty acid transport protein-1; SLC27A2, very long chain fatty acid Co-A synthetase; SPE, solid phase extraction; TG, triglyceride; WAT, white adipose tissue.

Abstract

Obesity is believed to be associated with a dysregulated endocannabinoid system which may reflect enhanced inflammation. However, reports of this in human white adipose tissue (WAT) are limited and inconclusive. Marine long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) have anti-inflammatory actions and therefore may improve obesity-associated adipose tissue inflammation. Therefore, fatty acid concentrations, endocannabinoid concentrations, and gene expression were assessed in subcutaneous WAT biopsies from healthy normal weight individuals (BMI 18.5 to 25 kg/m^2) and individuals living with metabolically healthy obesity (BMI 30 to 40 kg/m^2) prior to and following a 12-week intervention with 3 g fish oil/day (1.1 g EPA + 0.8 g DHA) or 3 g corn oil/day (placebo). WAT from individuals living with metabolically healthy obesity had higher n-6 PUFAs and EPA, higher concentrations of two endocannabinoids (anandamide and eicosapentaenoyl ethanolamide), higher expression of *PLA2G2D* and *PLA2G4A*, and lower expression of *CNR1*. In response to fish oil intervention, WAT EPA increased to a similar extent in both BMI groups, and WAT DHA increased by a greater extent in normal weight individuals. WAT eicosapentaenoyl ethanolamide and docosahexaenoyl ethanolamide increased in normal weight individuals only and WAT 2-arachidonyl glycerol decreased in individuals living with metabolically healthy obesity only. Altered WAT fatty acid, endocannabinoid, and gene expression profiles in metabolically healthy obesity at baseline may be linked. WAT incorporates n-3 PUFAs when their intake is increased which affects the endocannabinoid system; however, effects appear greater in normal weight individuals than in those living with metabolically healthy obesity.

Keywords: Endocannabinoids, Adipose tissue, Obesity, LC n-3 PUFA, Lipids

Introduction

Obesity is characterised by a state of chronic low grade inflammation (1), contributed to by altered levels of many fatty acid (FA) metabolites in white adipose tissue (WAT) and the circulation, and loss of sensitivity to hormones and lipid signalling molecules (2). A family of FA-containing metabolites called endocannabinoids may become dysregulated during obesity, but current evidence of this in human WAT is limited and inconsistent (3, 4). Endocannabinoids play a role in adipose tissue expansion and inflammation, both of which are observed to be upregulated in obesity (5-7). There is some evidence of alterations in endocannabinoids based on measurements in human plasma, where the arachidonic acid (AA; 20:4n-6) containing endocannabinoids, anandamide (AEA) and 2-arachidonyl-glycerol (2-AG), are elevated in obesity and positively correlate with BMI and intra-abdominal adiposity (8-11). Mechanisms behind these elevated concentrations are under-reported and more comprehensive investigation of endocannabinoid concentrations and the expression of genes encoding enzymes involved in the metabolism of these metabolites may provide greater insight into the regulation of adipose tissue during the onset of obesity-associated inflammation and may identify targets for intervention. An overview of endocannabinoid synthesis, degradation, actions, observations in metabolically unhealthy obesity, and potential modulation by long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) is detailed in Figure 1.

The endocannabinoid system has been targeted for therapeutic modulation in obesity, as it is involved in the regulation of food intake and energy homeostasis, glucose and lipid metabolism, and inflammation (12-14). Endocannabinoids act predominantly via cannabinoid receptors but they also bind to peroxisome proliferator activated receptors (PPARs) and a range of G-protein coupled receptors (6). Cannabinoid receptor-1 antagonists improve adipocyte insulin sensitivity and glucose homeostasis through altered cytokine production from WAT macrophages (15), and the downregulation of cannabinoid receptor-1 led to weight loss and normalisation of lipid homeostasis in individuals living with obesity and type-2 diabetes (16, 17). Activation of PPARs and G-protein coupled receptors by endocannabinoids has anti-inflammatory actions and regulates FA metabolism and energy balance (6).

In addition to AEA and 2-AG, some lesser reported endocannabinoids which are present in lower concentrations contain the n-3 PUFAs eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3); these include eicosapentaenoyl ethanolamide (EPEA) and docosahexaenoyl ethanolamide (DHEA) (18). LC n-3 PUFAs have been shown to decrease inflammation, a key factor in the development of obesity-related metabolic dysfunction and co-morbidities (2, 19-21). This anti-inflammatory effect was linked to increases in LC n-3 PUFA containing endocannabinoids and decreases in the AA containing “classical” endocannabinoids in WAT in experimental animals (10, 22-24) and in plasma from humans with obesity (25). In addition, EPA and DHA can act as signalling

molecules by binding to PPARs (26) to regulate the expression of genes associated with adipose functions including expansion, lipid accumulation and energy homeostasis, and inflammation (27, 28). However, there is limited exploration of these effects in human WAT.

Modulation of the endocannabinoid system and response to dietary FAs have also been shown to differ by metabolic status (4, 29). Concentrations of circulating endocannabinoids, which may reflect spill over from peripheral tissues such as the WAT, have been shown to decrease postprandially or following an oral glucose load and euglycemic clamp in normal weight individuals (30, 31), but not in those individuals living with obesity accompanied by insulin resistance (31). These individuals, in addition to those living with diagnosed type 2 diabetes, have higher fasting concentrations of circulating endocannabinoids (4, 12, 30, 32-34). The endocannabinoid system is under the control of leptin, insulin, and PPAR-γ agonists including glitozones used in the treatment of type-2 diabetes and LC n-3 PUFAs (12, 30, 31, 35). The use of LC n-3 PUFAs to modulate the endocannabinoid system in the context of obesity with accompanying type-2 diabetes is of interest due to improvements seen in WAT inflammation and energy homeostasis (17). However, influencing individuals earlier in the causal pathway is desirable in order to potentially prevent the onset of serious non-communicable conditions like diabetes. Despite this, the effects of LC n-3 PUFAs on WAT in individuals living with metabolically healthy obesity (MHO) have not been studied. This study aimed to identify any differences in the WAT endocannabinoid system, and the potential contribution of differences in FA composition and expression of endocannabinoid related genes, in individuals living with metabolically healthy obesity compared to individuals of normal weight to investigate the onset of dysregulation in the WAT. Furthermore, the study aimed to investigate whether intervention with LC n-3 PUFAs could modify the endocannabinoid system in WAT which may have potential to slow or even reverse the onset of obesity-associated inflammation in the tissue.

Methods

All procedures involving human participants were approved by the National Research Ethics Service South Central–Berkshire Research Ethics Committee (submission no. 11/SC/0384) and the study is registered at www.isrctn.com (study ID: ISRCTN96712688). The trial was conducted according to the principles of the Declaration of Helsinki, and all participants gave written informed consent prior to enrolment.

Healthy normal weight individuals (BMI 18.5 to 25 kg/m^2) and healthy individuals living with obesity (BMI 30 to 40 kg/m^2, waist circumference ≥ 94 cm males and ≥ 80 cm females) aged 18-65 years who were able to provide written informed consent were recruited into a double blind placebo (comparator oil) controlled trial. Exclusion criteria included: being outside the defined age or BMI and waist circumference categories; having diagnosed metabolic disease (e.g. diabetes, cardiovascular disease) or chronic gastrointestinal problems (e.g. inflammatory bowel disease, celiac disease, and cancer); the

use of prescribed medicine to control inflammation, blood lipids or blood pressure; consumption of more than one portion of oily fish per week (140 g cooked); use of fish oil or other oil supplements; being pregnant or planning to become pregnant during the study period; or participation in another clinical trial.

Study design

Fasted blood and an abdominal subcutaneous white adipose tissue (scWAT) biopsy (~1 g) were collected at baseline (week-0) and following a 12-week intervention (week-12) during which participants were randomised to consume either 3 g of fish oil (1.1 g EPA + 0.8 g DHA) or 3 g of corn oil (1.65 g linoleic acid (LA) and 0.81 g oleic acid) per day (full composition of intervention oils are detailed in Supplementary Table 1). Blinding, randomization, and supplement packaging were completed by the Research Pharmacy at Southampton General Hospital, Southampton, United Kingdom, by individuals independent of the researchers involved in the study. Treatment group blinding was maintained until completion of statistical analysis of all data.

Sample preparation

Abdominal scWAT biopsies were collected by surgical removal under local anaesthetic (1% lidocaine) to provide ~1 g of intact tissue and were directly stored on ice. Tissue was divided into 5 x ~200 mg aliquots. scWAT designated for FA and lipid metabolite analyses was wrapped in foil, placed in cryovials and snap frozen in liquid nitrogen. scWAT designated for RNA analysis was placed into 4 mL of RNAlater (Sigma, St. Louis, Missouri, United States) and stored for 24 hours between 2-4°C and then at -20°C until RNA extraction.

Blood analyses

~5 mL of heparinised blood was collected and stored on ice. Plasma was prepared by centrifugation (1900 × g, 10 min, room temperature) and stored at −80 °C until analysis. Plasma triglyceride (TG), cholesterol, high density lipoprotein cholesterol (HDL-C), nonesterified fatty acid (NEFA), and glucose concentrations were measured using an iLAB 600 clinical chemistry analyzer and software (Instrumentation Laboratories, Bedford, Massachusetts) and enzyme-based kits (Wako, Osaka, Japan). Low density lipoprotein cholesterol (LDL-C) concentrations were estimated using the Friedwald equation. Plasma insulin concentrations were measured by ELISA (Dako, Agilent, Santa Clara, California) and HOMA2-IR was calculated as follows: (((insulin mmol/L) x (glucose IU/L)) / 22.5).

Anthropometry

Height was measured using a Seca stadiometer (Seca, Hamburg, Germany) with shoes removed, and weight and body composition measurements were made using digital bioelectric

impedance apparatus (TANITA BC-418) with shoes and socks, personal items, and heavy clothing removed. Waist and hip circumference measurements were made using a tape measure whilst participants wore loose clothing. The tape was placed flat around their waist between the ribs and hip bone, one centimetre under the umbilicus for waist measurement, and around the greatest area of protrusion of the buttocks for hip measurement.

Fatty acid composition

Frozen scWAT was homogenised in 800 µl ice cold 0.9% sodium chloride (NaCl) and total lipid was extracted from the homogenate with 5 mL chloroform:methanol (2:1: vol:vol) containing 0.2 M butylated hydroxytoluene (36). 1 mL of 1 M sodium chloride was added and the sample vortex mixed prior to centrifugation at 1500 x g for 5 min at room temperature. The lower solvent phase was aspirated and evaporated under nitrogen at 40°C. The lipid extract was re-dissolved in 0.5 mL toluene and fatty acids were released from esterified lipids and simultaneously derivatised to methyl esters by incubation with 2% H_2SO_4 in methanol for 2 h at 50°C to form fatty acid methyl esters (FAMEs) (36-38). The samples were then neutralised and FAMEs transferred into hexane for analysis by gas chromatography; FAMEs were separated on a BPX-70 fused silica capillary column (30 m x 0.2 mm x 0.25 µm; manufactured by SGE) in a HP6890 gas chromatograph fitted with a flame ionisation detector. GC run conditions were as described elsewhere (38).

Endocannabinoid analysis

~100 mg of frozen scWAT was homogenised in 650 µl chilled 100% methanol (-20°C) and stored at -80°C for 30 minutes. The following deuterated internal standards were added to aid identification of peaks: arachidonic acid-d8, docosahexaenoic acid-d5, eicosapentaenoic acid-d5, PGD_2-d4, 15(s)-hydroxy-eicosatetraenoic acid-d8, 12(s)-hydroxy-eicosatetraenoic acid-d8, 5(s)-hydroxy-eicosatetraenoic acid-d8, 6-keto-$PGF_{1\text{-alpha}}$-d4, 8-iso-$PGF_{2\text{-alpha}}$-d4, PGF2-d9, 15(s)-hydroxy-eicosatetraenoic acid-d8, and 8-9 dihydroxy-eicosatrienoic acid-d11 (Cayman Chemical Company, Michigan, United States). Following centrifugation of the homogenate at 12,000 x g for 10 minutes at 4°C, FA metabolites were isolated from the supernatant using solid phase extraction (SPE) (39, 40). Strata-X polymeric reversed phase 500 mg 6 ml capacity SPE cartridges (Phenomenex, California, United States) and SPE tank needles were activated with 2 x 6 ml ethyl acetate. The cartridges were further conditioned with sequential addition and removal of 2 x 6 ml chilled methanol, hexane and H_2O under vacuum keeping the cartridge sorbent saturated. Chilled H_2O was added to the supernatant and vortex mixed, followed by the addition of 300 µl of chilled 0.1 mM hydrochloric acid, and immediately applied to the SPE cartridge followed with 6 x 1 ml of chilled H_2O. All fatty acid metabolites (including endocannabinoids) were eluted into 10 µl of chilled 30% glycerol in methanol with the addition of 6 ml chilled methyl formate and 2 ml of methanol (39). The FA metabolites were dried, dissolved in chilled 30% acetonitrile, 60% H_2O, 10% methanol, and filtered through a

polyvinylidine fluoride micro filter (centrifuged at 12,000 x g for 2 minutes at 4°C). FA metabolites in the filtrate were separated on a Thermo Dionex Ultimate 3000 RSLC (Dionex corporation, California, United States) liquid chromatograph fitted with a Kinetex® UPLC column 1.7 µm particle size, C18 stationary phase, 100 A pore size, 150 x 2.1 mm (Phenomenex, California, United States) with mobile phase composition A = 70% H_2O, 30% acetonitrile and 0.02% acetic acid; B = 50% acetonitrile and 50% isopropanol, and identified on an AB SCIEX SelexION QTRAP 5500 (AB SCIEX, Massachusetts, United States) triple quadrupole mass spectrometer selecting for electrospray in negative modes for endocannabinoid measurement (40). Deuterated internal standards were used to orientate the resulting UPLC-MS outputs and identify correct FA metabolite peaks using Analyst software (version 1.6.2 2013, AB SCIEX, Massachusetts, United States). The limit of detection was $\geq$ 0.1 pg for all metabolites.

Gene expression

RNA was isolated from scWAT stored in RNAlater using the RNeasy lipid tissue mini kit™ (QIAGEN, Hilden, Germany). The kit protocol was carried out twice to yield RNA from ~2 x 75mg of scWAT in total from each participant yielding an average of 2.47 µg of total RNA determined by Nanodrop 2000 (Thermo Scientific, Waltham, MA). A subset of 20 samples (10 normal weight, 10 metabolically healthy obese) matched for age and sex with an RNA integrity (RIN) score of 6.5 were selected to be sequenced. Sequencing was performed on a Hiseq2000 platform with 5 samples per lane in a total of 8 lanes (SE50) with a total of 20 million reads. RNA-Seq reads were aligned to the hg38.0 reference genome using TopHat (open source, Johns Hopkins University, Center for Computational Biology, Baltimore, United States) (41), utilizing Bowtie2 (42), and a read count table produced using HTSeq (open source, Huber group, Heidelberg, Germany) (43). The read counts were filtered to include only genes with greater than 5 counts in a minimum of 10 samples and normalised based upon the depth of sequencing, biological coefficient of variation, and estimated tagwise dispersion in EdgeR (open source, Bioconductor.org) (44). The normalised counts per million (CPM) were used to evaluate gene expression. The results from this subset were validated via qRT-PCR of RNA extracted from the whole cohort which showed the subset to be representative of the whole cohort.

Reverse-transcription was performed to obtain cDNA using GoScript Reverse Transcription System (Promega, Southampton, UK) and gene expression was quantified with RT-qPCR using Applied Biosystems 7500 qRT-PCR (Applied Biosystems, California, USA) using 4 ng cDNA per 20 µl reaction with QuantiNova Probe PCR kit (GIAGEN, Hilden, Germany) and custom double dye taqman style primers (Primer Design, Southampton, UK). Primer sequences for genes validated by qRT-PCR are detailed in Supplementary Table 2. Gene expression data were normalized to *GABARAP*, *VPS36*, custom primer sequences (Supplementary Table 2) and YWHAZ housekeeping

gene data (analysed using geNorm, Primer Design, Eastleigh, UK) and analysed using the 2^ (−ΔΔ Ct) method.

Enzyme activity

~ 10 mg scWAT was homogenised on ice in 100 µL of fatty acid amide hydrolase (FAAH) lysis buffer. Homogenates were centrifuged at 12,000 x g for 5 mins at 4°C and supernatants collected. Protein concentrations were determined using the bicinchoninic acid assay read at 500nm using the Pierce™ BCA Protein Assay Kit (Fisher Scientific, Loughborough, UK) following the manufacturer's instructions. FAAH activity was measured using the FAAH Activity Assay kit (Abcam, Cambridge, UK) according to manufacturer's instructions read in loop mode at Ex/Em 365/ 415-445nm for 60 mins. Delta RFU data were calculated against the standard curve and adjusted to mg protein used to give activity in pmol/min/mg.

Statistics

Sample size was calculated considering the typical distribution and expected response of circulating cytokines – not reported here - (20% decrease following fish oil intervention) and participant drop out of 20%. A sample size of 25 participants per group (BMI and treatment subgroup) was determined to be able to detect changes in circulating cytokines at > 80% power and a 5% level of significance with consideration for 20% loss. No formal power calculation was performed specifically for the outcomes described herein. Not all data were normally distributed. Non-normal data could not be corrected with $\log_{10}$ transformation. Therefore, appropriate non-parametric tests were performed and all data are displayed as median and interquartile range (IQR).

Data can be openly accessed via GEO (https://www.ncbi.nlm.nih.gov/geo/).

Results

Participant characteristics

A Consolidated Standards of Reporting Trials (CONSORT) diagram illustrating participant inclusion and flow through the study is detailed in Figure 2. Individuals living with obesity had significantly greater BMI, waist circumference, hip circumference, % body fat, and body fat mass (kg), and higher blood concentrations of TG, total cholesterol, LDL-C, glucose, and insulin in comparison to normal weight individuals (Table 1). Individuals living with obesity had a significantly higher average HOMA2-IR score; however, this was still within the 'normal range' (HOMA-IR < 1.95). As no individuals with diagnosed metabolic or inflammatory complications were recruited and obese individuals did not exhibit clinical hypertriglyceridemia and had HOMA2-IR scores within the normal range, the individuals living with obesity were defined as living with metabolically healthy obesity.

Obesity is associated with an altered scWAT fatty acid composition, higher concentrations of scWAT endocannabinoids, and altered expression of fatty acid metabolite pathway genes

The most abundant FA present in scWAT was oleic acid (18:1n-9) followed by palmitic acid (16:0) and linoleic acid (LA; 18:2n-6) (Figure 3). The monounsaturated FAs (MUFAs) palmitoleic acid (16:1n-7) and vaccenic acid (18:1n-7) contributed between 2-6%, while the n-6 PUFA AA contributed 0.3-0.6%, and the LC n-3 PUFAs EPA, docosapentaenoic acid (DPA; 225n-3), and DHA, contributed <1% of total scWAT FAs.

scWAT from individuals living with metabolically healthy obesity had significantly higher proportions of the MUFAs 16:1n-7, 18:1n-7 and 18:1n-9, the n-6 PUFAs dihomo-gamma-linolenic acid (20:3n-6) and AA, and the n-3 PUFAs EPA and DPA (Figure 3). scWAT from individuals living with metabolically healthy obesity also had significantly lower proportions of the saturated fatty acids (SFAs) myristic acid (14:0), stearic acid (18:0), and arachidic acid (20:0), the MUFA 20:1n-9, and the n-3 PUFAs alpha-linolenic acid (18:3n-3) and eicosatetraenoic acid (20:4n-3) (Figure 3) in comparison with scWAT from normal weight individuals.

scWAT from individuals living with metabolically healthy obesity had significantly higher concentrations of AEA and EPEA in comparison to scWAT from normal weight individuals ($P \leq$ 0.001 and 0.05 respectively, Figure 4, and 5) which may be due to increased availability of AA and EPA as substrates for their synthesis, or to changes in expression of enzymes involved their metabolism.

Individuals living with metabolically healthy obesity had altered scWAT gene expression with significantly lower expression of *SLC27A2* ($P < 0.001$) and *CNR1* (data obtained by qRT-PCR, $P <$ 0.05), and significantly higher expression of *PLA2G2D* (phospholipase A_2 Group IID – secretory), *PLA2G4A* (phospholipase A_2 Group IVA - cytosolic) and *PLA2G7* (lipoprotein-associated phospholipase A_2 Group VII; data obtained by RNA-Seq, $P \leq 0.01$) (Figure 6). In addition to this, there was $\geq$ 50% lower expression of *DAGLA* (diacylglycerol-α lipase), *MGLL* (monoacylglycerol lipase), *FAAH* (fatty acid amide hydrolase), *SLC27A1* (long chain fatty acid transport protein-1), and *SLC27A2* (very long chain fatty acid Co-A synthetase) in scWAT from individuals living with metabolically healthy obesity, although the differences were not statistically significant (data obtained by qRT-PCR, Figure 6).

FAAH is involved in the degradation of endocannabinoids and as there was a $\geq$ 50% lower expression of mRNA encoding this enzyme in scWAT from individuals living with metabolically healthy obesity, which may result in greater concentrations of endocannabinoids in the tissue, the activity of

this enzyme in MHO individuals was of interest. However, FAAH activity was not detectable in WAT from either normal weight or individuals living with metabolically healthy obesity.

scWAT from individuals living with metabolically healthy obesity is resistant to modulation of LC n-3 PUFA containing endocannabinoids by fish oil

In response to 12-week fish oil intervention, the proportions of scWAT EPA, DPA and DHA significantly increased (by 59%, 29% and 36% respectively) in normal weight individuals ($P = 0.006$, <0.001 and <0.001 respectively) and the proportion of EPA significantly increased (by 56%) in individuals living with metabolically healthy obesity ($P < 0.001$) (Figure 7). The proportions of DPA and DHA also increased in individuals living with metabolically healthy obesity (by 9% and 17%) but this did not reach statistical significance (Figure 7). Despite a smaller increase in these FAs, DPA proportion remained higher (by 21%) in individuals living with metabolically healthy obesity ($P = 0.021$), EPA, DPA and DHA did not change in scWAT following the placebo intervention in either group (Figure 6). At the end of the 12-week fish oil intervention, EPA and DHA were higher in scWAT than at the end of the placebo intervention in both normal weight individuals ($P < 0.001$ for EPA and $P = 0.009$ for DHA) and individuals living with metabolically healthy obesity ($P < 0.001$ for both EPA and DHA).

The absolute concentrations of the EPA and DHA containing endocannabinoids EPEA and DHEA were significantly increased in the scWAT of normal weight individuals in response to 12-week fish oil intervention ($P = 0.006$ and 0.039 respectively, Figure 8). These were unchanged in the scWAT of individuals living with metabolically healthy obesity, but the concentration of 2-AG, an AA containing endocannabinoid, significantly decreased with fish oil in the individuals living with metabolically healthy obesity ($P = 0.006$, Figure 8). A similar pattern was also observed for scWAT AEA in individuals living with metabolically healthy obesity, but this was not statistically significant (Figure 8). Endocannabinoids did not change in scWAT following the placebo intervention in either group (Figure 8).

There were no significant changes in the expression of scWAT genes involved in fatty acid metabolite synthesis or degradation in either normal weight individuals or individuals living with metabolically healthy obesity in response to 12-week fish oil intervention.

Discussion

Compared with normal weight individuals, scWAT from individuals living with metabolically healthy obesity had alterations in FA composition and concentrations of endocannabinoids containing AA and EPA which may be linked to changes in the expression of genes involved in lipid and endocannabinoid metabolism.

Higher concentrations of AEA and EPEA in scWAT from individuals living with metabolically healthy obesity may be indicative of 'normal' tissue expansion in response to metabolic stress such as increased lipid accumulation and subsequent inflammatory signalling (45). These higher concentrations may be due to increased synthesis or to decreased degradation of these mediators. Consistent with this, higher expression of mRNA for three phospholipase A2 (PLA_2) enzymes and lower mRNA expression for the gene encoding the degradation enzyme FAAH were observed in scWAT from these individuals. PLA_2 isoenzyme expression may influence the availability of substrates for endocannabinoid synthesis. Of note is *PLA2G2D,* which has been recently reported to be crucial for breakdown of membrane phospholipids in WAT (46) and previously reported to be downregulated in murine models of high fat feeding and to be associated with lower levels of LC n-3 PUFAs (47). In contrast, upregulation of *PLA2G2D* was observed in individuals living with metabolically healthy obesity in the current study and may suggest enhanced mobilisation of membrane lipids as substrates for synthesis of AA and EPA containing endocannabinoids and other lipid metabolites. In addition, higher concentrations of AEA and EPEA may also be due in part to the higher proportions of AA and EPA in scWAT; this could reflect higher availability of substrate containing AA and EPA.

These data suggest alteration of both availability and mobilisation of FAs contributing to differences in endocannabinoid synthesis in human obesity. To our knowledge, this is the first report of higher concentrations of WAT AEA and EPEA, and the potential influence of *PLA2G2D* expression, in humans living with metabolically healthy human obesity. Thus, these findings are novel.

The effect of LC n-3 PUFA intervention on the endocannabinoid system appears to be limited in individuals living with metabolically healthy obesity.

In normal weight individuals, LC n-3 PUFA significantly increased EPEA and DHEA without affecting AEA or 2-AG, while in individuals living with metabolically healthy obesity, the biggest effect was on 2-AG, which was decreased. There were no significant changes to scWAT AA in either group of individuals following the intervention period resulting in a greater proportion of AA remaining in the scWAT of individuals living with metabolically healthy obesity in comparison to normal weight individuals. It may be that having a higher proportion of AA, as seen in those with metabolically healthy obesity, hinders the synthesis of EPA and DHA containing endocannabinoids, and a higher dose of LC n-3 PUFAs is required to alter the n-6: n-3 ratio and favour the synthesis of these endocannabinoids in such individuals. Rossmeisl *et al.* (18) report no change to AA or to AA containing endocannabinoids in scWAT of individuals living with obesity and diabetes following intervention with a higher dose of LC n-3 PUFAs containing a greater proportion of DHA and over a longer period time than used in the current study. Therefore, the metabolic health of the individual

306 may also play a role in the handling and metabolic effects of these FAs and this may relate to insulin
307 sensitivity of the WAT.

308 In the current study LC n-3 PUFAs did not modulate the expression of scWAT genes encoding
309 enzymes involved in endocannabinoid synthesis suggesting that the increased concentrations of EPEA
310 and DHEA following intervention may more likely result from increased availability of EPA and
311 DHA within substrates for synthesis of these metabolites. In contrast, lack of significant change in
312 synthesis of LC n-3 PUFA containing endocannabinoids in individuals living with metabolically
313 healthy obesity may be due to altered expression of enzymes involved in the metabolism of LC n-3
314 PUFA, particularly DHA, observed at baseline. There was 92% lower expression of *SLC27A2* (*P*
315 <0.001, Figure 5) in scWAT of individuals living with metabolically healthy obesity at study entry;
316 the enzyme encoded by this gene is responsible for activating DHA to its Co-A ester required for
317 further metabolism ultimately to DHEA (48). If there is a similar proportion of DHA in the scWAT of
318 normal weight individuals and those living with metabolically healthy obesity, but its activation is
319 lower in the latter, this could result in reduced ability to convert DHA into DHEA. This may
320 contribute to why an increase in EPEA (albeit not significant) but not DHEA is observed in these
321 individuals.

322 EPA and DHA induced regulation of the endocannabinoid system in human adipose tissue has been
323 reported previously in only one study. In that study, scWAT EPEA and DHEA increased with
324 increased n-3 PUFA intake and positively correlated with the n-3 index in the serum (18) The authors
325 studied 16 individuals with obesity and medication-controlled type 2 diabetes in response to a 24-
326 week fish oil intervention (2.8 g EPA + DHA/day). Although EPEA and DHEA increased, there was
327 no change in AA containing endocannabinoids (18). The current study also observed an increase in
328 EPEA in individuals living with metabolically healthy obesity, but this was less than that observed in
329 normal weight individuals and was not significant. Furthermore, the current study reports no increase
330 in the concentration of DHEA in these individuals. The composition of the fish oil used in the current
331 study differs from that used by Rossemeisl *et al.* in that it is richer in EPA than DHA. It may be that
332 by using an oil with a higher proportion of DHA than EPA is required in obese individuals to raise the
333 concentration of DHEA.

334 Why LC n-3 PUFAs affect the endocannabinoid system differently in normal weight individuals and
335 in individuals living with metabolically healthy obesity is not clear but may relate to the specific
336 metabolic changes induced by the FA or to differences in expression of genes at baseline between the
337 two groups. These data question the use of LC n-3 PUFAs in individuals living with metabolically
338 healthy obesity for the amelioration of scWAT inflammation and improvement of adipose function
339 via the endocannabinoid system. The greatest effects were limited to normal weight individuals,
340 suggesting a potential role for LC n-3 PUFAs in the prevention of adipose tissue dysfunction through

modulation of the endocannabinoid system in more healthy individuals. It seems that even in those with metabolically healthy obesity, lipid and endocannabinoid metabolism may already be dysregulated. Further exploration of the mechanisms resulting in these differences in individuals living with metabolically healthy obesity and the consequences of these observations on WAT functionality is required.

One limitation of this study is that dietary FA intake, which may be a possible explanation for baseline differences in FA composition (and lipid metabolite profile), was not measured. Despite this, individuals were recruited on the basis that they consumed no more than one portion of oily fish per week, limiting EPA and DHA intake. As full dietary data were not collected, intake of n-6 PUFA could not be examined and the contribution of these FAs to changes in FA composition (and contribution to endocannabinoid concentrations) could not be assessed. The current study has many strengths which are its sample size, its phenotyping of the individuals, the use of an intervention to potentially modify the WAT endocannabinoid profile, and compliance to this intervention which was > 90%. We have shown that 12-weeks of 1.9 g of EPA + DHA daily was adequate to increase EPA and DHA in human scWAT and to alter endocannabinoid concentrations in both normal weight and individuals living with metabolically healthy obesity. This dose of EPA + DHA is lower than previously used in a study of WAT endocannabinoids humans (18) and could be achievable by diet or a combination of diet and supplementation amongst the general public. Furthermore, we show that 12 weeks is sufficient time to increase scWAT LC n-3 PUFAs and to modulate the endocannabinoid system in normal weight individuals but we suggest that a longer intervention period or a higher LC n-3 PUFA dose is required to favour n-3 PUFA containing endocannabinoids in individuals living with metabolically healthy obesity.

Conclusion

Compared to WAT from heathy individuals, WAT from individuals living with metabolically healthy obesity has an altered FA composition, higher concentrations of two endocannabinoids (anandamide and EPEA), higher expression of *PLA2G2D* and *PLA2G4A*, and lower expression of *CNR1*. Intervention with LC n-3 PUFAs increases EPA and DHA in scWAT and alters concentrations of some endocannabinoids. Changes in scWAT endocannabinoids are different between normal weight individuals and those with metabolically healthy obesity. At the dose and duration used, LC n-3 PUFAs do not modulate the expression of genes involved in WAT endocannabinoid metabolism. Effects of LC n-3 PUFAs on scWAT appear greater in normal weight individuals than in those living with metabolically healthy obesity.

Clinical perspectives

- The endocannabinoid system has been targeted in obesity due to associations with appetite control, energy homeostasis, lipid metabolism and inflammation. Despite this, WAT of humans living with metabolically healthy obesity, in which metabolic complications including changes to lipid handling and storage (often exhibited by high blood TGs) are yet to manifest and therefore may pose the best target for intervention with LC n-3 PUFAs, is under-explored.
- An altered FA composition and endocannabinoid profile is observed in individuals living with metabolically healthy obesity. This demonstrates that the endocannabinoid system is modified in the early stages of obesity. Alteration to the endocannabinoid system may be attributed to increased expression of PLA_2 isoenzymes. LC n-3 PUFA intervention modulates WAT endocannabinoids containing EPA and DHA but individuals living with metabolically healthy obesity display some resistance to these effects.
- These findings provide novel information about the state and regulation of the endocannabinoid system in individuals living with metabolically healthy obesity and describes WAT at this stage of obesity to be somewhat responsive to dietary FA manipulation but somewhat resistant to metabolism of EPA and DHA for endocannabinoid synthesis. These data question the potential use of LC n-3 PUFA for prevention of adipose dysfunction via the endocannabinoid system.

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Figure 1. Overview of the endocannabinoid system, its regulation in obesity, and potential modulation by long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs).
AA, arachidonic acid; AEA, arachidonoylethanolamide (also called anandamide); CNR, cannabinoid receptor; DAGL, diacylglycerol lipase; DHA, docosahexaenoic acid; DHEA, docosahexaenoylethanolamide; EPA, eicosapentaenoic acid; EPEA, eicosapentaenoylethanolamide; FA, fatty acid; FAAH, fatty acid amide hydrolase; FAS, fatty acid synthase; FFA, free fatty acid; IL-2, interleukin-2; IFN-γ, interferon-gamma; LPL, lipoprotein lipase; MGL, monoacylglycerol lipase; NAPE-PLD, N-acyl phosphatidylethanolamine lipase; NAT, N-acyl transferase; PLA_2, phospholipase A_2; PLC, phospholipase C; PPAR-y, peroxisome proliferator activated receptor-gamma.

Figure 2. Consolidated Standards of Reporting Trials (CONSORT) diagram of participant inclusion and flow through the study.

Figure 3. Fatty acid composition of scWAT in normal weight individuals and individuals with metabolically healthy obesity at study entry. Data are median $\pm$ interquartile range weight % of each fatty acid as a contribution to total fatty acids measured. * $P < 0.050$, ** $P < 0.001$ between groups from Mann-Whitney U test, n-6, omega-6 fatty acids, n-3, omega-3 fatty acids, 20:4n-6, arachidonic acid, 20:5n-3, eicosapentaenoic acid (EPA), 22:6n-3, docosahexaenoic acid (DHA).

Figure 4. Concentrations of arachidonic acid containing endocannabinoids in scWAT in normal weight individuals and individuals with metabolically healthy obesity at study entry. Data are median $\pm$ interquartile range. **$P \leq 0.001$ between groups from Mann-Whitney U test. AEA, anandamide; 2-AG, 2-arachidonoyl glycerol.

Figure 5. Concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) containing endocannabinoids in scWAT from normal weight individuals and individuals with metabolically healthy obesity at study entry. Data are median $\pm$ interquartile range. *$P \leq 0.05$ between groups obtained from Mann-Whitney U test. EPEA, eicosapentaenoyl ethanolamide; DHEA, docosahexaenoyl ethanolamide.

Figure 6. Difference in gene expression (%) in scWAT from individuals with metabolically healthy obesity in comparison to normal weight individuals at study entry. Data are median + IQR. *$P < 0.05$, ** $P < 0.01$ between groups from Mann-Whitney U test. *PLA2G4A*, *PLA2G2D*, and *PLA2G7* data obtained by RNA-Seq; all other data obtained by qRT-PCR. *SLC27A1*, long chain fatty acid transport protein, *SLC27A2* very long chain acyl- CoA synthetase, are involved in long chain FA transport and activation, *PLA2G4A*, *PLA2G2D* and *PLA2G7* encode phospholipase A2 enzymes involved in fatty acid mobilisation from the membrane, *CNR1*, cannabinoid receptor 1; *FAAH*, fatty acid amide

hydrolase, *MGLL*, monoacylglycerol lipase, *DAGLα*, diacylglycerol lipase-α, and *NAPE-PLD*, N-acyl phospholipase-D encode enzymes involved in the synthesis and degradation of the endocannabinoids.

Figure 7. Percentage change in proportion of EPA, DPA, and DHA in scWAT from normal weight individuals and individuals with metabolically healthy obesity in response to 12-week fish oil or corn oil intervention. Data are median + interquartile range. *$P < 0.05$, **$P < 0.001$ from Wilcoxon model comparing week-12 vs week-0 data split by treatment and BMI group. % is calculated as the % of EPA, DHA and DPA as a proportion of total fatty acids measured. EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Figure 8. Percentage change in concentrations of 2-AG, AEA, EPEA and DHEA in scWAT from normal weight individuals and individuals with metabolically healthy obesity in response to 12-week fish oil or corn oil intervention. Data are median + interquartile range. *$P < 0.05$ from a Wilcoxon model comparing week-12 vs week-0 data split by treatment and BMI group. AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; EPEA, eicosapentaenoyl ethanolamide; DHEA, docosahexaenoyl ethanolamide.