Gastric bypass in morbid obese patients is associated with reduction in adipose tissue inflammation via N-oleoylethanolamide (OEA)-mediated pathways

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Summary

Paradoxically, morbid obesity was suggested to protect from cardiovascular co-morbidities as compared to overweight/obese patients. We hypothesise that this paradox could be inferred to modulation of the "endocannabinoid" system on systemic and subcutaneous adipose tissue (SAT) inflammation. We designed a translational project including clinical and *in vitro* studies at Geneva University Hospital. Morbid obese subjects (n=11) were submitted to gastric bypass surgery (GBS) and followed up for one year (post-GBS). Insulin resistance and circulating and SAT levels of endocannabinoids, adipocytokines and CC chemokines were assessed pre- and post-GBS and compared to a control group of normal and overweight subjects (CTL) (n=20). *In vitro* cultures with 3T3-L1 adipocytes were used to validate findings from clinical results. Morbid obese subjects had baseline lower insulin sensitivity and higher hs-CRP, leptin, CCL5 and anandamide (AEA) levels as compared to CTL. GBS induced a massive weight and fat mass loss, improved insulin sensitivity and lipid profile, decreased C-reactive protein, leptin, and CCL2 levels. In SAT, increased expression of resistin, CCL2, CCL5 and tumour necrosis factor and reduced MGLL were shown in morbid obese patients pre-GBS when compared to CTL. GBS increased all endocannabinoids and reduced adipocytokines and CC chemokines. In morbid obese SAT, inverse correlations independent of body mass index were shown between palmitoylethanolamide (PEA) and N-oleoylethanolamide (OEA) levels and inflammatory molecules. In vitro, OEA inhibited CCL2 secretion from adipocytes via ERK1/2 activation. In conclusion, GBS was associated with relevant clinical, metabolic and inflammatory improvements, increasing endocannabinoid levels in SAT. OEA directly reduced CCL2 secretion via ERK1/2 activation in adipocytes.

Keywords

Endocannabinoids, obesity, subcutaneous adipose tissue, chemokine

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Introduction

Although some recent controversies on the paradoxical cardiovascular protection in subjects with an extreme stage of obesity (1, 2), this disease has been described as a pandemic condition that has to be treated also by surgical strategies to reduce the associated cardio-metabolic risk (3). The reduction of body weight and fat mass was shown to improve systemic inflammation underlying metabolic and cardiovascular co-morbidities in obese subjects (4). Several soluble molecules produced by visceral, perivascular or subcutaneous adipose tissues (SAT) and released in the circulation were shown to accelerate arterial dysfunction, atherogenesis and increase insulin resistance (5–7). To further complicate this panel, elevated plasmatic levels of endogenous cannabinoids (endocannabinoids) were also shown to be associated with coronary dysfunction in obesity and atherosclerotic plaque vulnerability (8–10). The "endocannabinoid" system is composed of active molecules (such as anandamide N-arachidonoylethanolamide [AEA],

2-arachidonoylglycerol [2-AG], N-palmitoylethanolamide [PEA], N-oleoylethanolamide [OEA]) that bind transmembrane (cannabinoid receptor type 1 (CB_1) and –2 (CB_2), GPR55, GPR119) or intracellular (PPAR-alpha) receptors and are degraded by enzymes (fatty acid amide hydrolase [FAAH] and monoacylglycerol lipase [MGLL]) (11). The role of this system on SAT inflammation in morbid obesity and weight loss after gastric bypass surgery (GBS) remains to be elucidated. Here, we aimed at investigating the systemic and SAT expression of the endocannabinoid system in morbid obese patients before and one year after GBS as compared to a control group of normal body weight or overweight subjects. Then, we assessed potential correlations between systemic and SAT levels of endocannabinoids and inflammatory biomarkers (such as insulin, adipocytokines and CC chemokines). Finally, we explored *in vitro* on cultured adipocytes if stimulation with endocannabinoids might directly affect the release of inflammatory molecules and the activation of underlying intracellular pathways.

Methods

Study population and design

This is a pilot, prospective, longitudinal case-control study, enrolling eleven subjects (cases: 7 females, 4 males) with morbid obesity (defined as body mass index [BMI] ≥ 40 kg/m^2) pre- and post-gastric bypass surgery (GBS) and a control group (control: 9 females, 11 males) of normal body weight or overweight subjects (BMI <30 kg/m^2) (8). All subjects enrolled had no arterial hypertension (blood pressure $<140/90$ mmHg), smoking, and diabetes mellitus (fasting plasma glucose obtained on more than two occasions ≤ 126 mg/dl). Similarly to previous studies by our group (8, 9, 12), all cases and controls underwent an initial screening visit that comprised a physical examination, electrocardiogram (ECG), blood pressure measurements, euglycaemic hyperinsulinaemic clamp test, routine blood chemistry as well as plasma and serum sampling in a fasting state. Exclusion criteria were: taking any cardiac or vasoactive medication (such as angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, calcium channel blockers, statins, or beta-blockers), any history of variant angina, family history of premature coronary artery diseases or clinically manifest cardiovascular disease. Exclusively morbid obese subjects underwent to GBS within one month from first visit. The standard operation involved Roux-en-Y gastric bypass surgery with an isolated gastric pouch <2 ounces and a 100 cm distal bowel limb. One year post-GBS, morbid obese subjects were submitted to a final physical examination, ECG, blood pressure measurements, euglycaemic hyperinsulinaemic clamp test, SAT biopsy, routine blood chemistry as well as plasma and serum sampling in a fasting state. Nobody between these morbid obese patients rejected the consent to any of these procedures after GBS. The study protocol was approved by the local ethical committee. All participants gave their written informed consent before study enrolment.

Measurement of cardiometabolic and anthropometric parameters

Sitting blood pressure and heart rate are the mean values of three measurements (OMRON 705 cp, OMRON Healthcare Europe, The Netherlands). Waist circumference was measured at the midpoint between the iliac crest and costal margin, while the hip circumference was taken at the widest point around the hips. Fat mass, fat free mass and lean body mass were measured by dual-energy X-ray absorptiometry.

Insulin sensitivity

Insulin sensitivity was evaluated by euglycaemic hyperinsulinaemic clamp (13). Insulin was continuously infused at a rate of 240 pmol·min^{-1}·m^{-2}, whereas a glucose solution (20%) was infused at variable rates in order to maintain a constant plasma glucose concentration between 4.5 and 5.5 mmol/l. Plasma glucose was measured at 5– to 10-minute (min) intervals to ensure it remained within the target glucose concentration. The steady-state period (for calculation of insulin sensitivity) was between 80 min to 120 min. Glucose uptake, measured during the steady state period, was expressed in mmol/kg fat free mass/min (mmol/kg ffm/min).

Inflammatory markers and lipid profile assessment

Human serum CCL2, CCL5, resistin and leptin were measured by colourimetric enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA), following manufacturer's instructions. The limits of detection were 15.6 pg/ml for CCL2, 15.60 pg/ml for CCL5, 31.25 pg/ml for resistin, 31.25 pg/ml for leptin. Mean intra- and inter-assay coefficients of variation (CV) were below 8% for all markers.

Mouse resistin, CCL2, and CCL5 in adipocyte supernatants were measured by colourimetric enzyme-linked immunosorbent assay (R&D Systems), following manufacturer's instructions. The limits of detection were 15.6 pg/ml for resistin, 3.91 pg/ml for CCL2 and 31.2 pg/ml CCL5. Mean intra- and inter-assay coefficients of variation (CV) were below 6% for all markers.

Total cholesterol, high-density lipoprotein cholesterol (HDL-c) and triglycerides were measured by Roche enzymatic colourimetric methods for modular systems (Hoffmann La-Roche Ltd, Basel, Switzerland). Low-density lipoprotein cholesterol (LDL-c) concentration was calculated by the Friedewald formula (14). Glucose, insulin and high sensitivity-C-reactive protein (hs-CRP) were routinely measured.

Endocannabinoids measurement

Endocannabinoid plasma levels in humans were determined from peripheral arm vein blood samples. AEA, 2-AG, OEA and PEA were extracted from 100 µl of human plasma by liquid–liquid extraction and separated by liquid chromatography (Ultimate 3000 RS, Dionex, CA, USA). Analyses were performed on a 5500

QTrap (triple quadrupole/linear ion trap) mass spectrometer equipped with a TurboIon-Spray™ interface (AB Sciex, Concord, ON, Canada) (9, 15). In human SAT, the EC concentrations were measured as described above, with the exception 20 mg of tissue that was homogenised by a ball mill during LLE. The adipose tissue samples were obtained by needle biopsy. The abdominal region at the level of the left lower quadrant was sterilized, and 5 ml 2% lidocaine without adrenalin was injected for local anesthesia. The biopsy was made by a 14-gauge needle connected to a 50 ml syringe. Fat tissue was aspirated as suction was applied while the syringe and needle were repeatedly rotated. Five to eight ml of fat and fluid were aspirated, washed, and immediately placed in liquid nitrogen at –180 °C.

Real-time PCR

Total RNA was isolated with Tri-reagent (MRC Inc.) from specimens of human SAT and mice differentiated 3T3-L1 cells. Reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Real-time PCR (StepOne Plus, Applied Biosystems, Foster City, CA, USA) was performed with the ABsolute™ QPCR Mix (ABgene). Specific human primers and probes (Suppl. Table 1, available online at www.thrombosis-online.com) were used to determine the mRNA expression of FAAH, MGLL, CB1 and CB2 receptors, human PPARα, leptin, leptin receptor, resistin CCL2, CCL5, tumour necrosis factor (TNF), and RPS13 (housekeeping gene). Specific mouse primers and probes (Suppl. Table 2, available online at www.thrombosis-online.com) were also used to determine the mRNA expression of Fabp4 and HPRT (housekeeping gene). The fold change of mRNA levels was calculated by the comparative C_t method. The resultant C_t values were first normalised to the internal control. This was achieved by calculating a delta C_t (ΔC_t) by subtracting the internal control C_t values from the RPS13 or HPRT C_t value. A delta delta C_t ($\Delta\Delta C_t$) was calculated by subtracting the designated control ΔC_t value from the other ΔC_t values. The $\Delta\Delta C_t$ was then plotted as a relative fold change with the following formula: $2^{-\Delta\Delta Ct}$.

Cell differentiation and culture

The immortalised cell line 3T3-L1 of murine fibroblasts was stored in liquid nitrogen, at -120°C until thawed, plated and grown in DMEM-HG (Dulbecco's Modified Eagle Medium high glucose, Gibco, Life Technologies, Carlsbad, CA, USA) culture medium supplemented with 10% new born calf serum (10% NBCS, Gibco). On reaching confluence, the cells were harvested after trypsinisation and distributed into 6- or 12-well plates. The fibroblasts were grown to 100% confluence, at which time differentiation into adipocytes step then initiated, according to a modified established protocol (16). In order to induce 3T3-L1 cell differentiation into adipocytes, the culture medium was substituted with DMEM-HG medium containing 10% fetal bovine serum (FBS), 5 µg/ml insulin (Sigma-Aldrich, Saint Louis, MO, USA), 1 µM dexamethasone (Sigma) and 500 µM 3-isobutyl 1-methylxanthine (IBMX, Sigma). After 72 hours (h) of incubation, differentiation medium was replaced by DMEM-HG medium containing 10% of FBS and 5 µg/ml insulin. This medium was refreshed every 2–3 days and after 7–10 days, about 80% of the fibroblasts had differentiated into adipocytes. The formation of lipid droplets and the increase of the fatty acid binding protein-4 (Fabp4) (17), characteristics of 3-T3-L1 cell differentiation into adipocytes, were confirmed by Oil Red O (Sigma) staining and real-time PCR (Suppl. Table 2, available online at www.thrombosis-online.com).

In parallel experiments, after 12-h starvation in serum-free DMEM supplemented with antibiotics, differentiated 3T3-L1 adipocytes were incubated for 0–1 h (time-course experiments) or 24 h (secretion experiments) in the presence of different concentrations (10^{-7} M to 10^{-5} M) from of endocannabinoids AEA, OEA, PEA and 2-AG (Cayman Chemical Company, Ann Arbor, USA) in serum-free DMEM-HG with antibiotics. The kinase inhibitors LY294002 (PI3K inhibitor, at 1 µM, from Sigma-Aldrich), U0126 (MEK inhibitor, at 0.1 µM, from Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA) and WP1066 (Stat3 inhibitor, at 10 µM, from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were pre-incubated for 1 h before the administration of endocannabinoids. After the stimulations, conditioned media and cells were collected and stored at – 80 °C until further analyses. In all experimental conditions, we observed by trypan blue (Sigma) exclusion that cell viability was near 100% (data not shown).

PCR

Total RNA was extracted from 3T3-L1 cells and adipocytes with Tri-reagent (MRC Inc.) following the manufacturer's instructions. The concentration and purity of RNA were determined spectrophotometrically, and cDNA was prepared from 500–1000 ng total RNA using the ImProm-II Reverse Transcription System (Promega) as suggested by the manufacturer. Amplification by PCR was performed in a Peqstar thermocycler (Peqlab, Erlangen, Germany), using CNR1, CNR2, GPR55, GPR119, PPARA and HPRT (house-keeping gene) specific primers (Suppl. Table 2, available online at www.thrombosis-online.com). After 35 PCR cycles, amplicons were analyzed by electrophoresis on a 2% agarose gel.

Western blot analysis

Proteins from 3T3-L1 adipocytes were extracted in lysis buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.05% SDS, 10 mM NaF, 1 mM PMSF, 2 mM Na3VO4, and complete protease inhibitor cocktail tablet (Roche, Basel, Switzerland). Proteins (10–20 µg per lane) were electrophoresed through polyacrylamide/SDS gels and transferred by electroblotting onto PVDF membranes. Membranes were blocked for 1 h in 5% (w/v) nonfat milk before O/N incubation with appropriate dilutions of primary phospho-specific anti-mouse ERK 1/2 (R&D Systems), anti-mouse AKT (Santa Cruz Biotechnology) or anti-mouse STAT-3 serine 472 or tyrosine 705 antibodies (both from Cell Signaling Technology, Danvers, MA, USA), as well as corresponding secondary antibodies. The blots were developed using the ECL system

(Immobilion™ Western, Millipore, Billerica, MA, USA). To verify equal loading of the total proteins, membranes were stripped (for 15 min at 50 C in Tris–HCL pH 6.7, 2% SDS and 0.1 M β-mercaptoethanol), reblocked and reprobed to detect total ERK 1/2 (R&D Systems), AKT (Santa Cruz Biotechnology) or STAT-3 (Cell Signaling Technology). Relative intensities were calculated by dividing the phosphorylated protein through the total protein.

Statistical analyses

Pearson's Chi-square test or Fisher's exact test, when appropriate, for the comparison of qualitative variables and Mann-Whitney nonparametric test (the normality assumption of the variables' distribution in both groups was violated) for comparisons of continuous variables were used to compare differences between Controls and Pre-GBS. Wilcoxon test for related samples was used for analysing differences between Pre-GBS and Post-GBS. Qualitative variables for in clinical and laboratory data of human results were expressed as absolute frequencies. Quantitative continuous variables were expressed as medians (interquartile range [IQR]: 25th to 75th percentile: quartile 1, quartile 3). Linear relationships among SAT endocannabinoids and SAT mRNA inflammatory molecule expression (ΔCt) were computed by Spearman's correlations. Multivariable analysis was performed with the linear regression model. *In vitro* results were expressed as mean ± SEM or medians (IQR). One-way ANOVA was used for multiple group comparison, while Mann-Whitney nonparametric test for two group comparison. All statistical analyses were performed with the STATA software (v.10, Stata Corporation, College Station, TX, USA). Values of $p<0.05$ (two-tailed) were considered significant.

Results

GBS is associated with improvements in clinical and metabolic features in morbid obese patients

Differences on anthropometrical characteristics, metabolic and inflammatory serum profile, and plasma endocannabinoids between controls and baseline morbid obese patients (pre-GBS) and between pre-GBS and one-year post-GBS were detailed in ▶Table 1. No difference on age and sex were shown between CTL and morbid obese subjects (▶Table 1). As an additional control of patient characteristics, CTL (normal and overweight patients) has significantly reduced BMI, fat mass, waist and hip circumferences as compared to baseline morbid obese (pre-GBS) (▶Table 1). Baseline fasting serum levels of glucose, total cholesterol, LDL-c, HDL-c and triglycerides were similar in CTL and pre-GBS morbid obese patients (▶Table 1). As expected, the pre-GBS group showed lower insulin sensitivity and higher fasting insulin levels as compared to CTL. GBS in morbid obese subjects induced a massif weight loss (BMI: 43.3 kg/m^2 [41.2–44.7] vs 27.9 kg/m^2 [25.7–32.1]), with decrease of fat mass (50% [47–54] vs 30% [25–34]), and both waist (122 cm [109–131] vs 92 cm [84–102]) and hip circumferences (130 cm [124–134] vs 106 cm [100–114]) (▶Table 1). On the other hand, GBS in morbid obese was shown to exclusively reduce total cholesterol and LDL-c serum levels at one-year follow up (in post-GBS as compared to pre-GBS) (▶Table 1). The alterations in insulin-related parameters in morbid obese pre-GBS were significantly improved one year post-GBS (▶Table 1).

GBS is associated with partial reduction in systemic inflammatory biomarkers, but does not affect endocannabinoid serum levels

Considering circulating levels of endocannabinoids and inflammatory biomarkers potentially related with adipose tissue inflammation in obesity (18), CTL and morbid obeses pre-GBS had comparable plasma levels of endocannabinoids, except for AEA levels that were increased in pre-GBs group. Pre-GBS morbid obese group had increased serum levels of CRP, leptin, and CCL5 as compared to CTL. No difference between CTL and pre-GBS was reported in CCL2 levels, while a significant reduction in resistin levels was shown in pre-GBS as compared to CTL. GBS did not alter endocannabinoid circulating levels in morbid obesity, showing similar levels in the pre-GBS and post-GBs group (▶Table 1). Post-GBS patients had decreased levels of CRP, leptin, CCL2, CCL5 and increased serum resistin as compared to pre-GBS (▶Table 1).

GBS is associated with enhancement of endocannabinoid levels and reduction in inflammatory molecule expression in SAT

In SAT, no significant differences were shown in AEA, 2-AG, PEA and OEA levels between CTL and pre-GBS groups (▶Figure 1A-D). Post-GBS group had significant increases in all endocannabinoid SAT levels as compared to pre-GBS (▶Figure 1A-D). Considering endocannabinoid degrading enzymes, CTL and pre-GBS groups had similar mRNA expression levels of FAAH (Suppl. Figure 1A, available online at www.thrombosis-online.com). However, MGLL mRNA levels were reduced in pre-GBS morbid obeses when compared to CTL (Suppl. Figure 1B, available online at www.thrombosis-online.com). Interestingly, in patients post-GBS, the mRNA levels of FAAH and MGLL were reduced when compared to pre-GBS (Suppl. Figure 1A and B, available online at www.thrombosis-online.com). Regarding endocannabinoid receptors, we focused on CB_1, CB_2 and PPAR-alpha mRNA expression (19–21). No difference was observed in the expression of these three receptors between CTL and pre-GBS morbid obeses (Suppl. Figure 2A-C, available online at www.thrombosis-online.com). Post-GBS group had significant reduction in both CB_1 and PPAR-alpha mRNA expression as compared to CTL and significant reduction in PPAR-alpha mRNA as compared pre-GBS (Suppl. Figure 2A and C, available online at www.thrombosis-online.com). No effect was induced by GBS on CB_2 mRNA expression (Suppl. Figure 2B, available online at www.thrombosis-online.com). Considering inflammatory molecules in SAT, no differences were shown in leptin and leptin receptor mRNA levels between CTL and pre-GBS (Suppl. Figure 3A and B, available online at

www.thrombosis-online.com). In contrast, resistin mRNA expression levels were increased of pre-GBS morbid obeses as compared to CTL (Suppl. Figure 3C, available online at www.thrombosis-online.com). Interestingly, both leptin and resistin levels were reduced in post-GBS as compared to pre-GBS (Suppl. Figure 3A and C, available online at www.thrombosis-online.com). CC chemokines (CCL2 and CCL5) and pro-inflammatory cytokines (TNF) were up regulated in SAT of morbid obese pre-GBS as compared to CTL (►Figure 2A-C). SAT levels of these three inflammatory molecules were abrogated in post-GBS as compared to pre-GBS (►Figure 2A-C). Strikingly, post-GBS mRNA levels of TNF were lower than in CTL (►Figure 2C). Finally, we explored potential correlation between endocannabinoid concentrations and inflammatory mediator mRNA expression in human SAT. First, baseline values of morbid obese (pre-GBS, n=11) and CTL (n=20) were included in these calculations (Suppl. Tables 3 and 4, available online at www.thrombosis-online.com). Then, SAT values of morbid obese (pre-GBS and post-GBS, n=22) were also investigated (►Table 2 and ►Table 3). As a parameter for mRNA gene expression we used ΔCT values defined as the distance between cycles of the target and hosekeeping genes, as previously performed (22). Thus, high ΔCT value represents a low expression of the target gene. At baseline in pre-GBS and CTL, SAT levels of AEA correlated inversely with SAT resistin ΔCT (r=-0.38, p=0.04), while 2-AG levels did not correlate with any of the parameter assessed (Suppl. Table 3, available online at www.thrombosis-online.com). SAT PEA and OEA levels positively correlated with resistin ΔCT, CCL2 ΔCT and CCL5 ΔCT, indicating that high levels of

Table 1: Characteristics of the study population.

Characteristics	CTL* (n=20)	Pre-GBS† (n=11)	P-value vs CTL	Post-GBS‡ (n=11)	P-value vs Pre-GBS
Clinical					
Age, years (IQR)	40 (33–47)	35 (32–42)	0.444	-	-
Males, n (%)	11 (55.0)	4 (36.4)	0.109	4 (36.4)	-
BMI§, kg/m² (IQR)	25.2 (22.0–26.7)	43.3 (41.2, 44.7)	<0.0001	27.9 (25.7–32.1)	0.001
Fat mass, % (IQR)	23 (19–27)	50 (47–54)	<0.0001	30 (25–34)	0.0002
Waist circumference, cm (IQR)	80 (72–91)	122 (109–131)	<0.0001	92 (84–102)	0.001
Hip, cm (IQR)	95 (91–99)	130 (124–134)	<0.0001	106 (100–114)	0.001
Serum metabolic and lipid profile					
Fasting glucose, mg/dl (IQR)	99.1 (91.9–106.3)	95.5 (84.7–102.7)	0.948	87.3 (85.5–105.5)	0.848
Fasting insulin, mUI/l (IQR)	4.4 (1.9–8.1)	22.0 (21.1–28.5)	0.011	4.1 (1.9–15.2)	0.004
M/Insulin value, mmol/min/kg ffm/mmol ins (IQR)	11.3 (9.9–13.4)	4.8 (4.4–5.8)	0.002	8.7 (6.3–9.6)	0.009
Cholesterol, mg/dl (IQR)	207 (181–238)	197 (176–207)	0.562	144 (117–164)	0.027
LDL-c‖, mg/dl (IQR)	126 (116–146)	124 (108–144)	0.703	78 (59–87)	0.010
HDL-c#, mg/dl (IQR)	49 (41–61)	43 (40–47)	0.201	49 (40–62)	0.391
Triglycerides, mg/dl (IQR)	83 (53–118)	98 (64–155)	0.330	75 (52–102)	0.391
Plasma endocannabinoids					
AEA**, ng/ml (IQR)	0.52 (0.48–0.67)	0.71 (0.59–0.80)	0.009	0.69 (0.48–0.91)	0.896
2-AG††, ng/ml (IQR)	1.82 (0.67–5.40)	3.53 (1.30–7.70)	0.173	2.06 (0.98–9.01)	0.491
PEA‡‡, ng/ml (IQR)	2.56 (2.40–3.00)	2.84 (2.50–3.12)	0.311	2.17 (1.83–2.72)	0.066
OEA§§, ng/ml (IQR)	2.46 (2.07–2.85)	2.40 (2.28–2.97)	0.522	2.86 (2.73–3.69)	0.158
Serum inflammatory biomarkers					
hs-CRP‖‖, mg/l (IQR)	0.9 (0.9–3.0)	8.5 (3.3–11.9)	0.001	1.5 (0.9–2.6)	0.018
Leptin, ng/ml (IQR)	11.2 (5.3–25.1)	122.1 (81.2–153.1)	0.001	4.3 (3.2–17.2)	0.004
Resistin, ng/ml (IQR)	8.1 (6.0–15.1)	3.8 (1.9–5.5)	0.007	9.3 (6.7–12.5)	0.03
CCL2, pg/ml (IQR)	44.6 (30.1–74.4)	46.1 (25.0–60.3)	0.647	15.6 (15.6–15.6)	0.003
CCL5, ng/ml (IQR)	33.4 (20.8–54.7)	59.7 (42.2–90.7)	0.042	22.2 (14.6–27.5)	0.008

Continuous variables are expressed as median (interquartile range [IQR]). * CTL: controls. † Pre-GBS: before gastric bypass surgery. ‡ Post-GBS: after gastric bypass surgery. § BMI: body mass index. ‖ LDL-c: low-density lipoprotein cholesterol. # HDL-c: high-density lipoprotein cholesterol. ** AEA: anandamide. †† 2-AG: 2-arachidonoylglycerol. ‡‡ PEA: N-palmitoyl-ethanolamide. §§ OEA: Oleoylethanolamide. ‖‖ hs-CRP: high sensitivity C-reactive protein.

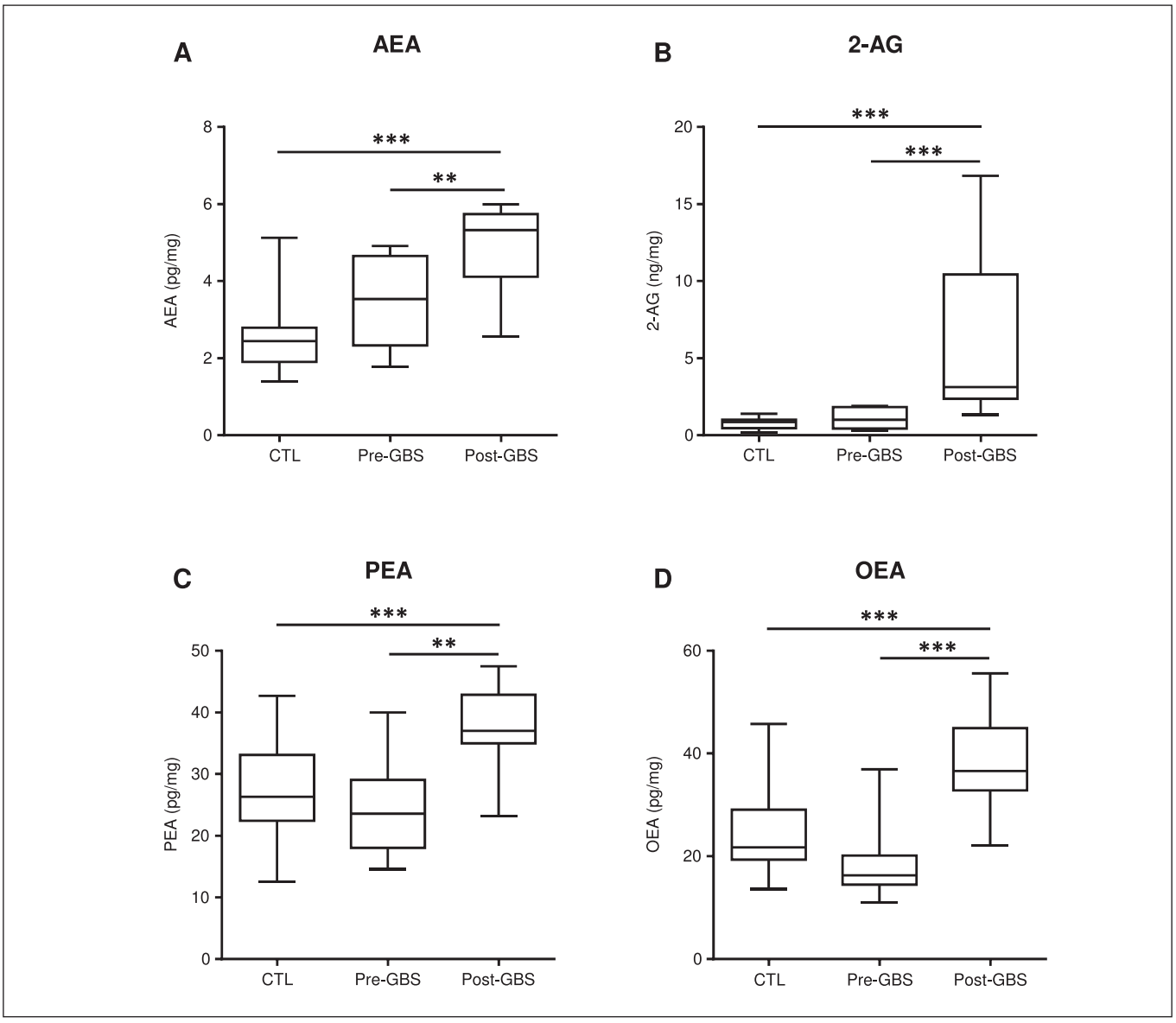

Figure 1: GBS is associated with increased SAT levels of endocannabinoids. Levels of endocannabinoids AEA (A), 2-AG (B), PEA (C), OEA (D) in human SAT of controls (CTL, n=20), morbid obese patients before gastric bypass surgery (GBS) (Pre-GBS, n=11) and morbid obese patients after GBS (Post-GBS, n=11), were determined by mass spectrometry after liquid–liquid extraction and separation by liquid chromatography. Data are expressed as medians (interquartile range). *** $p<0.001$.

PEA and OEA in SAT are associated with low mRNA expression levels of resistin and CC chemokines (Suppl. Table 3, available online at www.thrombosis-online.com). Univariate linear regression model confirmed positive association between SAT PEA and OEA and inflammatory molecules (Suppl. Table 4, available online at www.thrombosis-online.com). Multivariate analysis adjusted for age, gender and BMI did not confirm any significant association between SAT endocannabinoids and mRNA expression levels of resistin and CC chemokines, indicating that these parameters are important confounding factors in a cohort enrolling morbid obese patients pre-GBS and CTL (Suppl. Table 4, available online at www.thrombosis-online.com). Considering these associations in morbid obese patients pre- and post-GBS, significant positive associations between SAT PEA and OEA levels and resistin ΔCT, CCL2 ΔCT and CCL5 ΔCT were showed (▶Table 2). SAT OEA also positively correlated with leptin ΔCT, indicating that high SAT levels of endocannabinoids were better associated with low mRNA expression levels of several inflammatory molecules in morbid obese patients pre- and post-GBS (▶Table 2). Linear regression model confirmed these associations also when adjusted for age, gender and body mass index, indicating that SAT PEA and SAT OEA levels in morbid obese subjects pre- and post-GBS were associated with low mRNA expression levels of inflammatory molecules independently of these potential confounding factors (▶Table 3).

Among different endocannabinoids, OEA reduces CCL2 *in vitro* secretion from adipocytes via activation of ERK1/2-mediated pathways

Based upon our clinical results, we tested whether PEA and OEA could behave as anti-inflammatory molecules by down regulating secretion of resistin, CCL2 and CCL5 in cultured adipocytes. In all experimental conditions, we did not show any effect on cell viability that was near 100% (trypan blue staining, data not shown). We used a validated *in vitro* model of murine adipocytes, differentiated from 3T3-L1 cells, as previously described (23). Adipocyte differentiation was verified by testing Fabp mRNA expression and by Oil Red O staining (Suppl. Figure 4A and B, available online at www.thrombosis-online.com). Adipocytes displayed a significant Fabp mRNA up regulation and were positive at Oil Red O staining as compared to undifferentiated 3T3-L1 cells (Suppl. Figure 4A and B, available online at www.thrombosis-online.com). Similarly to 3T3-L1 cells, adipocytes were shown to express CB type 1 recep-

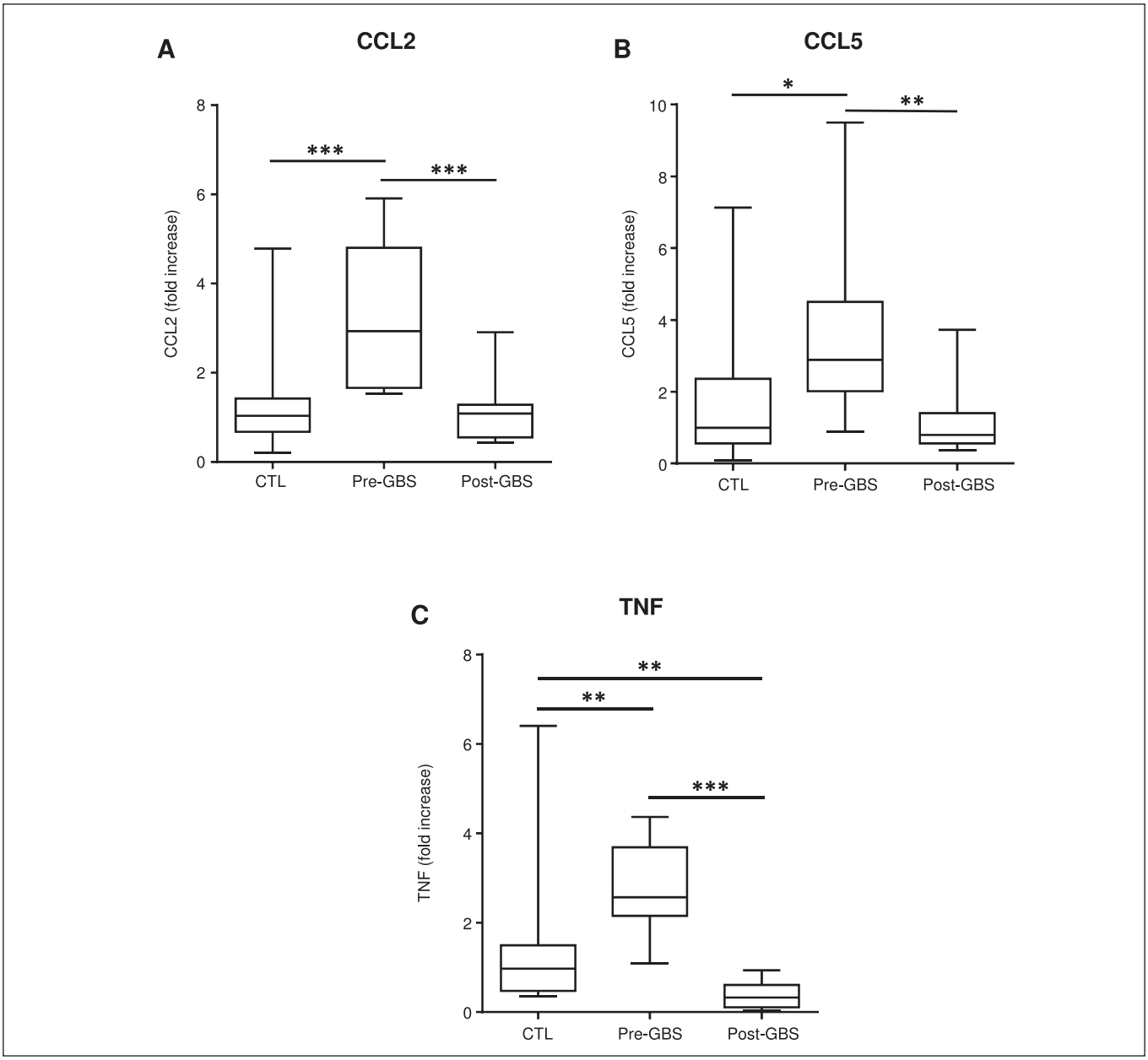

Figure 2: GBS is associated with reduction in SAT mRNA levels of CCL2, CCL5 and TNF. mRNA levels of CCL2 (A), CCL5 (B) and TNF (C) in human SAT of controls (CTL, n=20), morbid obese patients before gastric bypass surgery (GBS) (Pre-GBS, n=8) and morbid obese patients after GBS (Post-GBS, n=9), were determined by real time RT-PCR. Relative expression normalised to RPS13 (housekeeping gene) was calculated with the comparative Ct method and shown as fold change of mRNA levels. Data are expressed as medians (interquartile range). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Table 2: Spearman rank correlation between levels of endocannabinoids and inflammatory mediators in human SAT in morbid obese patients pre-GBS and post-GBS.

	Spearman's correlation coefficient (r)	P-value
SAT AEA*		
vs leptin mRNA, DCT	0.29	0.18
vs resistin mRNA, DCT	0.60	0.003
vs CCL2 mRNA, DCT	0.38	0.07
vs CCL5 mRNA, DCT	0.61	0.002
SAT 2-AG†		
vs leptin mRNA, DCT	0.57	0.004
vs resistin mRNA, DCT	0.35	0.10
vs CCL2 mRNA, DCT	0.53	0.009
vs CCL5 mRNA, DCT	0.41	0.04
SAT PEA‡		
vs leptin mRNA, DCT	0.38	0.07
vs resistin mRNA, DCT	0.67	<0.001
vs CCL2 mRNA, DCT	0.48	0.02
vs CCL5 mRNA, DCT	0.60	0.003
SAT OEA§		
vs leptin mRNA, DCT	0.53	0.01
vs resistin mRNA, DCT	0.74	<0.001
vs CCL2 mRNA, DCT	0.73	<0.001
vs CCL5 mRNA, DCT	0.59	0.003

* AEA: anandamide. † 2-AG: 2-arachidonoylglycerol. ‡ PEA: N-palmitoyl-ethanolamide. § OEA: oleoylethanolamide.

Table 3: Linear regression between PEA and OEA and mRNA expression levels of resistin, CCL2 and CCL5 in SAT in morbid obese patients pre- and post-GBS.

	Univariate model		Multivariate model	
	β	P-value	β	P-value
SAT PEA*				
resistin mRNA, DCT	3.76	0.001	3.42	0.01
Age, years	-023	0.50	-0.06	0.78
Gender, male	7.98	0.15	-1.22	0.76
BMI†	0.03	0.91	-0.11	0.52
SAT PEA				
CCL2 mRNA, DCT	4.62	0.025	3.37	0.19
Age, years			0.08	0.73
Gender, male			-0.53	0.91
BMI			-0.17	0.40
SAT PEA				
CCL5 mRNA, DCT	4.23	0.001	4.27	0.004
Age, years			0.23	0.25
Gender, male			2.68	0.51
BMI			-0.10	0.49
SAT OEA‡				
resistin mRNA, DCT	5.62	<0.001	4.33	0.01
Age, years	-0.22	0.47	0.01	0.98
Gender, male	9.26	0.05	-0.85	0.87
BMI	-0.07	0.73	-0.34	0.11
SAT OEA				
CCL2 mRNA, DCT	9.35	<0.001	7.52	0.01
Age, years			0.20	0.44
Gender, male			2.07	0.70
BMI			-0.28	0.20
SAT OEA				
CCL5 mRNA, DCT	4.89	0.009	4.07	0.04
Age, years			0.32	0.26
Gender, male			2.41	0.67
BMI			-0.41	0.07

* PEA: N-palmitoyl-ethanolamide. † BMI: body mass index. ‡ OEA: oleoylethanolamide.

tor and cannabinoid orphan receptors (GPR55 and GRP119), but not CB type 2 receptor (Suppl. Figure 4C, available online at www.thrombosis-online.com). On the other hand, only differentiated adipocytes were shown to express PPAR-alpha (a validated receptor for endocannabinod-like compounds) (Suppl. Figure 4C, available online at www.thrombosis-online.com). Stimulation with different concentrations of endocannabinoids (i.e. AEA and 2-AG) or the endocannabinoid-like compounds PEA and OEA did not affect resistin secretion (Suppl. Figure 5A-D, available online at www.thrombosis-online.com). All supernatants were below the low range limit of detection (31.25 pg/ml) for CCL5 (data not shown). Also, incubation with different concentrations of endocannabinoids (i.e. AEA and 2-AG) or the endocannabinoid-like compound PEA did not affect CCL2 secretion in cultured adipocytes (►Figure 3A-C). On the contrary, stimulation with OEA dose-dependently inhibited CCL2 release from culture adipocytes (►Figure 3D). In order to identify the potential pathways involved in OEA-mediated inhibition of inflammatory molecule release, we investigated the intracellular kinases Akt, Stat3 and ERK1/2 (24, 25). Incubation with the effective concentration of OEA (10^{-5} M) for up to 60 min did not induce any phosphorylation on Akt and Stat3 (Suppl. Figure 6A-D, available online at www.thrombosis-online.com). On the other hand, stimulation with OEA for 2–15 min markedly activated ERK1/2 phosphorylation (►Figure 4A-C). Accordingly, pre-incubation with LY294002 (PI3K/Akt inhibitor) or WP1066 (Stat3 inhibitor) did

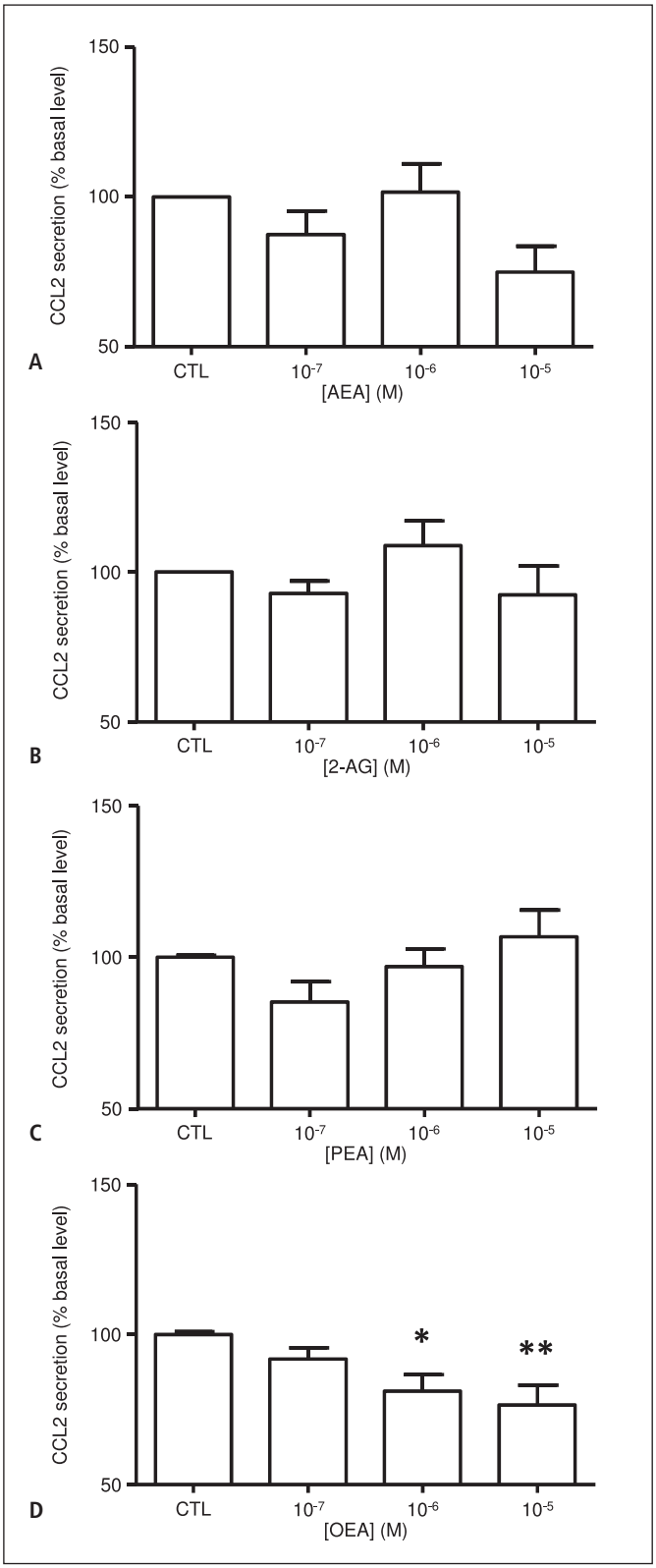

Figure 3: OEA inhibits CCL2 release *in vitro* from adipocytes. Release of CCL2 in supernatants of cells incubated in polystyrene dishes in the presence or absence of control medium (CTL), or different concentrations of AEA (A), 2-AG (B), PEA (C), OEA (D). Data are expressed as mean ± SEM of percentage of CTL, defined as 100%, n=12–14. *p<0.05 vs CTL; **p<0.01 vs CTL.

not affect OEA-mediated inhibition of CCL2 release (►Figure 5). However, pre-incubation with U0126 (MEK 1/2 inhibitor, a kinase directly activating EKR1/2) abrogated OEA-mediated effects on release of both mediators (►Figure 5). These results suggest that OEA-mediated inhibition of CCL2 release from culture adipocytes is dependent on ERK1/2-mediated pathways and does not affect phosphorylation of Akt and Stat3.

Discussion

The main results from this translational research highlighted a critical role for the endocannabinoid OEA in the inhibition of CCL2 release in adipocytes via activation of an intracellular pathway dependent on ERK1/2. Importantly, this endocannabinoid was markedly up regulated in SAT in subjects after GBS. Thus, this surgical procedure was shown to potentially play a beneficial role not only improving quality of life, metabolic and lipid profiles in morbid obese patients, but also reducing adipose tissue inflammation and related cardiovascular risk (26). In the following subparagraphs, we discussed on main items from clinical and basic research approaches.

Clinical study

As expected, our study confirmed that GBS in morbid obese patients was associated with relevant clinical benefits on fat mass, insulin resistance, lipid profile. Interestingly, we also showed marked improvements in systemic levels of CRP, adipocytokines and the CC chemokine CCL5. Post-GBS serum levels of inflammatory mediators were similar to those observed in the CTL group of normal body weight and overweight patients, indicating that this procedure could restore those inflammatory alterations associated with morbid obesity. Surprisingly, resistin serum levels were found to be reduced in morbid obese patients pre-GBS as compared to CTL or post-GBS. Those results are in contradiction with previous studies indicating that resistin serum concentrations are increased in obesity (27). The reason for this discrepancy is still elusive and could be related to several factors, such as different bioactivities in insulin resistance and extreme morbid obesity (28, 29). These speculations merit further confirmation in larger clinical trials investigating levels and activities of resistin in morbid obesity.

As a main clinical result, we did not confirm any increase of endocannabinoid levels in both plasma and SAT of morbid obese pre-GBS as compared to CTL. Several studies previously showed both activation and increase plasma and tissue levels of endocannabinoids in obese humans (30, 31) and atherosclerotic mice (32). However, morbid obesity was often mixed with obese subjects and thus, underestimated as specific obesity category. Recent evidence by our research group showed that final endocannabinoid-mediated protection on cardiovascular vulnerability were more likely to be due to CB_2 receptor up regulation rather than increase in plasma and intraplaque concentrations of endocannabinoids (22). Finally, different endocannabinoids were shown to exert dual (both protective and detrimental) activities depending on the sub-

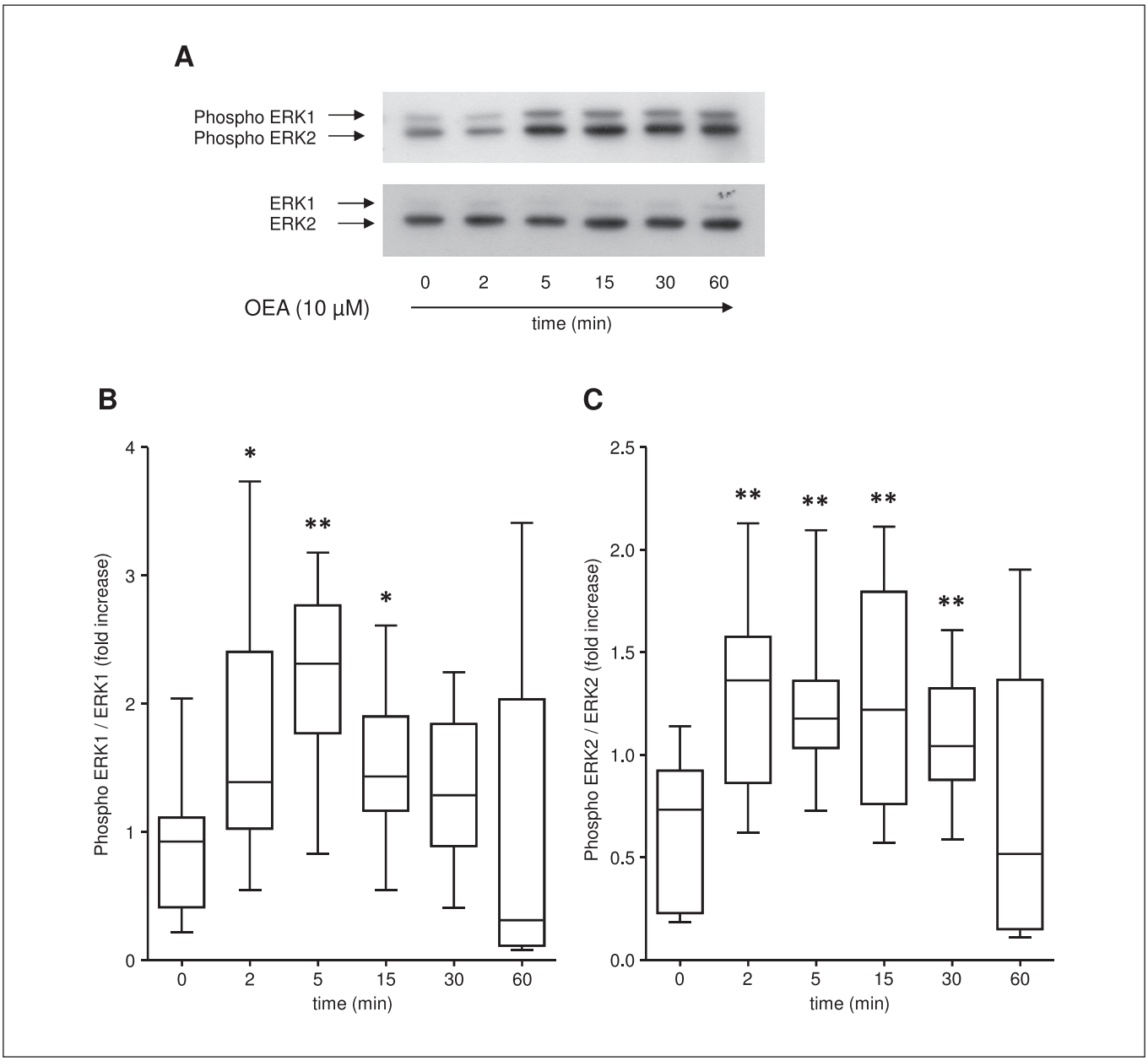

Figure 4: OEA increases ERK1/2 phosphorylation in adipocytes. Representative western blot (A) and densitometric analysis (B, C) of ERK1/2 phosphorylation in the presence or absence of control medium (time 0), or OEA (time course: 2 min, 5 min, 15 min, 30 min and 60 min, all at 10^{-5} M). Values were normalised to total amounts of ERK1/2, respectively, and expressed as percentage of control medium (time 0), defined as 1. Data are expressed as median (interquartile range) (n=11). *p<0.05 vs time 0; **p<0.01 vs time 0.

class of cannabinoid receptors they bind to, on the organ involved as well as the subset of inflammatory cells considered (11). Importantly, we showed that GBS was associated with a marked increase in the concentration of AEA, 2-AG, PEA and OEA in SAT as compared to pre-GBS samples in the same morbid obese patients. This general peak was potentially related with the concomitant modest down regulation of endocannabinoid degrading enzymes FAAH and MGLL in the same SAT samples. This endocannabinoid profile in SAT from patients post-GBS was also accompanied with down regulation of CB1 and PPAR-alpha mRNA expression as well as abrogation of inflammatory molecules (such as leptin, resistin, CCL2, CCL5 and TNF).

The different correlations between endocannabinoids and inflammatory molecules within human SAT suggested some potential pathophysiological mechanisms mediated by certain endocannabinoids (i.e. PEA and OEA) reducing adipose tissue inflammation. Considering that these associations were dependent on some critical confounding factors (such as age, gender and BMI) in a cohort enrolling both control and morbid obese subjects, but were independent when considering only morbid obese patients,

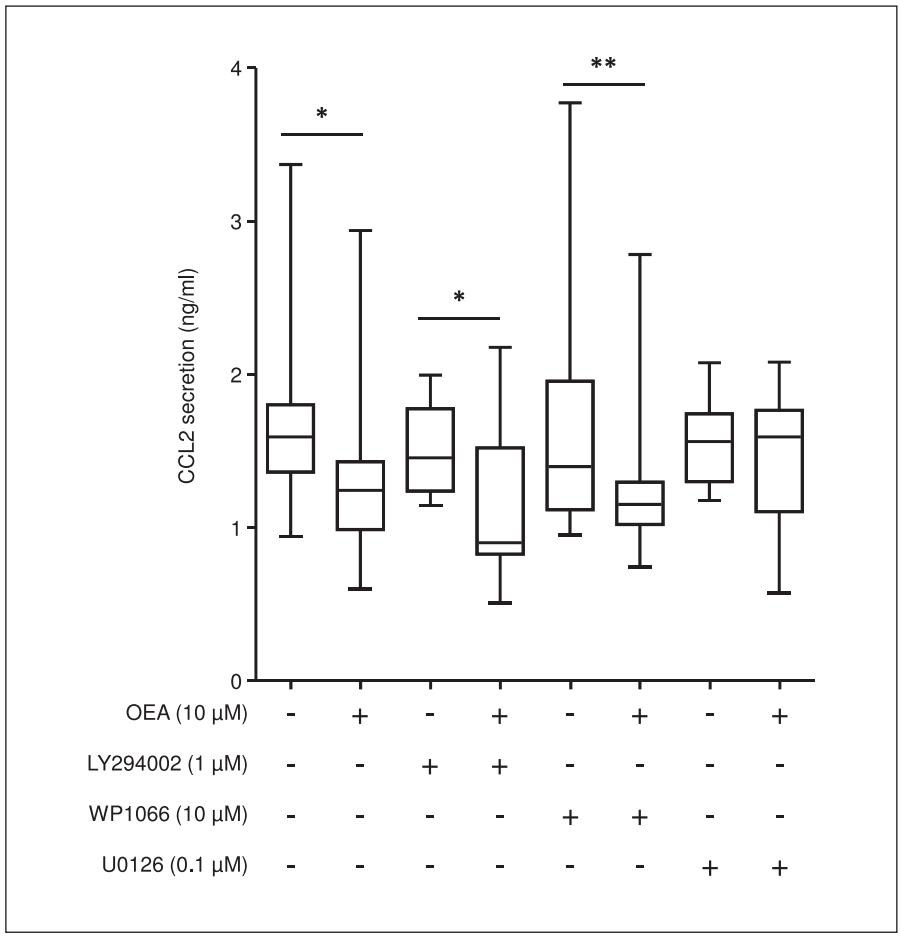

Figure 5: OEA-mediated inhibition of CCL2 release from adipocytes is abrogated by UO126 (inhibitor of MEK1/2, a kinase directly activating ERK1/2). Adipocytes were pre-incubated for 60 min in polystyrene dishes in the presence or absence of different concentrations of 1 µM LY294002, 10 µM WP1066 or 0.1 µM UO126. Then, without washing, cells were stimulated with in the presence or the absence of 10–5 M OEA for additional 24 h. Data are expressed as median (interquartile range), n=10–17. *p<0.05 vs absence of OEA; **p<0.01 vs absence of OEA.

our results only applied to morbid obese patients treated with GBS. Depending on its characteristics (white vs brown) and position (perivascular vs omental vs subcutaneous) adipose tissue has been widely described as a major source of pro-inflammatory (interleukin-6, TNF) and anti-inflammatory cytokines (adiponectin). However, targeting adipose tissue inflammation and infiltration of macrophage subsets in obesity is considered as a promising strategy against the development of cardiovascular and metabolic disorders (33). To clarify the clinical associations in our study, we investigated *in vitro* the role of endocannabinoids on adipocyte release of resistin, CCL2 and CCL5. Leptin secretion from cultured adipocytes was not tested in vitro due to lack of association with endocannabinoids.

In vitro study

To attend this goal we selected an *in vitro* model of adipocytes differentiated from the murine cell line 3T3-L1. Although no activity was shown on resistin release for any of the different compounds and no significant release of CCL5 was detected in any sample, we were able to show that incubation with OEA dose-dependently reduced CCL2 secretion in adipocyte supernatants. OEA has been recently identified as a natural ligand for PPAR-alpha that is capable of triggering intracellular signal cascades (34). After demonstrating the expression of PPAR-alpha in our differentiated adipocytes, we supposed a selective anti-inflammatory activity mediated by OEA on adipocyte CCL2 release. Our data are in partial contrast with evidence from rat adipocytes, showing that OEA induced detrimental effects via the inhibition of insulin actions impairing cell glucose uptake (35). However, the in vitro cell models used in these studies are very different, allowing potential discrepancies due to technical issues. In addition, our *in vitro* data are in agreement and partially explain clinical results, suggesting that our murine adipocytes might be an appropriate model to study human adipose tissue pathophysiology. The selective inhibitory role of OEA on CCL2 release has also a relevant pathophysiological impact, since this CC chemokine is known to potently recruit monocyte-macrophages within inflamed tissues, such as adipose tissue in obesity (33).

We finally investigated the intracellular pathways involved in OEA-mediated effects. Although stimulation with OEA did not affect Akt and Stat3 activation, this compound increased ERK1/2 phosphorylation in adipocytes within few minutes of exposure.

Accordingly, pre-incubation with the selective inhibitor or MEK1/2-ERK1/2 pathway abrogated OEA-mediated reduction of CCL2 secretion. These results showed that OEA-mediated inhibition of CCL2 secretion from murine adipocytes was dependent on EKR1/2 activation, suggesting this intracellular pathway as

a potential therapeutic target for protective endocannabinoid-related strategies to reduce adipose tissue inflammation.

Study limitations and strength

There are limitations worthy to consider. It is important to note that the results reported were obtained from a relatively small sample size of eleven morbidly obese individuals undergoing gastric-bypass for weight loss, which may not necessarily allow definite answers but may lend further promise for clinical and experimental investigations in this emerging research field. In addition, we did not include morbid obese patients with metabolic co-morbidities that represent the majority of morbid obese patients. Although this approach limited relevance of our results to a selected population, we decided on this study design in order to investigate the effects of morbid obesity on adipose tissue inflammation without any potential metabolic confounder. Another potential limitation was represented by the fact that our sample collection was limited to SAT, but not visceral or perivascular fat. Considering the potential different activities of adipose tissues in obesity (36, 37), further investigations are needed to investigate the role of visceral and perivascular fat and their interactions with SAT. Finally, the demonstration that OEA reduced CCL2 production within adipose tissue has a pathophysiological relevance with limited potential clinical implications. Additional clinical studies enrolling a bigger number of patients are needed to validate our findings and understand if OEA might be used as a potential biomarker of reduced SAT inflammation in morbid obesity. Conversely, the study is also unique in investigating the effects of GBS on expressions of endocannabinoids, adipocytokines, CCL2, CCL5, TNF and MGLL in SAT in morbid obesity at baseline and one year after GBS-induced weight loss. SAT biopsies were surely less invasive than VAT sampling that was only acceptable at the moment of GBS, but not after a prolonged clinical follow up. The *in vitro* experiments were essential to confirm clinical results, showing mechanistic insight underlying clinical observation and conferring to our study the valuable aspect of a "translational" research.

Conclusion

This study confirmed that GBS is associated with relevant clinical and metabolic improvements in morbid obese subjects. This was accompanied by a marked amelioration of both serum and SAT inflammation and a surprising increase in SAT levels of endocannabinoids. From clinical correlations within SAT parameters, PEA and OEA were identified as a potential anti-inflammatory molecules reducing adipose tissue inflammation. However, the fact that these associations were independent of age, gender and BMI only in morbid obese patients pre- and post-GBS, limited the potential clinical relevance of these molecules as SAT inflammatory biomarkers. On the other hand, only OEA was able to dose-dependently and selectively abrogate the *in vitro* release of CCL2 release from adipocytes via the activation of ERK1/2-mediated pathways. Considering the pivotal role of CCL2 on macrophage activation and recruitment (38), treatments potentially increasing levels of the endocannabinoid-like compound OEA within adipose tissue might selectively improve detrimental activities of these cells in morbid obesity.

What is known about this topic?

- Obesity is a pandemic disease that increases the cardiovascular risk.
- Paradoxically, morbid obesity was suggested to protect from cardiovascular co-morbidities as compared to overweight/obese patients.

What does this paper add?

- In morbid obese patients, gastric bypass (GBS) reduced body weight and fat mass and was associated with relevant improvements in subcutaneous adipose tissue (SAT) inflammation, potentially due to the increase in endocannabinoid levels.
- The endocannabinoid N-oleoylethanolamide (OEA) directly reduced CCL2 secretion via ERK1/2 activation in adipocytes.

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Conflict of interest

None declared.

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