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Changes in levels of endocannabinoidome mediators in mice with cancer cachexia: links with steatosis and gut microbial dysbiosis

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BACKGROUND: Cachexia is a debilitating syndrome associated with involuntary weight loss, often occurring in cancer patients. In both humans and animal models, alterations in endocannabinoid (eCB) signaling occur in association with both metabolic disorders and several types of tumors. The wider signaling system, including the two eCBs, anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG), their congeners and other long chain fatty acid amides, as well as their metabolic enzymes and receptors, is known as endocannabinoidome (eCBome). The eCBome is involved, among others, in the control of energy balance and cancer and interacts with the gut microbiome.

METHODS: Using mass spectrometry-based targeted lipidomics, we measured the hepatic and intestinal concentrations of eCBome mediators in mice injected with colon carcinoma 26 (C26) cells, a model of cancer cachexia characterized, among others, by weight loss, hepatic dyslipidemia, and gut microbiome dysbiosis.

RESULTS: We report that, 10 days after C26 cell injection, concomitant with >10% weight loss, eCBome lipids levels, namely 2-AG, AEA, and some of its *N*-acyl-ethanolamine congeners, as well as *N*-oleoyl-glycine, *N*-acyl-serotonins, and *N*-acyl-taurines (NATs), are altered in intestinal sections and the liver of C26 mice (e.g., hepatic 2-AG -30%, jejunal 2-AG + 30%, jejunal AEA -55%, hepatic *N*-oleoyl-ethanolamine (OEA) + 223%, hepatic NATs +144%, +141%, +216%). Gut dysbiosis was evident in these mice (PERMANOVA at the family level: $R^2 = 69\%$, $p < 0.001$), with altered levels in 3 phyla (mainly the *Proteobacteria*, +1484%), 12 families, and 12 genera (all with adjusted $p < 0.05$). Additionally, 2-AG, AEA, OEA, *N*-arachidonoyl-serotonin, and NAT levels in the liver positively correlated with hepatic total lipids, triglycerides, and cholesterol, whereas *N*-docosahexaenoyl-ethanolamine and *N*-docosahexaenoyl-serotonin showed negative correlations. Jejunal AEA negatively, and hepatic OEA and NATs positively, correlated with weight loss. Intestinal eCBome mediators correlated with several cecal microbial taxa, including genera known to include strains beneficial in metabolic disorders, such as *Bacteroides*, *Parabacteroides*, *Dysosmobacter*, and *Prevotella*.

CONCLUSIONS: These observations pinpoint eCBome mediators as new multi-functional players in the hepatic complications and gut dysbiosis accompanying cancer cachexia.

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INTRODUCTION

Cancer cachexia is a complex wasting syndrome accompanying cancer and characterized by the malfunctioning of several organs, leading to body weight reduction, loss of muscle mass with or without loss of fat mass, altered metabolism, and systemic inflammation [1, 2]. It is very frequent in cancer patients (up to 70%, depending on the type of cancer), in whom it may lead to death [1, 2]. Cancer cachexia also results in increased co-morbidity and more side effects of anti-cancer treatments [S1]. Among other factors, it is driven by systemic inflammation and altered hormone production; however, alterations in liver metabolism, which are often overlooked, also are likely to contribute to increased energy dissipation, particularly through hepatic futile cycles [3] and unbalanced amino acid metabolism, leading to muscle atrophy [4] [S2]. Other changes in liver metabolism also occur in mouse models of cachexia, including altered mitochondrial function, reduced oxidative phosphorylation [5][S3, S4] and increased hepatic triglyceride levels, altogether leading to hepatic steatosis,

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collagen deposition, and fibrosis [4, 6] [S5, S6]. Yet, the molecular mechanisms through which hepatic dysfunction contributes to cancer cachexia remain to be fully understood. One potential contributor is the gut microbiome. Imbalance in gut microbiota has been associated with cancer cachexia through microbial metabolites such as bile acids and short-chain fatty acids that could contribute to gut barrier dysfunction, cholesterol accumulation, inflammation, and muscle wasting [4, 7, 8] [S7-S9], although evidence of this in humans is still limited.

Among the endogenous signaling pathways deeply involved in both cancer and its consequences, on the one hand, and energy metabolism and liver function, on the other hand, the endocannabinoid (eCB) system has received great attention. The tissue concentrations of the two endogenous ligands of cannabinoid receptor type 1 (CB1) and 2 (CB2), anandamide (*N*-arachidonoyl-ethanolamine, AEA) and 2-arachidonoyl-glycerol (2-AG), also known as endocannabinoids (eCBs), were shown to undergo changes in biopsies and tissues affected by various tumors (breast, colorectal and prostate carcinoma, glioblastoma, etc.) [9][S10-S12], respectively, in human studies and murine models, with a role that has been proposed to be mostly protective. Additionally, circulating AEA and 2-AG levels are elevated in patients with refractory cancer cachexia [10]. CB1 expression and AEA levels are instead reduced in the skeletal muscle of mice injected with C26 colon carcinoma cells (C26), a widely used model of cancer cachexia, where lower and higher expression levels of the enzymes that biosynthesize and degrade AEA and its congener *N*-acyl-ethanolamines (NAEs), the *N*-acyl-phosphatidyl-ethanolamine-selective phospholipase D-like enzyme (NAPE-PLD) and fatty acid amide hydrolase (FAAH), respectively, were also found [11]. Whether the eCB system is altered in cachexia beyond serum and muscle is unclear. AEA and/or 2-AG levels are also altered (usually elevated) in the serum of individuals with obesity and steatosis, and in energy metabolism-regulating tissues, including the liver and small intestine, of rodent models of these metabolic disorders [12, 13] [S13, S14]. Likewise, eCB levels are elevated in the liver of mice with steatosis [14, 15] and in the small and large intestine of mice and rats with obesity [16, 17] and disrupted food intake, altered intestinal epithelial barrier, and gut microbiome dysbiosis [18, 19]. Blockade of hepatic CB1 receptors in mice ameliorates insulin resistance, dyslipidemia, steatosis, and related liver inflammation in diet-induced obesity [14] as well as alcoholic steatosis [15], and CB1 receptor antagonists counteract metabolic endotoxemia and obesity-related gut dysbiosis [18–20], suggesting that eCB signaling at CB1 receptors exacerbates these dysmetabolic conditions.

AEA and 2-AG are accompanied in tissues by their congeners, the NAEs and 2-mono-acyl-glycerols (2-MAGs), respectively, as well as by other bioactive amides of long chain fatty acids, such as the *N*-acyl-aminoacids (or lipoaminoacids [S15]), the *N*-acyl-serotonins [21], and the *N*-acyl-taurines (NATs) [22], which usually act via non-cannabinoid receptors [23], including orphan G protein-coupled receptors, transient potential receptor of vanilloid type (TRPV) channels, and peroxisome proliferator-activated receptors (PPAR) α and γ. While AEA and NAEs, on the one hand, and 2-AG and 2-MAGs, on the other hand, share at least the same respective anabolic and catabolic enzymes, other eCB-like molecules are produced and inactivated via yet to be fully identified mechanisms. The eCBs, their endogenously occurring bioactive congeners and analogues, and the receptors and metabolic enzymes of all these mediators, form a more complex and pleiotropic signaling system, known as the endocannabinoidome (eCBome) [23]. Although being in some cases regulated and/or acting via proteins different from those involved in the regulation and action of eCBs [23], other eCBome mediators can also intervene in energy metabolism and cancer control [S16, S17]. However, the regulation of the levels of eCB-like molecules in cancer cachexia and their relationships with its metabolic consequences have never been investigated.

Based on the hepatic and intestinal (including gut microbiota dysbiosis) sequelae of cancer cachexia, we have investigated here, using liquid chromatography coupled to mass spectrometry (LC-MS) and liquid chromatography coupled to tandem mass spectrometry (LC-MS^2)-based targeted lipidomics methods, the levels of eCBome mediators (including AEA and 2-AG) in the liver and various intestinal sections of the C26 mouse model of this condition. We report that most of the detected mediators, and in particular 2-AG, AEA, and members of the lipoaminoacid, *N*-acyl-serotonin, and NAT families of lipids, are altered in the studied tissues, and correlate either positively or negatively with hepatic dyslipidemia parameters, body weight loss, and the relative abundance of several taxa of the cecal microbiome of these mice.

MATERIALS AND METHODS

Cell culture

Murine colon carcinoma 26 cells (kindly provided by Dr. Mario Colombo, Fondazione IRCCS Instituto Nazionale Tumori, Italy) were maintained in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C with 5% CO_2.

Mouse experiments

Male CD2F1 mice (7 weeks old, Charles River Laboratories, Italy) were kept in specific pathogen-free conditions and housed 2 mice per cage in individually ventilated cages with a 12 h light/dark cycle and fed an irradiated chow diet (AO4-10, Safe, France). After 1-week acclimatization, six and ten mice were randomly assigned in the CT and C26 mice groups, respectively, based on their body weight, and either a saline solution [CT group] or C26 cells (1×10^6 cells in 0.1 ml saline) [C26 group] were subcutaneously injected intrascapularly. Body weight and food intake (average of consumption for 2 mice) were recorded on defined occurrences depicted in Fig. 1. Ten days after sham injection or C26 cells inoculation, mice were fasted from 7 A.M. to 1 P.M., and tissue samples were harvested following anesthesia (isoflurane gas, Abbot, Belgium). Tissues were weighed and frozen in liquid nitrogen. All samples were stored at −80 °C until further analyses. One C26 mouse died before the day of the necropsy, and no tissues were collected. The pair-feeding experiment followed the same timeline and was described previously [4]. All the experiments performed in Belgium were approved by and performed in accordance with the guidelines of the local ethics committee from the UCLouvain, Belgium. Housing conditions were as specified by the Belgian Law of 29 May 2013, regarding the protection of laboratory animals.

Hepatic and serum lipids analysis

Lipids, triglycerides, and cholesterol were measured in serum and in liver tissue after extraction with chloroform–methanol according to the Folch method, as previously described [4]. Details are provided in the supplemental material and methods.

Endocannabinoidome analysis

Endocannabinoids and related mediators were extracted and analyzed by LC-MS or LC-MS^2 [24]. Details are provided in the supplemental material and methods.

Gut microbiome analysis

DNA extraction, total bacteria quantification, 16S rRNA gene sequencing (sequencing, bioinformatics, biostatistics) were performed as previously described [25]. Full details are provided in the supplemental material and methods.

Statistical analyses and correlations

All data were checked for normality using the Shapiro–Wilk normality test. Outliers were identified in normally distributed data using the Grubbs' test and removed. Normally distributed data were analyzed using a Student's *t*-test. Data determined to be non-normal even after log-transformation were analyzed using a Mann–Whitney *U* test. Body weight and food intake evolution were analyzed using a two-way ANOVA with Tukey's post-tests and mix model with Sidak's multiple comparison tests, respectively. Correlation analyses were performed using Spearman correlations followed by FDR correction using the BH procedure. Statistical analyses

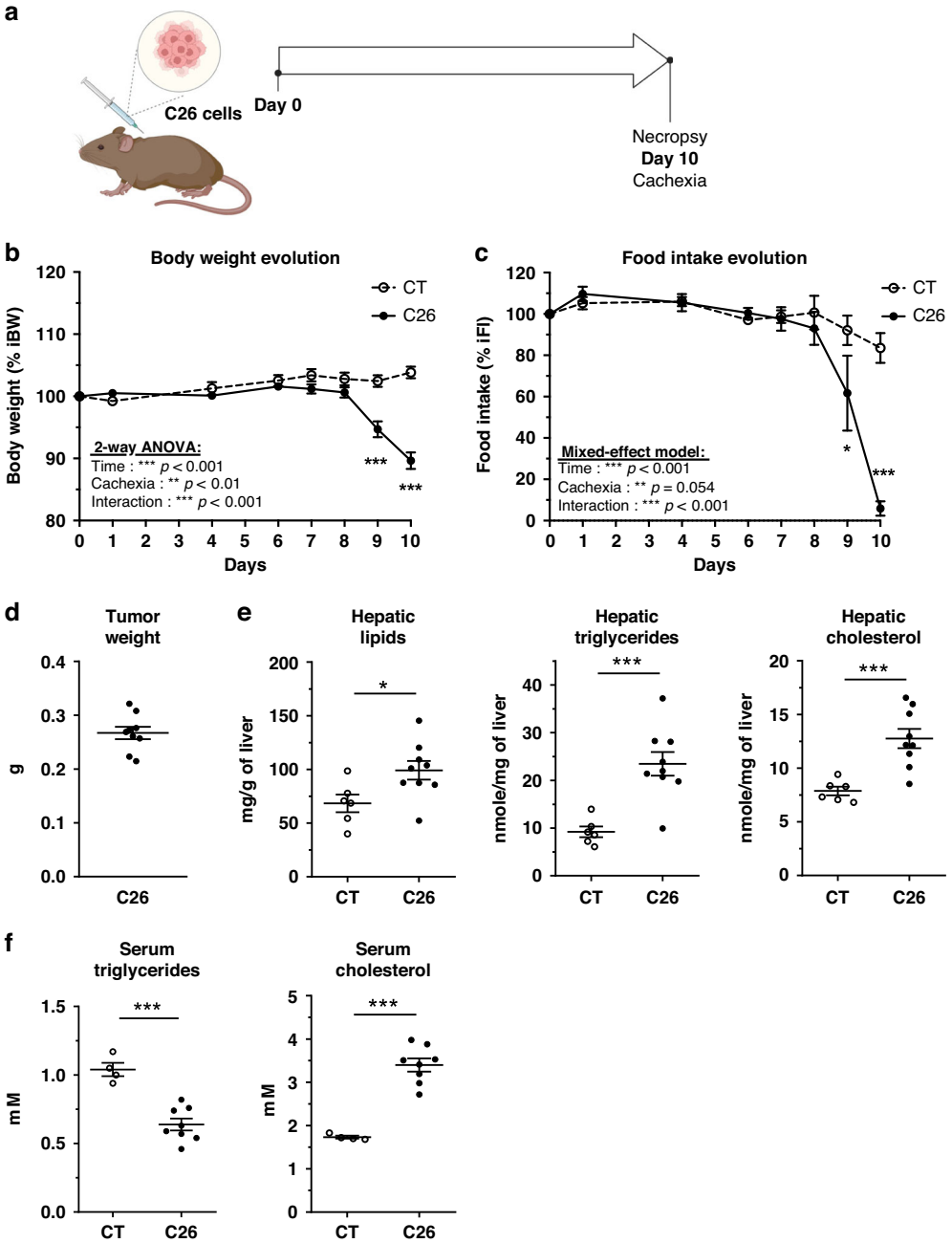

Fig. 1 C26 mice display cachexia and hepatic steatosis. a Experimental design of the *in vivo* experiment. Mice were either inoculated with C26 colon carcinoma cells ($n = 9$) or vehicle ($n = 6$). Created with Biorender.com. **b**, **c** Body weight and food intake evolution of control (CT) and cachectic mice (C26). **d** Tumor weight at the time of necropsy. **e** Hepatic lipid, triglyceride, and cholesterol content. **f** Serum triglyceride and cholesterol levels. $n = 6$–9 mice per group (except for serum results), data are presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. iBW, initial body weight. iFI initial food intake.

were carried out using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA) and R. Endocannabinoids and related eCBome mediators were analyzed by GraphPad Prism version 10.0.3. Data were analyzed using the Student *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Development of cachexia and liver dysmetabolism in C26 mice

Injection of C26 cells (Fig. 1a) resulted in the appearance, as compared to sham (CT) mice, of weight loss and food intake reduction after 9 days, which were stronger and highly statistically significant after 10 days (Fig. 1b, c). When the mice were euthanized, their tumors were weighed (Fig. 1d), and their liver and intestinal sections (duodenum, jejunum, ileum, and colon) were dissected for further analysis. The liver of C26 mice showed significantly elevated lipid, triglyceride, and cholesterol contents as compared to CT mice (Fig. 1e). Serum triglyceride levels were reduced while serum cholesterol levels were increased (Fig. 1f). Serum and hepatic levels of cholesterol and triglycerides were correlated (Supplementary Fig. 1).

The levels of several eCBome mediators are altered in the intestine and liver of C26 mice independently of anorexia

We found that AEA levels are reduced in the jejunum of C26 mice, although they tend to be strongly increased in the liver, whereas

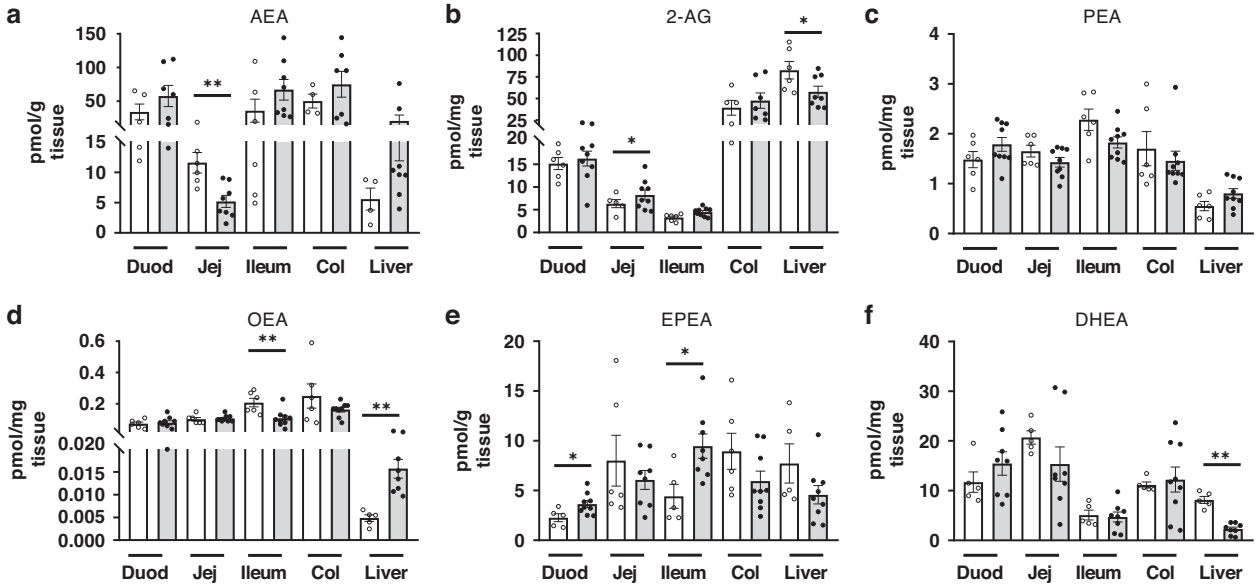

Fig. 2 eCBome mediators are altered in cachectic mice. AEA (**a**), 2-AG (**b**), PEA (**c**), OEA (**d**), EPEA (**e**), and DHEA (**f**) levels in the duodenum, jejunum, ileum, colon, and liver of control (CT, white bars) and cachectic mice (C26, black bars). $n = 4$–9 mice per group, data are presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$. ND not detected.

2-AG levels are increased in the jejunum and reduced in the liver (Fig. 2a, b), in agreement with several previous studies showing the two eCBs are often regulated in opposing manners during physiopathological conditions [19, 26] [S18-S20]. Some previous studies had also shown that AEA levels are reduced in the skeletal muscle of C26 mice [11], whereas circulating AEA and 2-AG levels are elevated in patients with refractory cancer cachexia [10]. In the present study, we also measured AEA saturated and monounsaturated congeners, i.e., *N*-palmitoyl- and *N*-oleoyl-ethanolamine (PEA and OEA), which only tended to be or were significantly altered, respectively, in C26 mice in a manner similar to AEA, although, in the case of OEA, a statistically significant decrease was observed in the ileum instead of the jejunum, and the increase in the liver was significant (Fig. 2c, d). OEA produces anorexic effects by acting in the small intestine [27] and anti-steatotic [28] actions in the liver. Conversely, the n-3 polyunsaturated anti-inflammatory NAEs, i.e., *N*-eicosapentaenoyl- and *N*-docosahexaenoyl-ethanolamines (EPEA and DHEA) [29] were found here to be, respectively, increased in the duodenum and ileum, and reduced in the liver (Fig. 2e, f).

Unlike OEA, *N*-oleoyl-glycine (OlGly) levels tended to be increased in the intestine, the effect being statistically significant for the ileum and colon, with no effect in the liver (Fig. 3a). Also, *N*-oleoyl-serotonin (OA5HT) levels were increased in the ileum (Fig. 3b), whereas, instead, those of *N*-arachidonoyl-, *N*-docosahexaenoyl- and *N*-eicosapentaenoyl- serotonins (AA5HT, EPA5HT and DHA5HT, an anti-inflammatory mediator [30]) were significantly decreased, respectively, only in the duodenum, only in the colon, and in the duodenum, ileum and liver (Fig. 3c).

Finally, the levels of all three NATs measured (i.e., *N*-palmitoyl-taurine (*N*-PalmTau), *N*-oleoyl-taurine (*N*-OleTau), and *N*-arachidonoyl-taurine (*N*-AraTau)) were significantly, or tended to be, increased in all sections of the small intestine and the liver, and those of *N*-PalmTau also in the colon (Fig. 3d). A graphical summary of tissue-specific changes is presented in Supplementary Fig. 2.

Pair-feeding (PF) was used to delineate the contribution of anorexia to the alterations in liver metabolism and eCBome. Using this approach, we found that reduced food intake did not modify hepatic cholesterol levels and induced a modest increase in hepatic triglycerides and lipid content, which did not replicate the levels of the alterations found in cachectic mice (Supplementary Fig. 3A). The same also applies to most of the hepatic eCBome mediators measured. Anorexia did not explain the pattern of eCBs but fully explained the increase in *N*-AraTau, most of the increase in *N*-PalmTau, and only minorly the increase in *N*-OleTau (Supplementary Fig. 3B, C). Of note, in this second experiment, we did not see again the increase in hepatic DHEA levels observed in the first experiment, possibly because this experiment was performed at an earlier time, with some little differences in tissue harvesting. Along these lines, we previously showed that, although food intake restriction alters the microbiota, these alterations do not mirror the ones found in C26 mice [34]. We concluded that anorexia is not the main or only driver of the hepatic steatosis and eCBome changes in C26 mice and cannot explain alone the alterations in the gut microbiota composition.

The hepatic levels of eCBome mediators correlate with hepatic dyslipidemia parameters

In agreement with previous studies on the role of AEA in mouse non-alcoholic steatosis and of 2-AG in alcoholic steatosis [14, 15], we found that eCB levels directly correlated with hepatic triglyceride and cholesterol levels, in the case of AEA, and total liver lipids, in the case of 2-AG (Fig. 4). Interestingly, however, the levels of OEA, which is known for its anti-lipogenetic and anti-steatotic properties [28], were also positively correlated with triglycerides and cholesterol, as were the levels of AA5HT and the three NATs, which are reported to exert anti-hyperlipidemic and insulin-sensitizing effects [31]. Unlike AEA and OEA, on the one hand, and AA5HT, on the other hand, the levels of DHEA and DHA5HT were instead negatively correlated with lipid weight and cholesterol, respectively (Fig. 4).

The cecal microbiota is altered in C26 mice

In agreement with previous results [4, 7, 32] [S9], the cecal microbiota was dramatically altered in cachectic C26 mice, at the phylum, family, and genus levels (Fig. 5). The cachexia status explained 69% of the variance of the dataset (PERMANOVA $R^2 = 0.69$, $p < 0.001$). Although the total bacterial number was unchanged with respect to CT mice, a significant increase of *Enterobacteriaceae* was detected (Supplementary Fig. 4A).

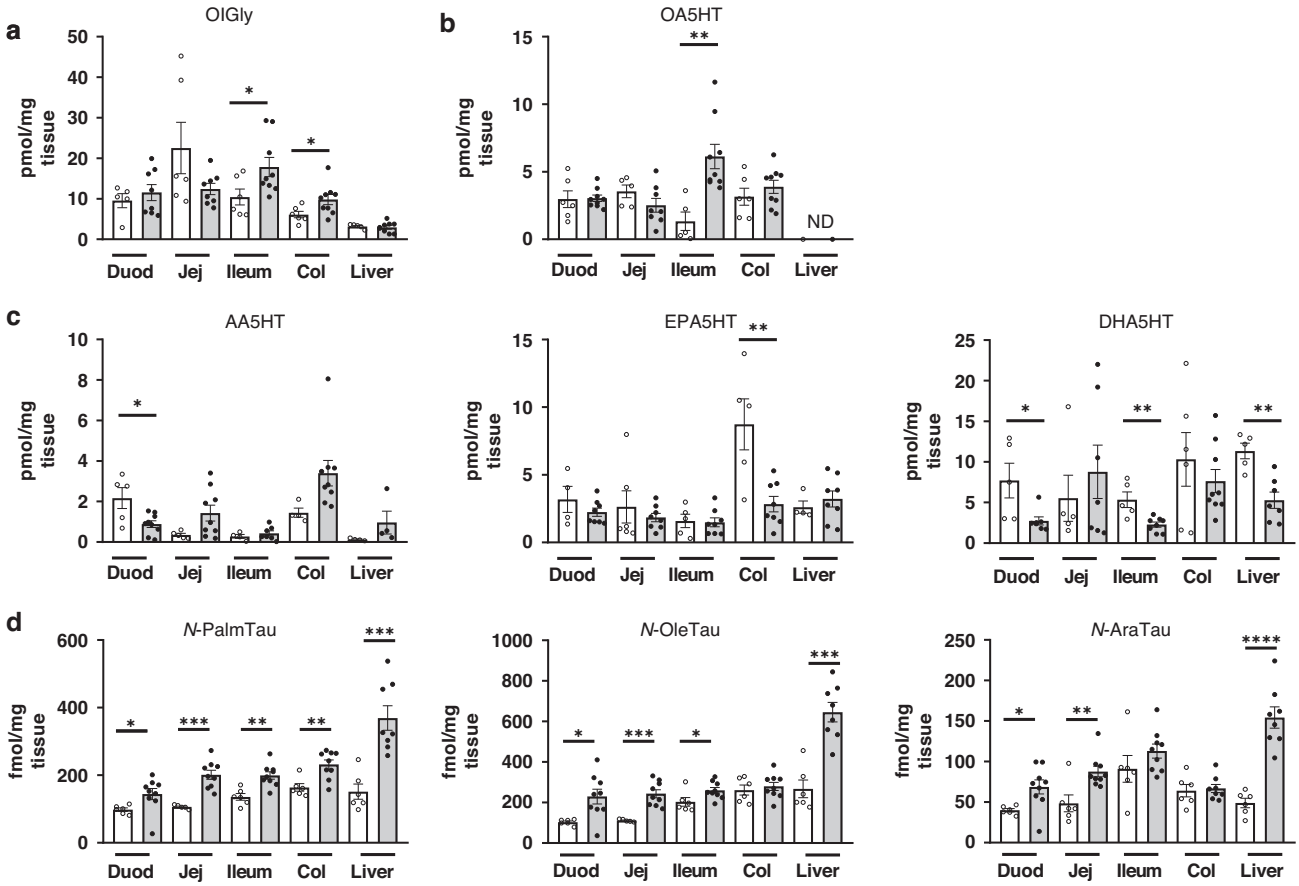

Fig. 3 eCBome mediators and *N*-acyl-taurine (NAT) levels are altered in cachectic mice. OlGly (**a**), OA5HT (**b**), AA5HT, EPA5HT, and DHA5HT (**c**), as well as *N*-PalmTau, *N*-OleTau, and *N*-AraTau (**d**) levels measured in the duodenum, jejunum, ileum, colon, and liver of control (CT, white bars) and cachectic mice (C26, black bars). $n = 4$–9 mice per group, data are presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Additionally, α-diversity (Simpson and Shannon) was reduced in C26 mice as a result of reduced evenness (Heip and Simpson) (Supplementary Fig. 4B). Finally, the relative abundance of 3 phyla (e.g. *Proteobacteria*, increased), 12 families (e.g. *Enterococcaceae*, increased; *Lachnospiraceae*, decreased) and 12 genera (e.g. *Bacteroides, Escherichia/Shigella, Parabacteroides*, increased; *Dysosmobacter*, *Oscillibacter*, *Prevotella*, decreased) was also significantly altered (Fig. 6 and Supplementary Fig. 5).

The intestinal levels of eCBome mediators correlate with several taxa of the cecal microbiota

When looking at the correlations between eCBome mediators and cecal microbial taxa, at the genus level, two clusters can be evidenced (Fig. 7a, upper panel): (1) taxa that were positively correlated with eCBome mediators (with the exception of DHA5HT, OEA and PEA in the ileum, and EPA5HT in the colon, which were instead negatively correlated), i.e., *Bacteroides, Enterococcus, Streptococcus, Escherichia/Shigella, Staphylococcus, Schaedlerella, Parabacteroides* and *Anaerotruncus*; and (2) taxa that were instead negatively correlated (except for DHA5HT and OEA in the ileum, which were instead positively correlated), i.e., *Oscillobacter, Dysosmobacter, Duncaniella* and *Prevotella*. This was true also at the family level (Fig. 7a, lower panel). Interestingly, the molecules that behaved in an opposite manner to the others in this clustering are mediators for which there is strong evidence for a solely anti-inflammatory and/or anti-steatotic effect (DHA5HT, PEA, and OEA), whereas for the other mediators measured in this study, a pro-inflammatory/steatotic action can also be envisaged.

The levels of eCBome mediators variedly correlate with body weight loss

Finally, looking at the correlations between eCBome mediators in the intestinal tissues and liver and body weight loss, we found that hepatic NATs were positively correlated to body weight loss, with *N*-PalmTau being positively correlated to body weight loss across all considered segments (Fig. 7b). Conversely, jejunal levels of AEA, OlGly, and DHA5HT were negatively correlated with body weight loss.

DISCUSSION

We are reporting here for the first time alterations in several eCBome mediators [33], with activity in the context of food intake and body weight control, hepatic fat accumulation, and inflammation, in the liver and intestinal sections of the C26 mouse model of cancer cachexia. To discuss the potential role of the observed changes in the signs and consequences of cancer cachexia, we have listed in Table 1 the molecular targets and some of the subsequent reported effects in the above-mentioned contexts, of the 14 mediators measured.

The first novel finding of this study is that the two eCBs, AEA and 2-AG, which can activate also targets other than CB1 and CB2 receptors (Table 1), are regulated in an opposing manner in the

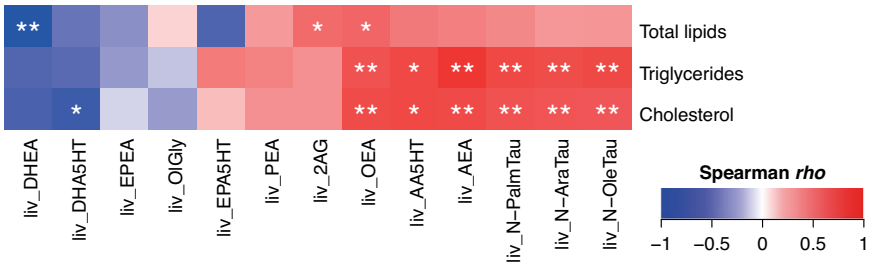

Fig. 4 Hepatic eCBome mediator levels correlate with hepatic lipid content. Spearman correlations between hepatic eCBome mediator levels and hepatic triglycerides, cholesterol, and lipid content, as depicted in Figs. 1–3. $n = 15$ mice. *$p < 0.05$ and **$q < 0.05$.

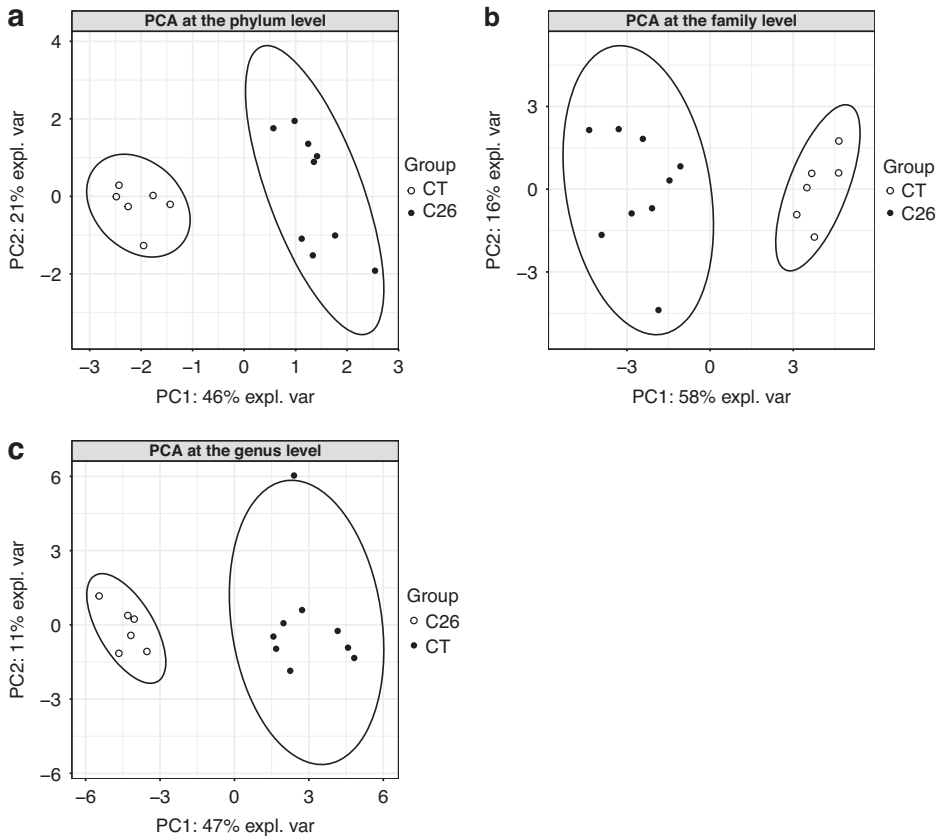

Fig. 5 C26 mice display altered cecal bacterial composition. Principal component analysis (PCA) at the phylum (**a**), family (**b**), and genus (**c**) levels (PERMANOVA at the phylum level: $R^2 = 61\%$, $p < 0.001$; PERMANOVA at the family level: $R^2 = 69\%$, $p < 0.001$; PERMANOVA at the genus level: $R^2 = 62\%$, $p < 0.001$). $n = 6–9$ mice/group.

liver and the jejunum, and in an opposite way from each other, the former mediator being reduced in the jejunum and strongly trending to be enhanced in the liver, and the latter compound being increased in the jejunum and reduced in the liver. This finding should be considered in view of the previously reported enhancement of blood AEA and 2-AG levels in human refractory cancer cachexia [10], and reduction of AEA levels in the skeletal muscle of C26 mice [11]. This latter effect, although observed in the previous study at a more advanced cachectic stage, might suggest a similar regulation of AEA levels in the jejunum and skeletal muscle, but not in the liver. In this latter tissue, the enhancement of AEA might be related to the significant enhancement of hepatic fat found in C26 mice, given the role of this mediator in non-alcoholic fatty liver disease, whereas 2-AG has been reported to participate in alcoholic fatty liver disease in mice [15]. Since 2-AG, as compared to AEA, is a full agonist at anti-inflammatory CB2 receptors (Table 1), its decreased levels might have contributed to the increased hepatic inflammation of C26 mice [34]. Conversely, in the jejunum, up-regulated 2-AG, acting at CB1 receptors, and down-regulated AEA, acting at TRPV1, might have contributed to increased intestinal permeability in these mice [7] (Table 1). In general, AEA and 2-AG levels have often been reported to be regulated in opposing manners in both mice and humans, which is likely to reflect their different capabilities at activating also non-cannabinoid receptors, and hence play only partially overlapping functions (Table 1).

The finding that eCB-like molecules never linked before with cancer cachexia, are also strongly altered in the intestine and liver of C26 mice, is another novel observation of the present study. This is particularly important as these mediators have been previously associated, by virtue of their molecular targets or pharmacological activities, to complications of metabolic disorders similar to those present in cancer cachexia (i.e., fatty liver, deregulated food intake, and reduced intestinal barrier, etc.) and

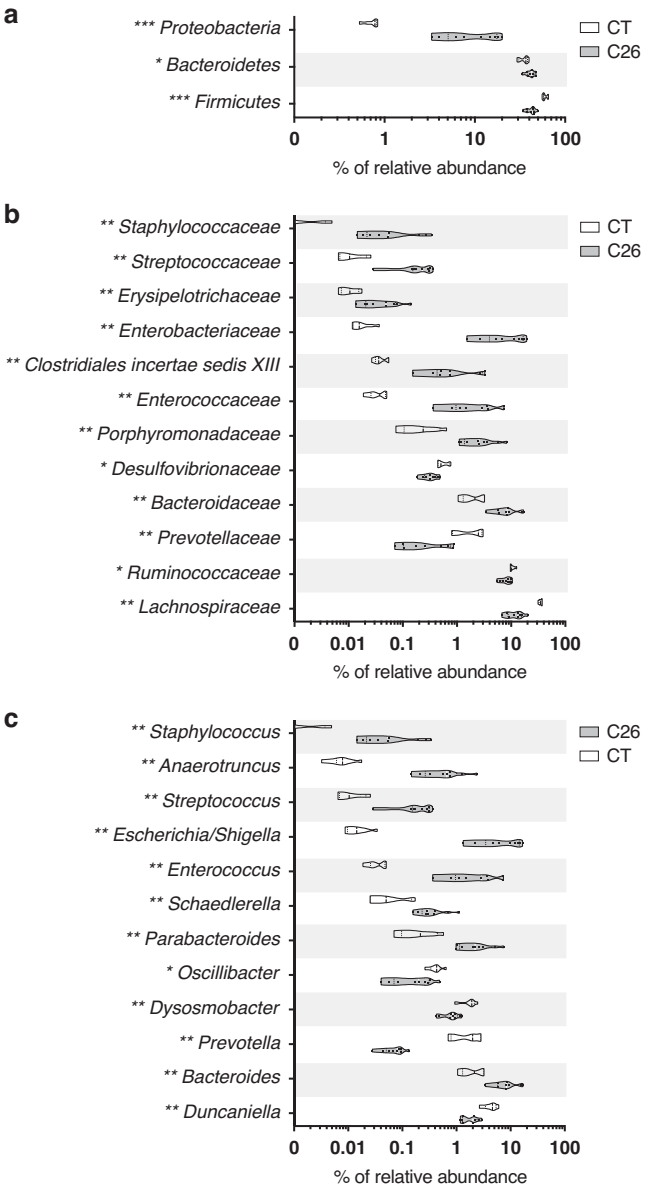

Fig. 6 C26 mice display altered cecal bacterial levels. Violin plots depicting taxa whose levels are significantly modified in cachectic mice (C26) compared to controls (CT) at the phylum (**a**), family (**b**), and genus (**c**) levels. $n = 6–9$ mice/group. Data are presented as violin plot, $*q < 0.05$ and $**q < 0.01$.

to inflammation. The levels of the endogenous NAE and PPARα ligand, OEA, known for its anorexic, anti-lipogenetic, anti-steatotic effects and anti-dysbiotic properties [27, 28] [S21], were reduced in the ileum and enhanced in the liver. Instead, the omega-3 fatty acid-derived EPEA and DHEA, known for their anti-inflammatory and anti-cancer effects [29, 30] [S22], were regulated in a manner different from AEA and OEA, being increased in the duodenum and ileum (EPEA) and reduced in the liver (DHEA). The decrease of OEA signaling at PPARα in the ileum could represent an adaptive response to reduced food intake, but, at the same time, it could also contribute to worsened intestinal permeability (which is reduced by both PPARα and TRPV1, another OEA receptor, Table 1). Increased OEA and decreased DHEA levels in the liver could, respectively, counteract or contribute to hepatic fatty liver and inflammation.

The oleic acid-derived mediator, OlGly, which, among others, inhibits food addiction and counteracts high-fat diet-induced obesity in mice via PPARα, a receptor also known to counteract the inflammation-induced increase of intestinal permeability [7, 35], was increased in the ileum and colon of C26 mice. This finding might thus represent again an adaptive feedback response aimed at reducing cachexia-induced weight loss and gut barrier dysfunction.

The levels of the four *N*-acyl-serotonins detected in this study were altered in C26 mice in a manner that appeared to depend on whether they derive from oleic or polyunsaturated fatty acids. We observed a decrease of AA5HT (in the duodenum), EPA5HT (in the colon), and DHA5HT (in all tissues analysed except the colon, where, however, a strong trend was observed), which are known to inhibit FAAH, the main hydrolytic enzymes of unsaturated NAEs, and to antagonize TRPV1 channels (Table 1). This effect might be related to maladaptive or negative feedback adaptive responses exerted, respectively, through lack of inhibition of NAE hydrolysis and subsequent decreased NAE levels (as indeed observed for AEA and OEA), or enhanced TRPV1 tone and corresponding reduction of food intake and increase of intestinal barrier integrity [S23]. The enhancement of OA5HT levels in the ileum could counterbalance the effect, in this intestinal section, of reduced levels of DHA5HT, which is also known to exert anti-inflammatory effects [36].

NATs are a family of eCB-like mediators known to activate TRPV1 and TRPV4 channels and, also depending on the type of their fatty acid chain, to exert a protective role against post-prandial and high fat diet-induced blood and hepatic hypertriglyceridemia and to increase glucose clearance through mechanisms that have not yet been ascribed to these targets [31, 37]. Here we report that the oleic, arachidonic, and palmitic acid-derived members of this family of bioactive lipids are extremely abundant in the mouse intestine and liver, and that their levels in these organs are significantly increased in C26 mice. This might represent a negative feedback reaction against fat accumulation in the liver, and, possibly through TRPV1 [35], impaired intestinal epithelial barrier (Table 1).

The above hypotheses regarding the potential function of the observed changes in eCBome mediators in C26 mice are in line with the strong correlations found between hepatic lipids, triglycerides, and cholesterol and the levels of such mediators. For example, the positive correlations between AEA, 2-AG, and OEA with parameters of hepatic dyslipidemia agree with a contributing role of the former two compounds (via CB1 receptors), and a potential negative feedback response for the latter one (via PPARα), in this co-morbidity of cancer cachexia. The negative correlations between two anti-inflammatory mediators, DHEA and DHA5HT, and the above parameters are also indicative of a potential protective (and deficient, given the reduction in their hepatic levels) role for these two molecules. Finally, the strong positive correlations between all NATs and liver triglycerides and cholesterol would suggest again a potential negative feedback response by these mediators, aimed at trying to counteract hepatic inflammation and lipogenesis. This effect is unlikely to be exerted via TRPV1 channels (which are poorly expressed in the liver and would rather mediate inflammation) but could be exerted via other receptors. Indeed, some of the authors of this paper have evidence that NATs activate PPARα at the same concentrations previously described to activate TRPV1 channels (V. Di Marzo, F.A. Iannotti, unpublished observations). Alternatively, NATs could attempt to exert tonic hepatoprotective effects against lipid accumulation via TRPV4 (whose activation was shown to possess this property [S24]), particularly as PPARα-mediated responses are blunted in the livers of C26 mice [4, 34]. This blunted response to PPARα may also explain why the increased hepatic tone of OEA and NAT is not sufficient to prevent fatty liver

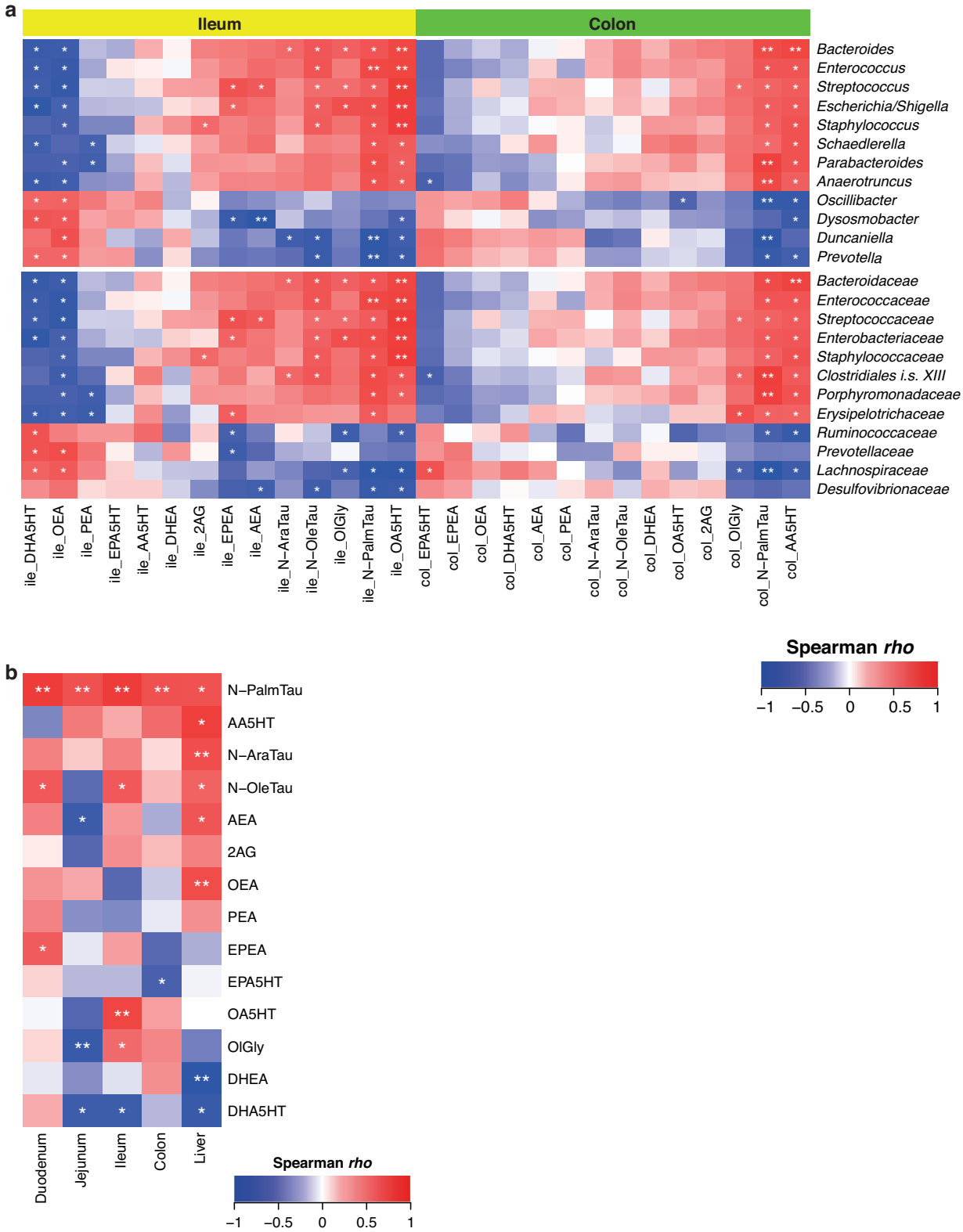

Fig. 7 eCBome mediator levels correlate with the relative abundance of bacteria in the cecal content and with body weight loss. **a** Spearman correlation between hepatic eCBome mediator levels and cecal relative abundance of significantly affected genera (top) and families (bottom). **b** Spearman correlations between eCBome mediator levels (in the duodenum, jejunum, ileum, colon, and liver) and body weight loss (expressed as % of initial body weight). $n = 15$ mice. * $p < 0.05$ and **$q < 0.05$.

Table 1. Molecular targets and subsequent observed or potential effects, in the context of metabolism and inflammation, of the eCBome lipids detected in this study.

eCBome signal or its target	2-AG	AEA	OEA	PEA	EPEA	DHEA	OlGLY	AA5HT	OA5HT	EPA5HT	DHA5HT	N-OleTau	N-AraTau
CB1	↑Food intake, AFLD, Int. perm.	↑Food intake, AFLD, Int. perm.						Indirect ago via FAAH inhib.[a]	Indirect ago via FAAH inhib.[a]	Indirect ago via FAAH inhib.[a]	Indirect ago via FAAH inhib.[a]		
CB2	↓ Infl.	↓ Infl.			↓ infl. (?)	↓ infl. (?)		Indirect ago via FAAH inhib.[b]	Indirect ago via FAAH inhib. [b]	Indirect ago via FAAH inhib. [b]	Indirect ago via FAAH inhib. [b]		
TRPV1	weak ago	ago[c]	↓Food intake, Int. perm.					antag[ab]	antag[ab]	antag[ab]	antag[ab]	↑Insulin secretion	↑Insulin secretion
TRPV2			antag[b]										
TRPV4												ago[c]	ago[c]
GPR18													
GPR55	ago[c] ↓Int. perm.	ago[c] ↓Int. perm.		ago[c] ↓Int. perm.									
GPR110						↓ Infl.							
GPR119			↓Food intake, ↑Insulin sens.									↓Food intake ↑Insulin sens.	
PPARα			↓Food intake, N/AFLD, Int. perm.	↓ Infl.			↓Food addiction						
PPARγ		↑adipo-genesis (high conc.)				↓Infl. ↑Insulin sens.							

When not mentioned otherwise, the mediators always produce their effects on food intake, inflammation, intestinal permeability, N/AFLD, adipogenesis, and insulin sensitivity or secretion by acting as agonists at the receptors indicated, except for GPR119, the action of which is likely to be due to glucagon-like peptide-1 release. *N*-palmitoyl-taurine was also investigated here, but its molecular targets and biological actions are currently unknown.

AFLD alcoholic fatty liver disease, *ago* agonist, *antag* antagonist, *CB1* cannabinoid receptor type-1, *CB2* cannabinoid receptor type-2, *FAAH* fatty acid amide hydrolase, *GPR G* protein-coupled receptor, *Infl.* inflammation, *inhib.* inhibition, *Int. perm.* intestinal permeability, *NAFLD* non-alcoholic fatty liver disease, *N/AFLD* both alcoholic and non-alcoholic fatty liver disease, *PPARα or γ* peroxisome proliferator-activated receptor α or γ, *sens.* sensitivity, *TRPV1-4* transient receptor potential of vanilloid type 1-4.

[a]Potential for food intake and body fat stimulation (not reported so far).

[b]Potential for anti-inflammatory effects (not reported so far).

[c]Potential for pro-inflammatory effects (not reported so far).

in this condition. Future studies should address the possibility that TRPV4 expression is altered in the liver of C26 mice.

Importantly, most of the eCBome mediator alterations observed in the liver, and in parallel those of hepatic lipids, were less strong or absent in mice fed with the same amount of food consumed by C26 mice. This suggests that in mice, anorexia is not the major or only determinant of hepatic eCBome levels and reinforces the hypothesis of a role in hepatic dyslipidemia of these mediators.

Also in view of the strong effects exerted by gut bacteria on the eCBome, and particularly on the levels of NAEs and their receptors in all intestinal sections [38], we attempted to understand the potential meaning of the observed changes in eCBome mediators in intestinal barrier function and, as a possible consequence, hepatic dyslipidemia, by correlating the levels of these molecules with the relative abundance of taxa found to be altered in C26 mice, some of which have been implicated in the control of intestinal permeability [S9]. Indeed, these mice exhibit profound taxonomic alterations of their cecal bacteriome, as evidenced from altered relative abundances of 12 out of the 20 considered families and of many of their parent genera. We found correlations between the ileal or colonic levels of protective eCBome mediators (OEA, OlGly, OA5HT, AA5HT, DHA5HT, and the NATs) and *Bacteroides*, *Parabacteroides*, *Prevotella*, and *Dysosmobacter*. The beneficial effects of many strains belonging to these genera on intestinal barrier protection and hepatic function [S25-S30], seem to support the hypothesis of the existence of an eCBome-gut microbiome axis in the regulation or dysregulation of metabolism (via adaptive or maladaptive changes in the signaling of these mediators at their receptors), also in the context of cancer cachexia. The frequent opposite correlations of the same mediators with potentially harmful bacteria, such as those belonging to the genera *Enterococcus*, *Streptococcus*, *Escherichia/Shigella, Anaerotruncus*, and *Staphylococcus*, also reinforce this hypothesis.

The alterations in eCBome lipids in the duodenum and jejunum, on the one hand, and in the ileum and colon, on the other hand, might also be linked, either directly or via effects on the gut microbiome, to the decreased food intake and nutrient absorption, respectively, typical of cachectic mice. For example, the reduction of AEA in the jejunum could underlie in part the reduced food intake. In the ileum, the reduction of OEA and the increase of OlGly, two mediators that are known to reduce food intake and preference for palatable food, respectively, and to modulate gut microbiota composition in the process [27, 39], might have affected both food intake and nutrient absorption. Interestingly, we found that a mediator known for its CB1-mediated orexigenic activity, such as AEA, was negatively correlated (in the jejunum) with weight loss, as was DHA5HT, which by antagonizing TRPV1 would be expected to also induce food intake; conversely, hepatic OEA and NATs, which activate receptors (TRPV1 and/or PPARα, Table 1) with anorexiant activity, were positively correlated with weight loss. Yet, exceptions to this rule were also noted: hepatic AEA and jejunal OlGly correlated positively and negatively with weight loss, respectively, despite their activities as CB1 and PPARα agonists, whereas AA5HT and OA5HT correlated positively with this measure, despite their antagonistic activity at TRPV1 and their FAAH inhibitory effects. As the members of two families of lipids acting on TRPV1, namely *N*-acyl-serotonins (antagonists) and NATs (agonists) [21, 22, 40], correlated in rather similar manners with microbial taxa, the role of the intestinal microbiome in these putative effects of TRPV1 activation/inactivation would be dubious in this case.

In conclusion, we report here that the intestinal and hepatic levels of the eCBs, AEA and 2-AG, and other eCB-like mediators belonging to the eCBome, are altered in the C26 mouse model of cancer cachexia. Given the multi-faceted, and often multi-target-mediated, effects of these bioactive lipids in the control of food intake and absorption, energy expenditure and fat accumulation, altered intestinal barrier and metabolic endotoxemia, hepatic inflammation and lipid accumulation, these alterations are likely to represent adaptive negative feedback or maladaptive exacerbating reactions that participate in the symptoms (Fig. 8). This hypothesis, which was also suggested by the strong correlations found here between the tissue concentrations of these mediators with hepatic lipids, on the one hand, and several metabolically relevant taxa of the cecal microbiome, on the other hand, will need to be investigated in future studies. Such studies will require employing additional mouse models of cachexia and several pharmacological and/or genetic tools to dissect the role, in the many co-morbidities of cancer cachexia, of each one of the eCBome members detected here and of their several receptors. In parallel, prospective clinical studies evaluating the link between

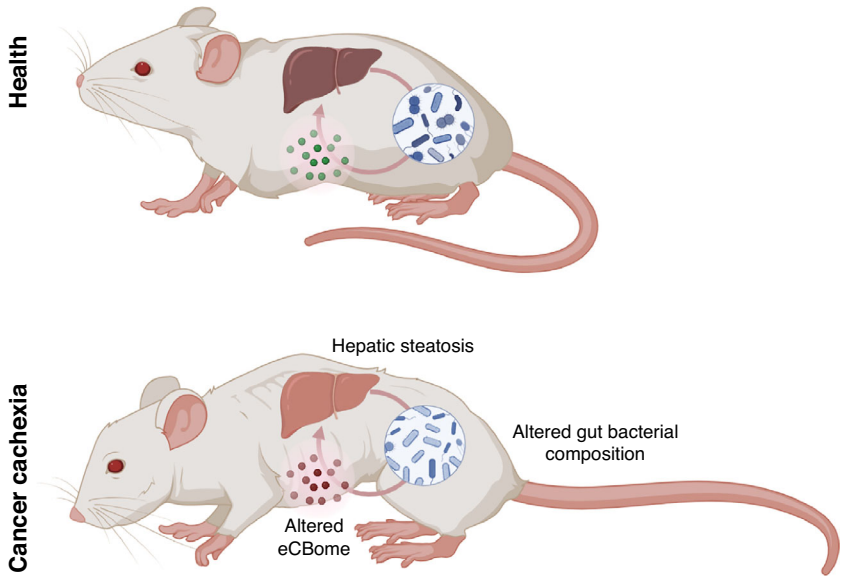

Fig. 8 Graphical abstract. eCBome mediator levels correlate with the relative abundance of bacteria in the cecal content and with with hepatic lipid content.

eCBome, microbial dysbiosis, and hepatic metabolism in cancer patients with and without cachexia will be needed. Finally, the potential relationship between the enterohepatic alterations described here and muscular atrophy, an important symptom of cachexia, should also be explored and constitutes an important research perspective.

DATA AVAILABILITY

Raw 16S rRNA gene sequences can be found in the SRA database (project ID: PRJNA1093036). The remaining data supporting the findings of this study are available within the article and its supplementary materials.

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AUTHOR CONTRIBUTIONS

Conception and design of the work: LBB and VD. Sample collection: MMT. Sample and data analysis: ALD, AC, FB, GDS, RVe. Chemical synthesis of deuterated standards for lipidomics: RVi. Data integration: LBB. Data interpretation: VD with the help of LBB. Acquisition of funding: LBB and VD. Drafting the article: VD. Critical revision of the article: all. Final approval of the version to be published: all. LBB and VD are corresponding authors.

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CONFLICT OF INTEREST

Alexandra L. Degraeve, Adele Cutignano, Fabiana Piscitelli, Rosaria Villano, Giulia De Simone, Roberta Verde, Morgane M. Thibaut, Laure B. Bindels, and Vincenzo Di Marzo declare that they have no conflict of interest.

ADDITIONAL INFORMATION

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