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Cancer cachexia induces morphological and inflammatory changes in the intestinal mucosa

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Abstract

Background Cachexia is a multifactorial and multiorgan syndrome associated with cancer and other chronic diseases and characterized by severe involuntary body weight loss, disrupted metabolism, inflammation, anorexia, fatigue, and diminished quality of life. This syndrome affects around 50% of patients with colon cancer and is directly responsible for the death of at least 20% of all cancer patients. Systemic inflammation has been recently proposed to underline most of cachexia-related symptoms. Nevertheless, the exact mechanisms leading to the initiation of systemic inflammation have not yet been unveiled, as patients bearing the same tumour and disease stage may or may not present cachexia. We hypothesize a role for gut barrier disruption, which may elicit persistent immune activation in the host. To address this hypothesis, we analysed the healthy colon tissue, adjacent to the tumour.

Methods Blood and rectosigmoid colon samples (20 cm distal to tumour margin) obtained during surgery, from cachectic (CC = 25) or weight stable (WSC = 20) colon cancer patients, who signed the informed consent form, were submitted to morphological (light microscopy), immunological (immunohistochemistry and flow cytometry), and molecular (quantification of inflammatory factors by Luminex® xMAP) analyses.

Results There was no statistical difference in gender and age between groups. The content of plasma interleukin 6 (IL-6) and IL-8 was augmented in cachectic patients relative to those with stable weight ($P = 0.047$ and $P = 0.009$, respectively). The number of lymphocytic aggregates/field in the gut mucosa was higher in CC than in WSC ($P = 0.019$), in addition to those of the lamina propria (LP) eosinophils ($P < 0.001$) and fibroblasts ($P < 0.001$). The area occupied by goblet cells in the colon mucosa was decreased in CC ($P = 0.016$). The M1M2 macrophages percentage was increased in the colon of CC, in relation to WSC ($P = 0.042$). Protein expression of IL-7, IL-13, and transforming growth factor beta 3 in the colon was significantly increased in CC, compared with WSC ($P = 0.02$, $P = 0.048$, and $P = 0.048$, respectively), and a trend towards a higher content of granulocyte-colony stimulating factor in CC was also observed ($P = 0.061$). The results suggest an increased recruitment of immune cells to the colonic mucosa in CC, as compared with WSC, in a fashion that resembles repair response following injury, with higher tissue content of IL-13 and transforming growth factor beta 3.

Conclusions The changes in the intestinal mucosa cellularity, along with modified cytokine expression in cachexia, indicate that gut barrier alterations are associated with the syndrome.

Keywords Colon cancer; Cancer cachexia; Inflammation; Gut barrier; Intestine

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Background

Cachexia is a multifactorial and multiorgan condition associated with cancer and various other systemic diseases, such as sepsis, renal failure, and chronic heart failure, and characterized by severe and involuntary loss of skeletal muscle and adipose tissue.[1] Other cachexia-associated manifestations include altered immune function, anorexia, disrupted metabolism, nausea, fatigue, poor physical function, and diminished quality of life.[2,3]

Around 50% of patients with colon cancer are affected by the syndrome,[4] which, owing to the multiplicity of its clinical symptoms, is often underdiagnosed and seldom treated.[1,5] Systemic inflammation is commonly observed in patients with cancer cachexia, having been postulated to play a key role in the aetiology of the condition and in the determination of the clinical symptoms.[1,6] The source(s) of inflammatory factors are many, including the tumour[7] and the various peripheral organs, tissues, and cells.[8] Yet the compartment that triggers the systemic inflammatory response has not been so far appointed.

It is possible to hypothesize that inflammation may arise, at least partly, from failure in gut barrier function, in association with perturbations in the intestinal microbiota composition, yielding persistent immune activation, as previously proposed.[9] Local intestinal inflammation may induce the expression of proinflammatory cytokines, tissue immune cell infiltration, and other inflammatory changes, which can further exacerbate mucosal damage and gut permeability.[10]

Gut barrier dysfunction is a syndrome marked by failure of the gut epithelial barrier, leading to systemic inflammation, because of tissue transposition of bacterial cell wall components (endotoxin or lipopolysaccharide), or intact bacteria into the blood circulation.[8] Moreover, gut barrier dysfunction may exacerbate systemic inflammation in the presence of other sources of inflammation and further contribute to anorexia, muscle wasting, and other hyper-metabolic changes observed in cachexia.[5]

This study aims to investigate and characterize local inflammation in the gut of cachectic and weight stable patients with colon cancer and to briefly discuss the potential contribution of the immune response of gut mucosa to systemic inflammation in cancer cachexia.

Methods

Patient recruitment

Patients with colorectal cancer (n = 45) were recruited at the University Hospital of the University of São Paulo. This study was approved by Biomedical Sciences Institute Ethics Committee (CAAE: 15492013.0.0000.5467) of the University of São Paulo and by the University Hospital Ethics Committee (CEP-HU/USP: 1385/14), and written informed consent was obtained from each patient before admission to the protocol. At the time of assessment, patients were not under continuous anti-inflammatory treatment and they had not received anticancer treatment (radiotherapy or chemotherapy) prior to the application of the research protocol. All patients received the first cancer diagnosis immediately before being recruited. Subjects with liver failure, renal failure, AIDS, inflammatory bowel disease (IBD), and autoimmune disorders were excluded. Cancer patients were divided into two groups: weight stable cancer (WSC = 20) and cachectic cancer (CC = 25). Patient group division was based on 'Cachexia a new definition',[1] in which cachexia is diagnosed in patients with involuntary weight loss of at least 5% in the past 12 months or body mass index < 20 kg/m^2 and, at least, three of the five following criteria: decreased muscle strength, fatigue, anorexia, low fat-free mass index, and abnormal biochemistry, with increased circulating inflammatory markers such as interleukin 6 (IL-6) > 4.0 pg/mL or C-reactive protein (CRP) > 5.0 mg/L; anaemia (haemoglobin < 12 g/dL); or low serum albumin (<3.2 g/dL). The WSC included colorectal cancer patients who did not fulfil the referred criteria to be enrolled in the cachectic group.

Clinical and biochemical parameters assessment

At the time of admission to the hospital for preoperative procedures (within the 24 h prior to surgery), patients were interviewed with specific questionnaires: EORTC QLQ-C30,[11,12] presenting a compendium of questions assessing functionality, general health, and clinical symptoms, thus enabling evaluation of the patient's quality of life. The FAACT-ESPEN questionnaire[11,12] was adopted to assess the presence and degree of anorexia. Anthropometric measurements (height and weight) of the patients were performed, and blood samples were collected for biochemical analysis in the automatic LABMAX 240® equipment, employing commercial kits (Labtest Diagnóstica SA, Lagoa Santa, Brazil) for CRP, albumin, and haemoglobin. Most of the blood samples were obtained in the afternoon (90% in the WSC group and 72% in the CC group) because of pre-established hospital admission schedules for patients whose surgeries would be performed the following morning. The majority of patients were fasted for more than 3 h at the time of blood collection (85% in the WSC group and 88% in the CC group).

Histological analysis of the colon

Rectosigmoid colon mucosal biopsies, about 20 cm distant from the tumour, were obtained during colectomy, fixed in 4% paraformaldehyde, and embedded in paraffin and non-serial 5 μm sections were obtained for histological evaluation. The slides were stained with haematoxylin, eosin, and periodic acid–Schiff (PAS) and examined by light microscopy at ×100, ×400, and ×1000 magnification, employing an integrative eyepiece with an ocular grid (Zeiss Integration Eyepiece I Kpl 8, Zeiss, Hamburg, Germany).

Detection of lymphocyte aggregates and determination of the number and distribution of eosinophils, plasma cells, and fibroblasts in sections of the colonic crypts were carried out. The number of lymphocytic aggregates in colonic mucosa (five fields/slide from each patient, stained with haemotoxylin–eosin, ×400 magnification) was compared between study groups. For eosinophils, plasma cells and fibroblasts infiltrated in the mucosa counts, 10 fields of each section were evaluated, under ×1000 magnification. Quantification was based on the morphology of cell types by light microscopy with an ocular grid.

For the determination of the area occupied by goblet cells in the colonic mucosa, three fields of each slide stained with PAS were photographed, with Image ProPlus v.5.2 (Media Cybernetics, Bethesda, USA), and evaluated (×200 magnification) for the calculation of percentage of positive area for PAS, with ImageJ software.

Immunohistochemistry of Ki-67 (cell proliferation marker) and CD68 (macrophage surface marker) in the colon

For immunohistochemical localization and quantification of nuclear protein Ki-67, a cell proliferation marker[13] and CD68, a macrophage surface marker, paraffin-embedded sections of 5 μm were deparaffinized and rehydrated, and peroxidase activity was blocked (0.3% H_2O_2 in methanol). Antigen retrieval was performed with citric acid (pH 6.0) at 95 °C for 20 min. The immunohistochemical reaction was performed with a commercial kit (Histostain-plus HRP, Life Technologies), following manufacturer's recommendations. After antigen retrieval, non-specific binding was blocked with serum blocking solution, followed by incubation with anti-Human Ki-67 primary antibody (SP6, M3062, Spring Bioscience, Pleasanton, CA, USA) at 1:100 and anti-human CD68 primary antibody [KP1] (ab955, Abcam, Cambridge, UK) at 1:200, overnight, at 4 °C.

After the addition of the biotinylated secondary antibody and streptavidin-peroxidase conjugate, peroxidase activity was detected with immPACT™DAB substrate (Vector Laboratories, Burlingame, CA, USA) and slides were counterstained with Mayer's haematoxylin. Negative controls were incubated with serum to replace the primary antibody.

Sections were observed under light microscope (Olympus, Montreal, Canada) at ×1000 magnification. Ki-67 labelled and non-labelled cells were identified along the crypt and the number of nuclei (labelled or not), counted in a total of at least 1000 cells per section, while the macrophages (CD68 positive cells) and unlabelled cells were counted from the observation of the colonic LP (10 fields/section), with an ocular grid. Representative photomicrographs were acquired with Image ProPlus v.5.2 (Media Cybernetics, Bethesda, MD, USA). For both immunohistochemical analysis of Ki-67 and CD68, the labelling index was obtained as the number of positive cells/total cells × 100.

Immunophenotyping of colonic lamina propria macrophages by flow cytometry

Rectosigmoid colon mucosal biopsies were collected in RPMI medium 1640 (Gibco, ThermoFischer Scientific). Underlying muscular layers and fat were carefully removed with scissors and the remaining tissue was cut into small fragments. For isolation of colonic LP immune cells, the fragments were digested for 25 min at 37 °C in R10 medium (RPMI medium 1640 supplemented with foetal bovine serum at 10%) containing collagenase type II (0.25 mg/mL) (Sigma Aldrich), under continuous stirring.

The cell suspensions were pooled after filtering through a 70 μM cell strainer and washed in R10 medium, and pelleted twice at 600g for 5 min at 4 °C. The samples of isolated cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco, ThermoFischer Scientific) with foetal bovine serum and dimethyl sulfoxide and stored in liquid nitrogen until analysis by flow cytometry.

Cell samples were thawed rapidly in a water bath at 37 °C, washed in DMEM, and pelleted at 500g for 10 min at 4 °C. The fluorochrome-conjugated antibodies from the macrophage panel (Supporting Information, *Table* S1) were then added to the samples, and these were incubated for 30 min at 4 °C, in the dark. One of the tubes with cell suspension did not receive antibodies. The cells were washed, centrifuged 400g for 5 min, resuspended in DMEM, and detected by flow cytometer (FACSCanto II, BD Biosciences), based on the fluorescence emitted by the cells labelled with antibodies.

Flow cytometer compensation was performed with compensating beads (BD™ CompBead, BD Biosciences) added to the fluorochrome-conjugated antibodies suspensions, individually. The gates were delimited for the analysis of macrophage subpopulations (M1, M2, and M1M2 phenotypes), as previously described by our group.[7] Data acquisition and immunophenotyping analysis were performed in Flow Jo software.

Multiplex protein analysis

The concentration of plasma and colonic mucosa cytokines/chemokines was analysed employing Luminex®

xMAP technology, following the manufacturer's instructions (human cytokine/chemokine panel I, Merckmillipore, MA, USA); quantification of the transforming growth factor beta (TGF-β) family proteins in the colon samples was also performed (multi-species TGF-β 3-plex, Merck Millipore, MA, USA). Samples of proteins of the colon mucosa (25 μL; 1:20 dilution; approximately 20 μg of colon lysate) and plasma samples (25 μL) were incubated with antibodies conjugated to Magplex microspheres for 2 h, under stirring, at room temperature. For detection of target antigens bound to the microspheres, the samples were incubated with biotinylated antibodies for 1 h, followed by incubation with streptavidin-phycoerythrin fluorescent conjugate for 30 min. The Magpix® instrument (Life Technologies, Grand Island, NY, USA) detected the intensity of the signal for each microsphere added to the protein samples. The results are reported in Median Fluorescent Intensities and the observed concentration of each analyte was calculated against standard curve regression, using MILLIPLEX™ Analyst. Finally, each value was corrected to total protein concentration. The analysed proteins are listed in *Table* 1.

Statistical analysis

Statistical analysis was carried out with the GraphPad Prism software version 5.0. Kolmogorov–Smirnov test were performed to determine whether the data were normally distributed. Parametric data were expressed as means ± standard errors, while non-parametric data as median [first quartile; third quartile]. The means of WSC and CC were compared using Student's *t*-test or Mann–Whitney's test, for parametric and non-parametric data, respectively; χ^2 and Fisher exact tests were used to compare nominal variables. Differences were considered significant if *P*-values were <0.05.

Table 1 Analysed proteins

Analyte	Abbreviation
Cytokine/chemokine	
Tumour necrosis factor alpha	TNF-α
Tumour necrosis factor beta	TNF-β
Interleukin 1 alpha	IL-1α
Interleukin 1 beta	IL-1β
Interleukin 1 receptor antagonist	IL-1RA
Interleukin 5	IL-5
Interleukin 7	IL-7
Interleukin 8	IL-8
Interleukin 10	IL-10
Interleukin 13	IL-13
Interleukin 15	IL-15
Interferon alpha	IFN-α
Interferon gamma	IFN-γ
Monocyte chemotactic protein 1	MCP-1/CCL2
Macrophage inflammatory protein-1 alpha	MIP-1α/CCL3
Macrophage inflammatory protein-1 beta	MIP-1β/CCL4
Chemokine (C-C motif) ligand 5	RANTES/ CCL5
Growth and differentiation factors	
Epidermal growth factor	EGF
Granulocyte-colony stimulating factor	G-CSF
Granulocyte-macrophage colony-stimulating factor	GM-CSF
Vascular endothelial growth factor	VEGF
Transforming growth factor beta 1	TGF-β1
Transforming growth factor beta 2	TGF-β2
Transforming growth factor beta 3	TGF-β3

Results

Characteristics of the study population

The characteristics of the study population are shown in *Table* 2. Considering the inclusion criteria, 45 patients with colorectal cancer were studied. Of these, 25 patients were classified as cachectic and 20 patients as weight stable.

No significant differences were observed in regard to age, gender, and current body mass index between WSC and CC patients. As expected, cachectic patients showed higher weight loss and lower serum albumin and haemoglobin content, as compared with patients without cachexia. The tumour stage variation and presence of metastasis was similar between WSC and CC. There was statistical difference between groups in regard to CRP plasma concentration ($P = 0.048$). Additionally, the CRP/albumin ratio was increased more than twice in CC, as compared with WSC. We have found this ratio to be more predictive of cachexia than the isolated parameters, taking in consideration the great variability of CRP concentration, normally detected among patients within the same groups.

Interleukin 6 and IL-8 expression in the plasma were found to be higher in CC, in relation to WSC ($P = 0.047$ and 0.009, respectively, *Table* 2). All values of cytokines, growth factors, and chemokines in plasma are shown in Supporting Information, *Tables* S2 and S3. The assessment of anorexia by specific questionnaires yielded lower scores for CC, which means that cachectic patients presented a higher degree of anorexia in relation to the weight stable counterparts. Cachectic patients also showed worsened quality of life, in the presence of systemic inflammation, when compared with WSC. Figure 1

Histological and immunological analysis of the colon

To characterize the morphological changes in the healthy segment of rectosigmoid colonic mucosa of cachectic patients, we analysed the tissue sections by light microscopy. The average number of lymphocyte aggregates found in the LP of the colon was higher in sections of CC ($P = 0.016$), compared with those obtained from WSC (*Figure* 2A and 2C).

Table 2 Characteristics of the study population

Characteristics		WSC	CC	*P*
N		20	25	
Male/female		11/9	14/11	0.947
Age (years)		64.5 ± 2.87	63.88 ± 2.43	0.658
BMI (kg/m^2)		23.95 ± 0.8	25.37 ± 0.99	0.753
Weight loss (kg)		0 [0; 0]	6 [0; 9.65]*	0.0004
Weight loss (%)		0 [0; 0]	8.8 [0; 14.35]*	0.0006
Tumour stage	I–II	12	10	
	III–IV	8	15	
Metastasis	Presence	1	5	0.205
	Absence	19	20	
Largest tumour dimension (cm)		4.35 ± 0.41	5.52 ± 0.53	0.097
CRP (mg/L)		6.53 ± 0.97	9.37 ± 0.98*	0.048
Albumin (g/dL)		4.22 ± 0.24	3.52 ± 0.22*	0.039
CRP/albumin		1.14 [0.77; 2.64]	3.21[1.33; 3.9]*	0.021
Haemoglobin (g/dL)		13.19 ± 0.49	11.1 ± 0.44*	0.003
IL-6 (pg/mL)		0.83 [0.005; 1.75]	2.89 [0.91; 7.52]*	0.047
IL-8 (pg/mL)		6.39 [3.41; 7.52]	30 [7.38; 61.92]*	0.009
TNF-α (pg/mL)		5.03 [4.03; 8.61]	7.91 [6.18; 10.97]	0.065

Data expressed as mean ± SE or as median [frst quartile; third quartile]. *P* = significance of Student's *t*-test or Mann–Whitney's test. Sample number (*n*). BMI, body mass index; CRP, C-reactive protein; IL-6, interleukin 6; IL-8, interleukin 8; TNF-α, tumour necrosis factor alpha. (WSC = 16; CC = 14 for cytokine analysis). CC, cachectic cancer; WSC, weight stable cancer.
*Significant difference WSC vs. CC ($P < 0.05$).

Cell proliferation in the colon crypt was investigated by immunohistochemistry, and an augmented number of epithelial cells labelled for Ki-67 was found in the cachectic patient's colon crypts, compared with the weight stable patients (*Figure* 2D and 2G). The area occupied by goblet cells in the colon mucosa was decreased in CC ($P = 0.016$), in comparison with WSC (*Figure* 3).

Figure 4A and 4B shows increased density of infiltrating immune cells in the LP in histological sections of patients with cancer and cachexia (CC), compared with non-cachectic cancer patients (WSC) ($P < 0.0001$).

To study the distribution of immune cells in the colon mucosa/submucosa of the patients, specific cell counting was performed by light microscopy, based on the morphology of cells. The results indicate an increase in the number of fibroblasts ($P < 0.0001$) and eosinophils ($P < 0.0001$) and a decrease in plasma cell density ($P = 0.0457$) in the CC patients, as compared with the findings in the sections of WSC (*Figure* 4C and 4D).

There was no significant difference in the quantification (labelling index) of macrophages by immunohistochemistry among the groups (WSC = 14.88 ± 1.66; CC = 13.78 ± 2.13; $P = 0.69$); (*Figure* 5A and 5D). We also did not detect statistical difference in the total macrophage population by flow cytometry. However, we observed an increase in macrophages with both phenotypes (M1M2 population) ($P = 0.042$) and a trend of higher M1 population ($P = 0.067$) in the colon of CC (*Figure* 5E and 5G).

Protein expression analysis in the colon

The concentration of growth factors and differentiation factors in the colon of patients is shown in *Table* 3. For these

Figure 1 (A) Functional assessment of anorexia/cachexia therapy–anorexia/cachexia subscale (FAACT–A/CS). (b) Quality of life score–quality of life questionnaire (QLQ)-C30. (WSC = 19; CC = 24). Data expressed as mean ± SE. CC, cachectic cancer; WSC, weight stable cancer. * Significant difference WSC vs. CC ($P < 0.05$).

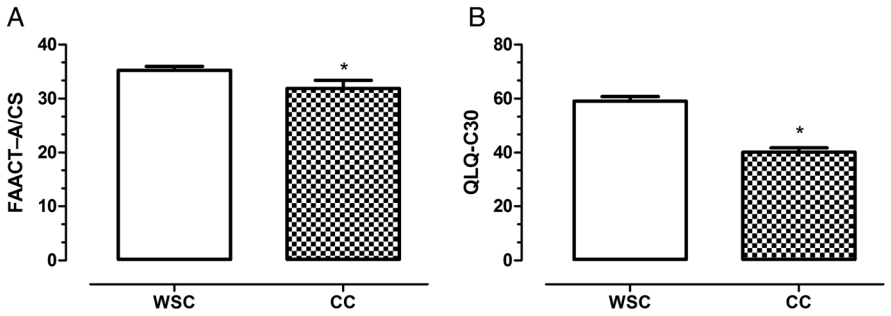

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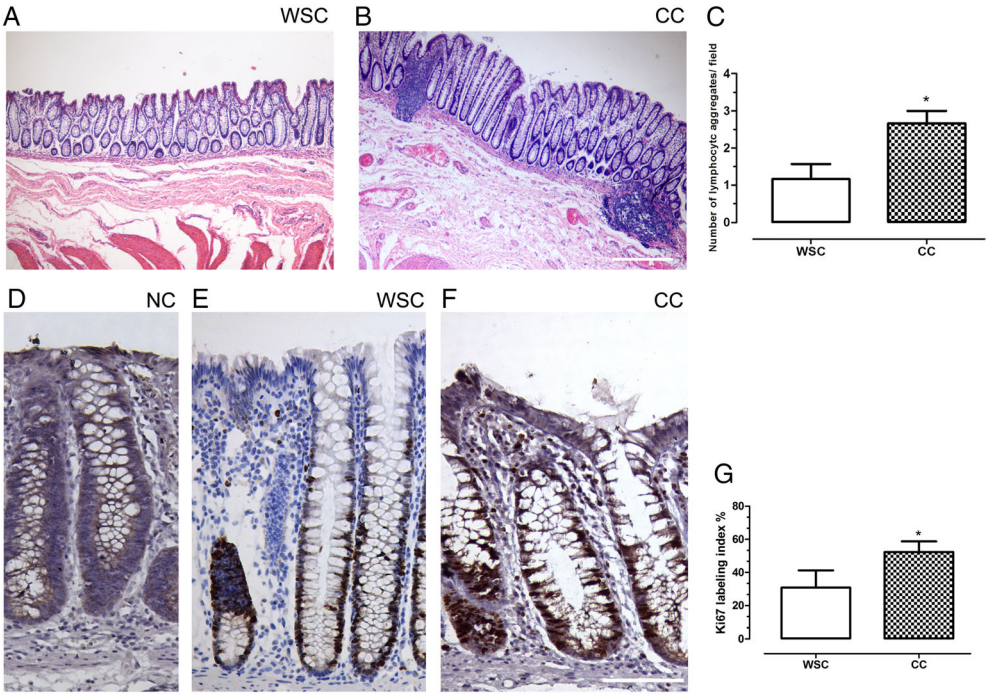
Figure 2 (A–C) Lymphocytic aggregates in the rectosigmoid colon mucosa. (A) WSC; (B) CC. Tissues were stained with haematoxylin and eosin and images represent ×40 magnification; (C) quantification of lymphocyte aggregates (WSC = 6; CC = 6); magnification bar: 500 μm. (D–G) Ki-67 immunostaining for epithelial cells in colonic mucosal crypts. (D) negative control; (E) WSC; (F) CC. Slides were counterstained with Meyer's haemotoxylin and images represent ×200 magnification; (G) Ki-67 labelling index. (WSC = 4; CC = 4); magnification bar: 100 μm. Data expressed as mean ± standard error. CC, cachectic cancer; WSC, weight stable cancer. * Significant difference WSC vs. CC ($P < 0.05$).



factors, there was a trend towards higher content of granulocyte-colony stimulating factor (G-CSF) in CC, compared with WSC ($P = 0.061$). There was no statistically significant difference for chemoattractants between WSC and CC (*Table* 3).

Interleukin 7 and IL-13 protein expression was significantly increased in CC, when compared with WSC ($P = 0.02$ and $P = 0.048$, respectively; *Table* 4; *Figure* 6A and 6B).

In addition, the analysis of the TGF-β family proteins in the colon of patients revealed that TGF-β3 concentration was higher in CC (*Table* 5; *Figure* 6C), compared with WSC ($P = 0.048$). No difference was found for TGF-β1 and TGF-β2 concentration between WSC and CC, as illustrated by *Table* 5.

Discussion

Cachexia is a multifactorial syndrome that affects around 50% of patients with colon cancer,[4] and there is an inverse correlation between the degree of cachexia and patient survival. This syndrome is also related to reduced response to therapy

Figure 3 Detection of mucus glycoproteins in the rectosigmoid colon mucosa. (A) WSC; (B) CC. Tissues were stained with periodic acid–Schiff (PAS) and images represent ×200 magnification; (C) quantification of PAS positive area (%) (WSC = 6; CC = 8); magnification bar: 100 μm. Data expressed as mean ± standard error. CC, cachectic cancer; WSC, weight stable cancer. * Significant difference WSC vs. CC ($P < 0.05$).

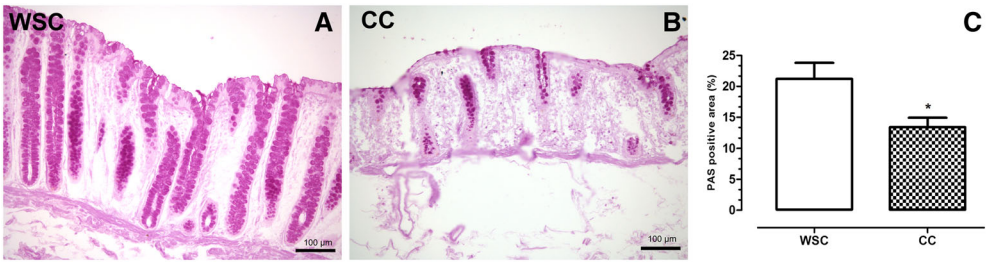


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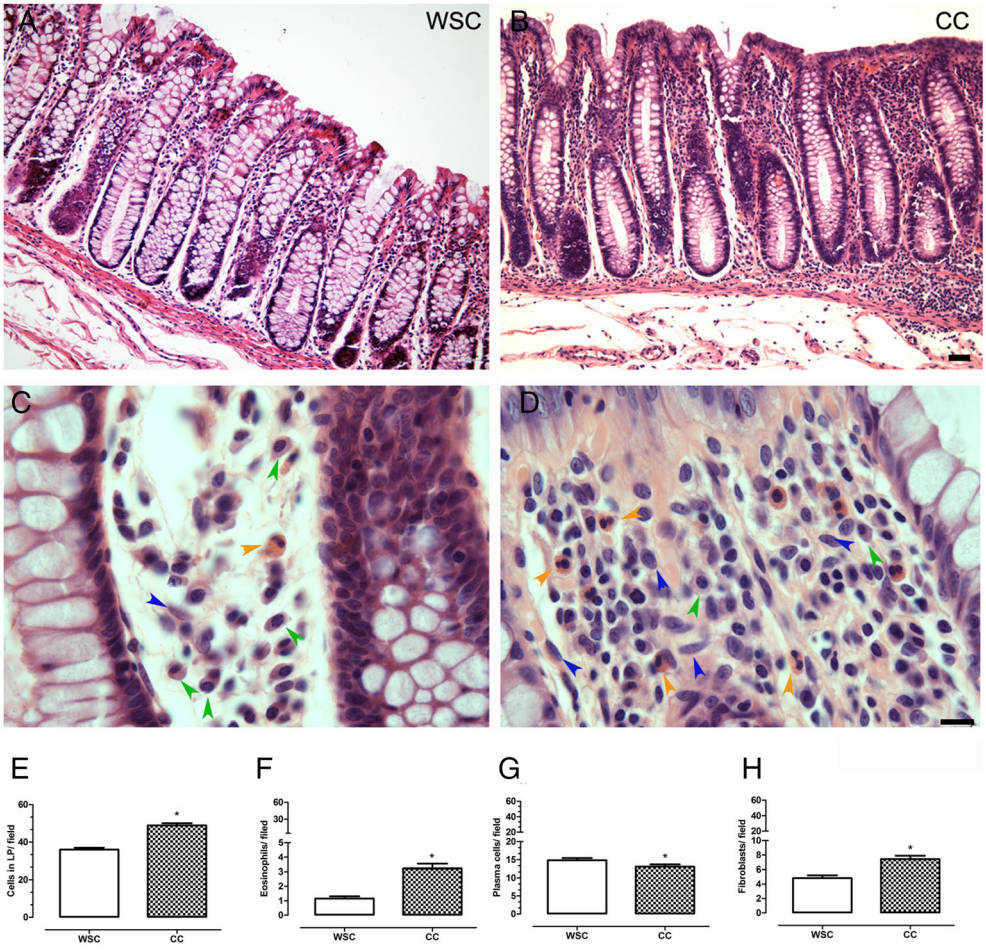
Figure 4 (A, B, E) Cellularity in the lamina propria (LP) of rectosigmoid colon mucosa. (A) WSC; (B) CC. Tissues were stained with haemotoxylin and eosin and images represent ×200 magnification; (E) number of LP cells in 10 fields of each sample (WSC = 6; CC = 6); magnification bar: 40 μm. (C, D, F, G, H) Cell infiltration in the LP of rectosigmoid colon mucosa. (C) WSC; (D) CC. Tissues were stained with haemotoxylin and eosin and images represent ×1000 magnification; arrowhead: eosinophil (orange); plasma cell (green); fibroblast (blue). (WSC = 6; CC = 6); magnification bar: 10 μm. Data expressed as mean ± standard error. CC, cachectic cancer; WSC, weight stable cancer. * Significant difference WSC vs. CC ($P < 0.05$).

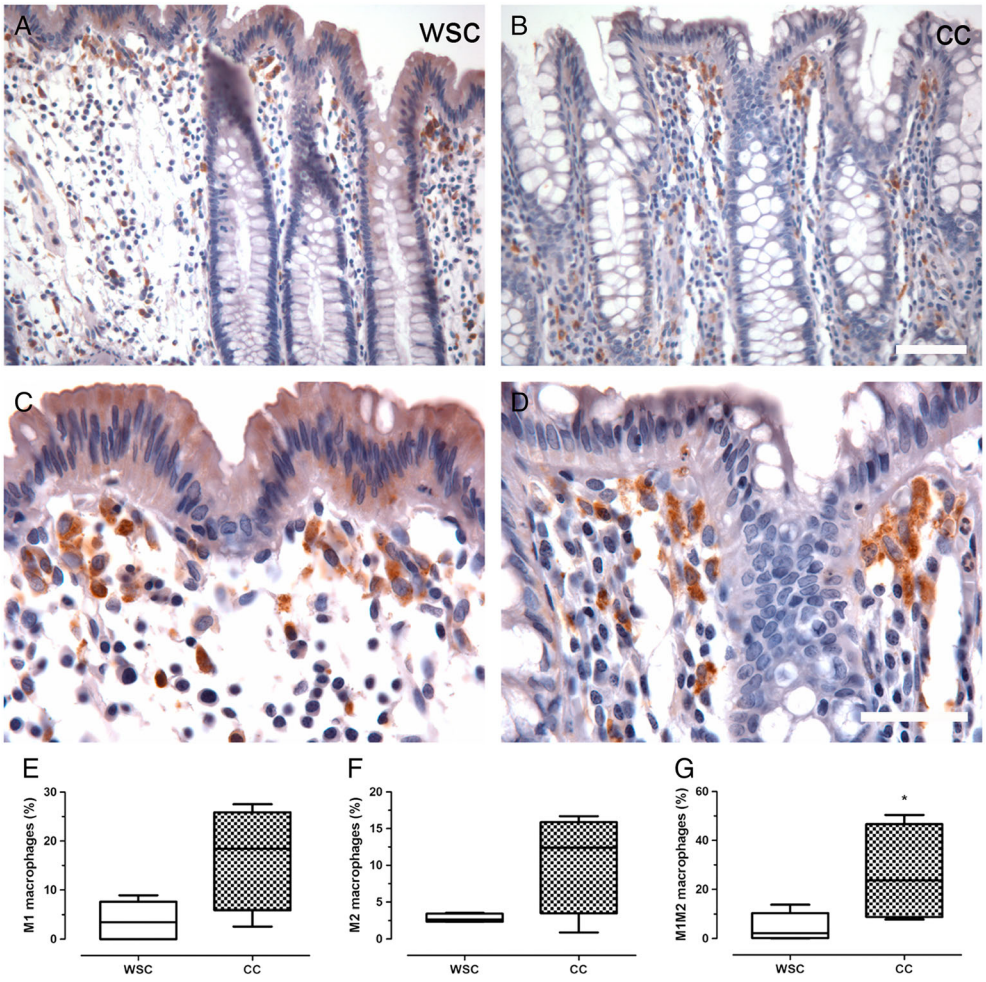


(surgery and/or chemotherapy) and decreased quality of life of patients.[14] Despite that, cachexia is often underdiagnosed and seldom treated, while its aetiology is not at the moment fully understood.[1,15]

Because cachexia may be envisaged as a syndrome that affects all the compartments of the organism, understanding of the molecular and morphological changes in different tissues or organs in cachectic patients is essential to reveal therapeutic targets. It is today clear that inflammation plays a central role in the syndrome and both the parenchymal and immune cells of the most organs could contribute to this component of the disease. The intestine, compared with any tissue in the body, contains the largest number of immune cells and the local immune response is modulated by a wide variety of antigens and potential immunological stimuli.[16] Barry[17] reported major changes in the small bowel in cancer patients that were associated with cancer-induced malnutrition. Creamer had already suggested that mucosal alterations could be found in the gut of patients with extra-gastrointestinal (GI) tract tumours.[18] Gilat *et al*. proposed the existence of a so called 'cancer enteropathy'.[19] These studies, nevertheless, examined the changes present in the small intestine, not those of the large intestine, an organ involved in many physiological processes, including water reabsorption, phenolic acids-derivatives absorption,[20] and transformation of bile acids,[21] among many others. Another aspect with potential consequences for cachexia is the disruption of colonic mucosa integrity, potentially leading to increased permeability, bacterial translocation, and the establishment of inflammatory and infectious reactions. Indeed, it has been recently suggested that intestinal barrier function is disrupted in cachexia.[10]

This study investigated inflammation-related parameters in the healthy segments of colon of patients with colon cancer,

Figure 5 (A–D) Representative images of immunohistochemistry for CD68 in the lamina propria (LP) of rectosigmoid colon mucosa. (A, C) WSC; (B, D) CC. Tissues were counterstained with Mayer's haematoxylin. Images represent ×400, magnification bar: 100 μm (A, B) and ×1000, magnification bar: 50 μm (C, D); (WSC = 4; CC = 4). (E–G) Percentage of macrophage subpopulations M1, M2, and M1M2 in cells isolated from the colonic LP; (WSC = 6; CC = 4). Data expressed as a minimum; 1st quartile; median; 3rd quartile; maximum. CC, cachectic cancer; WSC, weight stable cancer. * Significant difference WSC vs. CC ($P < 0.05$).



comparing cachectic and stable weight individuals. We have shown that the morphology and cytokine expression in the colon are altered in CC patients. The results show higher concentration of IL-13 and TGF-β3, as well as major increment in the cellularity and recruitment of immune cells, particularly of eosinophils, fibroblasts, and M1M2 macrophages, to the colonic LP in the cachectic patients. Genton *et al.*[22] stressed the role of inflammation in inducing epithelial changes in wasting diseases, with consequences on absorption and gut permeability. The same authors suggested that protein-energy wasting stimulates systemic and intestinal immune responses, as reported for patients with chronic heart failure.[23] However, to the best of our knowledge, no studies have been published on the modifications induced by cancer cachexia in the colon.

In the present study, the analysis of the colon tissue revealed an increased number of lymphoid aggregates (LA) in the submucosa of CC, in relation to WSC, a common histopathological finding in IBDs, such as ulcerative colitis (UC).[24] LA in the human colon seem to be involved in mucosal regeneration following tissue injury, in a process called mesenchymal-to-epithelial transition.[25] Bone marrow-derived mesenchymal stem cells migrate to the epithelial layer of the gut, where they acquire the epithelial-like phenotype, renewing the epithelium.[26] Growth factor receptors, such as epithelial growth factor receptors, and epithelial healing markers are expressed in subepithelial LA, which may indicate the direct involvement of these tissues in epithelial regeneration, mainly via mesenchymal-to-epithelial transition.[27]

Table 3 Protein expression of growth and differentiation factors and chemokines in the colon (whole tissue samples)

pg/mg total protein	WSC		CC		*P*
Growth and differentiation factors					
EGF	0.007 [0.0045; 0.0255]	*n* = 5	0.006 [0.0049; 0.0254]	*n* = 7	0.876
G-CSF	0.272 ± 0.0616	*n* = 5	47.81 ± 17.69	*n* = 8	0.061
GM-CSF	0.224 ± 0.0648	*n* = 5	0.311 ± 0.0754	*n* = 7	0.427
VEGF	0.470 [0.180; 2.207]	*n* = 4	0.984 [0.668; 148.1]	*n* = 7	0.412
Chemokines					
Eotaxin	49.25 ± 26.74	*n* = 5	73.92 ± 18.61	*n* = 7	0.451
CCL2	119.6 ± 53.37	*n* = 4	189.2 ± 54.19	*n* = 8	0.439
CCL3	0.065 ± 0.02	*n* = 5	0.15 ± 0.05	*n* = 8	0.209
CCL4	12.89 ± 7.043	*n* = 5	40.30 ± 14.61	*n* = 8	0.189
CCL5	2050 ± 709.7	*n* = 5	2643 ± 567.8	*n* = 8	0.529

Data expressed as mean ± standard error or as median [first quartile; third quartile]; *P* = significance of Student's *t*-test or Mann–Whitney's test. Sample number (*n*). The concentration of each protein of interest was normalized to total protein in the colon samples; CC, cachectic cancer patients; EGF, epidermal growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor; WSC, weight stable cancer patients.

The augmented cell proliferation in the intestinal crypts of cachectic patients, compared with the colon of weight stable cancer patients, suggests an attempt to repair the mucosa, injured by local inflammation, similarly to what occurs in colonic diverticulitis.[28]

The differentiation of epithelial cells is also affected during chronic colitis, as atrophy of the mucous glands and mucin depletion have been suggested as diagnostic markers for UC.[29] The epithelium responds to crypt epithelial damage during inflammation, especially with increased proliferation, in detriment of cell differentiation. Therefore, epithelial differentiation plays an important role in the protection of epithelial integrity and defects in the mucus barrier allow a greater exposure of the intestinal epithelium to antigens and may trigger local mucosal inflammation.[29,30]

Depletion of goblet cells in the colon epithelium was observed in cachectic patients, compared with weight stable counterparts (*Figure* 3). The integrity of the mucus barrier can be affected by several factors. Some of these factors, such as bacteria or toxins and cytokine response, may stimulate or inhibit mucin production and secretion, alter the chemical composition of mucins, or degrade the mucus layer.[31]

The increment in proliferation was paralleled by greater cellularity in the LP of CC, as compared with WSC. The number of eosinophils and fibroblasts was markedly increased in the colon of the cachectic patients. A study investigating the involvement of eosinophils in colonic mucosa of patients with IBD showed an augmented number of activated eosinophils in the LP of patients with UC, compared with control patients, which is associated with the development of IBD.[32]

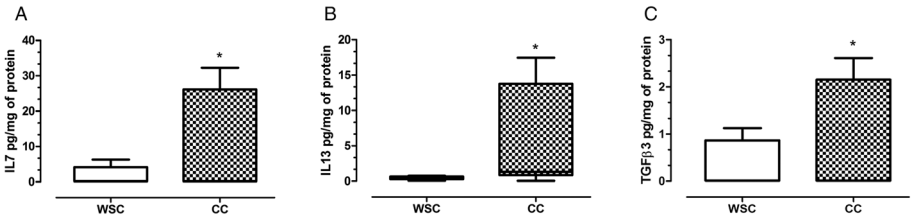
In response to proinflammatory cytokines in the colon, eosinophils and neutrophils are the first types of inflammatory cells recruited and are potentially important modulators of intestinal tumorigenesis.[33] Mucosal eosinophils may participate in both inflammatory cascades as well as in tissue

Table 4 Protein expression of cytokines in the colon (whole tissue samples)

pg/mg total protein	WSC		CC		*P*
IFN-α2	39.390 ± 21.12	*n* = 4	149.4 ± 57.27	*n* = 8	0.221
IFN-γ	0.106 ± 0.033	*n* = 5	0.204 ± 0.08	*n* = 7	0.340
IL-1α	0.133 ± 0.03	*n* = 5	0.281 ± 0.11	*n* = 7	0.291
IL-1ra	32.17 ± 23.63	*n* = 4	305.3 ± 95.75	*n* = 8	0.079
IL-1β	0.358 [0.16; 0.64]	*n* = 5	0.521 [0.195; 3.98]	*n* = 8	0.524
IL-2	0.273 ± 0.08	*n* = 5	0.326 ± 0.068	*n* = 7	0.636
IL-4	0.015 ± 0.005	*n* = 5	0.016 ± 0.005	*n* = 7	0.980
IL-5	0.180 ± 0.05	*n* = 5	0.251 ± 0.06	*n* = 7	0.411
IL-7	4.14 ± 2.14	*n* = 5	26.14 ± 6.15*	*n* = 8	0.020
IL-8	38.44 [20.43; 61.82]	*n* = 5	26.97 [11.79; 390.3]	*n* = 8	0.943
IL-13	0.528 [0.27; 0.64]	*n* = 5	1.324 [0.84; 13.78]*	*n* = 7	0.048
IL-15	0.458 ± 0.18	*n* = 5	0.446 ± 0.093	*n* = 7	0.949
IL-17	0.122 ± 0.029	*n* = 5	0.339 ± 0.09	*n* = 7	0.089
TNF-α	0.309 ± 0.09	*n* = 5	0.530 ± 0.179	*n* = 7	0.358
TNF-β	0.535 ± 0.27	*n* = 5	0.328 ± 0.08	*n* = 7	0.420

Data are expressed as mean ± standard error or as median [first quartile; third quartile]; *P* = significance of Student's *t*-test or Mann–Whitney's test. Sample number (*n*). The concentration of each protein of interest was normalized to total protein in the colon samples; CC, cachectic cancer; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor; WSC, weight stable cancer.
*Significant difference WSC vs. CC ($P < 0.05$).

Figure 6 (A, B, C) Protein expression in the colon (whole tissue samples). (A) Interleukin 7 (IL-7). (B) IL-13. (C) Transforming growth factor beta 3 (TGF-β 3). Protein expression of cytokines was normalized by the total protein content in the colon samples. (WSC = 5; CC = 8); CC, cachectic cancer; WSC, weight stable cancer. Data expressed as mean ± standard error or as minimum; first quartile; median; third quartile; maximum. * Significant difference WSC vs. CC ($P < 0.05$).



remodelling and repair.[34] In the circumstance of tissue repair, damage to intestinal epithelial cells or necrosis is potent signals for eosinophil recruitment and the release of cytokines that act by regulating tissue regeneration, such as TGF-β and fibroblast growth factors.[35] Thus, it is possible to speculate that CC patients' mucosa is in a condition similar to that of tissue damage.

We also focused on LP macrophages because they are one of the most abundant leukocytes in the colon[36] and because they contribute to homeostasis through an anti-inflammatory phenotype, in the steady state.[37] However, in conditions such as IBD, proinflammatory macrophages are increased in the colon and have been linked to disease severity and progression.[38,39] In colorectal cancer, anti-inflammatory macrophages prevent immune responses against tumour cells to promote tumour growth and dissemination, whereas proinflammatory macrophages may antagonize tumour growth.[40] A previous publication of our group showed a lower presence of M2 macrophages in tumours of cachectic colorectal cancer patients, as compared with the weight stable group.[7] Interestingly, this study evidenced an increase in the macrophage population exhibiting markers for both phenotypes (M1M2) in the colon of cachectic patients.

Increased immune cell density, nevertheless, is just one aspect of tissue inflammation. Hence, we performed protein expression analysis of growth and differentiation factors, cytokines, and chemokines, as to characterize the secretory profile of the immune cells populating the colon mucosa. IL-7 protein expression was increased in CC, in relation to WSC. This cytokine plays a regulatory role in differentiation and growth of intraepithelial lymphocytes in the intestinal mucosa.[41] Additionally, the concentration of IL-7 has been described to enhance in murine acute response,[42] HIV infection and chemotherapy,[43] as well as in rheumatoid arthritis,[44] all conditions that have been previously associated with cachexia. Furthermore, in the clinical setting of IBD, the persistence of IL-7-dependent colitogenic memory $CD4^+$ T cells is critical to the maintenance of colitis.[45]

Furthermore, we found increased concentration of IL-13 and TGF-β3 in the colon of cachectic patients, as compared with the weight stable subjects. This finding adds to the assumption that a tissue repair response-like process[46] is present in the cachectic patients, as these two factors present a synergistic action. IL-13 activates TGF-β, meaning this growth factor could be a downstream mediator of the cytokine actions.[47] IL-13 and TGF-β3 seem to act in concert in wound healing in intestinal inflammation,[46] corroborating the hypothesis of intestinal barrier impairment in CC.

A study by Suzuki and colleagues[48] showed increased mRNA expression of TGF-β in the colon of mice, in a model of chronic colitis induced by DSS, and the authors suggested that TGF-β stimulates fibrogenic mesenchymal cells to produce more collagen, acting as a profibrogenic cytokine.[48]

Contributing to the premise of the ongoing wound healing-related process owing to barrier disruption, CC showed a trend ($P = 0.061$) towards greater concentration of G-CSF expression in the colon G-CSF, IL-13 together with IL-5 have been recognized as activators of eosinophil function, including migration to the site of inflammation.[49] TGF-β concentration correlated positively with IL-7, IL-5, and IL-8

Table 5 Protein expression of TGF-β family proteins in the colon (whole tissue samples)

pg/mg total protein	WSC		CC		*P*
TGF-β1	196.8 ± 60.88	$n = 5$	184.2 ± 41.60	$n = 8$	0.8633
TGF-β2	17.13 [5.45; 56.92]	$n = 5$	19.55 [13.39; 28.75]	$n = 8$	0.9433
TGF-β3	1.172 [0.24; 1.34]	$n = 5$	2.189 [1.64;3.31]*	$n = 7$	0.0480

Data are expressed as mean ± standard error or as median [first quartile; third quartile]; P = significance of Student's *t*-test or Mann–Whitney's test. Sample number (n). The concentration of each protein of interest was normalized to total protein in the colon samples; CC, cachectic cancer; TGF, transforming growth factor; WSC, weight stable cancer.
*Significant difference WSC vs. CC ($P < 0.05$).

concentration in the cachectic group. IL-5 production has been associated with fibrosis-inducing mechanisms, together with IL-13 and IL-4.[50]

The microbiota may be comprehensively involved in the alterations herein reported, and Jiang *et al*.[51] have described increased bacterial translocation in patients with colon cancer; yet it was not our aim to address this matter presently.

Conclusions

In summary, our data point out that there is tissue repair-like process as a result of local inflammation in the colon of cachectic patients. Major changes in the number of immune cells, particularly of eosinophils, fibroblasts, and macrophages, and in cytokine secretion in the colon mucosa suggest an attempt to heal the tissue during cachexia. Further investigation is required concerning the intestinal immune response during cancer cachexia, in order to establish the contribution of the colon in the pathogenesis of this syndrome and to propose new therapeutic targets, ameliorating intestinal inflammation and its consequences for the patient.

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The authors certify that they comply with the ethical guidelines for authorship and publishing of the Journal of Cachexia, Sarcopenia and Muscle.[52]

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethics approval and consent to participate

This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. This study was approved by Biomedical Sciences Institute Ethics Committee (CAAE: 15492013.0.0000.5467) of the University of São Paulo and by the University Hospital Ethics Committee (CEP-HU/USP: 1385/14) in Brazil, and written informed consent was obtained from each patient before admission to the protocol.

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Panel of fluorochrome-conjugated antibodies for flow cytometry.
Table S2 Protein expression of cytokines in the plasma.
Table S3 Protein expression of growth and differentiation factors and chemokines in the plasma.

References

1. Evans WJ, Morley JE, Argilés J, Bales C, Baracos V, Guttridge D, et al. Cachexia: a new definition. *Clin Nutr* 2008;**27**:793–799.
2. Donohoe CL, Ryan AM, Reynolds JV. Cancer cachexia: mechanisms and clinical implications. *Gastroenterol Res Pract* 2011;**2011**:1–13.
3. Laviano A, Seelaender M, Rianda S, Silverio R, Rossi Fanelli F. Neuroinflammation: a contributing factor to the pathogenesis of cancer cachexia. *Crit Rev Oncog* 2012;**17**:247–252.
4. Argilés JM, Busquets S, Stemmler B, López-Soriano FJ. Cancer cachexia: understanding the molecular basis. *Nat Rev Cancer* 2014;**14**:754–762.
5. Klein GL, Petschow BW, Shaw AL, Weaver E. Gut barrier dysfunction and microbial translocation in cancer cachexia: a new therapeutic target. *Curr Opin Support Palliat Care* 2013;**7**:361–367.
6. Argilés JM, Busquets S, López-Soriano FJ. Anti-inflammatory therapies in cancer cachexia. *Eur J Pharmacol* 2011;**668**: S81–S86.
7. de Matos-Neto EM, Lima JD, de Pereira WO, Figuerêdo RG, Riccardi DM, Radloff K, et al. Systemic inflammation in cachexia—is tumor cytokine expression profile the culprit? *Front Immunol* 2015;**6**:629.
8. Argilés JM, Stemmler B, López-Soriano FJ, Busquets S. Nonmuscle tissues contribution to cancer cachexia. *Mediators Inflamm* 2015;**2015**:1–9, 182872.
9. Natividad JMM, Verdu EF. Modulation of intestinal barrier by intestinal microbiota: pathological and therapeutic implications. *Pharmacol Res* 2013;**69**:42–51.
10. Watson CJ, Hoare CJ, Garrod DR, Carlson GL, Warhurst G. Interferon-gamma selectively increases epithelial permeability to large molecules by activating different

populations of paracellular pores. *J Cell Sci* 2005;**118**(Pt 22:5221–5230.
11. Arezzo di Trifiletti A, Misino P, Giannantoni P, Giannantoni B, Cascino A, Fazi L, et al. Comparison of the performance of four different tools in diagnosing disease-associated anorexia and their relationship with nutritional, functional and clinical outcome measures in hospitalized patients. *Clin Nutr* 2013;**32**:527–532.
12. Chang VT, Xia Q, Kasimis B. The functional assessment of anorexia/cachexia therapy (FAACT) appetite scale in veteran cancer patients. *J Support Oncol* 2005;**3**:377–382.
13. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000;**182**:311–322.
14. Argilés JM, Busquets S, López-Soriano FJ, Figueras M. Pathophysiology of neoplasic cachexia. *Nutr Hosp* 2006;**21**:4–9.
15. Penet MF, Bhujwalla ZM. Cancer cachexia, recent advances, and future directions. *Cancer J* 2015;**21**:117–122.
16. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. *Nat Rev Immunol* 2014;**14**:667–685.
17. Barry RE. Malignancy, weight loss, and the small intestinal mucosa. *Gut* 1974;**15**: 562–570.
18. Creamer B. Malignancy and the small-intestinal mucosa. *Br Med J* 1964;**2**(5422):1435–1436.
19. Gilat T, Fischel B, Danon J, Loewenthal M. Morphology of small bowel mucosa in malignancy. *Digestion* 1972;**7**(3):147–155.
20. Williamson G, Clifford MN. Role of the small intestine, colon and microbiota in determining the metabolic fate of polyphenols. *Biochem Pharmacol* 2017;**139**:24–39.
21. Staley C, Weingarden AR, Khoruts A, Sadowsky MJ. Interaction of gut microbiota with bile acid metabolism and its influence on disease states. *Appl Microbiol Biotechnol* 2017;**101**:47–64.
22. Genton L, Cani PD, Schrenzel J. Alterations of gut barrier and gut microbiota in food restriction, food deprivation and protein-energy wasting. *Clin Nutr* 2015;**34**: 341–349.
23. Sandek A, Anker SD, von Haehling S. The gut and intestinal bacteria in chronic heart failure. *Curr Drug Metab* 2009;**10**:22–28.
24. Yeung MM, Melgar S, Baranov V, Oberg A, Danielsson A, Hammarström S, et al. Characterisation of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TcR-gammadelta expression. *Gut* 2000;**47**:215–227.
25. Sipos F, Muzes G, Galamb O, Spisák S, Krenács T, Tóth K, et al. The possible role of isolated lymphoid follicles in colonic mucosal repair. *Pathol Oncol Res* 2010;**16**: 11–18.
26. Matsumoto T, Okamoto R, Yajima T, Mori T, Okamoto S, Ikeda Y, et al. Increase of bone marrow-derived secretory lineage epithelial cells during regeneration in the human intestine. *Gastroenterology* 2005;**128**:1851–1867.
27. Sipos F, Muzes G, Valcz G, Galamb O, Tóth K, Leiszter K, et al. Regeneration associated growth factor receptor and epithelial marker expression in lymphoid aggregates of ulcerative colitis. *Scand J Gastroenterol* 2010;**45**:440–448.
28. Ulmer TF, Rosch R, Mossdorf A, Alizai H, Binnebösel M, Neumann U. Colonic wall changes in patients with diverticular disease—is there a predisposition for a complicated course? *Int J Surg* 2014;**12**:426–431.
29. Koch S, Nusrat A. The life and death of epithelia during inflammation: lessons learned from the gut. *Annu Rev Pathol* 2012;**7**:35–60.
30. Johansson MEV. Mucus layers in inflammatory bowel disease. *Inflamm Bowel Dis* 2014;**20**:2124–2131.
31. Cornick S, Tawiah A, Chadee K. Roles and regulation of the mucus barrier in the gut. *Tissue Barriers* 2015;**3**:e982426.
32. Carvalho AT, Elia CC, de Souza HS, Elias PR, Pontes EL, Lukashok HP, et al. Immunohistochemical study of intestinal eosinophils in inflammatory bowel disease. *J Clin Gastroenterol* 2003;**36**:120–125.
33. Slattery ML, Fitzpatrick FA. Convergence of hormones, inflammation, and energy-related factors: a novel pathway of cancer etiology. *Cancer Prev Res (Phila)* 2009;**2**: 922–930.
34. Powell N, Walker MM, Talley NJ. Gastrointestinal eosinophils in health, disease and functional disorders. *Nat Rev Gastroenterol Hepatol* 2010;**7**:146–156.
35. Stenfeldt AL, Wennerås C. Danger signals derived from stressed and necrotic epithelial cells activate human eosinophils. *Immunology* 2004;**112**:605–614.
36. Lee SH, Starkey PM, Gordon S. Quantitative analysis of total macrophage content in adult mouse tissues. Immunochemical studies with monoclonal antibody F4/80. *J Exp Med* 1985;**161**:475–489.
37. Isidro RA, Appleyard CB. Colonic macrophage polarization in homeostasis, inflammation, and cancer. *Am J Physiol Gastrointest Liver Physiol* 2016;**311**: G59–G73.
38. Thiesen S, Janciauskiene S, Uronen-Hansson H, Agace W, Högerkorp CM, Spee P, et al. CD14(hi)HLA-DR (dim) macrophages, with a resemblance to classical blood monocytes, dominate inflamed mucosa in Crohn's disease. *J Leukoc Biol* 2014;**95**: 531–541.
39. Tamoutounour S, Henri S, Lelouard H, de Bovis B, de Haar C, van der Woude CJ, et al. CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur J Immunol* 2012;**42**:3150–3166.
40. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *J Leukoc Biol* 2009;**86**:1065–1073.
41. Okazawa A, Kanai T, Nakamaru K, Sato T, Inoue N, Ogata H, et al. Human intestinal epithelial cell-derived interleukin (IL)-18, along with IL-2, IL-7 and IL-15, is a potent synergistic factor for the proliferation of intraepithelial lymphocytes. *Clin Exp Immunol* 2004;**136**:269–276.
42. Sawa Y, Arima Y, Ogura H, Kitabayashi C, Jiang JJ, Fukushima T, et al. Hepatic interleukin-7 expression regulates T cell responses. *Immunity* 2009;**30**:447–457.
43. Fry TJ, Mackall CL. The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *J Immunol* 2005;**174**: 6571–6576.
44. van Roon JA, Verweij MC, Wijk MW, Jacobs KM, Bijlsma JW, Lafeber FP. Increased intraarticular interleukin-7 in rheumatoid arthritis patients stimulates cell contact-dependent activation of CD4(+) T cells and macrophages. *Arthritis Rheum* 2005;**52**:1700–1710.
45. Kanai T, Nemoto Y, Kamada N, Totsuka T, Hisamatsu T, Watanabe M, et al. Homeostatic (IL-7) and effector (IL-17) cytokines as distinct but complementary target for an optimal therapeutic strategy in inflammatory bowel disease. *Curr Opin Gastroenterol* 2009;**25**:306–313.
46. Scharl M, Frei S, Pesch T, Kellermeier S, Arikkat J, Frei P, et al. Interleukin-13 and transforming growth factor β synergise in the pathogenesis of human intestinal fistulae. *Gut* 2013;**62**:63–72.
47. Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, et al. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med* 2001;**194**: 809–822.
48. Suzuki K, Sun X, Nagata M, Kawase T, Yamaguchi H, Sukumaran V, et al. Analysis of intestinal fibrosis in chronic colitis in mice induced by dextran sulfate sodium. *Pathol Int* 2011;**61**:228–238.
49. Műzes G, Molnár B, Tulassay Z, Sipos F. Changes of the cytokine profile in inflammatory bowel diseases. *World J Gastroenterol* 2012;**18**:5848–5861.
50. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med* 2012;**18**:1028–1040.
51. Jiang Y, Lin J, Zhang D, Yu Z, Li Q, Jiang J, et al. Bacterial translocation contributes to cachexia and its possible pathway in patients with colon cancer. *J Clin Gastroenterol* 2014;**48**:131–137.
52. von Haehling S, Morley JE, Coats AJS, Anker SD. Ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2017. *J Cachexia Sarcopenia Muscle* 2017;**8**: 1081–1083.