ORIGINAL ARTICLE

Journal of Cachexia, Sarcopenia and Muscle 2016; **7**: 37–47
Published online 4 June 2015 in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/jcsm.12037

Cachexia-associated adipose tissue morphological rearrangement in gastrointestinal cancer patients

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Abstract

Background and aims Cachexia is a syndrome characterized by marked involuntary loss of body weight. Recently, adipose tissue (AT) wasting has been shown to occur before the appearance of other classical cachexia markers. We investigated the composition and rearrangement of the extracellular matrix, adipocyte morphology and inflammation in the subcutaneous AT (scAT) pad of gastrointestinal cancer patients.

Methods Surgical biopsies for scAT were obtained from gastrointestinal cancer patients, who were signed up into the following groups: cancer cachexia (CC, $n = 11$), weight-stable cancer (WSC, $n = 9$) and weight-stable control (non-cancer) (control, $n = 7$). The stable weight groups were considered as those with no important weight change during the last year and body mass index <25 kg/m^2. Subcutaneous AT fibrosis was quantified and characterized by quantitative PCR, histological analysis and immunohistochemistry.

Results The degree of fibrosis and the distribution and collagen types (I and III) were different in WSC and CC patients. CC patients showed more pronounced fibrosis in comparison with WSC. Infiltrating macrophages surrounding adipocytes and CD3 Ly were found in the fibrotic areas of scAT. Subcutaneous AT fibrotic areas demonstrated increased monocyte chemotactic protein 1 (MCP-1) and Cluster of Differentiation (CD)68 gene expression in cancer patients.

Conclusions Our data indicate architectural modification consisting of fibrosis and inflammatory cell infiltration in scAT as induced by cachexia in gastrointestinal cancer patients. The latter was characterized by the presence of macrophages and lymphocytes, more evident in the fibrotic areas. In addition, increased MCP-1 and CD68 gene expression in scAT from cancer patients may indicate an important role of these markers in the early phases of cancer.

Keywords Adipose tissue; Extracellular matrix; Fibrosis, Inflammation, Cachexia

Received: 19 December 2014; Revised: 11 March 2015; Accepted: 30 March 2015
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Introduction

Cachexia is a wasting condition, directly associated with 22–40% of all cancer deaths.[1,2] It has been defined as 'a multifactor syndrome', characterized by ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment.[3,4] Although muscle wasting has been the main focus of cachexia-related research,[5,6] studies show that fat loss occurs more rapidly and more precociously than the reduction of lean mass in cancer cachexia (CC)[7–9] and may extend up to 80% within a very short interval, especially in the immediate period preceding death. Still, more recently, we have shown that the adipose tissue (AT) of cachectic cancer patients, in particular the subcutaneous AT (scAT) depot, is a possible relevant systemic source of inflammatory molecules during the development of the disease.[10]

Several factors have been demonstrated to contribute to cachexia-related loss of AT, such as (i) increased lipid mobilization due to enhanced adipocyte triglyceride lipolysis,[8,11] (ii) reduced lipogenesis and fatty acid esterification due to decrease of both fatty acid synthase and lipoprotein lipase activity[12] and (iii) impairment of fat cell turnover (pre-adipocyte/mature adipocyte), resulting in a disruption in the organization and

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development of AT.[13] In addition to that, several morphological and molecular changes in AT depots of rats during the progression course of CC have been described.[9,14–16] Most of the alterations, nevertheless, are perceived before any detectable morphological disruption, notably the down-regulation of genes related with adipogenesis, metabolism and mature adipocyte function. Furthermore, AT inflammation is a well-characterized aspect of the syndrome.[9,17,18] Such condition seems to be most apparent at the final stages of cachexia (cachexia to refractory cachexia).

AT has been described as a heterogeneous tissue that possesses marked anatomically-related depot specialization in regard to many parameters, including cellularity, form of growth and expansion, metabolism, production of and response to cytokines, hormones, density and distribution of innervation, as well as to fatty acid composition (reviewed by Pond[19]). Several chronic diseases, such as obesity,[20] metabolic syndrome and lipodystrophy,[21] induce AT remodelling, involving two major processes: adipose cell morphometric changes (hypertrophy or atrophy) and immune cells accumulation. Inflammation, endoplasmatic reticulum stress and hypoxia are also part of the general biologic alterations subsidizing the attraction and retention of inflammatory cells in AT.[22] AT extracellular matrix (ECM) remodelling has been found to play an essential role in adipogenesis[22] and tissue structure establishment.[23] In obesity, these alterations were demonstrated to be crucial to accommodate cellular alterations.[24] In db/db obese mice, epididymal AT morphological changes occur concomitantly to up-regulation of various types of collagens, such as I, IV and VI.[25] The former is related with increased adipocyte size.[26] This feature is generally followed by persistent inflammatory stimulus in AT that may underline excessive synthesis of ECM components. Consequently, subsequent interstitial deposition of fibrotic material (fibrosis) and enhanced expression of ECM proteins seem to be a result of a pervasive tissue response to unresolved chronic inflammation.[20] Divoux *et al*.[20] have reported increases in ECM components secretion induced by products synthesized by macrophages infiltrated in the AT (ATMϕs), suggesting a possible interaction between inflammatory cells and AT remodelling. In the same study, other inflammatory cells, including T-lymphocytes and mast cells, were shown to participate in one such process. However, despite AT remodelling being well described in obesity, few studies have addressed this topic in CC.

Thus, to obtain broader insight into AT remodelling in the setting of CC, we have analysed the scAT depot of cancer patients. The choice of scAT, rather than visceral AT, relies on the fact that we have previously shown[10] that this pad responds more precociously to the presence of the tumour in the organism, acting as a source of inflammatory markers. Additionally, it is an ideal tissue for the obtainment of biopsies.

Firstly, we evaluated fibrosis in a qualitative and quantitative manner and in particular, collagen density in scAT of weight-stable and cachectic cancer patients. Secondly, we characterized the inflammatory cells surrounding fibrotic depots. Finally, we determined scAT inflammatory gene expression in cancer patients. The analysis shows discrete early morphological modifications in scAT, followed by increased inflammatory cell infiltration and fibrosis, which are more evident in the cachectic cancer patients.

Materials and methods

Patients and sample collection

Patients were recruited between November 2008 and July 2010 at University Hospital of the University of São Paulo ($n = 26$). The inclusion criteria were as follows: 1, not having received prior anticancer treatment and 2, willingness to participate. The exclusion criteria were as follows: chemotherapy at the time of the study; continuous anti-inflammatory therapy; and kidney or liver failure, acquired immunodeficiency syndrome, inflammatory bowel disease or chronic inflammatory processes not related with cachexia, such as autoimmune disorders. Patients with body mass index (BMI) greater than 29.9 were also excluded from the study. The study was approved by the Ethics Committee from the Institute of Biomedical Sciences and by the Human Ethics Committee of the University of São Paulo Hospital (CEP-ICB/USP 1117/13, CEP-HU/USP 752/07 and 1117/13, CAAE 0031.0.198.019-07). The investigation was explained in detail to each patient, and written informed consent was obtained. They were separated into three groups, based on diagnosis after surgery. The subjects were subdivided into CC (CC, $n = 12$) and weight-stable cancer (WSC, $n = 7$) and weight-stable control (non-cancer) (control, $n = 7$). Patients were considered cachectic based on criteria from the international consensus.[2] Cachexia is recognized in patients with weight loss $>5\%$ in the past 6 months or any degree of weight loss $>2\%$ in the last 6 months + BMI $<20\ kg/m^2$. The stable weight groups were considered as those with no important weight change during the previous year and BMI $<25\ kg/m^2$. In the cancer groups (CC and WSC), the tumour primary location was colon ($n = 8$), stomach ($n = 5$), pancreas ($n = 2$) and other ($n = 4$). The control group included patients undergoing surgery for incisional hernia ($n = 5$) and chronic cholecystitis ($n = 2$). The study was designed as 'intention to compare'; therefore, all subjects were kept in the analyses despite a few missing values of the measurements. *Table* 1 presents the general characteristics of patients in each group.

Clinical parameters assessment

Height and weight were determined, and approximately 10 mL of blood was collected after overnight fast, within the

DOI: 10.1002/jcsm.12037

Table 1 Characteristics of study groups

Measure	Control	WSC	CC	*P*
n	7	7	12	
Gender (male/female)	3–4	3–4	8–4	
Age (years)	59.0 ± 12.7	68.2 ± 13.3	61.5 ± 16.9	0.722
Weight (kg)	59.9 ± 8.5	63.4 ± 10.6	69.5 ± 9.2	0.105
Height (m)	1.62 ± 0.04	1.56 ± 0.07	1.63 ± 0.06	0.053
BMI (kg/m^2)	21.9 ± 1.2	24.2 ± 1.7	21.1 ± 3.6	0.073
Weight loss (kg)	1.4 ± 1.8	2.7 ± 0.7	13.6 ± 4.1	<0.01
Weight loss (%)	2.8 ± 0.3	4.3 ± 0.7	19.9 ± 6.6	<0.01
Tumour stage				
IA–B		1 (14%)	1 (8%)	
IIA–B		3 (43%)	3 (25%)	
IIIA–B		2 (29%)	5 (42%)	
IV		1 (14%)	3 (25%)	
S-Hg (g/dL)	12.6 ± 1.2	10.8 ± 1.9	11.4 ± 1.8	0.147
P-urea (mg/dL)	35.2 ± 11	34.3 ± 14	30.2 ± 15	0.622
P-creatinine (mg/dL)	0.88 ± 0.2	0.90 ± 0.3	0.84 ± 0.3	0.892
S-TAG (mg/dL)	148 ± 19	133 ± 15	101 ± 13	<0.01
S-cholesterol (mg/dL)	205 ± 14	203 ± 16	173 ± 18	<0.01
S-LDL (mg/dL)	105 ± 12	102 ± 13	83 ± 15	<0.01
P-IL-6 (pg/mL)	9.2 ± 8.2	36.6 ± 20	96.4 ± 99	0.034
P-CRP (pg/mL)	2.1 ± 2.2	4.4 ± 3.5	28.2 ± 20	<0.01

BMI, body mass index; CC, cancer cachexia; CRP, C-reactive protein; Hg, haemoglobin; IL, interleukin; LDL, low-density lipoprotein levels; *n*, number of patients; P, plasma; S, serum; TAG, triglyceride serum levels; WSC, weight-stable cancer.
Values are mean ± standard deviation.
Statistical analysis, *P* vs. control subjects.

venous access procedure for anaesthesia during the surgery, allowing the measurement of plasma C-reactive protein, serum urea, creatinine and haemoglobin. Plasma and serum samples were then immediately frozen at −80°C, until further analysis. Tumour staging was determined postoperatively, according to the guidelines of the Union for International Cancer Control TNM.[27]

Adipose tissue biopsies

Approximately 1 g of subcutaneous white AT (by approximate anatomical site) was collected in within a 5 min interval, similarly to that described by Agustsson *et al*.[11] A portion of AT biopsy was immediately transferred into liquid nitrogen and kept at −80 °C before RNA analysis. The other part was fixed overnight at 4 °C in 4% paraformaldehyde and processed for standard paraffin embedding.[10] This procedure presents a minimal degree of risk and does not interfere with the standard surgery procedure.

Morphological analysis

The sequential 5 μm sections obtained were first stained with haematoxylin and eosin and then, with picro sirius red. The sections were analysed with a Leica microscope (DM 750), equipped with filters to provide circularly polarized light. For the analysis of morphometric aspects, the area, average diameter, perimeter and shape were measured by Imagen Pro-Plus 6.0 (100 adipocytes per stained section). Picro sirius red staining sections allowed collagen fibres detection with different colours.[28] Type I collagen fibres appear orange to red, whereas the thinner type III collagen fibres are stained with a yellow to green hue. Tissue images were obtained with a ×40 objective lens, recorded on a digital camera (DFC 295, Leica), displayed on a high-resolution monitor (LG, Flatron, E1941) and analysed with SigmaScan Pro image analysis (Chicago, IL, USA). For the different morphological analysis, different histological slices (non serial sections) from the same group were employed.

Immunohistochemistry

Immunohistochemistry of AT was carried out with sections fixed in buffered formalin and embedded in paraffin. Deparaffinized sections (5 μm) were stained with haematoxylin and eosin. After quenching of endogenous peroxidase activity with 0.3% H2O2 in methanol and blocking of free protein-binding sites with 5% normal goat serum, sections were immunostained for immune cells: macrophages with CD68 anti-mouse KP-1 monoclonal antibody (DAKO, Denmark—Ref m 0814), neutrophils with CD15 (DAKO, Denmark—Ref IR062) and T lymphocytes with CD3 (Cell Marque, Rocklin—Ref 103A-76) antibodies. Specific secondary antibodies were peroxidase (horseradish peroxidase) conjugated. Histochemical reactions were performed employing Vecta stain ABC Kit

(Vector Laboratories) and Sigma Fast 3,3-diaminobenzidine as substrate (Sigma, St. Louis, MO). Sections were counterstained with haematoxylin.

Gene expression analysis

Total RNA of the samples was isolated with TriPure Isolation Reagent (Roche®), following the recommendations of manufacturer,[29] and total RNA concentration, quantified by spectrophotometry (Nanodrop ND-1000). Complementary DNA synthesis was carried out employing 13 μL assay mix containing 3 μg total RNA, 10U RNAse inhibitor, 2 μL random primers, 2 μL dNTP (10 nmol), 2 μL dithiothreitol, 10U Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and 4 μL of ×10 reaction buffer (100 mM Tris-HCl, 500 mM KCl; 150 nM $MgCl_2$ in nuclear free water) (Invitrogen). Gene expression of CD68 (NM_001040059.1, forward 5'ACT GAA CCC CAA CAA AAC CA3', reverse 5'TTG TAC TCC ACC GCC ATG TA) and MCP-1 (CCL2) (NM_002982.3, forward 5' CCC CAG TCA CCT GCT GTT AT 3' and reverse 5'TGG AAT CCT GAA CCC ACT TC 3'). Five microlitres of cDNA (25 ng) were mixed with 2x SYBR Green PCR master mix (Applied Biosystems) and primers (Invitrogen). Quantitative real-time PCR was performed with an ABI 7300 real-time systems (Applied Biosystems). The mRNA levels were determined by comparative Ct method for each sample. A ΔCt value was obtained by subtracting 18 S values from those of the gene of interest. The average ΔCt value of the control group was then subtracted from the sample to derive a ΔΔCt value. The expression of each gene was evaluated by $2^{-(\Delta\Delta Ct)}$.

Statistical analysis

The statistical analysis was performed with the commercially available statistical package from SigmaStat (version 3.1, Sigma Stat, SYSTAT, Point Richmond, CA). Data are expressed as mean ± standard error and analysed by one-way analysis of variance. When a significant *F*-value was found by one-way analysis of variance, a Bonferroni's *post hoc* test was performed to demonstrate all pairwise multiple comparisons between the means. All calculated *P*-values were two sided, and a $P < 0.05$ was considered significant.

Results

Clinical findings

Baseline characteristics of the patients are shown in *Table* 1. The subjects in the three groups were of similar age and BMI. The body mass 6–12 months before inclusion in the study, as reported by patients, showed difference among the groups. CC mass was significantly lower, as compared with WSC and control, considering weight loss, both as absolute values (13.6 kg, $P < 0.01$, compared with control) as relative values (19.9%, $P < 0.01$, compared with control). Plasma lipid profile was affected by CC, with decreased level of triglyceride serum levels (31.8%, $P < 0.01$), total cholesterol (15.6%, $P < 0.01$) and low-density lipoprotein levels (14.3%, $P < 0.01$) when compared with the control patients. There was no difference with respect to other biochemical parameters (i.e. plasma creatinine and urea and serum haemoglobin values).

We did not evaluate lean body mass, although groups were matched by BMI. CC patients showed values ranging from 8.9% to 30.8% of body weight loss during the previous 6 months and C-reactive protein higher than 5 μg/mL, as well as increased levels of interleukin (IL)-6 (12.6-fold and 9.4-fold, respectively, $P < 0.001$) compared with control, indicating that the studied cachectic patients demonstrated weight loss and increased plasma inflammatory markers. WSC patients showed values ranging from 3.2% to 5.4% of body weight loss during the previous 6 months and C-reactive protein values ranging from 1.2 to 10.7 5 μg/mL. However, there was no statistical difference when compared with the control group.

Morphological analysis and collagen diversity in scAT

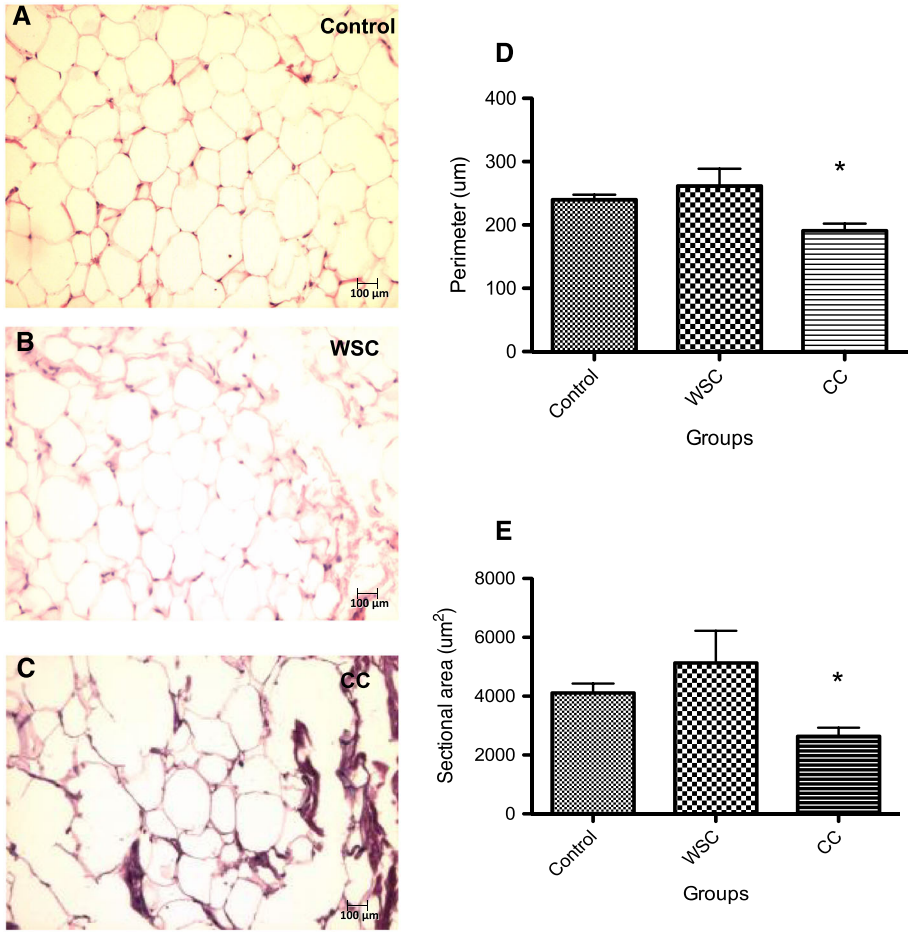
Cancer cachexia patients showed body weight loss and BMI reduction, suggesting the possibility that modification of AT cell morphology may be a response to CC. In order to address this question, we determined cell perimeter and the cross-sectional area in samples of the subcutaneous depot (*Figure* 1). Light microscopy examination indicates that morphometric alteration of scAT is only evident in the CC group (*Figure* 1A–C). In scAT from CC, adipocyte size, determined as sectional area (*Figure* 1D), and cell perimeter (*Figure* 1E) showed a decrease of 32.9% ($P < 0.05$) and 18.5% ($P < 0.05$), respectively, compared with the control samples.

When examined under polarized light microscopy, the same samples from CC patients demonstrated that types I (red label) and III (green label) collagen fibres were present around the adipocytes, in organized bundles of various thicknesses (*Figure* 2A–F). As compared with control patients, CC samples showed an increase of 1.7-fold ($P < 0.01$) of total type I collagen, no changes in type III collagen ($P = 0.10$) and an increase in collagen I–III ratio ($P = 0.0159$) (*Figure* 2G–H).

Inflammatory profile and cell infiltration

Taking into account the scope of data regarding AT remodelling induced by cachexia, notably the modification in total collagen density (fibrosis), we also examined the presence

Figure 1 Morphological characteristics of subcutaneous adipose tissue depot in cachexia and control patients. Haematoxylin and eosin stained sections of subcutaneous tissue from (A) weight-stable subjects and control, (B) weight-stable cancer (WSC) patients and (C) cancer cachexia (CC) patients. Morphometric analysis of sectional area (D) and (E) perimeter of adipocytes from different experimental groups. Photomicrographs (A–C) illustrate the most representative images considering data related to morphological analysis (D, E). Values are mean ± SEM. * $P < 0.05$ vs. control subjects.



of inflammation and infiltrated cells in scAT from the patients. Immunostaining for macrophages (CD68), T-lymphocytes (CD3) and neutrophils (CD15) and analysis of gene expression of MCP-1 and CD68 were carried out (*Figures* 3–5).

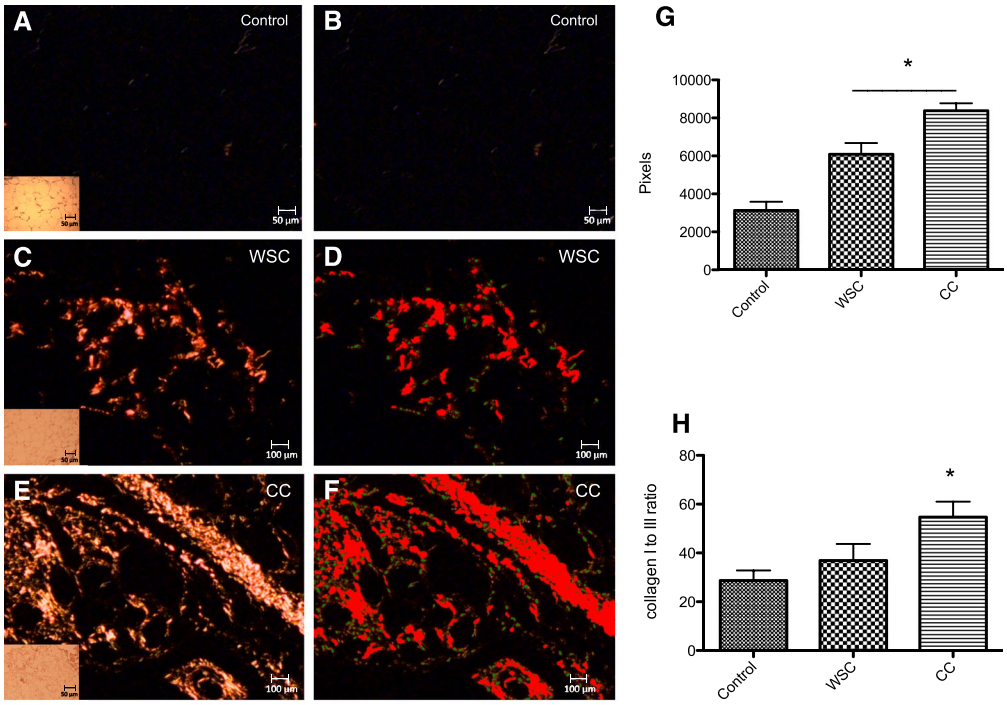
Although the presence of CD15 neutrophils was minimal in CC, we observed an abundant number of macrophages stained with CD68 surface markers in the groups (CC and WSC) (*Figure* 3). Additionally, in tissue sections stained for CD68 positive cells, we found higher intensity that was more pronounced in the surrounding of adipocytes [crown-like structures (CLS)] in CC (*Figure* 4A, C). However, crown aggregates were not extensive, and there was no signal of syncytial giant cells. Less frequently, sections for CD3 positive cells showed irregular distribution more often observed in the scAT fibrotic areas from CC (*Figure* 4B, D).

CD68 mRNA expression increased in scAT obtained from CC patients (109-fold, $P = 0.029$, respectively), when compared with the control group (control vs. CC). In scAT of WSC, an increase of 48-fold in MCP-1 mRNA levels was detected, when compared with the control group ($P = 0.010$) (*Figure* 5).

Discussion

The present study documents, for the first time, increased number of ATMϕs in scAT obtained from gastrointestinal cachectic cancer patients. One such change was accompanied by ECM remodelling and associated with adipocyte size reduction and AT atrophy. Fibrosis was detected, as well as the presence of distinct patterns of fibrous material surrounding adipocytes, while collagen fibre organization was altered in scAT samples obtained from cancer patients.

Figure 2 Picro sirius red staining sections of subcutaneous adipose tissue in cachexia and control patients. Collagen fibres are presented in different colours. Type I collagen fibres are orange to red, whereas the thinner type III collagen fibres appear yellow to green from (A, B) weight-stable subjects and control, (C, D) weight-stable cancer (WSC) patients and (E, F) cancer cachexia (CC) patients. Total collagen quantification in cachexia and control patients of (G) type I collagen and (H) types I–III ratio. Values are mean ± SEM. * $P < 0.05$ vs. control subjects.



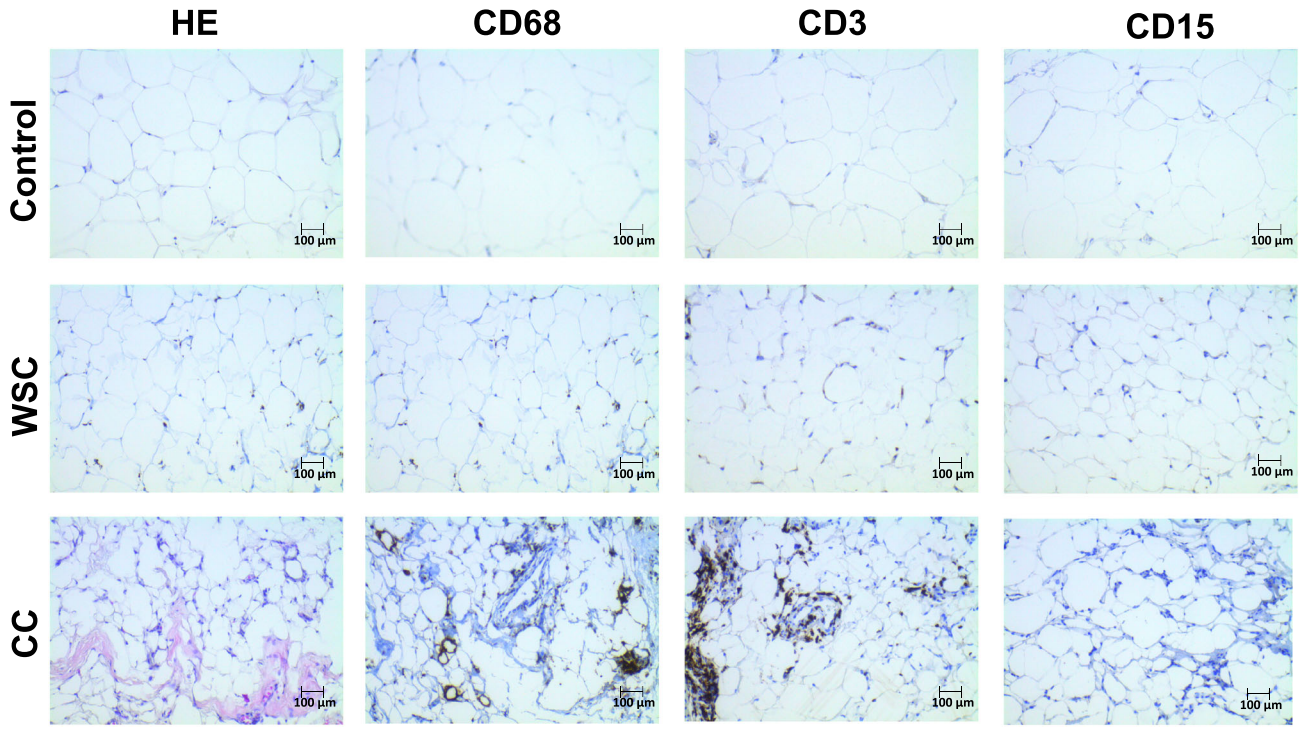
In this aspect, markedly increased total collagen amounts were found in the cachectic patients while being already mildly increased in the cancer (weight stable) patients. Interestingly, such structural modification of scAT was accompanied by increased inflammatory cells subset presence, which was more conspicuous in the cachectic patients. CD68-labelled ATMϕs localized to crowns surrounding adipocytes and expressed higher mRNA levels of scavenger receptor (CD68), as well as chemokine attractant for macrophages (MCP-1). Additionally, CD3 Ly was more abundant in the fibrotic areas in scAT samples from cachectic patients, in a different fashion compared with infiltrated ATMϕs.

The hallmark of CC is body weight loss.[30,31] Loss of muscle and AT mass, anorexia, anaemia and alterations in metabolism are also evident during the development of the disease. In particular, AT catabolism followed by tissue mass depletion (atrophy) and metabolic dysregulation are consistent and prominent findings in CC patients.[1,9,10,31,32] In this aspect, the present study shows adipocyte size reduction in scAT of cachectic cancer patients. This aspect was not evident in the WSC patients. AT atrophy induced by cachexia has been consistently demonstrated in both patients[7,33] and animal models.[9,34] In CC, longitudinal investigation has suggested that fat loss is not only due to loss of adipocyte volume (lipolysis)[29,34,35] but also may be attributed to a decrease in lipid accretion in these cells,[36] both resulting in a smaller cell.[37] Recently, a well-designed investigation[8] showed consistent evidence that lipases in AT breakdown stored fat, contributing to cancer-associated cachexia. However, despite several studies having consistently shown AT atrophy as a result of cachexia, the associated mechanisms are still not fully elucidated.

Another aspect addressed was ECM fibrosis, in order to assess whether there was AT remodelling in this scenario. CC patients showed ECM increased fibrosis in AT, with evident collagen-fibril staining. Simultaneously, an increase in both types I and III collagen content was observed in these patients, whereas in WSC, only a discrete change detected in type I collagen was found. A recent microarray data analysis from scAT of cachectic patients demonstrated a possible connection between regulation of energy turnover, cytoskeleton and ECM, with loss of AT.[38] In an animal model of cachexia, AT fibrosis was demonstrated by ultrastructural analysis showing severe delipidation and alterations in cell membrane conformation, irregular cytoplasmic projections and increased electron-dense mitochondria. An enhanced level of collagen-fibril staining was also evident in the tumour-bearing group.[39]

In fact, the ECM is one of the most important regulators of cellular and tissue function in the body.[40] Disruption in ECM leads to marked metabolic dysregulation and failure to

Figure 3 Identification of different immune cell types present in subcutaneous adipose tissue obtained from cancer cachexia patients. Serial sections of weight-stable subjects and control, weight-stable cancer (WSC) patients and cancer cachexia (CC) patients were stained with markers of macrophages (CD68), T-lymphocytes (CD3) and for neutrophils (CD15). Nuclei were stained with haematoxylin (blue staining). HE, haematoxylin and eosin staining.



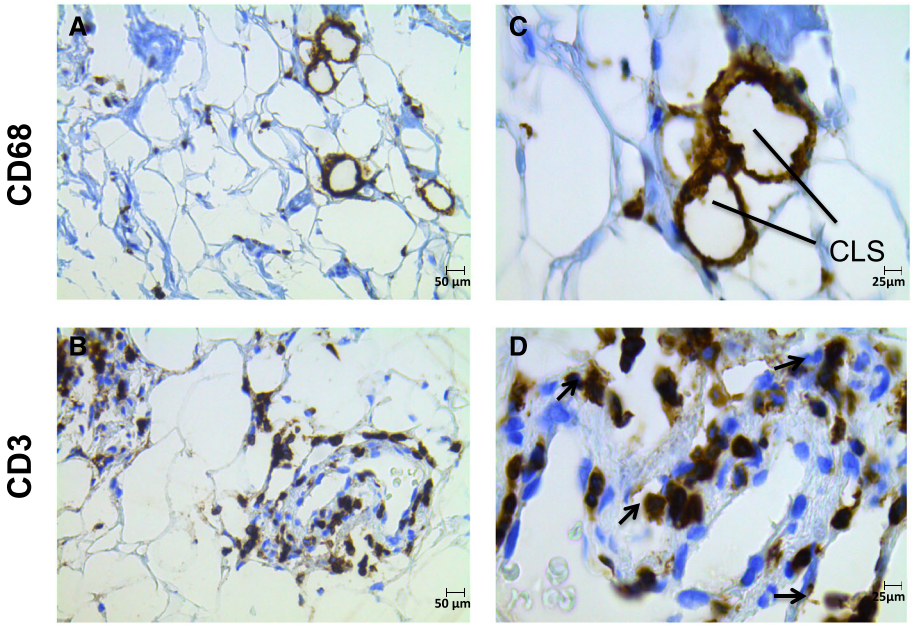
expand AT in both obese patients and experimental obesity (high fat diet and ob/ob knockout).[41] In the same way, AT reduction as induced by a body weight reduction programme was shown to result from modified ECM gene expression profile.[42] In obese patients, body fat loss as a consequence of gastric bypass was associated with increased fibrosis in fat depots after a 3 month to 1 year post-surgery period.[43] However, even considering the important role of ECM to AT homeostasis, there is limited information regarding quantitative EMC analysis in cachectic cancer patients. The few studies that have addressed this question propose that AT solely adjusts its extracellular environment to adapt to the shrinking volume of the fat cells.[11,38] Contrary to this view, we show a robust increase in total collagen content and additionally, that, even before any morphofunctional disruption in AT occurs, as reported for WSC patients, AT is already affected by the syndrome. Indeed, it seems that the process of AT remodelling induced by cachexia is triggered before any of the clinical signs of the disease. Additional studies should take place to fully confirm this hypothesis.

As described above, several researchers have shown that AT ECM remodelling is a crucial event to accommodate obesity-induced cellular alterations. One such process has also been described to be the end point of a persisting inflammatory stimulus (unresolved chronic inflammation) in AT, which may be responsible for the excessive synthesis of ECM components and subsequent interstitial deposition of fibrotic material.[44,45] There are, however, no studies, to our knowledge, that describe the nature of the inflammatory infiltrate in the AT of cachectic patients.

One previous study by our group demonstrated that AT, in particular, scAT, contributes in a significant manner to systemic inflammation, as a potent source of inflammatory factors.[10] We presently show an accentuated modification in gene expression, as well as in plasma concentration of adipokines and pro-inflammatory molecules in cachectic gastrointestinal cancer patients, in relation to their non-cachectic counterparts. These include adiponectin, leptin, tumour necrosis factor-α, IL-6 and IL-10. Furthermore, in the present study, we provide new information in regard to the inflammatory cells found in the AT of CC patients. Tissue sections stained for CD68 ATMϕs positive cells show a higher intensity for the macrophage marker, especially in the surrounding surface of adipocytes (CLS) in CC. However, crown aggregates were not as cell dense as those normally described in obesity, and there was no signal of occasional

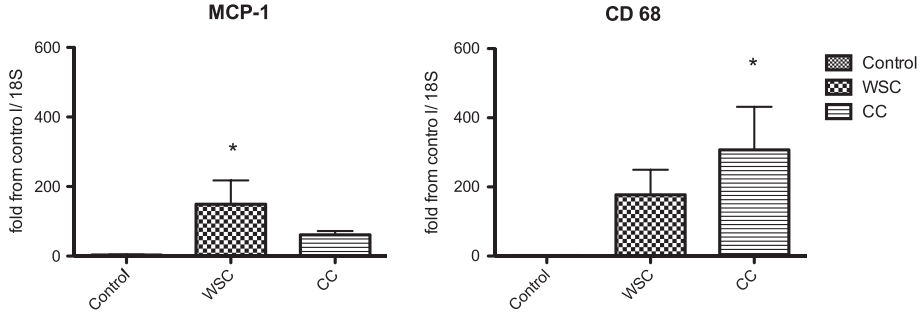
DOI: 10.1002/jcsm.12037

Figure 4 Immunohistochemistry for immune cells from weight-stable subjects and control, weight-stable cancer (WSC) patients and cancer cachexia (CC) patients. Subcutaneous adipose tissue (scAT) macrophages localize to crown-like structures (CLS) around individual adipocytes, which increase in frequency with cancer cachexia. Light microscopy of scAT of CC patients showing CD68 immunoreactive macrophages (brown colour) aggregated to numerous (C, CC patients) CLS among unilocular adipocytes. Note that almost all CD68 immunoreactive macrophages are organized to form CLS. CD3 immunoreactive T-lymphocyte shows positive cells in fibrotic areas, stained with DAB (brown colour) (arrow). Nuclei were stained with haematoxylin (blue labelling). Bar (μm): 50 μm for A and B; 25 μm for C and D.



coalescence to form syncytial giant cells. In a different way, CD3 Ly positive cells were the main immune cells found in the fibrotic areas. We previously hypothesized that infiltrating ATMϕs may play an important role(s) in cachexia-associated AT remodelling, based on our observation that ATMϕs in cachectic rats and humans are placed surrounding or in close proximity to the adipocytes.[46] In addition, we demonstrated that ATMϕs aggregates display a CLS pattern. This may be a consequence of an increased lipid scavenging function of ATMϕs, induced by CC. In obesity, a positive relationship between infiltrating ATMϕs and AT remodelling has been demonstrated.[41,47–49] In particular, CLS are described at sites of adipocyte death. Such structures are related with envelopes and ingest the moribund adipocyte and its

Figure 5 Expression levels of genes involved in the inflammation of subcutaneous adipose tissue depots in subjects from different experimental groups. Real-time PCR analysis of RNA isolated from weight-stable subjects and control, weight-stable cancer (WSC) patients and cancer cachexia (CC) patients. mRNA levels of target genes were normalized to 18 S. Values are mean ± SEM for five to nine samples per group. * $P < 0.05$ vs. control subjects.



potentially cytotoxic remnant lipid droplet.[47] Consequently, CLS become lipid-laden 'foam cells'[47] frequently observed in AT in obesity. In an animal model of cachexia, we have shown[46] that foam-cell resembling macrophages are present in the AT. During CC, increased lipolysis has been consistently demonstrated in both patients[7,11] and animal models[9,32] and that might be an important source of lipids to ATMϕs. In tumour-bearing animals, lipid profile was modulated by the cachectic state in an anatomical region-related pattern. Visceral AT composition is changed and presents increased storage of monounsaturated fatty acids (16:1), as well as decreased percentage of stearic acid, a saturated fatty acid.[50] In the same animal model, abundant lipid inclusions were found in infiltrated ATMϕs in the mesenteric depots obtained from cachectic rats. Those cells also exhibited increased tumour necrosis factor-α secretion when stimulated with lipopolysaccharide.[45] However, this aspect was not assessed in the present study.

Infiltrating inflammatory cells secrete pro-inflammatory mediators that are enhanced in the scAT of CC patients.[10] Up-regulation of IL-6 has been a consistent finding in several studies, notably in cachectic patients, independent of the aetiology of the syndrome.[51] We previously demonstrated that plasma IL-6 is markedly increased during cachexia, a result that may represent an interesting tool for diagnostic purposes. Although our previous study reported increased inflammatory cytokine profile gene expression in scAT samples from CC patients, we herein show that CD68 gene expression was also increased in scAT from these patients, corroborating the observation that CD68 ATMϕs positive cells were more abundant in the same situation. Interestingly, MCP-1 gene expression was solely augmented in WSC samples, with no changes in CC patients. ATMϕs infiltrate to AT as circulating monocytes in response to AT secretion of MCP-1, which recruits monocytes expressing the C-C chemokine receptor type 2. This is one of the initial steps to trigger inflammatory response and consequently increase Mϕs subset in AT. In CC, high MCP-1 levels found in WSC might be suggestive of a pre-cachexia stage, once increased gene expression of CD68, IL-6 and IL-10 was already demonstrated in such CC patients[11]. However, the relationship between MCP-1-associated ATMϕs augment and the initial signals of cachexia, as well as the importance of this process for the development and progression of cachexia complications, remains unclear.

Limitations of this study should be acknowledged. Our investigation was carried out with relatively small study groups. The Brazilian patients enrolled may not be representative of a typical cancer patient population from Europe or North America and some differences concerning total fat mass of patient in the cancer groups, and overweight and obesity prevalence should be considered. Cachectic groups showed values ranging from 9% to 31% of body weight loss in the previous 6 months and C-reactive protein ranging from 4.9 to 77 μg/mL, indicating that we studied patients staging between cachexia and refractory cachexia. Regarding the WSC patients, some of them could be perhaps considered pre-cachectic, once they showed values ranging from 3.2% to 5.4% of body weight loss in the previous 6 months and C-reactive protein values ranging from 1.2 to 10.75 μg/mL. However, there was no difference when compared with controls, and we have not attempted to adopt this staging system to classify CC, due to the relatively small sample. The current study was addressed to assess the effect of cachexia and tumour *per se* but was not empowered to detect differences between different tumour types. However, the study was powered to detect the observed differences in fibrosis and inflammation in scAT.

Conclusion

We report consistent modification consisting of fibrosis and inflammatory cell presence in scAT induced by cachexia in gastrointestinal cancer patients. The latter was characterized by the presence of CLS composed of CD68 positive ATMϕs surrounding adipocytes, and increased CD3 Ly, more evident in the fibrotic areas. In addition, some of these changes were already present in the cancer group, suggesting that AT disruption may occur at a precocious stage of cachexia, even before the detection of pre-cachexia clinical features. In this particular, increased MCP-1 and CD68 gene expression in scAT of cancer patients may indicate an important role of these inflammatory mediators as early biomarkers of cancer.

Acknowledgements

We thank Emilia Ribeiro for technical assistance. We also would like to thank Alex Y. Shimura for initial conception of this study. The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of FAPESP. Grant sponsor: FAPESP; Grant numbers: 2010/51078-1 (Miguel L. Batista, Jr) and 2008/54091-9 and 2012/50079–0 (Marília Seelaender). The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle 2010;1:7–8 (von Haehling S, Morley JE, Coats AJ and Anker SD).

Conflict of interest

None declared.

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