

Cancer cachexia differentially regulates visceral adipose tissue turnover

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Abstract

Cancer cachexia (CC) is a progressive metabolic syndrome that is marked by severe body weight loss. Metabolic disarrangement of fat tissues is a very early event in CC, followed by adipose tissue (AT) atrophy and remodelling. However, there is little information regarding the possible involvement of cellular turnover in this process. Thus, in this study, we evaluated the effect of CC on AT turnover and fibrosis of mesenteric (MEAT) and retroperitoneal (RPAT) adipose tissue depots as possible factors that contribute to AT atrophy. CC was induced by a subcutaneous injection of Walker tumour cells (2×10^7) in Wistar rats, and control animals received only saline. The experimental rats were randomly divided into four experimental groups: 0 days, 4 days, 7 days and 14 days after injection. AT turnover was analysed according to the *Pref1/Adiponectin* ratio of gene expression from the stromal vascular fraction and pro-apoptotic CASPASE3 and CASPASE9 from MEAT and RPAT. Fibrosis was verified according to the total collagen levels and expression of extracellular matrix genes. AT turnover was verified by measurements of lipolytic protein expression. We found that the *Pref1/Adiponectin* ratio was decreased in RPAT (81.85%, $P < 0.05$) with no changes in MEAT compared with the respective controls. CASPASE3 and CASPASE9 were activated on day 14 only in RPAT. Collagen was increased on day 7 in RPAT (127%) and MEAT (4.3-fold). The *Collagen1A1*, *Collagen3A1*, *Mmp2* and *Mmp9* mRNA levels were upregulated only in MEAT in CC. Lipid turnover was verified in RPAT and was not modified in CC. We concluded that the results suggest that CC affects RPAT cellular turnover, which may be determinant for RPAT atrophy.

Key Words

- ▶ cancer cachexia
- ▶ adipose tissue
- ▶ cellular turnover
- ▶ extracellular matrix
- ▶ apoptosis

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Introduction

Cancer cachexia (CC) is a progressive multifactorial syndrome that affects approximately 60–80% of all patients with advanced cancer and is directly associated with 22–40% of all cancer deaths (Blum *et al.* 2014). Body weight loss is the main marker of cachectic syndrome, followed by atrophy of both skeletal muscle and adipose tissue (AT) (Das *et al.* 2011, Batista *et al.* 2012,

Seelaender & Batista 2014). In this regard, several studies have postulated that AT atrophy is caused by (1) increased lipolysis in adipocytes (Agustsson *et al.* 2007, Ryden *et al.* 2008, Arner & Langin 2014); (2) reduction of lipogenesis (Ebadi & Mazurak 2014); (3) downregulation of adipogenic and lipogenic gene expression (Bing *et al.* 2006, Batista *et al.* 2012) and (4) disruption of the

formation and development of AT (Arner & Spalding 2010). However, few studies have addressed AT turnover in CC.

Recently, some murine models of cancer cachexia have shown a heterogeneous response from different AT depots, considering its remodelling process induced by cachexia syndrome (Bing *et al.* 2006, Batista *et al.* 2012, Petruzzelli *et al.* 2014). In particular, such studies have focused on inflammation, rearrangements of the extracellular matrix (ECM) and metabolic chaos, which seem to occur differently in AT depots (i.e., visceral and subcutaneous) (Bertevello & Seelaender 2001, Machado *et al.* 2004, Bing *et al.* 2006, Batista *et al.* 2012, 2013, 2016, Beluzi *et al.* 2015). In our previous study, cachectic animals (Walker-256-induced) showed more accentuated atrophy in the retroperitoneal adipose tissue (RPAT) compared with another visceral depot, such as mesenteric adipose tissue (MEAT) (Batista *et al.* 2012).

In AT, cellular turnover is regulated by a balance between the growth/differentiation (adipogenesis) and death of its cells (generally by apoptosis) (Warne 2003, Arner & Spalding 2010). Some studies have elucidated the adipogenic marker profile during development of cachexia syndrome (Bing *et al.* 2006, Batista *et al.* 2012); however, few studies have addressed the apoptotic processes and/or AT turnover during cachexia (Ryden *et al.* 2008). It has been known that adipogenic genes are downregulated in CC from epididymal AT (Bing *et al.* 2006) and RPAT (Batista *et al.* 2012). On the other hand, subcutaneous AT apoptosis did not change in cancer patients (Ryden *et al.* 2008). Thus, considering that AT depots respond heterogeneously to CC and several metabolic and inflammatory pathways are involved in AT remodelling, additional studies are needed to achieve a deeper understanding of the possible role of AT turnover in cachexia.

The ECM plays a key role in angiogenesis (Lafontan 2014) and modulates adipogenesis, cell death and inflammation (Mariman & Wang 2010). Among the components of the ECM, type 1 collagen has unique importance in structuring of mesenchymal tissue (Sun *et al.* 2013). The level of extracellular matrix components depends on a fine balance between synthesis and degradation. Collagen degradation is controlled by matrix metalloproteinases (MMPs), which are modulated by tissue inhibitors of metalloproteinases (TIMPs) (Su *et al.* 2016). Finally, the primary modulator for balance between MMPs and TIMPs appears to be TGFB (Su *et al.* 2016), an important cytokine that regulates fibrosis (Allison 2014, Craft 2015) and inflammation (Sanjabi *et al.* 2009, Craft 2015). The balance of these

ECM components is important for the regulation of collagen deposition, and this balance maintains normal tissue function (Iwayama *et al.* 2015). In obesity, ECM from AT is subjected to a remodelling process that occurs as a change in size and/or number of adipocytes (Craft 2015). On the other hand, in CC, increased fibrosis in AT in response to syndrome development has recently been demonstrated in a murine model (Bing *et al.* 2006) and in humans (Batista *et al.* 2016), which contributes to AT rearrangement (Batista *et al.* 2016).

Therefore, to study AT turnover during the development of CC, we analysed the visceral depots in two stages of cachexia (early and cachexia stages) after tumour induction. The analysis showed the downregulation of adipogenic markers as well as apoptotic markers, indicating a possible change in RPAT cellular turnover. This condition becomes even more evident in the last stages of the disease. Furthermore, the results suggested that RPAT atrophy induced by CC appears to be largely due to failure of cellular turnover than other metabolic mechanisms or remodelling of AT.

Materials and methods

Animals and adipose tissue collection

Male adult Wistar rats (200–280 g), obtained from the University of Mogi das Cruzes Animal Facility, were maintained in plastic cages in a 12-h light:12-h darkness cycle (lights on at 07:00h) under controlled temperature conditions ($22 \pm 1^\circ\text{C}$) and received water and food (NuvilabCR1-Nuvital S/A, Colombo, PR, Brazil) *ad libitum*. The Ethical Committee for Animal Research from the University of Mogi das Cruzes approved all of the adopted procedures, which were carried out in accordance with the ethical principles stated by the Brazilian College of Animal Experimentation (Filipecki *et al.* 2011).

To induce CC, Walker 256 tumour cells (2×10^7 cells) were injected s.c. into the right flank of animals (Bertevello & Seelaender 2001). Walker 256 cells were obtained from the American Type Culture Collection (ATCC) constituting our repository of cancer cell lines. The cell preparation protocol included: (1) initial cells submitted to sub-culture (*in vitro* phase); (2) intraperitoneal cells injection ranging 5–7 days (doubling time, *in vivo* phase); and (3) isolation of viable cells, considering cells morphology and growth rate. Control rats received saline injections on the same day of tumour inoculation. Animals were held in a collective

Table 1 General characteristics of animal groups during the progression of cancer cachexia.

| | Tumour cells inoculation (days) | | | |
|-------------|---------------------------------|------------|------------|-------------|
| | 7 | | 14 | |
| | Control | TB | Control | TB |
| <i>n</i> | 5 | 8 | 6 | 8 |
| BW (g) | 264 ± 14 | 247 ± 11 | 285 ± 13 | 255 ± 12* |
| BW loss (%) | – | 6.3 | – | 10.7 |
| Δ BW (g) | 19.7 ± 2.1 | 6.5 ± 1.1* | 29.3 ± 3.0 | 15.5 ± 1.5* |
| TM (g) | – | 4.4 ± 1.8 | – | 12.1 ± 3.1 |
| TM (%) | – | 1.8 ± 0.5 | – | 4.7 ± 1.0 |
| FI (g/day) | 22.1 ± 0.3 | 21.7 ± 0.4 | 24.1 ± 0.7 | 22.0 ± 0.5 |

Values are mean ± s.e.m. for five to eight animals per group.

**P* < 0.05 relative to the control group.

BW, body weight; FI, food intake; TB, tumour-bearing; TM, tumour mass; Δ, delta of body weight of the animals from days 7 to 14 in relation to day 0.

cage, containing five animals/cage. Weight was assessed daily and food intake was assessed on days 7 and 14 after injection, always in the afternoon. Cachectic syndrome was observed in rats bearing Walker 256 tumour cells after 10–15 days (Machado *et al.* 2004). To evaluate the development of CC, experiments were carried out in the time course study when rats were killed by decapitation (12 h fasting) on days 0, 4, 7 or 14 after injection (five to eight animals for each time point). Animals without tumour inoculation and with only vehicle solution were used as controls. Because of the absence of food intake (Table 1), all experimental results were compared

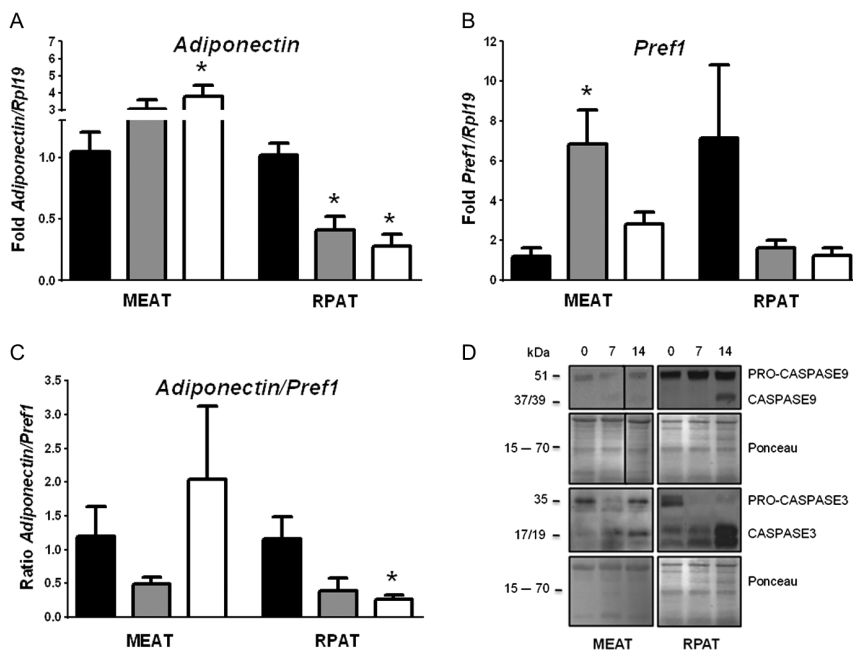
with the control group. Mesenteric adipose tissue (MEAT, after careful removal of adjacent lymph nodes), retroperitoneal adipose tissue (RPAT) and tumours were removed, weighed, snap-frozen in liquid nitrogen and stored at –80°C.

PicroSirius red staining

MEAT and RPAT were fixed by HistoChoice MB (Amresco, Solon, OH, USA) at pH 7.4 for 2 h. Samples were processed as described by Junqueira and Brentani (Junqueira *et al.* 1979) with minor modifications. Five micrometre sections were obtained with a Leica microtome (Aotec Scientific Instruments LTDA, São Paulo, SP, Brazil).

Light microscopy and software analysis

The slides were visualised via microscopy using a Leica DM 750 (Aotec Scientific Instruments) and captured with a Leica DFC 295 camera (Aotec Scientific Instruments) and LAS software (Aotec Scientific Instruments). After image capture, images were analysed with ImageJ software (<http://rsb.info.nih.gov/ij/>). The numbers of positive collagen staining per area were counted under 400 magnification, in at least 3 independent sections, with at least 10 fields per section for each adipose tissue sample, by 2 independent investigators who did not know the origin of the samples.

**Figure 1**

Adipogenesis markers and CASPASE cleavage during cancer cachexia. qPCR of mRNA gene expression of *Adiponectin* (A) and *Pref1* (B) from the stromal vascular fraction of MEAT and RPAT. qPCR was normalized to *Rpl19* (housekeeping gene). Values are the mean ± s.e.m. for five animals per group. *Ratio between *Pref1* and adiponectin expression; *Pref1/Adiponectin* ratio (C); (D) Western blot analysis of CASPASE3 and CASPASE9 relative to Ponceau red staining; days after tumour cells injection (or saline – control): 0 (control), 7 and 14. **P* < 0.05 relative to the control group. MEAT, mesenteric adipose tissue; RPAT, retroperitoneal adipose tissue.

Stromal vascular fraction (SVF) cells and adipocyte isolation

SVF cells and adipocyte isolation were adapted from Neves and coworkers (Neves *et al.* 2015), as well as Rodbell's method (Rodbell 1964) with minor modifications.

Gene expression

Real-time PCR was performed as described by Batista and coworkers (Batista *et al.* 2012). The gene sequences are shown in Supplementary Table 1 (see section on supplementary data given at the end of this article). All the primer sequences were obtained from Invitrogen (Life Technologies). Samples were normalised to the 18S and *Rpl19* values, and the results are expressed as fold changes of the C_t values relative to those of controls using the $2^{-\Delta\Delta C_t}$ formula.

Western blot

Western blot analyses were performed as described in Batista and coworkers (Batista *et al.* 2012). The primary antibodies were against CASPASE3 (1:1000 dilution), CASPASE9 (1:1000 dilution), HSL LIPE (1:4000 dilution)

and HSLphos563, 565 and 660 (1:1000 dilution). All primary incubations were conducted overnight, and secondary anti-rabbit IgG antibodies were conjugated to HRP (1:3000 dilution); anti-mouse IgG was conjugated to HRP (1:3000 dilution) for 1 h at room temperature. All antibodies were obtained from Cell Signalling Technology (Cat. number 9662S, 9508S, 4139, 4137, 4126, 7074 and 7076) or Abcam (Cat. number ab45422).

Statistical analysis

Data were analysed with one-way or two-way ANOVA followed by Bonferroni's *post hoc* comparisons tests using GraphPad Prism software for Macintosh, version 5.0 (GraphPad). $P < 0.05$ was considered significant for all statistical tests. Data are expressed as the mean value \pm S.E.M.

Results

The progression of CC was assessed on days 7 (early stage) and 14 (cachectic stage). Walker tumour cells induced CC as expected, in particular considering AT atrophy and tumour mass growth (Machado *et al.* 2004,

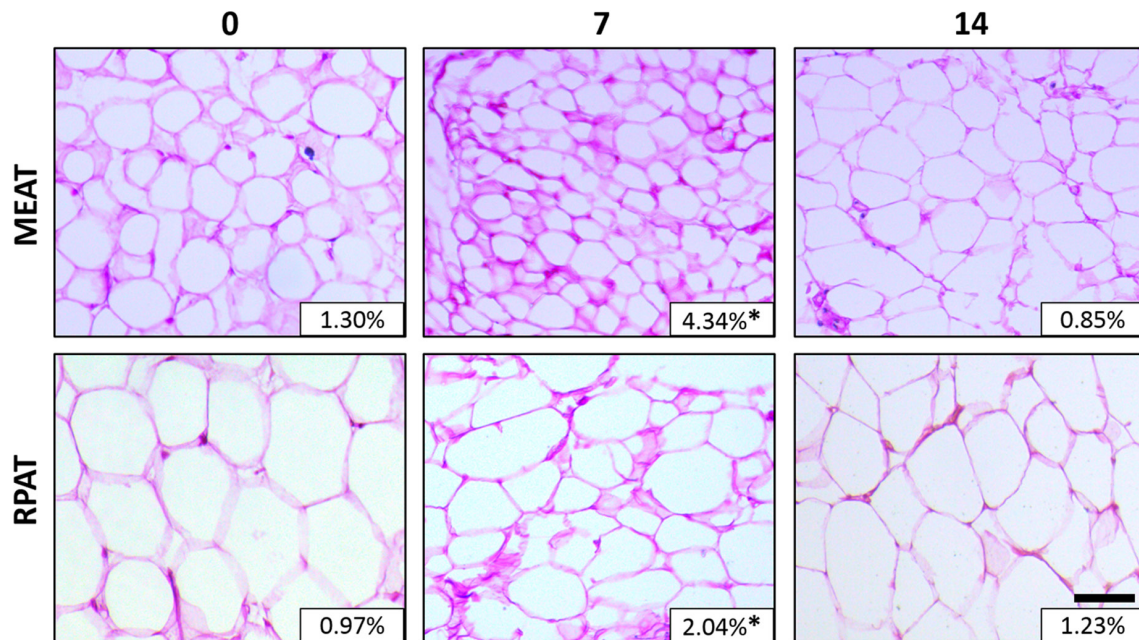
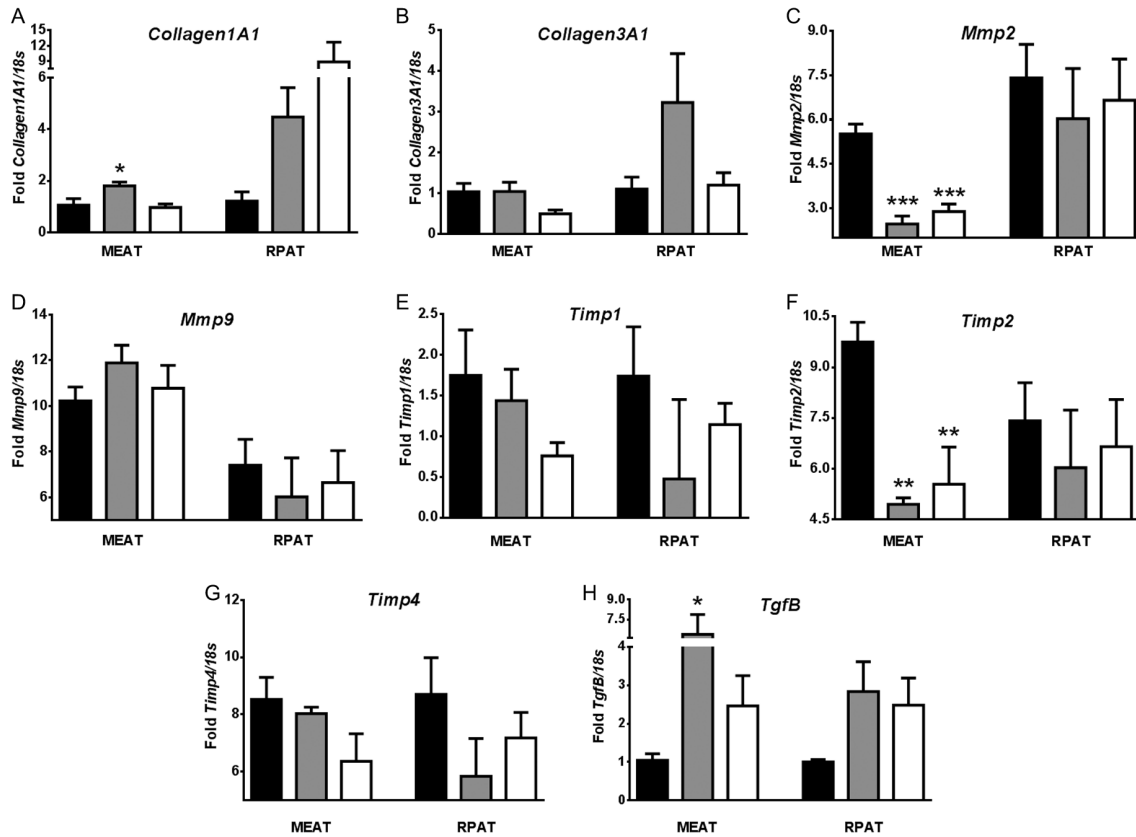


Figure 2

Adipose tissue total COLLAGEN content during cancer cachexia. PicroSirius staining (total COLLAGEN marker) micrographs of MEAT and RPAT during cachexia development; days after tumour cells injection (or saline control): 0 (control), 7 and 14. The quantification of the total COLLAGEN content is shown at the bottom of each micrograph, and the values are expressed as the percentage of the total area. * $P < 0.05$ relative to the control group. MEAT, mesenteric adipose tissue; RPAT, retroperitoneal adipose tissue; Scale bar, 100 μ m. A full colour version of this figure is available at <http://dx.doi.org/JOE-16-0305>

**Figure 3**

qPCR of extracellular matrix components during cachexia development. *Collagen1A1* (A), *Collagen3A1* (B), *Mmp2* (C), *Mmp9* (D), *Timp1* (E), *Timp2* (F), *Timp4* (G), *TgfB* (H). The targets were normalized to *18s* (A and B) and *Rpl19* (C, D, E, F, G and H) housekeeping genes. Values are the mean \pm s.e.m. for five animals per group. * $P < 0.05$ and ** $P < 0.01$ vs day 0 (control group). MEAT, mesenteric adipose tissue; RPAT, retroperitoneal adipose tissue.

Batista *et al.* 2012, Beluzi *et al.* 2015). Body weight loss was evident on day 14, showing a reduction of 10.7%, when compared with the same day control. Food intake for the four groups over the 14 days of treatment was similar (Table 1). To evaluate cell turnover, we measured the mRNA levels of *Pref1* (pre-adipocyte marker) because its expression is linked to adipogenesis inhibition (O'Connell *et al.* 2011) and the mRNA levels of *Adiponectin*, which have been used as a marker of mature adipocytes found in the SVF (Martella *et al.* 2014). There was no change in *Pref1* expression in the SVF from RPAT samples at days 7 and 14 (Fig. 1B). However, *Pref1* expression increased 4.5-fold ($P < 0.05$) only on day 7 in the SVF MEAT (Fig. 1B). Gene expression of the *Adiponectin* from the SVF decreased 61.6% ($P < 0.05$) on day 7 and 78.6% ($P < 0.05$) on day 14 (Fig. 1A) in RPAT. On the other hand, *Adiponectin* increased 2.2-fold ($P < 0.05$) in the SVF from MEAT only on day 14 (Fig. 1A). Although the ratio of *Adiponectin* and *Pref1* gene expression in the SVF cells from RPAT decreased 81.8% ($P < 0.05$) on day 14, no change was detected in MEAT (Fig. 1C). Considering the apoptotic parameters, cleavage

of CASPASE3 increased on day 14 in RPAT, with no change in MEAT. In the same period (day 14), CASPASE9 cleavage dramatically increased in RPAT, whereas it was slightly increased on day 7 in MEAT (Fig. 1D).

Once we determined that AT atrophy may result from the decrease in the turnover of the adipocyte cell number and/or lipid reserves or both, we verified the involvement of some of the main enzymes related to lipolytic pathways, such as HSL (total and phosphorylated serine forms 563, 565 and 660). There was no change in the protein expression of HSL during the experimental period (Supplementary Fig. 1). To better understand the changes related to AT remodelling during CC, some extracellular matrix components were evaluated. Total COLLAGEN showed an increase only on day 7 with a 1.3-fold increase ($P < 0.05$) in RPAT and 4.3-fold increase ($P < 0.05$) in MEAT (Fig. 2). *Collagen* and its gene expression modulators (*Mmp2*, *Mmp9*, *Timp1* and *Timp2*) revealed no changes in RPAT at any of the time points evaluated. On the other hand, in MEAT, there was increased expression of *Collagen1A1* (69.8% $P < 0.05$) only on day 7 (Fig. 3A).

Collagen3A1 gene expression did not differ at any of the experimental time points (Fig. 3B). *Mmp2* gene expression increased 6.7-fold ($P < 0.01$) only on day 7 (Fig. 3C). The *Mmp9* mRNA levels were not significantly different at any of the examined times (Fig. 3D). The *Timp1* mRNA levels increased 4.5-fold ($P < 0.05$) on day 14, whereas *Timp2* increased 62.9-fold ($P < 0.01$) on day 7 and 81.7-fold ($P < 0.05$) on day 14 (Fig. 3E and F). However, *Timp4* did not change at any of the analysed time points (Fig. 3G). Finally, expression of the *Tgfb* gene increased 5.7-fold ($P < 0.05$) only on 7 day (Fig. 3H).

Discussion

We previously demonstrated that the RPAT depot is most affected during CC syndrome and is accompanied by the marked atrophy of adipocytes (Batista *et al.* 2012). In this study, we also evaluated the morph-functional aspects with the aim of comparing the RPAT and MEAT profiles during CC. Thus, the cachexia model adopted here induced a decrease in the gene expression of adipogenic markers, as well as increased CASPASE3 and CASPASE9 cleavage (apoptotic markers) in RPAT, indicating a possible impairment in cell turnover during the syndrome. Additionally, lipid turnover parameters in RPAT, as well as an ECM component profile, showed no changes during cancer. Thus, these data indicated an additional importance in cell turnover for the occurrence of RPAT atrophy.

Considering the *Adiponectin* to *Pref1* ratio as an adipogenic parameter in AT depots, we showed a reduced adipogenic rate at the cachexia stage (day 14) from RPAT cells, indicating that adipogenesis may be reduced in this tissue. This fact confirmed previous data demonstrating that the expression of adipogenic genes are more affected in RPAT than MEAT (Batista *et al.* 2012), as well as an *in vitro* adipogenesis and co-culture study with tumour cell assays (Lopes *et al.* 2015). In this aspect, Bing and coworkers showed that adipogenic genes and their protein levels were decreased in epididymal AT from tumour-bearing mice (Bing *et al.* 2006). Along these lines, our group recently showed that treatment with pioglitazone, a thiazolidinedione drug (TZD), was effective at increasing the survival of animals and was also positively correlated with the preservation of RPAT mass during cachexia, possibly through lipogenic and adipogenic positive modulation (attenuation), at least in the early stages of cachexia (Beluzi *et al.* 2015). Interestingly, this effect was only evident in the RPAT depot without an effect on MEAT. It is well established that TZDs are capable of

enhancing the differentiation of pre-adipocytes (Kang *et al.* 2010, Bjorndal *et al.* 2011) and increasing body weight (Knights *et al.* 2014). Consequently, TZDs may be candidates for anti-cachectic treatment. However, further studies should address this issue to achieve a deeper understanding of possible interventions in the cachectic state.

For more information regarding AT turnover, the activation of some apoptosis pathway protein expressions, in particular, CASPASE3 and CASPASE9, were addressed in visceral depots of cachectic rats. CASPASE3 cleavage activation was only increased in RPAT, condition that seems to begin at the early stage of cachexia. CASPASE9 cleavage was also sharply increased in RPAT at the cachexia stage and slightly increased in MEAT. This condition was only evident during the cachexia stages. CASPASE9 activation by an intrinsic pathway is related to oxidative metabolic impairment (Zuo *et al.* 2009), a condition that is already seen in CC. Considering the information presented previously, our data corroborated the hypothesis proposed earlier by our group (Batista *et al.* 2012) that MEAT and RPAT respond differently during CC. RPAT is the most affected depot, with a decrease in the cell perimeter and area (Batista *et al.* 2012), and the increased of apoptotic marker adds new information regarding the plethora of events that results in AT atrophy. In fact, DiGirolamo and coworkers (DiGirolamo *et al.* 1998) showed the heterogeneity of fat depots during their expansion in normal development and RPAT expansion mainly occurs through hyperplasia than hypertrophy, unlike MEAT (DiGirolamo *et al.* 1998). This characteristic highlights the importance of cell proliferation for the maintenance/expansion of RPAT (DiGirolamo *et al.* 1998, Hausman *et al.* 2001).

In this way, cell turnover appears to be impaired in RPAT, possibly due to a decrease in the adipogenic process and an increase in apoptosis. Under physiological conditions, cell turnover is the balance between cell death and renewal (proliferation and differentiation) (Spalding *et al.* 2008). Fat mass increases in overweight and obesity are mainly due to hypertrophy, whereas in morbid obesity, hyperplasia is predominant (Arner & Spalding 2010). Therefore, these two parameters are important to the health of adipose tissue. However, as far as we know, this is the first study that showed that AT atrophy induced by CC in RPAT might be due to reduced AT turnover in this setting.

In addition, taking into account the important role of lipid turnover to induce AT atrophy in response to CC, we verified the main lipolytic enzymes in adipocytes cells from RPAT. In these cells, no changes were demonstrated in the phosphorylated and total levels of HSL

(Supplementary Fig. 1), indicating that lipolysis seems to not be modified. However, further analyses considering additional lipid turnover parameters, such as ATGL activation, *de novo* lipogenesis and fat acid re-esterification, should be addressed by investigating adipocyte atrophy mechanisms in RPAT. Recently, our group showed that a drastic reduction in the *Srebp1* expression occurs in RPAT in CC, whereas no change occurs in MEAT (Batista *et al.* 2012). Taking into account that SREBP1 is a transcription factor that acts as a positive regulator of lipogenesis processes (Shimano *et al.* 1999), this metabolic pathway might be downregulated in RPAT. Based on this context, we hypothesised that atrophy in RPAT occurs because of impairment in cellular AT turnover and adipocyte atrophy, which mainly occurs according to decreases in lipogenesis with or without discrete alterations in lipolysis.

Because AT turnover appears to be impaired in CC and considering that adipogenesis and apoptosis are modulated by the ECM (Mariman & Wang 2010), we analysed some ECM components. The level of COLLAGEN deposition in the ECM is dependent on the balance of regulators. *Mmp* and *Timp* gene expression changes took place only in MEAT, and *Tgf β* changes also occurred, which among their other roles, indirectly contribute to collagen deposition (Maurer *et al.* 2010). This change in ECM components is evident only in MEAT, which was previously shown to be more resistant to the effects of cachexia (Batista *et al.* 2012). The changes in ECM components from MEAT may represent a pathway to maintain the normal physiological state, which does not occur in RPAT. However, more studies are needed to understand the possible role of ECM responses in AT during the development of CC. In this way, despite the ECM rearrangement of epididymal (mice) (Bing *et al.* 2006) and subcutaneous (patients) (Batista *et al.* 2016) AT, which has already been demonstrated to be induced by CC, our study provided new data regarding MEAT and RPAT rearrangements, showing a heterogeneity of fibrosis induced by CC. However, the consequence of such changes requires further investigation. In summary, our study demonstrated that the occurrence of the impairment of cellular turnover in RPAT occurred throughout the decrease in adipogenesis and increase in apoptotic markers, suggesting the additional importance of maintaining the cellularity of RPAT mass. Such a modification was not followed by robust impairment of lipid turnover or ECM component modification. On the other hand, in the MEAT depot, despite the lack of changes in cell turnover markers, it was shown that ECM component changes occurred, demonstrating the heterogeneous response of visceral adipose depots during

CC. Finally, the data demonstrated that the RPAT is particularly affected by CC, and a deeper understanding of AT heterogeneity responses due to cachexia may be an important topic to develop a deeper understanding of AT remodelling.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0305>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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