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## Original article

# Autophagy and proinflammatory cytokine expression in the intestinal mucosa and mesenteric fat tissue of patients with Crohn's disease

Raquel Franco Leal<sup>a,b,c,\*</sup>, Marciane Milanski<sup>b</sup>, Cláudio Saddy Rodrigues Coy<sup>a,c</sup>,  
Mariana Portovedo<sup>b</sup>, Viviane Soares Rodrigues<sup>a,b</sup>, Andressa Coope<sup>b</sup>,  
Maria de Lourdes Setsuko Ayrizono<sup>a,c</sup>, João José Fagundes<sup>a,c</sup>, Lício Augusto Velloso<sup>b</sup>

<sup>a</sup>Coloproctology Unit, Surgery Department, Escola de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), São Paulo, SP, Brazil

<sup>b</sup>Laboratory of Cell Signaling, Internal Medicine Department, Escola de Ciências Médicas, UNICAMP, São Paulo, SP, Brazil

<sup>c</sup>Brazilian Society of Coloproctology (TSBC), Rio de Janeiro, RJ, Brazil

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## ABSTRACT

**Background:** Recently, mesenteric fat has been proposed to play a role in the pathophysiology of Crohn's disease (CD), as fat hypertrophy is detected close to the affected intestinal area; however, there are few studies regarding autophagy and creeping fat tissue in CD.

**Objective:** Evaluate autophagy-related proteins and proinflammatory cytokines in intestinal mucosa and mesenteric fat in patients with CD and controls.

**Patients and methods:** Ten patients with CD, eight with non-inflammatory disease who underwent surgery, and eight with normal ileocolonoscopy were studied. The expression of LC3-II, TNF- $\alpha$  and IL-23 was determined by immunoblot of protein extracts. In addition, total RNA of LC3 and Atg16-L1 were determined using RT-PCR.

**Results:** The expression of LC3-II was significantly lower in the mesenteric tissue of CD when compared to controls ( $p < 0.05$ ). In contrast, the intestinal mucosa of the CD group had higher levels of LC3-II ( $p < 0.05$ ). However, mRNA expression of autophagy-related proteins was similar when compared to mesenteric fat groups. TNF- $\alpha$  and IL-23 expressions were higher in intestinal mucosa of CD than in control ( $p < 0.05$ ).

**Conclusion:** These findings suggest a defect in the autophagic activity of the creeping fat tissue in CD, which could be involved with the maintenance of the inflammatory process in the intestinal mucosa.

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\* Corresponding author.

E-mail: raquellleal@mpc.com.br / raquelfl@fcm.unicamp.br (R. F. Leal)

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## Autofagia e expressão de citocinas pró-inflamatórias na mucosa intestinal e no tecido mesenterial de pacientes com doença de Crohn

### R E S U M O

#### Palavras-chave:

Doença de Crohn  
Doença inflamatória intestinal  
Autofagia  
Citocinas  
Gordura mesenterial

**Introdução:** Recentemente, tem se proposto que o tecido mesenterial possa participar da fisiopatologia da DC, uma vez que é notória a hipertrofia da gordura mesenterial próxima ao segmento intestinal afetado pela doença. Entretanto, há poucos estudos relacionando autofagia e tecido mesenterial na DC.

**Objetivo:** Avaliar autofagia e citocinas na mucosa intestinal e no mesentério de pacientes com DC.

**Pacientes e métodos:** Dez pacientes com DC, oito sem doença inflamatória intestinal que foram submetidos à cirurgia, e oito com ileocolonosopia normal, foram estudados. As expressões de LC3-II, TNF- $\alpha$  e IL-23 foram determinadas por imunoblot de extrato protéico total. Além disso, expressão gênica de LC3 e de Atg16-L1 foi realizada por RT-PCR.

**Resultados:** A expressão de LC3-II foi significativamente menor no tecido mesenterial de pacientes com DC quando comparada à dos controles ( $p < 0,05$ ); as amostras de tecido intestinal do grupo DC apresentaram maior expressão de LC3-II ( $p < 0,05$ ). Entretanto, as expressões gênicas relacionadas à autofagia foram similares nos grupos de tecido mesenterial. Os níveis de TNF- $\alpha$  e de IL-23 foram maiores na mucosa intestinal do grupo CD ( $p < 0,05$ ).

**Conclusão:** Estes achados sugerem alteração da autofagia no mesentério na DC, o que pode estar envolvido com a manutenção da inflamação na mucosa intestinal.

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## Introduction

Autophagy seems to be essential for intestinal mucosal immunity, and recent studies show that mutations in autophagy-related genes are associated with CD.<sup>1-4</sup> The autophagy process has important functions, such as regulation of cell survival and cell death. When properly activated, the process promotes survival through the degradation of cytoplasmic organelles and content via lysosomes, thereby providing metabolic fuel for mitochondrial oxidation,<sup>5,6</sup> as well as accounting for the removal of proteins generated from cellular stress. However, exacerbated autophagy may also lead to cell death.<sup>7,8</sup> Autophagy can be induced by extracellular and intracellular signals, including oxidative stress, growth factors, ceramide, endoplasmic-reticulum (ER) stress, and glucose and amino acid serum starvation.<sup>9</sup>

Association of CD with the gene loci ATG16L1 was referred and patients who had more mutant alleles, develop more severe course of disease (stenosis and fistulae), compared to those patients with inflammatory CD phenotype only.<sup>10</sup>

Although there is phenotypic variation in surgical specimens from CD patients, macroscopic aspects are notorious; particularly regarding mesenteric fat thickening near the affected intestinal area.<sup>11</sup> Mesenteric fat hypertrophy and bowel wrapping are characteristic of CD.<sup>12</sup> Histological analyses revealed abnormalities in adipose tissue, including infiltration of macrophages and T cells, perivascular fibrosis and inflammation. Moreover, there are increased numbers of adipocytes in CD creeping fat, which have been reported to be smaller in CD than in controls.<sup>13,14</sup> Similar to macrophages and epithelial cells, adipocytes from normal individuals are able to synthesize several proinflammatory and anti-inflammatory cytokines, and fat hormones.<sup>15</sup> Indeed, adipocytes

can express TLR4 for the recognition of local or systemic bacterial antigens.<sup>16-18</sup>

There have been few reports discussing the role of the mesenteric fat tissue in CD.<sup>19-24</sup> However, there are no studies evaluating autophagy in this tissue. Therefore, in order to compare the autophagic activity (called 'macroautophagy') and the expression of proinflammatory cytokines in fat and intestinal tissue between CD patients and controls, we used immunoblotting to determine LC3, TNF- $\alpha$ , and IL-23 protein expression. In addition, we performed RT-PCR assays to determine the relative RNA quantification related to the step of membrane elongation and autophagosome formation (LC3II and Atg16L1) in mesenteric fat tissue groups.<sup>25,26</sup>

## Patients and methods

Mucosal biopsies were taken from 10 patients with ileocecal CD [mean age 34.9 (range, 14-60) years; male 50%; female 50%]. The presence of disease activity was assessed by colonoscopy before surgery, and all patients had Crohn's disease activity index (CDAI)<sup>27</sup> over 250 points. The control groups were composed of eight patients who underwent resectomy for non-inflammatory disease (megacolon), with normal distal ileum (ileum mesenteric fat tissue control group) [mean age 55.6 (range, 39-70) years; male 62.5%; female 37.5%], and eight patients with normal ileocolonoscopy (intestinal tissue control group) [mean age 50.4 (range, 33-60) years; male 37.5%; female 62.5%].

The study was approved by the local Ethical Committee. All biopsies were performed after obtaining informed consent from the patients. The study was carried out at the Coloproctology Unit and at the Cell Signaling Laboratory of the Universidade Estadual de Campinas (UNICAMP).

Mucosal biopsies from the terminal ileum and mesenteric fat tissue near the affected intestinal area were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until ready to be used.

### Western blotting analysis

For total protein extract preparation, the fragments were homogenized in solubilization buffer at  $4^{\circ}\text{C}$  [1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mg aprotinin/mL] with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY) operated at maximum speed for 30 sec. Insoluble material was removed by centrifugation (20 min at 110,000 rpm at  $4^{\circ}\text{C}$ ). Protein concentration in the supernatants were determined by the Bradford dye binding method.<sup>28</sup> Aliquots of the resulting supernatants containing 50  $\mu\text{g}$  total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-LC3, anti-TNF- $\alpha$  and anti-IL-23 antibodies.<sup>29</sup>

Reagents for SDS-PAGE and immunoblotting were from Bio-Rad Laboratories (Richmond, CA). Phenylmethylsulfonyl fluoride, aprotinin, Triton X-100, Tween 20, and glycerol were from Sigma (St. Louis, MO). Nitrocellulose paper (BA85, 0.2  $\mu\text{m}$ ) was from Amersham (Aylesbury, UK). The anti-LC3 (M115-3, mouse monoclonal) antibody was purchased from MBL International (MA). The anti-TNF- $\alpha$  (sc-1347, rabbit polyclonal) and anti-IL-23 (sc-50303, rabbit polyclonal) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein molecular weight was assessed by the PageRuler™ from Fermentas (Glenburnie, MD). The signal was detected by chemiluminescent reaction (SuperSignal® West Pico Chemiluminescent Substrate from Pierce Biothechnology, Inc. Rockford, IL).

All numerical results are expressed as mean  $\pm$  SEM of the indicated number of experiments. Blotting results are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the Gel-Pro Analyzer 6.0 software (Exon-Intron Inc., Farrell, MD). Data were analyzed by the *t*-Test, comparing the mesenteric fat tissue of the CD group and its respective fat control group; and comparing, separately, the intestinal tissue of the CD group and its respective intestinal control group. The level of significance was set at  $p < 0.05$ .

### RT-PCR analysis

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer instructions. RNA purity and concentration were determined by UV spectrophotometry at 260 nm. RNA was treated with RNase-free Dnase (RQ1 RNase-free Dnase, Promega), then reverse transcribed using oligo (dT) primers and reverse transcriptase (RevertAid™ Kit, Fermentas). The reaction mixture (20  $\mu\text{l}$ ) was incubated at  $42^{\circ}\text{C}$  for 60 min, then 10 min at  $70^{\circ}\text{C}$ , and cooled on ice. RT-PCR was performed on resulting cDNA, using the manufacturer protocol, in 25  $\mu\text{l}$  reaction volume per capillary. Gene-specific primers (Applied Biosystems™) were: Hs00261291 (MAP1LC3-LC3); Hs00250530 (ATG16-L1); NM\_002046.3 (GAPDH). The reaction mixture contained SYBR®Premix Ex Taq™ II (2x), cDNA template, primer

pair mixture and  $\text{dH}_2\text{O}$ . RT-PCR amplification consisted of an initial denaturation step ( $50^{\circ}\text{C}$  for 2 min and  $95^{\circ}\text{C}$  for 10 min), 40 cycles of denaturation ( $95^{\circ}\text{C}$  for 15 seconds), annealing ( $53^{\circ}\text{C}$  for 20 seconds) and extension ( $72^{\circ}\text{C}$  for 20 seconds), followed by a final incubation at  $60^{\circ}\text{C}$  for 1 min. All measurements were normalized by the expression of GAPDH gene, considered as a stable housekeeping gene. Gene expression was determined using the delta-delta Ct method:  $2^{-\Delta\Delta\text{CT}}$

$$(\Delta\Delta\text{CT} = [\text{Ct}(\text{target gene}) - \text{Ct}(\text{GAPDH})]_{\text{patient}} - [\text{Ct}(\text{target gene}) - \text{Ct}(\text{GAPDH})]_{\text{control}})$$

Real-time PCR analysis of gene expression was performed in a 7500 SDS sequence detection system (Applied Biosystems). The optimal concentration of cDNA and primers, as well as the maximum efficiency of amplification, were obtained through five-point, two-fold dilution curve analysis for each gene. Real-time data were analyzed using the Sequence Detector System 1.7 (Applied Biosystems). The *t*-test was used for statistical analyses, comparing the mesenteric fat tissue of the CD group and its respective fat control group; and comparing, separately, the intestinal tissue of CD group and its respective intestinal control group. The level of significance was set at  $p < 0.05$ .

## Results

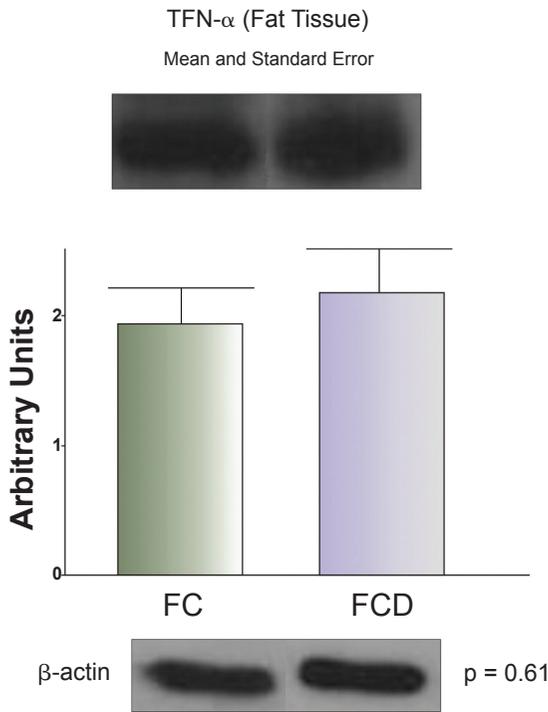
LC3-II protein expression was significantly lower in the mesenteric fat tissue of CD patients (FCD group), when compared to controls (FC group) ( $p < 0.05$ ). Patients with CD also had significantly higher levels of LC3-II, TNF- $\alpha$  and IL-23 in intestinal mucosa (ICD group) when compared to intestinal control (IC group) ( $p < 0.05$ ). No difference in cytokine levels was observed among the mesenteric groups. Results are showed in Figs. 1 to 6.

Regarding LC3 and ATG16L1 gene expressions, no statistically significant differences were detected when the mesenteric fat tissue groups were compared ( $p > 0.05$ ). Figs. 7 and 8 illustrate these findings.

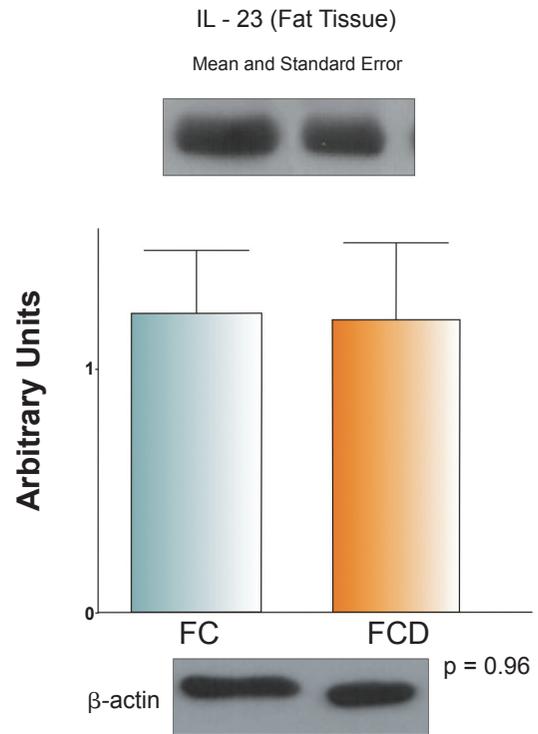
## Discussion

Recent genome-wide association studies (GWAS) have increased the number of CD associated susceptibility genes. Two of these genes (ATG16L1 and IRGM) modulate autophagy, and the variant T300A of ATG16L1 results in a specific defect in bacteria-induced autophagy.<sup>3,30,31</sup> ATG16L1 and NOD2 risk variants, in addition to Paneth cell defect, could modify intestinal epithelial cell antimicrobial responses.<sup>30</sup> However, there is no report of autophagy-related protein levels in distinct tissues affected by CD, such as intestinal and mesenteric fat tissues.

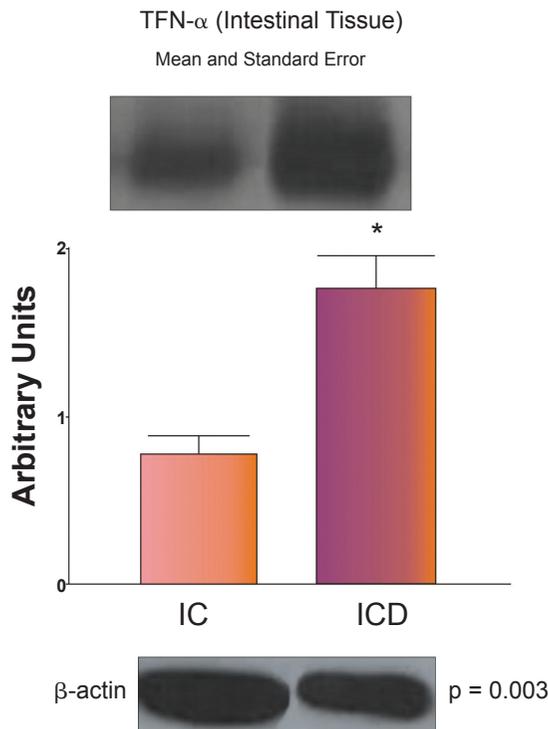
Autophagy is a cellular process by which a double-membrane structure (autophagosome) surrounds the cytoplasm to break captured proteins and cytoplasmic organelles.<sup>32</sup> Although Beclin-1 is a part of one of the core Atg (autophagy-related genes) complexes and participates in the initial autophagy process, in the formation of the isolated membrane, LC3-II conjugations are essential for membrane elongation and autophagosome formation and are often used as markers of autophagy. Whereas LC3-I is localized in the cytosol,



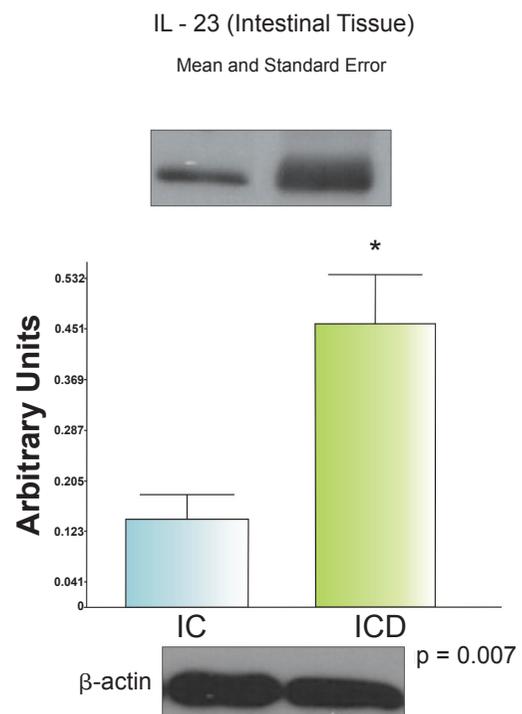
**Fig. 1 - Representative Western blot analyses and determination of TNF- $\alpha$  protein expression in the fat tissue of control (FC) and Crohn's disease (FCD) groups. For illustration purposes each band represents one patient. For the FCD Group, n = 10; for the FC Group, n = 8, \*p < 0.05 vs control.**



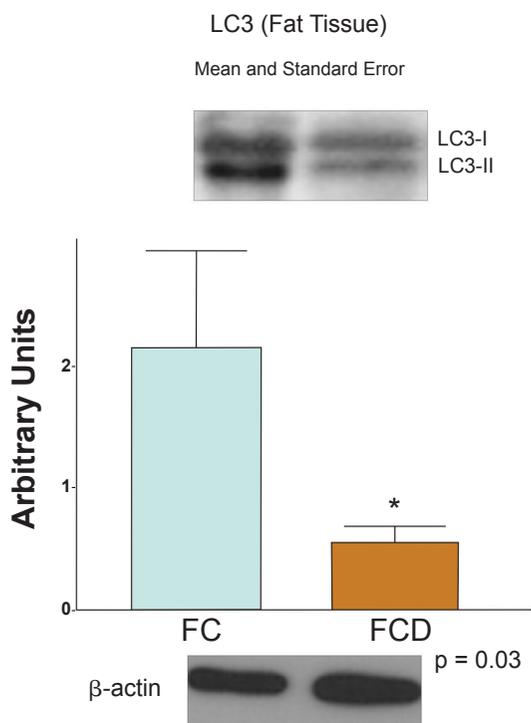
**Fig. 3 - Representative Western blot analyses and determination of IL-23 protein expression in the fat tissue of control (FC) and Crohn's disease (FCD) groups. For illustration purposes each band represents one patient. For the FCD Group, n = 10; for the FC Group, n = 8, \*p < 0.05 vs control.**



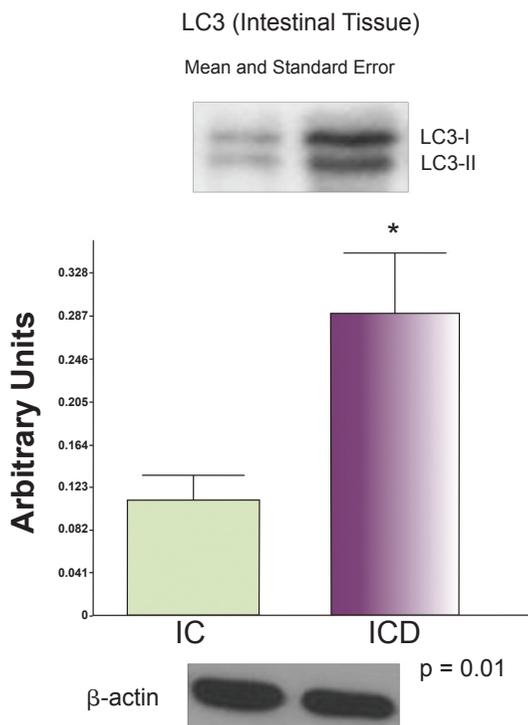
**Fig. 2 - Representative Western blot analyses and determination of TNF- $\alpha$  protein expression in the intestinal tissue of control (IC) and Crohn's disease (ICD) groups. For illustration purposes each band represents one patient. For the ICD Group, n = 10; for the IC Group, n = 8, \*p < 0.05 vs control.**



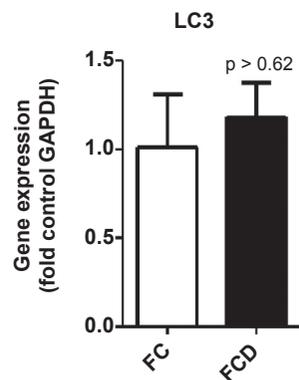
**Fig. 4 - Representative Western blot analyses and determination of IL-23 protein expression in the intestinal tissue of control (IC) and Crohn's disease (ICD) groups. For illustration purposes each band represents one patient. For the ICD Group, n = 10; for the IC Group, n = 8, \*p < 0.05 vs control.**



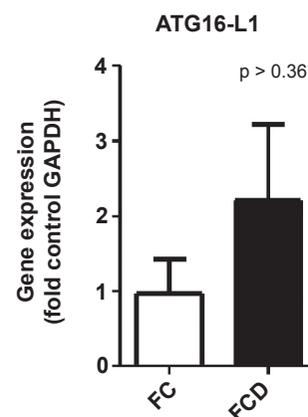
**Fig. 5 – Representative Western blot analyses and determination of LC3-II protein expression in the fat tissue of control (FC) and Crohn’s disease (FCD) groups. For illustration purposes each band represents one patient. For the FCD Group, n = 10; for the FC Group, n = 8, \*p < 0.05 vs control.**



**Fig. 6 – Representative Western blot analyses and determination of LC3-II protein expression in the intestinal tissue of control (IC) and Crohn’s disease (ICD) groups. For illustration purposes each band represents one patient. For the ICD Group, n = 10; for the IC Group, n = 8, \*p < 0.05 vs control.**



**Fig. 7 – Results of LC3 gene expression determined by RT-PCR. For FCD Group, n = 10; for FC Group, n = 8, \*p < 0.05 vs control.**



**Fig. 8 – Results of ATG16-L1 gene expression determined by RT-PCR. For FCD Group, n = 10; for FC Group, n = 8, \*p < 0.05 vs control.**

LC3-II is localized on the cytoplasmic surface of autophagosomes and is delipidated by Atg4B to recycle LC3-I for further autophagosome formation.<sup>33,34</sup> Indeed, Atg16L1 determines the site of LC3 conjugation, essential for autophagosome formation, suppressing inflammasome activity and maintaining Paneth cells.<sup>35,36</sup> Despite great progress since the identification of ATG1,<sup>37</sup> many questions regarding the molecular regulation of autophagy remain unresolved.<sup>38</sup>

Moreover, autophagy and inflammatory pathways are closely linked. Proinflammatory cytokines secretion, such as IL-1 $\beta$  and IL-18, were enhanced in ATG16L1 or ATG7 deleted macrophages in response to lipopolysaccharide (LPS), displaying increased susceptibility to a murine model of colitis.<sup>39</sup> Autophagy also suppresses endotoxin-induced inflammatory immune responses.<sup>40</sup> Defects of autophagy activity and bacterial receptors (NOD2) have been associated with impaired antigen presentation, engaged intracellular removal of pathogenic bacteria and, consequently, higher expression of proinflammatory cytokines.<sup>41-45</sup>

Autophagy contributes to cellular survival against nutrient starvation and the turn-over of injured organelles,<sup>46,47</sup> explaining why in the present study was verified high expression of autophagy protein in the intestinal tissue of CD patients when compared to controls. However, this ability was not verified in the hypertrophied mesenteric fat tissue of CD patients, other-

wise, was found low expression of LC3-II when compared to mesenteric tissue of patients without IBD. This LC3-II expression could lead to an impaired clearance of bacterial species, as well as the accumulation of unprocessed unnecessary proteins, which activate proinflammatory pathways involved in CD pathogenesis. The higher expression of proinflammatory cytokines in intestinal mucosa, such as TNF- $\alpha$  and IL-23, which signalize respective Th1 and Th17 lymphocyte response, could also explain the higher levels of autophagy protein seen in this study.

Although LC3-II protein expression was lower in the mesenteric fat tissue and higher in the intestinal tissue of CD patients, no statistical differences were seen among these groups, considering LC3 and ATG16L1 gene expressions. Reports on the role of microRNAs (miRNAs) in autophagy may lead to insights into these complex processes. MicroRNAs represent an important and still relatively unexplored manner of regulating protein synthesis. It does not encode for proteins, but exert catalytic, structural or regulatory activities by annealing to specific target RNAs, and downregulating their stability and/or translation.<sup>48,49</sup> It has been reported that miRNA-101 is a potent inhibitor of autophagy.<sup>50</sup> Regarding CD, Brest et al., showed that a family of miRNAs, miR-196, is overexpressed in the inflammatory intestinal epithelia of these individuals and there is subsequent loss of the tight regulation of IRGM expression, which compromises the control of intracellular replication of CD-associated adherent invasive *Escherichia coli* by autophagy. The association of IRGM with CD arises from a miRNA-based alteration in IRGM regulation that affects the efficacy of autophagy.<sup>51</sup> The results of the protein and gene expression in the present study suggest that miRNA alterations could be involved in the complete gene transcription, resulting in the defective production of the protein. Only one study reporting a miRNA (miR-204) regulating LC3-II protein has been published, which reported an important role for this mechanism in myocardial injury;<sup>52</sup> however, there are no studies of miRNA, autophagy regulation, and CD in the literature.

Recent studies, in mice, provide insights into how defective autophagy in cells, such as macrophages and Paneth cells, may contribute to CD,<sup>39,53</sup> although none of these studies describe these alterations in adipose tissue. It is possible that the defective autophagy in this tissue could be responsible for maintaining the local production of inflammatory mediators and lead to intestinal involvement, especially on mesenteric longitudinal side, forming ulcers, characteristic of CD, as described by Crohn et al.<sup>54</sup> In late stages of the disease, there is a decrease in adipocytes number, as these are substituted by impaired autophagic cells of the immune system, such as macrophages,<sup>55,56</sup> possible explaining why the present study showed a defective autophagy in the mesenteric adipose tissue of CD patients.

The importance in knowing the autophagy pathway in creeping fat tissue in CD can lead us to understand more about the phenotype and molecular biology involved in CD and its pathogenesis, as well as the active role of adipose tissue in the maintenance of the inflammatory process during the course of disease.

## Conclusion

The present study shows a defective expression of protein-related autophagy in the mesenteric fat tissue of CD patients,

when compared to controls. Data suggest that the primary defects of cell autophagy may not occur at all tissue involved by CD, however, it may occur mainly in the adipose tissue, close to the affected intestinal area. The mesenteric fat tissue could play an important role in the maintenance of local inflammation in these patients. Further studies will determine whether miRNA has a role in autophagy regulation in patients with CD, and whether autophagy can be used for therapeutic approaches in the treatment of CD.

## Conflict of interest

The authors declare no conflict of interest.

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