



## Relationship between IGF-1 and body weight in inflammatory bowel diseases: Cellular and molecular mechanisms involved

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### ABSTRACT

Inflammatory bowel diseases (IBD), represented by ulcerative colitis (UC) and Crohn's disease (CD), are characterized by chronic inflammation of the gastrointestinal tract, what leads to diarrhea, malnutrition, and weight loss. Depression of the growth hormone-insulin-like growth factor-1 axis (GH-IGF-1 axis) could be responsible of these symptoms. We demonstrate that long-term treatment (54 weeks) of adult CD patients with adalimumab

**Abbreviations:** IBDs, Inflammatory bowel diseases; UC, ulcerative colitis; CD, Crohn's disease; GH-IGF-1 axis, growth hormone-insulin like growth factor-1 axis; ADA, adalimumab; IGF1BP4, IGF-1 without changes in serum IGF-1 binding protein; AJs, adherers junctions; DAI, disease activity index; DSS, dextran sulfate sodium.

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(ADA) results in a decrease in serum IGF-1 without changes in serum IGF-1 binding protein (IGF1BP4). These results prompted us to conduct a preclinical study to test the efficiency of IGF-1 in the medication for experimental colitis. IGF-1 treatment of rats with DSS-induced colitis has a beneficial effect on the following circulating biochemical parameters: glucose, albumin, and total protein levels. In this experimental group we also observed healthy maintenance of colon size, body weight, and lean mass in comparison with the DSS-only group. Histological analysis revealed restoration of the mucosal barrier with the IGF-1 treatment, which was characterized by healthy quantities of mucin production, structural maintenance of adherens junctions (AJs), recuperation of E-cadherin and  $\beta$ -catenin levels and decrease in infiltrating immune cells and in metalloproteinase-2 levels. The experimentally induced colitis caused activation of apoptosis markers, including cleaved caspase 3, caspase 8, and PARP and decreases cell-cycle checkpoint activators including phosphorylated Rb, cyclin E, and E2F1. The IGF-1 treatment inhibited cyclin E depletion and partially protects PARP levels. The beneficial effects of IGF-1 in experimental colitis could be explained by a re-sensitization of the IGF-1/IRS-1/AKT cascade to exogenous IGF-1. Given these results, we postulate that IGF-1 treatment of IBD patients could prove to be successful in reducing disease pathology.

## 1. Introduction

The incidence of inflammatory bowel disease (IBD), i.e., ulcerative colitis (UC) and Crohn's disease (CD), has been increasing annually throughout the world [1]. Both CD and UC are characterized by chronic inflammation of the gastrointestinal tract [1,2]. This inflammation is caused by a T lymphocyte response that leads to the local increase of several inflammatory cytokines, such as TNF- $\alpha$  [3]. Thus, neutralization of TNF- $\alpha$  using therapeutic antibodies, like adalimumab (ADA), is useful in IBD treatment [3]. Inflammatory cytokines circulating in the gut lead to typical IBD symptomatology of diarrhoea, malnutrition, and weight loss [4]. Beyond the observed relation with an increase in pro-inflammatory cytokines, some of these IBD symptoms could be associated with depression of the growth hormone-insulin like growth factor-1 axis (GH-IGF-1 axis) [5,6] or with systemic GH-resistance [7]. Both IGF-1 and GH carry out important regulatory functions; these hormones serve as trophic factors [8] and they contribute to the regulation of various processes including, caloric intake, catabolic activity, intestinal absorption, and intestinal immune cell activity [9,10]. GH-IGF-1 axis dysregulation causes a reduction in serum levels of IGF-1 in patients with active IBD [11–13]. Consequently, IBD patients with depression of the GH-IGF-1 axis [5,6] or systemic GH-resistance can suffer dramatic changes in body composition throughout the lifespan [4]. Previous studies in rodents established a decrease in IGF-1 serum levels in experimental colitis [14]. The role of IGF-1 in the expansion of intestinal stem cells during epithelial regeneration and in intestinal immune homeostasis, has been determined using IGF-1 knockout mice [15]. Moreover, IGF-1 administration to adult rats resulted in the growth of gastrointestinal organs [16] and in a significant increase in gut weight accompanied by an increase in crypt cells (33%) and villus cell density (20%). It has also been demonstrated that serum levels of IGF-1 directly regulate bone growth [17] and skeletal muscle formation [18]. Clinical studies have translated these findings and investigated the efficacy of GH (NCT00109473, NCT00511329) or IGF-1 (NCT00764699) in the treatment of paediatric CD, which led to some encouraging results [19–21]. However, there is currently no research on the effects of exogenous IGF-1 on adult CD patients. In order to assess the potential of this line of clinical research, we carried out the present study in CD patients and in a preclinical model evaluating the following:

a) serum IGF-1 levels during adalimumab treatment of adult CD patients; b) IGF-1 effects on body composition, intestinal inflammation, and serum biochemical parameters in adult rats with experimental colitis; c) cellular and molecular mechanism involved in the effects of IGF-1.

## 2. Materials and methods

### 2.1. Patients and treatment

The present study was carried out using sera from CD patients ( $n = 68$ ) that participated in the PREDICROHN project, A prospective multicenter study of the Spanish IBD Group (GETECCU). Subjects ultimately included in the study had to fulfill the following criteria: be over 18 years of age; be diagnosed with luminal or perianal CD using clinical, radiological, endoscopic, and histological methods; require anti-TNF treatment. The exclusion criteria were as follows: pregnant or nursing women; patients infected with HBV, HCV, or HIV; patients receiving anti-TNF treatment or other biological drugs. All patients were naïve to anti-TNF therapy and received Adalimumab (ADA) according to standard clinical practice. ADA was administered throughout the study at the following doses, 160 mg (week 1), 80 mg (week 2), and 40 mg for the remaining weeks (6, 14, 22, 30, 38, 46, and 54). Patient sera were analyzed at baseline (week 0), and at weeks 14 and 54 of ADA administration. In CD patients, the clinical response was defined as a  $> 70$ -point reduction in CDAI score. Of the 68 subjects initially enrolled, a total of 28 maintained participation in the clinical trial through study completion (Table 1). The present study was performed in accordance with the guidelines of the Helsinki Declaration of 1975. The study obtained the Institutional Ethics Committees approval from all hospitals involved. All patients signed informed consent at the time of their clinical evaluations.

### 2.2. Animals and treatment

Male adult Wistar rats (12 weeks old) weighing 180–200 g were accommodated with light and temperature-controlled ( $23 \pm 1.5$  °C) rooms in the animal research facility of Alcalá University. The animals were fed as per the standard diet (Harlan 2014 2.9 kcal/g; 4% fat; 14.3% protein) throughout their lifespans. The animals were separated into two primary groups: a healthy group and an experimental colitis group. Experimental colitis was induced by treating rats with 5% dextran sulfate sodium (DSS) mixed in drinking water [22]. The animals were then subdivided into four experimental arms: i) Control group: rats were fed with the standard diet and tap water (5 rats); ii) Control + IGF-1 group: rats were fed with the standard diet and tap water and treated daily with IGF-1 (20  $\mu$ g/day/rat, i.p.) (6 rats); iii) DSS group: rats were fed with the standard diet and DSS (5%) in tap water (5 rats); iv) DSS + IGF-1 group: rats were fed with the standard diet and DSS (5%) in tap water and treated daily with IGF-1 (20  $\mu$ g/day/rat, i.p.) (6 rats). All the animals were sacrificed on the ninth day of experimental conditions. The Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health was referenced for proper handling of the laboratory animals. The study was conducted in accordance with the protocols approved by the Ethics Committee of Animal Experiments of Alcalá University. Table 2 and 3.

**Table 1**

A. Patients with Crohn's disease treated with Adalimumab (n = 28). B. Scoring system for the disease activity index (DAI) of the rats with experimental colitis. C. Biochemical parameters and symptomatology of rats with experimental colitis.

	Baseline	14 weeks	54 weeks	p-value
Age (years)	40.5 ± 12.0	–	–	
Male, n (%)	16 (57)	–	–	
Smokers, n (%)	8 (28)	–	–	
Harvey-Bradshaw	4 (3–5)	2 (1–3) (*)	1 (0–3) (***)	< 0.0001
CDAI	134 ± 81	80 ± 57 (**)	57 ± 44 (**)	0.002
Weight (kg)	70.5 ± 13.8	71.6 ± 13.3	71.2 ± 14.1	0.4
<b>Haematological indices</b>				
Leukocytes (10 <sup>9</sup> /L)	6.6 (5.5–8.4)	6.6 (4.7–7.8)	6.1 (4.7–8.2)	0.05
Neutrophils (10 <sup>9</sup> /L)	4.2 (3.8–6.9)	3.2 (2.7–4.5) (**)	3.5 (2.4–4.4) (*)	0.006
Platelets (10 <sup>9</sup> /L)	268.5 (222.8–343.3)	237.5 (198.5–327.0)	220.5 (201.3–318.5)	0.05
Haemoglobin (g/dL)	13.8 (13.3–14.7)	13.9 (13.5–14.7)	14.1 (13.6–14.9)	0.8
Ferritin (ng/mL)	91.2 ± 79.9	64.9 ± 63.1	65.9 ± 45.6	0.1
<b>Inflammatory indices</b>				
CRP (mg/dL)	0.5 (0.3–1.7)	0.2 (0.1–1.7) (**)	0.3 (0.1–0.4) (*)	0.003
ESR (mm/h)	30 (13–43)	21 (13–31)	22 (10–41)	0.6
IL6 (pg/mL)	12.2 (10.7–22.8)	12.1 (9.0–24.9)	10.6 (8.5–12.0)	0.1
TNFα (pg/mL)	3.7 (2.6–5.5)	3.1 (1.8–5.5)	3.4 (1.8–5.6)	0.3
<b>Hepatic biomarkers</b>				
Albumin (mg/dL)	4.1 ± 0.4	4.4 ± 0.4	4.3 ± 0.4	0.1
Fibrinogen (mg/dL)	422.5 (372.3–480.8)	356.5 (279.5–443.8)	380.0 (235.8–475.0)	0.2
<b>IGF1 axis</b>				
IGF1 (ng/mL)	94.5 (61.8–132.4)	74.2 (63.7–109.7)	56.5 (51.6–69.9) (**)	0.01
IGF1BP4 (ng/mL)	442.2 ± 150.7	412.8 ± 119.7	405.1 ± 140.0	0.6

The results were shown as mean and SD (or median and interquartile range [IQR]) for continuous variables, and absolute numbers and relative frequencies for categorical variables. Statistical differences were analysed using the one-way ANOVA or Friedman test. P-value from these tests are indicated in the last column. A post-hoc Tukey's or Dunn's multiple comparisons test was performed. \*Significant differences with the baseline found by post-hoc tests were indicated (\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001).

**Table 2**

Scoring system for the disease activity index (DAI) of the rats with experimental colitis.

Score	Weight loss (% of control)	Stool consistency	Occult/gross blood in stools
0	x ≤ 1		Normal
1	1 < x ≤ 5	Normal stool	
2	5 < x ≤ 10	Diarrhea	
3	10 < x ≤ 20		
4	20 < x		Gross bleeding

### 2.3. General assessment of colitis

Five or six animals were used in each experimental group. Every day, body weight, fecal counts, stool consistency, and rectal bleeding were recorded to obtain the disease activity index (DAI). Each rat's daily food consumption was also measured. DAI was assessed as previously described by Román et al. [23] with minor modifications (S Table 1. A). Immediately prior to sacrifice, body composition of each rat was

**Table 3**

Biochemical parameters and symptomatology of rats with experimental colitis.

	Control	DSS	Control+IGF1	DSS+IGF1
AST (U/L)	218.50 ± 154.90	307.30 ± 31.77	135.30 ± 17.04	235.50 ± 201.50
ALT (U/L)	38.50 ± 7.78	38.67 ± 5.69	41.67 ± 3.51	40.50 ± 9.19
GGT (U/L)	<4	5.00 ± 1.00	<4	<4
Alkaline phosphatase (U/L)	192.30 ± 4.51	132.50 ± 14.85	181.7 ± 51.83	119.50 ± 26.16
Phosphate (mg/dL)	11.10 ± 0.28	9.00 ± 0.46(*)	9.10 ± 0.52	10.25 ± 0.35
Calcium (mg/dL)	10.53 ± 0.40	9.23 ± 0.87	10.57 ± 0.06	10.30 ± 0.28
Glucose (mg/dL)	115.00 ± 8.48	42.50 ± 7.78 (**)	97.33 ± 13.32	133.00 ± 1.41 (##)
Albumin (g/dL)	1.37 ± 0.06	0.80 ± 0.10 (***)	1.33 ± 0.11	1.15 ± 0.07 (#)
Total proteins (g/dL)	5.93 ± 0.06	4.80 ± 0.20 (**)	6.03 ± 0.15	5.25 ± 0.35 (*) (#)
Diarrhea	No	Yes	No	No
Blood in stool	No	Yes	No	Yes

The results were shown as mean and SD (or median and interquartile range [IQR]) for continuous variables, and absolute numbers and relative frequencies for categorical variables. Statistical differences were analysed using the one-way ANOVA or Friedman test. P-value from these tests are indicated in the last column. A post-hoc Tukey's or Dunn's multiple comparisons test was performed. \*Significant differences with the baseline found by post-hoc tests were indicated (\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001).

analyzed using the DXA technique. After each rat was sacrificed, serum and colon tissue were obtained.

### 2.4. Biochemical parameters

Blood samples were procured in absence of anticoagulants from each rat at the time of sacrifice via cardiac puncture. Serum was collected following a 30 min incubation at room temperature and 5 min of centrifugation at 500 g. Biochemical parameters were obtained using a hematology analyzer (S Table 1. B).

### 2.5. Tissue preparation, immunohistochemistry, and immunofluorescence staining

Proximal colon samples were fixed with neutral formalin, washed with PBS, dehydrated with graded ethanol series and embedded in paraffin as previously described by Román et al. [23]. Five-micron thick sections were obtained and mounted on silanized glass slides.

Periodic acid Schiff (PAS) and alcian blue (AB) stains were used to differentiate between neutral and acidic mucins [23]. Sections were stained by incubation with AB for 15 min, washed with distilled water, incubated with periodic acid (1%) for 5 min and then washed with distilled water. The samples were then incubated with the Schiff's reactive for 5 min, washed with distilled water, and finally stained with hematoxylin. The tissue expression of mucins was determined by calculating optical density as previously described by Román et al. and Sanmartín-Salinas et al. [23,24].

For immunofluorescence staining, we used the following specific antibodies: rabbit anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-E-cadherin (BD-transduction labs, Madrid, Spain). After incubation with the primary antibody, samples were incubated with the chicken anti-rabbit-Alexa Fluor 488 antibody or the chicken anti-mouse-Alexa Fluor 594 antibody. In immunohistochemistry experiments, we used rabbit anti-AIF-1 (Wako, Osaka, Japan) antibody. After incubation with the primary antibody, samples were incubated with the peroxidase-conjugated secondary antibody (polymer-based system MasVision™ from Master Diagnostica, Spain). Nuclei were

stained with hematoxylin. Negative controls were performed by incubating the samples with non-immune mouse or rabbit serum instead of the primary antibody.

To analyze apoptotic nuclei, the DeadEnd Fluorometric TUNEL System (Promega, Madrid, Spain) was used in accordance with the manufacturer's instruction. Deparaffinized and hydrated sections were washed with a 0.85% solution of NaCl at room temperature for 5 min and sequentially washed with PBS (5 min), 4% methanol (15 min), and PBS again (2 washes, 5 min each). After protein digestion (proteinase K, 20 µg/mL), the samples were incubated with a buffered nucleotide solution and the TdT enzyme at 37 °C for 60 min. Slides were examined under an epifluorescence microscope (Olympus BX50, Olympus Australia, Windsor, Qld, Australia).

## 2.6. Protein extraction

Rat colonic tissue was homogenized in ice-cold lysis buffer (Tris-HCl 50 mM, pH 7.4, EDTA 5 mM, EGTA 1 mM, PMSF 1 mM, leupeptin 5 µg/mL, and aprotinin 5 µg/mL) by mechanical disruption. To remove connective tissue, a centrifugation (500 rpm for 10 min at 4 °C) was carried out. The supernatant was collected and centrifuged again (20,000 rpm for 30 min at 4 °C). The pellet was re-suspended in ice-cold lysis buffer plus Triton X-100 (1%) and stored at -80 °C until use. The amount of protein present was determined using a Bradford protein assay kit (Bio-Rad). For the determination of the protein concentration the samples were diluted 10 times to obtain a final concentration of Triton X-100 of 0.1% in order to avoid its interference with the Bradford reagent.

## 2.7. Immunoprecipitation protocol and immunoblot analysis

Colonic tissue extracts containing 500 µg of proteins were incubated with 2 µg of either anti-E-cadherin (BD-transduction labs, Madrid, Spain), anti-bcl-2 (Abcam, Cambridge, UK), or anti-bax (Abcam, Cambridge, UK) antibodies. After 1 h of gentle rocking at 4 °C, 30 µL of A/G plus Agarose were added to the lysates and incubation was continued for another 2 h. After washing, beads were re-suspended in 30 µL of 2X Laemmli buffer. For the negative control, the primary antibody was omitted.

Protein extracts were analyzed by SDS-PAGE and western blot assays as previously described by Sanmartín-Salinas et al. [24]. Whole extracts were resolved on 8–10% SDS polyacrylamide gels and transferred to nitrocellulose membranes at 25 V and 4 °C during 12–14 h. The immunoblots were developed as previously described by Sanmartín-Salinas et al. [24]. In some immunoblot assays joins between lanes are observed. When there is a high concentration of protein in the loading gel it is common for lateral diffusion to occur, causing the apparent joins between lanes. Additionally, anomalous patterns on immunoblot images may be observed on some assays. This is also a common occurrence that results from handling the delicate film. These artifacts have no effect on the integrity of our results.

## 2.8. Gelatin zymogram protocol

Rat colon protein extracts (50 µg) were electrophoresed at 4 °C in 10% SDS-PAGE containing 2 mg/mL of gelatin using a constant current of 40 mA at 100 V for approximately 4 h. The gel was washed two times with renaturation buffer (Tris-HCl 100 mM, NaCl 50 mM, CaCl<sub>2</sub> 10 mM, pH 7.5) containing 1% Triton X-100 for 30 min and was then incubated with renaturation buffer for 12–14 h at room temperature under gentle shaking. Subsequently, the gel was stained by incubation with a 0.1% Coomassie brilliant blue R-250 solution in methanol (40%) and acetic acid (10%) for 2 h under gentle shaking. Gel de-staining was carried out with distilled water changing the solution multiples times.

## 2.9. Statistical analysis

Descriptive analyses were performed for all the variables studied. The results were presented as mean and SD (or median and interquartile range [IQR]) for continuous variables, and absolute numbers and relative frequencies for categorical variables. Depending on data distribution, statistical differences among the groups were analyzed using a one-way, two-way ANOVA, or a Friedman test. In function on data distribution, Post-hoc Tukey's or Dunn's multiple comparisons test were performed. \*Significant differences from baseline found by post-hoc tests were indicated (\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001). Correlations between colon length and body weight or bone mineral content or lean body mass were analyzed using the Pearson's correlation coefficient (*r*). Differences were determined statistically significant when *p* < 0.05.

## 3. Results

### 3.1. Study of patient population

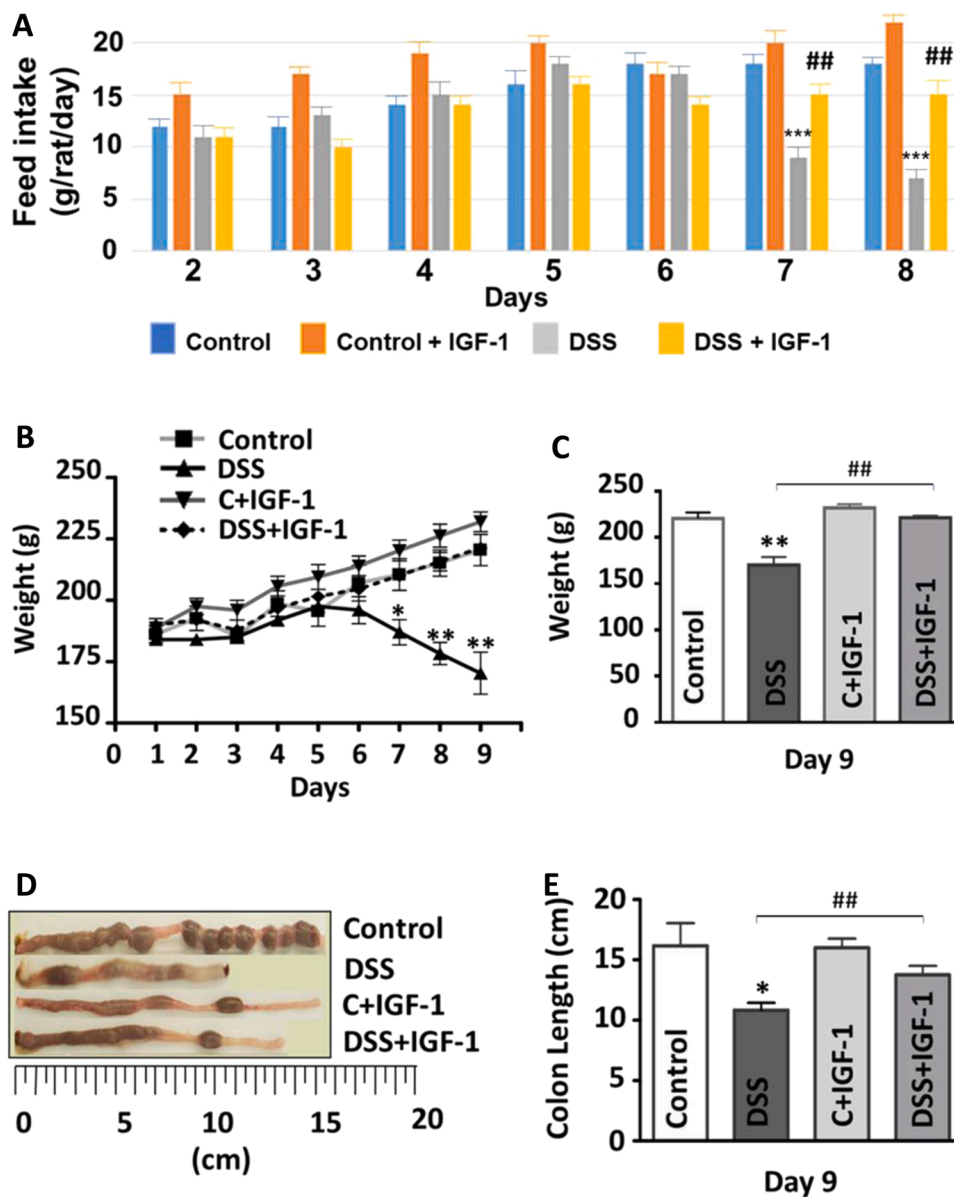
A total of 68 patients with Crohn's disease began treatment with ADA in a 54-week longitudinal study, of which only 28 patients participated through study completion. This article presents the results of these 28 patients at three time points: baseline, 14 weeks (induction of remission), and 54 weeks (maintenance therapy). The main characteristics of the study cohort are summarized in Table 1. Most of the patients presented ileocolic inflammation. The study cohort consisted of patients with mild to moderate CD. At baseline, the average CDAI score was 134 ± 81 and throughout the study it was shown to decrease to 57 ± 44 at 54 weeks of ADA treatment. Furthermore, the beneficial effect of ADA was observed without significant changes in patient weight as presented in Table 1. The observed pharmacological effect of ADA was correlated with a decrease in the number of neutrophils in conjunction with a decrease in CRP (nonspecific biomarker of inflammation). No significant changes were observed in other haematological (leukocytes, platelets, haemoglobin and ferritin) or in inflammatory indices (ESR, IL6 and TNFα), or in liver biomarkers (albumin and fibrinogen). Some of the observed changes have already been described; however, the decrease in serum IGF-1 levels during long-term treatment (54 weeks) with ADA of adult CD patients is a noteworthy, novel finding. Interestingly, no significant changes in serum IGF-1 binding protein (IGF1BP4) levels were observed during the study period. These novel clinical findings prompted us to further investigate the effect of IGF-1 on the enterohepatic axis in vivo which translated into the animal study described above.

### 3.2. Changes in feed intake, body weight and colon length after DSS treatment. Effect of concomitant IGF-1 administration

Seven days after the beginning of DSS administration we observed a significant decrease in food consumption in the treatment group (group iii) with respect to the control group (group i) (Fig. 1A). Simultaneously, in group iii we observed a significant decrease in body weight from day 7 onwards (Fig. 1B). Following autopsies at the end of the study period (day 9), we observed significant decreases in body weight (Fig. 1C) and colon length (Figs. 1D and 1E) of group iii compared to group i.

Notably, concomitant administration of IGF-1 (20 µg/rat/day) to rats with DSS-induced experimental colitis (group iv) was protective against the development of some colitis symptomatology. Specifically, increased food intake (Fig. 1A), body weight (Figs. 1B and 1C), and colon length (Figs. 1D and 1E) relative to animals treated with DSS only. IGF-1 treatment of healthy animals (group ii) did not produce a significant change in the feed behavior, body weight, or colon length in this group (Fig. 1A–E).





**Fig. 1.** - Changes in feed intake, body weight, and colon length for rats with experimental colitis compared to healthy controls. A. Food consumed per rat, per day; B. Body weight throughout the study; C. Body weight at study day 9; D. Colon lengths of 4 representative rats measured at study day 9; E. Colon lengths assessed on study day 9. Values are mean  $\pm$  SD from 4 to 6 animals per group and data were analyzed by two-way ANOVA. Results of Tukey's post-test between the four groups are shown (\*\* $p < 0.001$ ; \* $p < 0.01$ ; \* $p < 0.05$  control vs DSS) (## $p < 0.01$ ; DSS vs DSS+IGF-1).

### 3.3. Changes in body composition and disease activity index (DAI) after DSS treatment. Effect of concomitant IGF-1 administration

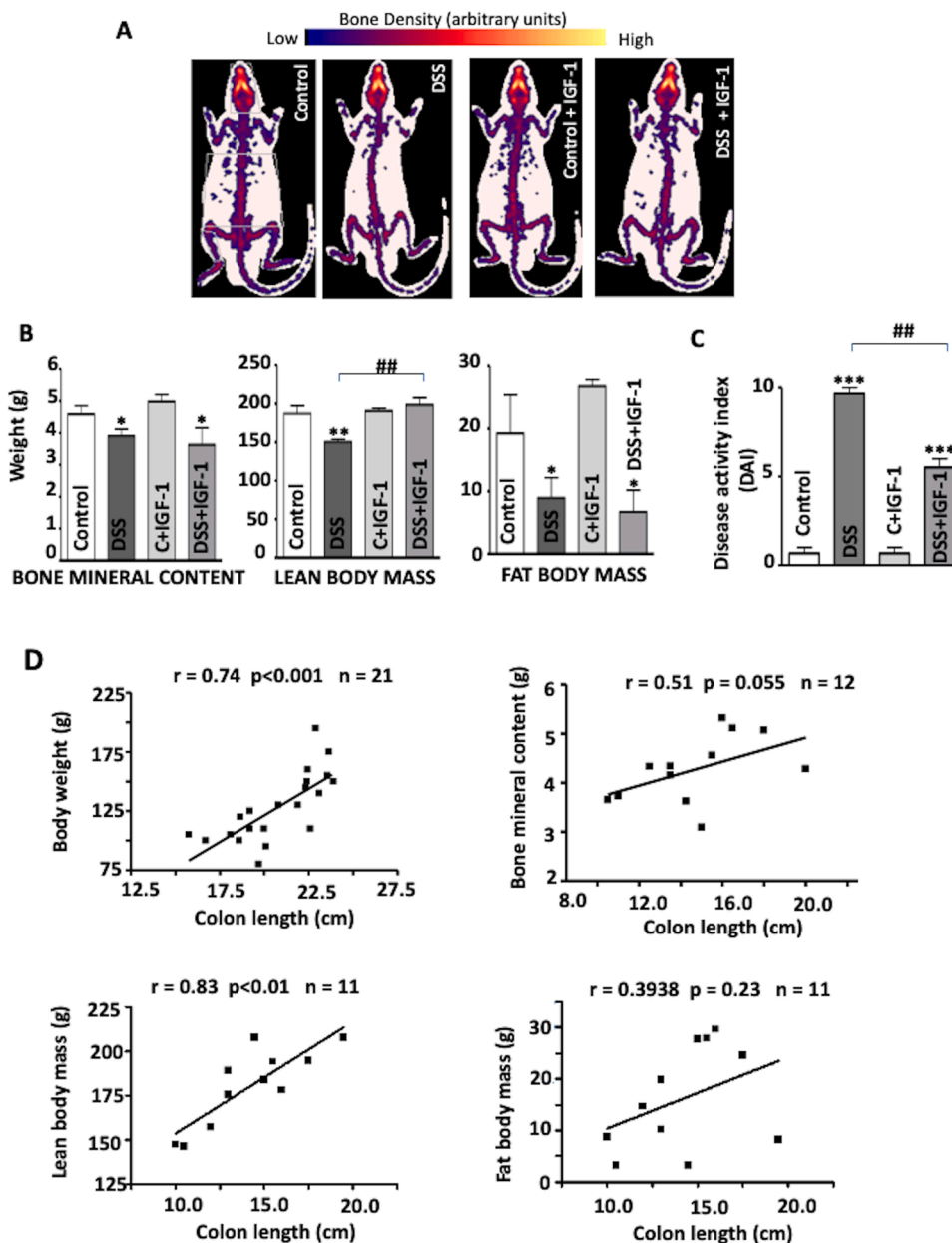
Fig. 2A shows bone mineral content, lean body mass, and fat deposits in four representative rats, one from each group: control (group i), DSS (group ii), control + IGF-1 (group iii), and DSS + IGF-1 (group iv). Animal body composition was determined by DXA (dual energy X-ray absorptiometry). Data indicate that in group ii bone mineral content, lean body mass, and fat body mass decrease significantly at day 9 of DSS treatment compared to controls (Fig. 2B). Our data suggest that IGF-1 administration (20  $\mu$ g/rat/day) to DSS treated animals (group iv) had a protective effect against lean body mass wasting observed in rats treated with DSS only (group ii); however, no protective effect is observed for the other studied parameters (Fig. 2B). IGF-1 treatment of healthy animals (group iii) had no effect on the parameters mentioned above (Fig. 2B).

As expected, DSS-treatment increased DAI score significantly, but when rats were co-treated with IGF-1 (20  $\mu$ g/rat/day) and DSS (group iv) the index score was significantly lower than in the DSS-only group (group ii) (Fig. 2C). IGF-1 treatment alone did not produce a significant

change in the DAI score of healthy rats (Fig. 2C). We observed a significant positive correlation between colon length and body weight when we used the data of all animals involved in the study (Fig. 2D). The same happened when we compared colon length and lean body mass (Fig. 2D). However, there were no significant correlations between colon length and bone mineral content, or between colon length and fat body mass of the rats (Fig. 2D).

### 3.4. Effect of IGF-1 on histological markers of colon damage after DSS treatment

PAS and AB staining were used to evaluate colon mucosal integrity (Fig. 3). Magenta corresponds to neutral mucins, while acid mucins are stained in blue. The amount of both types of dye was evaluated using the Image program as previously described by Sanmartín-Salinas et al. [24]. In the DSS treated rats (group ii), we observed a decrease in neutral mucins and an increase in acidic mucins when compared to control group rats (group i). The levels of mucins were very similar in DSS and IGF-1 co-treated rats (group iv) relative to healthy animals (group i) (Fig. 3, first lane). Alteration in the protective mucin layer affected the



**Fig. 2.** - Assessment of bone mineral content, lean body mass, and fat body mass of rats with experimental colitis using DXA. A. DXA scan of 4 representative rats performed at study day 9. B. Average of body composition analysis of all rats assessed on study day 9. C. The Disease Activity Index (DAI) of the four study groups. Data were obtained following the scoring system described in Table 1. C. Correlation between colon length and body weight and colon length and body composition components (bone mineral content, lean body mass, and fat body mass). The Pearson correlation coefficient (r) and the p-value are indicated in each panel. Values are mean  $\pm$  SD from 4 to 5 animals per group and data were analyzed by two-way ANOVA. Results of Tukey post-test between the four groups are shown (\*\*p < 0.01; \*\*\*p < 0.001; \*\*p < 0.01; \*p < 0.05 control vs DSS or control vs DSS + IGF-1) (##p < 0.01; DSS vs DSS+IGF-1).

adherens junctions (AJ), which we visualized by staining the proteins beta-catenin (green) and E-cadherin (yellow). In DSS-treated rats (groups ii and iv), we observed a decrease in beta-catenin/E-cadherin ratio. Administration of IGF-1 to animals with experimental colitis (group iv) did not show any protective effects against the protein ratio dysregulation (Fig. 3, second lane). Next, we assessed the levels of AIF-1 as a marker of macrophage presence in the sub-epithelial layer. We observed an increase of AIF-1 in the DSS treatment groups (ii and iv), especially in ulcerated regions of tissue. Again, IGF-1 treatment in experimental colitis rats did not produce any protective effect in the sub-epithelial area (Fig. 3, lane three). Furthermore, there is a visible increase in AIF-1 positive cells in the muscular layer of experimental colitis group (ii) when compared to the healthy control. In contrast, administration of IGF-1 to DSS-treated rats (group iv) reduced the number of AIF-1 positive cells in the muscularis externa (Fig. 3 lane four). Next, we measured TUNEL staining in colonic tissues to study apoptotic processes in inflamed tissue. TUNEL-positive nuclei (green) indicate the presence of apoptotic DNA fragmentation. Cell nuclei were counterstained in blue with Hoechst (Fig. 3, lane five). The TUNEL

positive cells (green) were divided by total number of cells (blue) to yield the apoptotic index. Our data show that DSS treatment increased the apoptotic index with respect to the healthy control. Moreover, co-treatment with IGF-1 did not have any appreciable protective effects against apoptosis (Fig. 3, lane five).

### 3.5. Effect of concomitant IGF-1 administration on the IGF-1 signaling cascade in the colon following DSS treatment

To study the acute effect of IGF-1 administration on the IGF-1 signaling cascade, animals were sacrificed 30 min after a single dose of IGF-1 (20  $\mu$ g/rat, i.p.) and the different components of the signaling cascade were analyzed in colon extract by immunoblot (Fig. 4A). The data suggest IGF-1 administration increases phosphorylation of the IGF-1 receptor beta subunit, of AKT, of mTOR, and of GSK-3. However, there was no significant difference in the total amount of IGF-1 receptor (IGF-1R), AKT, and GSK-3 between untreated and IGF-1-treated rats (Fig. 4A). The ratios of phosphorylated/total protein for IGF-1R, AKT, and GSK-3 is represented by a bar graph in Fig. 4B.

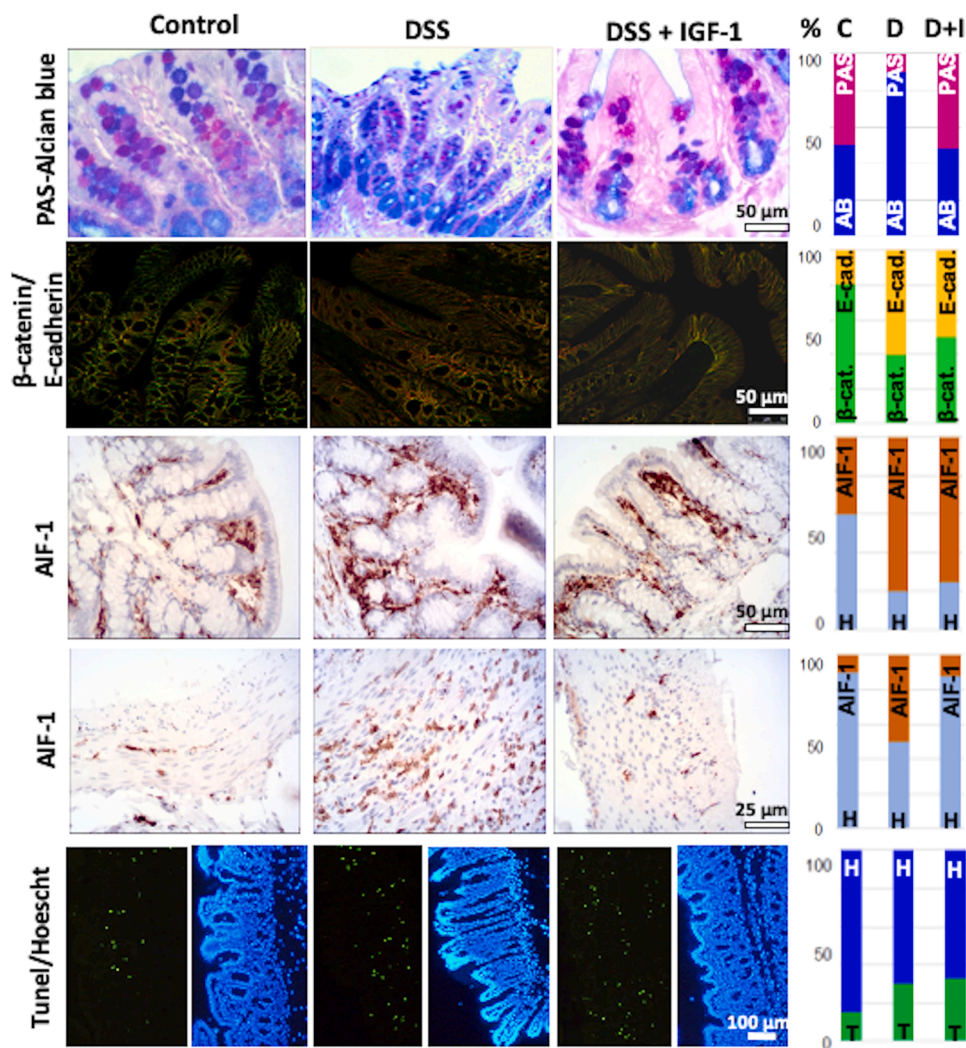


Fig. 3. - Histochemical staining of colon mucosa from rats with experimental colitis at study day 9. First lane: Images of PAS-alcian blue staining (20X magnification). Second lane: Immunostaining of  $\beta$ -catenin/E-cadherin (20X magnification). Third lane: Immunostaining of AIF-1 in mucosa and submucosa (20X magnification). Fourth lane: Immunostaining of AIF-1 in muscularis externa (40X magnification). Fifth lane: TUNEL assay (4X) and Hoechst staining (at 4X magnification). The ratio of each pair of histological markers is shown to the right of the figure. PAS = periodic acid Schiff; AB = alcian blue; H = haematoxylin; T = TUNEL; H = Hoechst.

The IGF-1 signaling cascade was also analyzed by immunoblot in colonic tissue after chronic treatment with IGF-1 (20  $\mu$ g/day/rat, i.p.) for 9 days (groups i to iv) (Fig. 4C). In experimental colitis (group ii), phosphorylation of IRS-1 at residues ser312 and tyr1179 decreased drastically. This reduction in phosphorylation was also observed at residue ser473 in AKT and at residue ser2448 in mTOR. In group ii there was also a notable decrease in the total amount of p85 protein. Nevertheless, there was no significant change in the amount of total IRS-1, AKT, and the beta subunit of IGF-1R (Fig. 4C). These data suggest that a desensitization to endogenous IGF-1 in experimental colitis is occurring. Treatment with IGF-1 (20  $\mu$ g/day/rat) appears to have a protective effect against desensitization in DSS-induced experimental colitis rats. Chronic administration of IGF-1 to healthy animals did not produce any significant effects on IGF-1 signaling cascade (Fig. 4C).

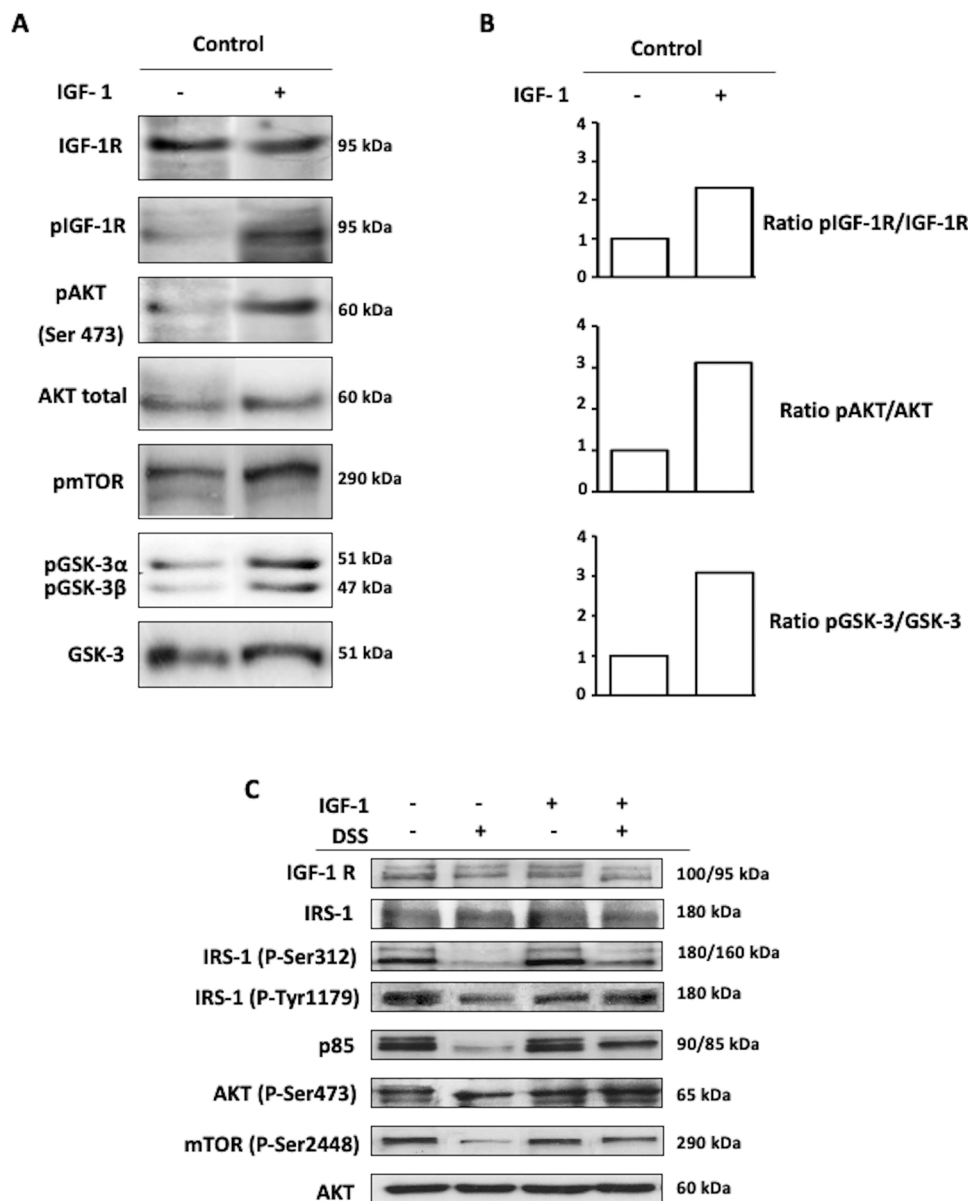
### 3.6. Effect of IGF-1 on molecular markers of colonic damage after DSS treatment

Observed alterations of the mucosal barrier in experimental colitis (group ii) were accompanied by an increase in metalloproteinase activity and in the amount of MMP-2 measured by immunoblotting in the colon (Fig. 5A). However, our data suggest that consistent treatment of experimental colitis animals with IGF-1 (group iv) was protective against increased MMP-2 levels as measured by zymography and western blot. Activation of metalloproteinases in colon epithelia of DSS-treated rats correlated with the increase of AIF-1 in the same tissue of

these animals. Normal metalloproteinase activity is maintained when experimental colitis rats are concomitantly treated with IGF-1 (Fig. 5A). We also observed a decrease in E-cadherin and  $\beta$ -catenin levels, the proteins primarily responsible for forming adherens junctions in the colon, in the experimental colitis (group ii) (Fig. 5B). Treatment with IGF-1 maintained normal levels of both proteins (Fig. 5B). In order to study the functionality of adherens junctions, we immunoprecipitated E-cadherin from colon samples and studied phosphorylation levels on its tyrosine residues as well as its association with  $\beta$ -catenin. As demonstrated in Fig. 5C, in the healthy control (group i) the majority of precipitated E-cadherin has a molecular weight of 140 kDa; although, we observed 120 kDa and 100 kDa bands, due to the fact that E-cadherin has an extracellular domain which consists of a repeated amino acid sequence of  $\sim$ 110 residues which correspond to 20 kDa cassettes that could be differentially cleaved. When we studied the phosphorylation profile of E-cadherin, we observed bands at 140 kDa and 100 kDa, corresponding to Tyr phosphorylated residues in the colon of rats from groups i, iii, and iv (Fig. 5C). However, in experimental colitis (group ii), the 140 kDa band is not present, instead a band of 120 kDa is detected, suggesting a proteolytic degradation of E-cadherin (Fig. 5C).

Moreover, the immunoprecipitation experiments reveal the interaction between E-cadherin and  $\beta$ -catenin, demonstrated by the presence of a 90 kDa band detected with an anti- $\beta$ -catenin antibody. In Fig. 5C, the 90 kDa band is detected in the groups i, iii, and iv but not in group ii, which correspond to rats with colitis. In summary, our data indicate that consistent concomitant treatment with IGF-1 (20  $\mu$ g/day/rat) was





**Fig. 4.** - IGF-1 signalling pathway in colon mucosa from rats with experimental colitis at study day 9. **A.** Study of IGF-1 signalling pathway by immunoblot after acute IGF-1 (20 µg/rat, i.p.) treatment of rat, 30 min before sacrifice. **B.** Ratio of phosphorylated/total protein obtained from the immunoblots of figure A. Representative results of that obtained from 4 to 5 rats. **C.** Analysis of IGF-1 signalling cascade by immunoblot after chronic treatment of rats with IGF-1 (20 µg/day/rat, i.p. for 8 days). Representative Immunoblots from each experimental group at study day 9. Control group = DSS/IGF-1 (-/-); Control + IGF-1 group = DSS/IGF-1 (-/+); DSS group = DSS/IGF-1 (+/-); DSS + IGF-1 group = DSS/IGF-1 (+/+).

protective against colitis-induced dysfunction of essential adherens junction proteins. Moreover, the treatment of the healthy animals with IGF-1 did not produce important changes in the levels of E-cadherin, Tyr phosphorylated E-cadherin and in its interaction with  $\beta$ -catenin (Fig. 5C).

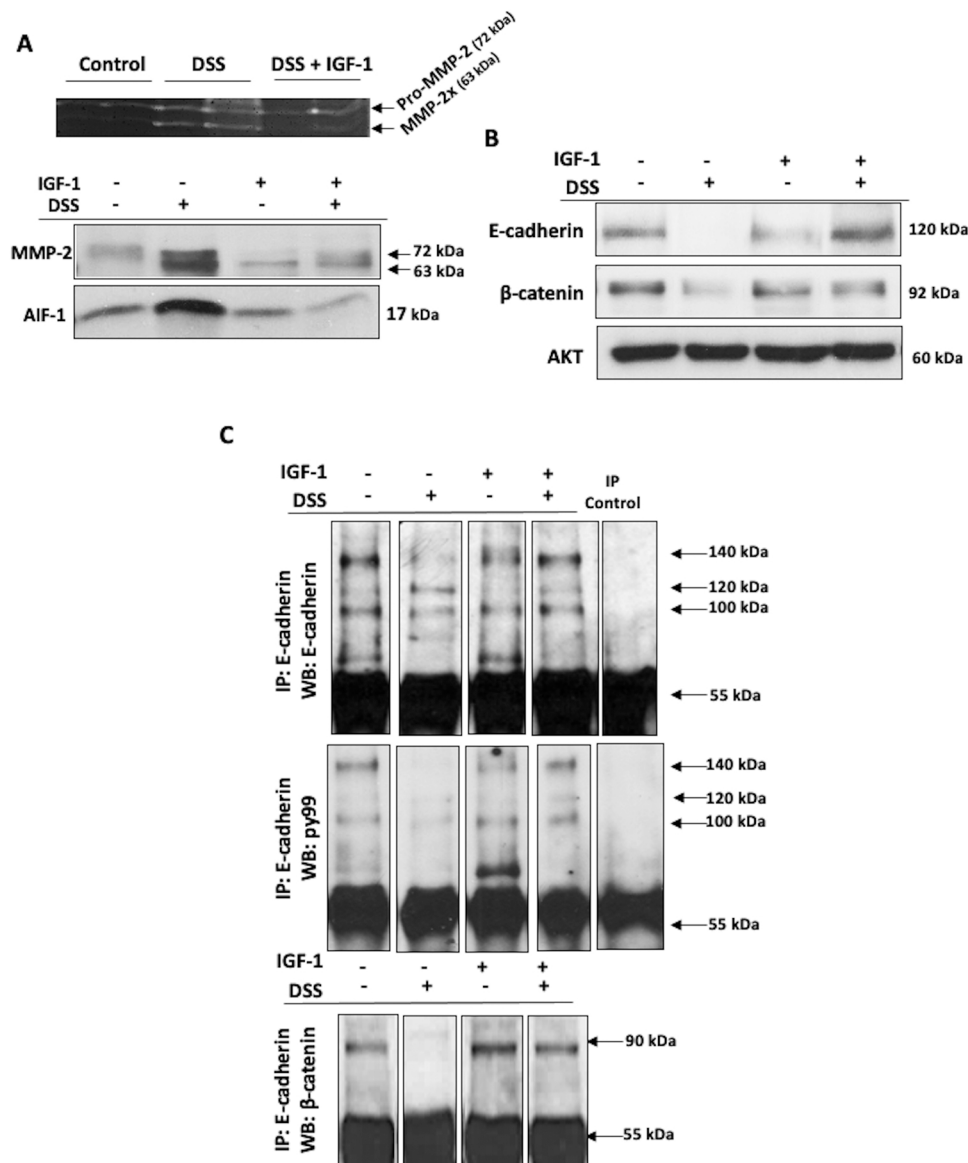
Furthermore, we evaluated the impact of weakening of the colon barrier on apoptosis and cell proliferation by analyzing biomarkers for both processes using immunoblotting. These assays revealed increased levels of Bid and of Bax/Bcl-2 heterodimers in the colons of experimental colitis rats compared to the healthy controls (Fig. 6A). Likewise, a small decrease in Bax and Bcl-2 levels was observed after treatment with IGF-1 (Fig. 6A). The presence of Bax/Bcl-2 heterodimers was demonstrated by Bax immunoprecipitation and subsequent Bcl-2 immunodetection studies and vice versa (Fig. 6B). These results correlated with the increase in caspase 8 fragments and with the degradation of procaspase 3 and PARP proteins which indicates the increase of extrinsic and intrinsic apoptotic pathways in the colons of experimental colitis rats (Fig. 6C). Chronic treatment with IGF-1 of colitis rats (group iv) was shown to protect against the formation of Bax/Bcl-2 heterodimers (Fig. 6A) as well as modestly protect against PARP degradation

(Fig. 6C). However, there were no appreciable protective effects against increased Bid levels (Fig. 6A), or against caspase -3 and -8 fragmentation (Fig. 6C). These data suggest that IGF-1 is protective against intrinsic pathway-induced apoptosis but not against extrinsic pathway-induced apoptosis. Treatment with IGF-1 of healthy animals did not lead to appreciable changes in any of the apoptotic parameters studied.

### 3.7. Effect of concomitant IGF-1-treatment on retinoblastoma-cyclin dependent kinase pathway in colon with DSS administration

Since IGF-1 is capable of activating the proliferation of colon epithelium through the retinoblastoma cascade, we decided to study this signaling pathway in experimental colitis, as well as the effects of chronic IGF-1 treatment. DSS administration produced a decrease in the phosphorylation of Rb in ser 807/811 residues without changes in total Rb levels, as well as a decrease in E2F1 levels in the colon, which resulted in a decrease in cyclin E with respect to control animals (Fig. 6D). However, we did not observe any changes in the total amount of other proteins in this cascade, such as total Rb, CDK2, cyclin D1, or CDK4 (Fig. 6D). Chronic co-treatment with IGF-1 of DSS-treated





**Fig. 5.** - Metalloproteinase, AIF-1,  $\beta$ -catenin, and E-cadherin levels in colon mucosa from experimental colitis and control groups at study day 9. The chronic treatment of rats with experimental colitis, was performed with IGF-1 at 20  $\mu$ g/day/rat, i. p. for 8 days. A. Evaluation of metalloproteinases by zymography (upper lane) and by immunoblot (middle lane). Immunoblot of AIF-1 from colon mucosa (lower lane). B. Immunoblot of E-cadherin,  $\beta$ -catenin, and AKT from colon mucosa. C. Immunoprecipitation of E-cadherin with subsequent immunoblot of E-cadherin, phosphotyrosine residues using PY99 antibody, and  $\beta$ -catenin. Representative immunoblots of the four animal study groups. IP = immunoprecipitation; WB = western blotting. Control group = DSS/IGF-1 (-/-); Control + IGF-1 group = DSS/IGF-1 (-/+); DSS group = DSS/IGF-1 (+/-); DSS + IGF-1 group = DSS/IGF-1 (+/+).

animals, maintained cyclin E levels in a normal range and, to a lesser extent, the phosphorylation levels of Rb (807/811 residues). Likewise, IGF-1 treatment of control animals did not produce a dramatic effect in Rb/E2F1 signaling pathway (Fig. 6D).

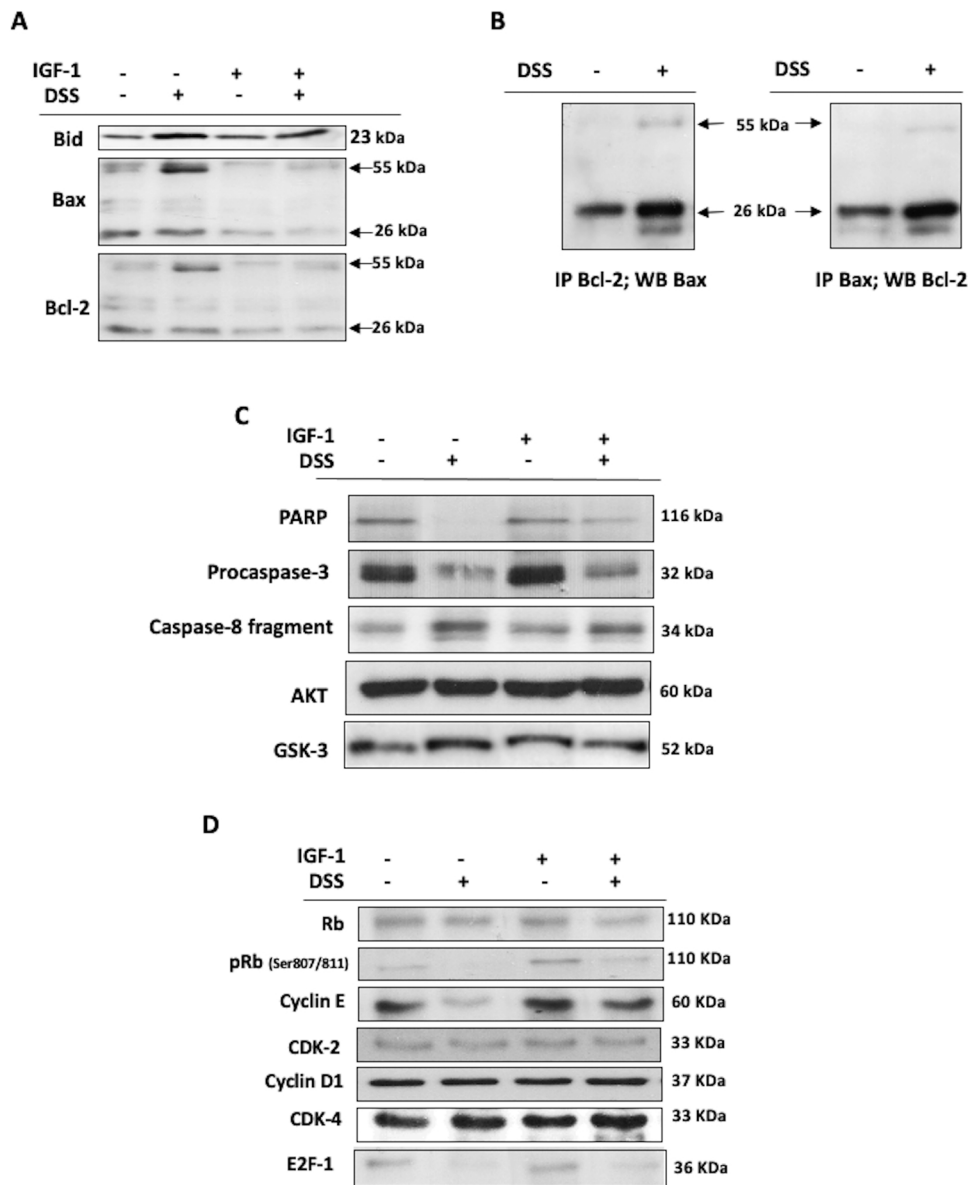
#### 4. Discussion

In 2005, the FDA approved recombinant IGF-1 (rhIGF-1) for the treatment of short stature caused by IGF-1 deficiency. Despite much evidence on the importance of the GH-IGF-1 axis in the pathogenesis of growth retardation in pediatric CD [25–27] and weight loss in adult CD [28–30], there are few ongoing clinical trials (NCT00109473, NCT00511329, NCT00764699) evaluating the clinical utility of these two hormones. Additionally, said clinical trials were carried out in the pediatric CD population, but not in adult CD patients. This prompted us to study IGF-1 serum levels in adult CD patients undergoing long-term treatment (54 weeks) with adalimumab (ADA). We further evaluated the effects of IGF-1 on IBD by using a preclinical model to research how IGF-1 administration affects animals with DSS-induced experimental colitis. Our observations in the clinical arm of our study are contrary to what was previously observed with long-term anti-TNF treatment in

pediatric CD patients [26] as we observed a decrease in circulating IGF-1 levels.

Previous studies in adult CD patients show that serum IGF-1 increased significantly following infliximab therapy during the induction phase (6 weeks of treatment). IGF-1 levels subsequently fell to baseline at the beginning of the maintenance therapy phase (16 weeks of treatment) [29]. Currently, there is no research evaluating the effect of long-term antibody therapy on IGF-1 serum levels in adult CD patients. Our study is the first to show that in adult CD patients receiving long-term ADA treatment, serum IGF-1 levels decrease. However, there were no changes in the level of serum IGF-1 in the same cohort. Circulating levels of IGF-1 are not only stimulated by pituitary GH, but also by gastrointestinal ghrelin [31], which decreases in circulation after chronic treatment with anti-TNF drugs for 53 weeks [32]. This phenomenon could explain the discrepancies observed. Therefore, it is necessary to consider the effect of ghrelin in GH secretion, as well as the sensitizing effects of TNF blocking on the GH and IGF-1 efficiency [28], especially in long-term antibody treatment of CD.

To pave the way for future studies in adult CD patients, we analyzed the effects of 9-day IGF-1 treatment in adult rats with experimental colitis. For the treatment of osteoporosis in human beings, great



**Fig. 6.** - Apoptosis and cell cycle analysis in colon mucosa from rats with experimental colitis at study day 9. Chronic treatment of rats with experimental colitis was performed with IGF-1 at 20  $\mu\text{g}/\text{day}/\text{rat}$ , i. p. for 8 days. A. Immunoblot of Bcl-2 family proteins. B. Immunoprecipitation of Bcl-2 with subsequent immunoblot of Bax and vice versa. C. Immunoblot of apoptotic markers. D. Analysis of G1 cell cycle markers by immunoblot. Representative immunoblots of the four animal study groups. Control group = DSS/IGF-1 (-/-); Control + IGF-1 group = DSS/IGF-1 (-/+); DSS group = DSS/IGF-1 (+/-); DSS + IGF-1 group = DSS/IGF-1 (+/+).

differences in the dose of IGF-1 have been reported (from 0.5 to 240  $\mu\text{g}/\text{kg}/\text{day}$ ) [33]. We have chosen an intermediate dose of 100  $\mu\text{g}/\text{kg}/\text{day}$  which correspond to 20  $\mu\text{g}/\text{rat}/\text{day}$ , taking into account an average weight of 0.2 kg per animal.

Treatment with IGF-1 was shown to be protective against decreased food consumption, weight loss and colon shrinkage in rats with intestinal inflammation. Likewise, IGF-1 treatment protected against diarrhea and DAI score decreases observed in rats with experimental colitis. Additionally, we showed a significant decrease in phosphate, glucose, albumin, and total proteins serum levels in colitis rat with respect to healthy animals, all factors indicating malnutrition. All of these metabolites maintained normal levels when colitis rats receive a treatment with IGF-1 (group iv), suggesting that this hormone protects against malnutrition in rats with colitis. However, this protective effect was not extended to alkaline phosphatase. Despite its structural analogy with insulin, previous studies indicate no relation between circulating IGF-1 levels and any form of hypoglycemia [34]. Our results were consistent with this data as IGF-1 did not induce a hypoglycemic state in any tested animals. Loss of body weight associated with experimental colitis can be explained by decreases in lean and fat body mass and a loss of bone mineral content. However, IGF-1 treatment only protected against

decreases in lean body mass. Although IGF-1 plays a role in bone formation [35] and in muscle growth [36], it has been speculated that the effect of IGF-1 on muscle growth precedes that of bone formation. Consequently, longer- longitudinal studies will be needed to assess the effects of IGF-1 on bone accretion. The statistically significant correlation among rat body weight or lean body mass with colon length, motivated us to investigate the colon as the potential primary cause of weight loss in IBD. Our histological data show that colitis in rats is due to mucosal barrier weakness, which is characterized by an imbalance in mucin production and decreased levels of the proteins constituting adherens junctions (AJs),  $\beta$ -catenin and E-cadherin. Moreover, immunoprecipitation experiments reveal the dramatic decrease in E-cadherin and  $\beta$ -catenin interaction in rats with experimental colitis. In the colons of rats with experimental colitis we observed an increase in AIF-1 as well as in the number of apoptotic cells, which could reflect an increase in luminal antigens in contact with sub-epithelial cells, activating an immune response. This process, in turn, induces metalloproteinase activity, including MMP-2. Increased activity of MMPs -1, -2, -3, and -9 in colonic mucosa of UC and CD patients [37] contributes to the maintenance and evolution of IBD.

The increased levels of Bid and of Bax/Bcl2 heterodimer formation,

characteristic of apoptosis, in experimental colitis rats allows for mitochondrial membrane permeabilization [38] and subsequent caspase 3 activation and PARP degradation.

IGF-1 co-treatment of animals with colitis protected against some of the pathological characteristics described, such as mucin imbalance and AJ protein depletion. However, the protective effects of IGF-1 treatment did not extend to apoptotic process in the colon. The experimental colitis model used in the present study is characterized by reduced cell proliferation [39] resulting from inadequate Rb phosphorylation at ser 807/811 residues together with a decrease in cyclin E and E2F1 levels. Co-treatment of experimental colitis rats with IGF-1 was only able to protect against decreases in cyclin E levels. Our results suggest that apoptosis activation and cell proliferation inhibition observed in DSS-induced colitis could result from a desensitization to endogenous IGF-1, characterized by a downregulation of the entire IGF-1 signaling cascade, from IRS-1, p85, AKT, to mTOR. Our study demonstrates potential protective effects of IGF-1 treatment on several components of the IGF-1 signaling cascade. These data imply that restoring previously decreased IGF-1 levels in CD pathology could be of clinical importance. While this potential avenue of clinical research is promising, IGF-1 treatment has not become widespread due to the hormone's implication in tumorigenesis [40]. To mitigate this risk, cell-specific modes of IGF-1 delivery are being investigated. A new generation of smart insulin growth factor-1 (SIGF-1) therapy has emerged as a promising treatment for targeted protection and repair of damaged cells. SIGF-1 is a fusion protein containing an IGF-1 domain and an annexin V domain that specifically targets phosphatidylserine, which is preferentially expressed in apoptotic cells [US20170096469A1]. Other strategies emphasizing precision treatment include intraperitoneal treatment using IGF-1 C hydrogel [41], recombinant *Lactococcus lactis* expressing IGF-1 [42], and embryonic stem cells, which promote colon epithelial integrity and regeneration via an IGF-1-dependent mechanism [43]. While there are several innovative therapies being evaluated for the treatment of CD, supplementation with IGF-1 in adult patients is not one of them. The present study establishes the scientific basis for the therapeutic potential of IGF-1 in adult CD patients.

#### Author contributions

Luis G Guijarro and Borja Hernández-Breijo: study design, Data collection, Data analysis, data interpretation, Writing the manuscript. Clinical data interpretation: all the group. All authors approved the final version of the manuscript. Luis G Guijarro, Miguel Ángel Ortega and Borja Hernández-Breijo are the guarantors of the article.

#### Conflict of interest statement

LG Guijarro: None. JL Cabriada has served as consultant for and received research funding from MSD, Abbvie, Pfizer and Kern Ph. M Chaparro has served as a speaker, or has received research or education funding from MSD, Abbvie, Hospira, Pfizer, Takeda, Janssen, Ferring, Shire Pharmaceuticals, Dr Falk Pharma, Tillotts Pharma. I Guerra has served as a speaker for Pfizer and Janssen and consultant and advisory member for Kern Pharma and MSD. M Iborra has received funding for educational activities, research projects and scientific meetings from Takeda and Abbvie. L Bujanda: none C Taxonera has served as a speaker, a consultant and advisory member for, or has received research funding from, MSD, Abbvie, Hospira, Pfizer, Takeda, Ferring, Faes Farma, Shire Pharmaceuticals, Dr Falk Pharma, Gebro Pharma. V García-Sánchez has served as a speaker, a consultant and advisory member for or has received research funding from MSD, Abbvie, Hospira, Kern Pharma, Takeda, Pfizer, Ferring, Faes Farma, Shire Pharmaceuticals, Dr Falk Pharma, Janssen, Gebro Pharma and Otsuka Pharmaceutical. I Marín-Jiménez has served as a consultant, advisory member, speaker, or has received research funding from Abbvie, Chiesi, Faes Farma, Falk-Pharma, Ferring, Gebro Pharma, Hospira, Janssen, MSD, Otsuka

Pharmaceutical, Pfizer, Shire, Takeda, Tillotts, and UCB Pharma. JP Gisbert has served as a speaker, a consultant and advisory member for, or has received research funding from, MSD, Abbvie, Hospira, Pfizer, Kern Pharma, Biogen, Takeda, Janssen, Roche, Ferring, Faes Farma, Shire Pharmaceuticals, Dr Falk Pharma, Tillotts Pharma, Chiesi, Casen Fleet, Gebro Pharma, Otsuka Pharmaceutical, Vifor Pharma. M Barreiro-de Acosta has served as a speaker, consultant and advisory member for or has received research funding from MSD, Abbvie, Janssen, Kern Pharma, Celltrion, Takeda, Gilead, Celgene, Pfizer, Sandoz, Biogen, Fresenius, Ferring, Faes Farma, Dr. Falk Pharma, Chiesi, Gebro Pharma, Adacyte and Vifor Pharma. I Vera has served as a speaker for Abbvie, Takeda and Shire MD Martín-Arranz has served as a speaker, a consultant and advisory member for MSD; Abbvie, Takeda, Janssen, Ferring, Faes Farma, Shire Pharmaceutical, Tillotts Pharma and Chiesi. B. Hernández-Breijo: None F. Mesonero: None L Sempere has served as a speaker, a consultant and advisory member for TAKEDA, ABBVIE, KERN, MSD Y TILLOTTS. Pfizer Gomollón has received fees for lectures from Janssen, Abbvie, Takeda, MSD and research grants (Group): MSD, Abbvie. Advisory Committees: None active at present. J Hinojosa Abbvie, MSD, Takeda, Janssen, Kern, Pfizer-Hospira, Otsuka, Faes, Ferring, Biogen, Shire. F Bermejo has served as a speaker, a consultant and advisory member for, or has received research funding from, MSD, Abbvie, Pfizer, Hospira, Takeda, Ferring, Faes Farma, Shire, Tillotts, Chiesi and Gebro.

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2021.112239](https://doi.org/10.1016/j.biopha.2021.112239).

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