

# The Differential Expression of the Inflammasomes in Adipose Tissue and Colon Influences the Development of Colon Cancer in a Context of Obesity by Regulating Intestinal Inflammation

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**Background:** Inflammasomes maintain tissue homeostasis and their altered regulation in the colon, and the adipose tissue (AT) leads to chronic activation of inflammatory pathways promoting colon cancer (CC) development. We aimed to analyze the potential involvement of inflammasomes in obesity-associated CC.

**Methods:** Ninety-nine volunteers [61 with obesity (OB) and 38 normoponderal (NP)] further subclassified according to the approved protocol for the diagnosis of CC (58 without CC and 41 with CC) were included in the case-control study.

**Results:** CC ( $P<0.01$ ) and obesity ( $P<0.01$ ) were accompanied by increased mRNA levels of *NLRP3*, *NLRP6*, *ASC*, *IL1B* and *NOD2* in VAT. Contrarily, patients with CC exhibited a downregulation of *NLRP6* and *IL18* in their colon. Additionally, we revealed that the decreased *Nlrp1* ( $P<0.05$ ), *Nlrp3* ( $P<0.01$ ) and *Nlrp6* ( $P<0.01$ ) mRNA levels in the colon from obese rats significantly increase ( $P<0.05$ ) after caloric restriction. Adipocyte-conditioned media obtained from subjects with obesity reduced ( $P<0.01$ ) the mRNA of *NLRP3* as well as molecules involved in maintaining the intestinal integrity (*MUC2*, *CLDN1* and *TJPI*) and the anti-inflammatory factors *FGF21*, *KLF4*, and *IL33* and in HT-29 cells. We also found that the knockdown of *NLRP6* in HT-29 cells significantly upregulated ( $P<0.05$ ) the mRNA of *NLRP1* and *NLRP3* and inhibited ( $P<0.05$ ) the expression levels of *MUC2*. Finally, we showed that the incubation of HT-29 with *Akkermansia muciniphila* influence ( $P<0.05$ ) the inflammasome expression profile as well as intestinal integrity-related genes and aberrant inflammation.

**Conclusions:** These findings provide evidence that the downregulated levels of *NLRP6* and *IL18* in the colon from patients with CC may be responsible for a reduced intestinal-barrier integrity, triggering local inflammation, which in turn acts on the dysfunctional AT in obesity, increasing the expression of different inflammasome components and flaring up a vicious cycle of uncontrollable inflammatory cascades that favours a pro-tumorigenic microenvironment.

**Keywords:** inflammasome, NLRP, inflammation, colon cancer, obesity, adipose tissue, *Akkermansia muciniphila*

## Introduction

Colon cancer (CC) is the third most common cancer worldwide and the second in terms of mortality, with more than one-half of all cases and deaths attributable to modifiable risk factors including an unhealthy diet, excess body weight and

physical inactivity.<sup>1</sup> Despite a huge body of epidemiological knowledge supporting the link between excess adiposity and cancer, cellular and molecular mechanisms insights explaining this association are scarce.<sup>2</sup> Different hallmarks of colon carcinogenesis are causally linked with obesity, being of special relevance to chronic and uncontrolled inflammation as well as the altered intestinal permeability and gut dysbiosis.<sup>3</sup> Specifically, the innate immune response required to impair early carcinogenesis is also dysregulated in the obese state.<sup>4</sup> In this sense, we previously demonstrated that the inflammasome, a cytosolic innate immune multi-protein complex, is involved in a diversity of metabolic diseases including obesity, type 2 diabetes (T2D) and nonalcoholic fatty liver disease (NAFLD) by regulating inflammation and extracellular matrix remodelling.<sup>5</sup> In the gastrointestinal tract, the inflammasomes are crucially implicated in maintaining the homeostasis of gut epithelial due to their capacity to recognize diverse pathogenic insults.<sup>6,7</sup> Although the main function of inflammasomes is to restrict damage, when inadequately controlled, intestinal inflammasomes promote a constant activation of inflammatory pathways prompting immune pathology, tissue damage and cancer development.<sup>7,8</sup>

The impact of the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRPs) family on tumorigenesis has been revealed over the last decade.<sup>9,10</sup> Inflammasomes exhibit complex and diverse roles in all stages of colon carcinogenesis, playing either pro-tumoral (perpetuating inflammation) or anti-tumoral (maintaining intestinal barrier integrity) functions and, therefore, conflicting results regarding CC development have been reported.<sup>10</sup> These apparently differing functions have been suggested to be context-dependent and tissue-specific.<sup>11,12</sup>

The colon is the main organ for host-microbiome crosstalk and a disturbance of the gut microbiota not only influences the development and progression of CC, but also promotes the progression of other major conditions including obesity, metabolic syndrome and T2D.<sup>13,14</sup> In this context, the inflammasomes tightly regulate intestinal microbiota and mice deficient in the different inflammasome components *Nlrp3*, *Nlrp6* or *Asc* exhibit intestinal dysbiosis promoting gut inflammation, metabolic syndrome and CC development.<sup>15–17</sup> Of note, the supplementation with *Akkermansia muciniphila* improved different metabolic parameters in both mice<sup>18</sup> and humans.<sup>19</sup>

Obesity-associated sustained inflammation is a recognized risk factor for colon carcinogenesis. Whether excess adiposity has an impact on CC via a direct mechanism is widely twilight and the function of the inflammasomes in cancer remains controversial. Therefore, we hypothesized that obesity and colon cancer have an impact on the expression of the main components of the inflammasomes as well as their different effectors in the colon and the visceral AT (VAT) from patients with obesity and CC. The potential changes in the expression of the inflammasome components after weight loss were evaluated in the colon of a diet-induced obese rat model. The regulatory role of *A. muciniphila* in the expression of the inflammasome components and key inflammation- and intestinal integrity-associated factors in HT-29 cells was evaluated.

## Materials and Methods

### Patient Selection

Ninety-nine subjects [38 normoponderal (NP) and 61 with obesity (OB)] were included in the case-control study. Patients were also subclassified according to the approved protocol for the diagnosis of colon cancer (58 without CC and 41 with CC) and were included in the case-control study ([Supplemental Table 1](#)). VAT samples were obtained from volunteers undergoing Nissen fundoplication (for normoponderal volunteers), Roux-en-Y gastric bypass (RYGB) (for morbid obesity treatment) and curative resection for primary colon carcinoma (for CC treatment). The experimental design was approved, from an ethical and scientific standpoint, by the Clínica Universidad de Navarra's Ethics Committee responsible for research (2018.094). The written informed consent of all participants was obtained. This study was conducted in accordance with the Declaration of Helsinki. A cohort of human colon total RNA samples (5 from normal colon tissue and 9 from CC tissue) were purchased from OriGene (Rockville, MD, USA). All the clinical information associated with each of these samples is available from the OriGene website.

### Experimental Models of Diet-Induced Obesity

#### Surgical Procedures

Eight-week-old male Wistar rats (n=40) were fed ad libitum either a normal chow diet (ND) (n=20) (Diet 2014S, Harlan Laboratories Inc., Barcelona, Spain) or a high-fat

diet (HFD) (n=20) (Diet F3282, Bio-Serv, Frenchtown, NJ, USA) for 16 weeks as previously described.<sup>20</sup> Body weight and food intake were weekly registered. Diet-induced obesity (DIO) rats were randomized to be submitted to sham surgery (n=10), sleeve gastrectomy (SG) (n=10) or pair-fed (n=10) to the amount of food eaten by animals undergoing SG in order to differentiate possible effects of the decrease in food intake after surgery. Anesthesia and surgical procedures were performed as previously described.<sup>20</sup> After interventions, animals were fed a ND.

### Caloric Restriction

Ten-week-old male Wistar rats (n=40) (breeding house of the University of Navarra) were used in the study. Experimental animals were housed in the same conditions as described above. Rats were randomized in four groups (n=10) to analyze the effect of the diet [ND (Diet 2014S) or HFD (Diet F3282)] and the effect of a 25% of caloric restriction (ND-R and HFD-R, respectively).

After all the interventions, rats were sacrificed by decapitation, and the colon was dissected out and immediately stored at -80°C until next experiments. All experimental protocols were approved by the Ethics Committee for Animal Experimentation of the University of Navarra (049/10) and conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU).

### Blood Processing and Assays

Blood samples were collected by venipuncture after an overnight fast and centrifuged at 12,000 g for 15 min at 4°C to obtain serum and plasma. Glucose and triglycerides, as well as carcinoembryonic antigen (CEA) and C-reactive protein (CRP), were determined as previously described.<sup>21,22</sup> An automated cell counter (Beckman Coulter, Inc., Fullerton, CA) was used to determine the white blood cell (WBC) count. Lipopolysaccharide-binding protein (LBP) (R & D Systems, Minneapolis, MN, USA), lactoferrin (R & D Systems), calgranulin A (S100A8) (R & D Systems) and IL-18 (R & D Systems) were measured by ELISA according to the manufacturer's instructions. The limulus amoebocyte lysate assay (Lonza, Basel, Switzerland) was used to detect and quantify circulating levels of bacterial lipopolysaccharide (LPS).

### RNA Isolation and Real-Time PCR

Total RNA isolation and purification were performed by homogenization with an Ultra-Turrax® T25 basic (IKA®

Werke GmbH, Staufen, Germany) using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) for colon samples and HT-29 cultured cells and QIAzol® Reagent (Qiagen, Hilden, Germany) for AT and then treated with DNase I (RNase Free DNase set, Qiagen). Transcript levels were determined by Real-Time PCR (7300 Real Time PCR System, Applied Biosystems, Foster City, CA, USA) as previously reported.<sup>21</sup> Primers and probes ([Supplemental Table 2](#)) were designed using the software Primer Express 2.0 (Applied Biosystems).

### Western-Blot Studies

VAT was homogenized, and protein content was measured as previously described.<sup>23</sup> Protein samples (20 µg) were separated by SDS-PAGE (Criterion™ TGX™ Precast Gels, Bio-Rad Laboratories, Inc., Hercules, CA, USA) under denaturing conditions and transferred to a nitrocellulose membrane (Bio-Rad) for immunoblotting. Blots were incubated overnight at 4°C with rabbit monoclonal anti-NLRP-3 antibody (#13158, Cell Signaling Technology, Danvers, MA, USA) diluted 1:2500. The antigen-antibody complexes were visualized using HRP-conjugated secondary antibodies (1:5000) and the enhanced chemiluminescence ECL Plus detection system (Amersham Biosciences, Buckinghamshire, UK). The intensity of the bands was determined using the ChemiDoc™ MP imaging system and the Image Lab 4.0.1 software (Bio-Rad) and normalized to total protein values.

### Cell Cultures

Human stromovascular fraction cells were isolated from VAT from patients with obesity and were grown to confluence in six-well plates and differentiated into mature adipocytes, as previously described.<sup>23</sup> The adipocyte conditioned media (ACM) were prepared by collecting the supernatant from differentiated adipocytes. After that, the supernatant was centrifuged and diluted (20% and 40%) in adipocyte medium. Two colorectal adenocarcinoma cell lines, HT-29 (HTB-38™) and Caco-2 (HTB-37™) were obtained from the ATCC® (Middlesex, UK) and cultured following manufacturer's instructions. Caco-2 cell line was a generous gift from Dr. Amaya Azqueta of the University of Navarra. HT-29 cells were serum-starved for 2 h and then treated with increasing concentrations of LPS (10, 100 and 1000 ng/mL) (Merck), TNF-α (1, 10 and 100 ng/mL) (Merck), IL-4 (1, 10 and 100 ng/mL) (R&D systems), IL-13 (1, 10 and 100 ng/mL) (R&D systems),

leptin (1, 10 and 100 ng/mL) (Merck), insulin (10, 100 and 1000 ng/mL) (Merck),  $\text{CoCl}_2$  (100 and 200 nmol/L) (Merck), palmitic acid (100 and 200 nmol/L) (Merck) and ACM (20% and 40%). Caco-2 cells were stimulated with ACM (20% and 40%).

## HT-29 Transfection with siNLRP6

HT-29 cells were serum-starved for 2 h, and then two pairs of small interfering RNAs (siRNAs) for blocking the expression of *NLRP6* (s46905 and s46907, Ambion, ThermoFisher Scientific, Waltham, MA, USA) were annealed and transfected into HT-29 cells (100 pmol/L siRNA/ $2 \times 10^5$  cells/well) using 40 nmol of Lipofectamine 2000 (Invitrogen, ThermoFisher). A scrambled siRNA was used as a negative control. Knockdown effectiveness was determined after 24 h by real-time PCR. The treatment with the *NLRP6*-s46905 resulted in an average knockdown of 49%, whereas no decreased expression was found after the transfection with the si*NLRP6*-s46907 ([Supplemental Figure 1](#)). Thus, the *NLRP6*-siRNA s46905 was selected for *NLRP6* knocking-down studies. HT-29 cells were also cultured in the presence of LPS (1000 ng/mL) for 24 h.

## Akkermansia muciniphila Cell Culture and HT-29 Treatment

*A. muciniphila* (ATCC<sup>®</sup> BAA-835<sup>TM</sup>) was cultured aseptically and anaerobically in 6 mL tubes of brain heart infusion broth (Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C for 7 days. Cultures were washed and concentrated in anaerobic phosphate buffered saline (PBS) (Merck). Additionally, an identical quantity of *A. muciniphila* was heat-inactivated for 30 minutes at 70°C. The bacteria-conditioned medium (BCM) was obtained by collecting the supernatant. The BCM was centrifuged and diluted at 20% and 40% in McCoy's 5A medium.

In the infection step, HT-29 cells were serum-starved for 2 h and incubated with alive and pasteurized *A. muciniphila* at a multiplicity of infection (MOI) of 100 as well as with the BCM (20% and 40%) for 4 hours. The brain heart infusion medium (diluted at 20% and 40% in McCoy's 5A medium) was used as a control medium. The HT-29 media was collected after treatment with different stimuli, centrifuged at 200 g for 10 min and stored at -80°C. In order to assess the concentrations of mucin-2 in the media, a commercially available ELISA kit

(MyBioSource, San Diego, CA, USA) was used according to the manufacturer's instructions.

## Statistical Analysis

Differences between groups were evaluated by Mann-Whitney *U*-test, two-way ANOVA and one-way ANOVA followed by Dunnett's or Tukey's post hoc tests as appropriate. The analysis of covariance (ANCOVA) was used to analyze differences between groups adjusted for age. Pearson's correlation coefficients (*r*) were used to evaluate the association between variables. The calculations were performed using the SPSS/Windows version 15.0 statistical package (SPSS, Chicago, IL), and the figures were created with GraphPad Prism version 8.3 (GraphPad Software, Inc., San Diego, CA). A *P* value <0.05 was considered statistically significant.

## Results

### VAT- and Colon-Specific Expression of the Inflammasome Components and Its Mediators in Obesity and CC

Clinical characteristics of the cohort are shown in [Table 1](#). All markers of adiposity were increased ( $P < 0.001$ ) in patients with obesity compared to the normoponderal volunteers. Both obesity ( $P < 0.001$ ) and CC ( $P < 0.001$ ) were associated with increased levels of CRP. Although patients with CC showed a higher number of leucocytes ( $P < 0.05$ ) compared to those without CC, no differences in global WBC regarding obesity were observed.

First, we analyzed whether both obesity and CC influence the expression of the inflammasome pathway in VAT given its relevance in the development of obesity-associated CC. Although no changes were found regarding the expression of *NLRP1*, significant differences in mRNA levels of *NLRP3*, *NLRP6* and *ASC* were observed, being significantly increased due to CC ( $P < 0.05$ ) and obesity ( $P < 0.05$ ) ([Figure 1A-D](#)). We also confirmed increased ( $P < 0.05$ ) protein expression levels of NLRP-3 in the VAT from patients with obesity ([Supplemental Figure 2](#)). Importantly, patients with CC showed an upregulation ( $P < 0.05$ ) of *IL1B* and *IL18*, the main inflammasome mediators, with the expression of *IL1B* being also increased ( $P < 0.05$ ) in patients with obesity ([Figure 1E and F](#)). Since NLRPs directly interact with NOD2, we also analyzed its mRNA levels, which were higher ( $P < 0.05$ ) in patients with obesity and CC ([Figure 1G](#)). Noteworthy, mRNA levels of *ADIPOQ*, an anti-inflammatory factor with proven

**Table 1** Anthropometric and Biochemical Characteristics of Subjects Included in the Study

|                                      | Lean        |             | Obese                                     |                                                           | P Obesity        | P Cancer         | P O x C          |
|--------------------------------------|-------------|-------------|-------------------------------------------|-----------------------------------------------------------|------------------|------------------|------------------|
|                                      | Non-CC      | CC          | Non-CC                                    | CC                                                        |                  |                  |                  |
| n (male, female)                     | 22 (9, 13)  | 16 (9, 7)   | 36 (16, 20)                               | 25 (16, 9)                                                | -                | -                | -                |
| Age (years)                          | 45 ± 6      | 60 ± 2      | 49 ± 2                                    | 64 ± 3                                                    | 0.238            | <b>&lt;0.001</b> | 0.934            |
| Body weight (kg)                     | 59.6 ± 2.8  | 64.5 ± 2.1  | 108.4 ± 3.2 <sup>**</sup> , <sup>§§</sup> | 80.3 ± 2.9 <sup>**</sup> , <sup>§§</sup> , <sup>††</sup>  | <b>&lt;0.001</b> | <b>0.003</b>     | <b>&lt;0.001</b> |
| Body mass index (kg/m <sup>2</sup> ) | 20.8 ± 0.5  | 21.8 ± 0.4  | 38.2 ± 1.1 <sup>**</sup> , <sup>§§</sup>  | 31.4 ± 0.7 <sup>**</sup> , <sup>§§</sup> , <sup>††</sup>  | <b>&lt;0.001</b> | <b>&lt;0.001</b> | <b>&lt;0.001</b> |
| Estimated BF %                       | 22.4 ± 2.2  | 28.2 ± 1.6  | 44.4 ± 1.4 <sup>**</sup> , <sup>§§</sup>  | 35.3 ± 1.7 <sup>**</sup> , <sup>§§</sup> , <sup>††</sup>  | <b>&lt;0.001</b> | <b>0.022</b>     | <b>&lt;0.001</b> |
| Fasting glucose (mg/dL)              | 88 ± 5      | 130 ± 4     | 125 ± 9                                   | 127 ± 8                                                   | 0.258            | 0.231            | 0.086            |
| Triglycerides (mg/dL)                | 71 ± 11     | 94 ± 2      | 133 ± 11                                  | 182 ± 19                                                  | <b>0.009</b>     | 0.369            | 0.598            |
| C-reactive protein (mg/L)            | 0.16 ± 0.06 | 1.02 ± 0.65 | 0.81 ± 0.22                               | 9.16 ± 0.67 <sup>**</sup> , <sup>§§</sup> , <sup>††</sup> | <b>&lt;0.001</b> | <b>&lt;0.001</b> | <b>&lt;0.001</b> |
| CEA (ng/mL)                          | 1.58 ± 0.32 | 2.55 ± 0.44 | 1.68 ± 0.28                               | 8.41 ± 2.60                                               | 0.267            | <b>0.021</b>     | 0.401            |
| Leucocyte (x10 <sup>9</sup> /L)      | 6.36 ± 0.51 | 6.82 ± 1.03 | 7.02 ± 0.44                               | 7.72 ± 0.81                                               | 0.568            | 0.473            | 0.541            |

**Notes:** Data are mean ± SEM. BF, body fat; CC, colon cancer; CEA, carcinoembryonic antigen. Statistical differences were analyzed by two-way ANCOVA and one-way ANOVA followed Tukey's post hoc tests in case of interaction between variables. <sup>\*\*</sup>*P*<0.01 vs Lean non-CC, <sup>§§</sup>*P*<0.01 vs Lean CC, <sup>††</sup>*P*<0.01 vs Obese non-CC. Bold values denote statistically significant *P* values.

decreased levels in obesity, were decreased in VAT from patients with obesity (*P*<0.01) and CC (*P*<0.05) (Figure 1H) and also negatively associated with BMI (*r*=−0.32; *P*=0.011). Gene expression levels of all inflammatory components were significantly associated between them (Supplemental Table 3). As major mediators of the innate immunity, *NLRP3* and *NOD2* were positively correlated with the percentage of monocytes (*P*<0.05) and negatively with the percentage of eosinophils (*P*<0.01). In addition, *NLRP3* (*r*=0.44; *P*=0.012) and *NOD2* (*r*=0.48; *P*=0.005) expression levels were positively correlated with the circulating concentrations of CRP.

In light of the highly tissue-dependent expression of the inflammasomes, we analyzed the expression of the inflammasome components and their effectors in colon samples from patients with and without CC. Although we found a tendency towards an increased colonic gene expression of *NLRP1*, *NLRP3* and *IL1B* in patients with CC, differences did not reach statistical significance. Importantly, the gene expression levels of *NLRP6* and its mediator *IL18* were downregulated (*P*<0.05) in the colon from patients with CC (Figure 2A). The results also showed reduced (*P*<0.01) *MUC2* together with increased *CLND1* gene expression levels in the colon from patients with CC compared with normal tissue colon samples (Figure 2B).

We further investigated the circulating levels of biomarkers of obesity and colon damage. In this line, subjects with obesity showed higher serum levels of S100A8 (*P*<0.001), lactoferrin (*P*<0.05), IL-18 (*P*<0.05) and the

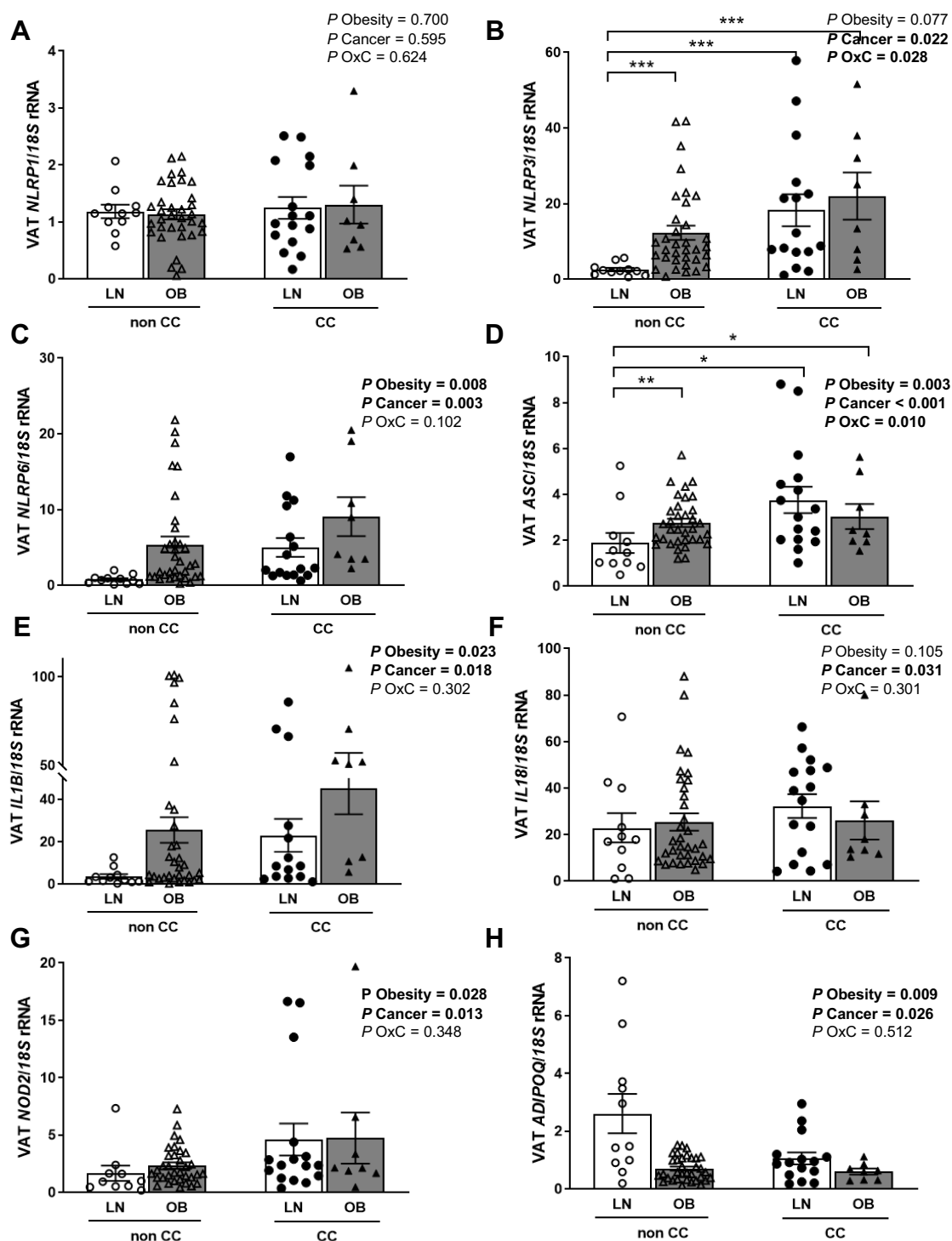
marker of LPS exposure LBP (*P*<0.001). However, no differences were found due to CC (Figure 3).

### Impact of Weight Loss Achieved by Surgery and Caloric Restriction on Colonic Expression of the Inflammasome Components in a DIO Rat Model

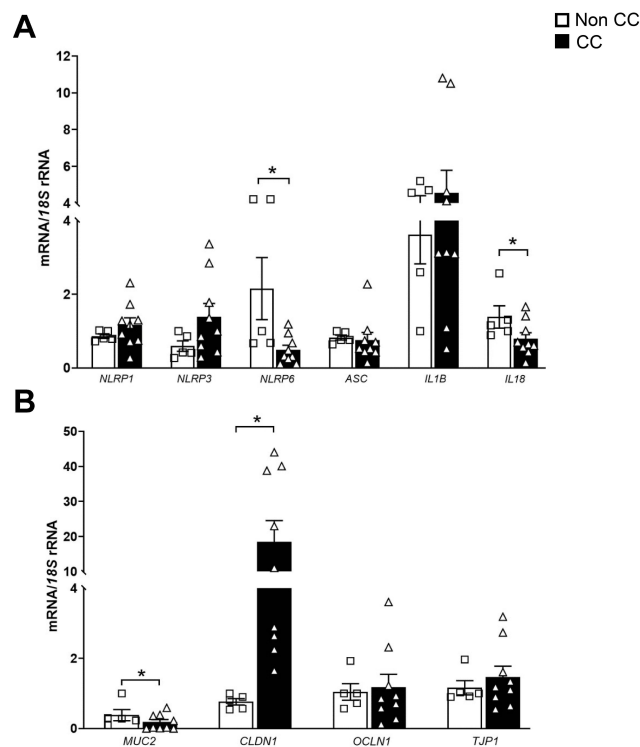
To gain further insight into the effect of obesity and weight loss on the regulation of the inflammasomes in the colon, we analyzed their expression levels in a DIO rat model submitted to SG and caloric restriction. Results showed decreased *Nlrp1* (*P*<0.05), *Nlrp3* (*P*<0.01) and *Nlrp6* (*P*<0.01) mRNA levels in the colon from obese rats in both animal models (Figure 4A-C). Although an increase in the expression levels of the components of the inflammasome after surgery in rats under a ND was detected, differences were not statistically significant (Figure 4A-C). However, an increase (*P*<0.05) in the colonic mRNA levels of *Nlrp1* after caloric restriction under both a ND and a HFD was observed together with an upregulation of *Nlrp6* in rats under a restricted HFD (Figure 4C).

### Effects of Inflammation-Related Factors and Hypoxia in the mRNA Levels of Inflammasome Components in Colon Cancer Cells

Since NLRPs recognize different inflammation-inducing stimuli, we further analyzed whether well-known inflammation-related markers that are increased in obesity influence



**Figure 1** The increased gene expression levels of different components of the inflammasome in visceral adipose tissue (VAT) due to both, obesity and colon cancer (CC) suggest that obesity-associated VAT inflammation create a microenvironment favourable for CC development. Bar graphs show the mRNA levels of (A) *NLRP1*, (B) *NLRP3*, (C) *NLRP6*, (D) *ASC*, (E) *IL1B* and (F) *IL18* (G) *NOD2* and (H) *ADIPOQ* in VAT from lean subjects (LN) and volunteers with obesity (OB) classified according to the presence or absence of CC. Bars represent the mean  $\pm$  SEM. Differences between groups were analyzed by two-way ANOVA or one-way ANOVA followed by Tukey's tests as appropriate. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . (LN non CC:  $n = 11$ ; OB non CC:  $n = 34$ ; LN CC:  $n = 16$ ; OB CC:  $n = 8$ ). Bold values denote statistically significant  $P$  values. **Abbreviations:** *ADIPOQ*, adiponectin; *ASC*, apoptosis-associated speck-like protein containing a CARD; *IL*, interleukin; *MUC2*, mucin 2; *NLRP*, nucleotide-binding oligomerization domain, leucine rich repeat and pyrin; *NOD2*, nucleotide binding oligomerization domain containing 2.



**Figure 2** Impact of colon cancer (CC) on colonic inflammasome- and intestinal integrity-associated gene expression levels. Downregulation of gene expression levels of *NLRP6*, *IL18* and *MUC2* in the colon from patients with CC. mRNA levels of (A) *NLRP1*, *NLRP3*, *NLRP6*, *ASC*, *IL1B* and *IL18* and (B) *MUC2*, *CLDN1*, *OCLN1* and *TJP1* in the colon from patients with and without colon cancer. Bars represent the mean  $\pm$  SEM. Differences between groups were analyzed by unpaired two-tailed Student's *t* tests. \* $P < 0.05$ . (non CC:  $n = 5$ ; CC:  $n = 9$ ).

**Abbreviations:** ASC, apoptosis-associated speck-like protein containing a CARD; *CLDN1*, claudin 1; IL, interleukin; *OCLN1*, occludin; *NLRP*, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin; *NOD2*, nucleotide-binding oligomerization domain containing 2; *TJP1*, tight junction protein 1.

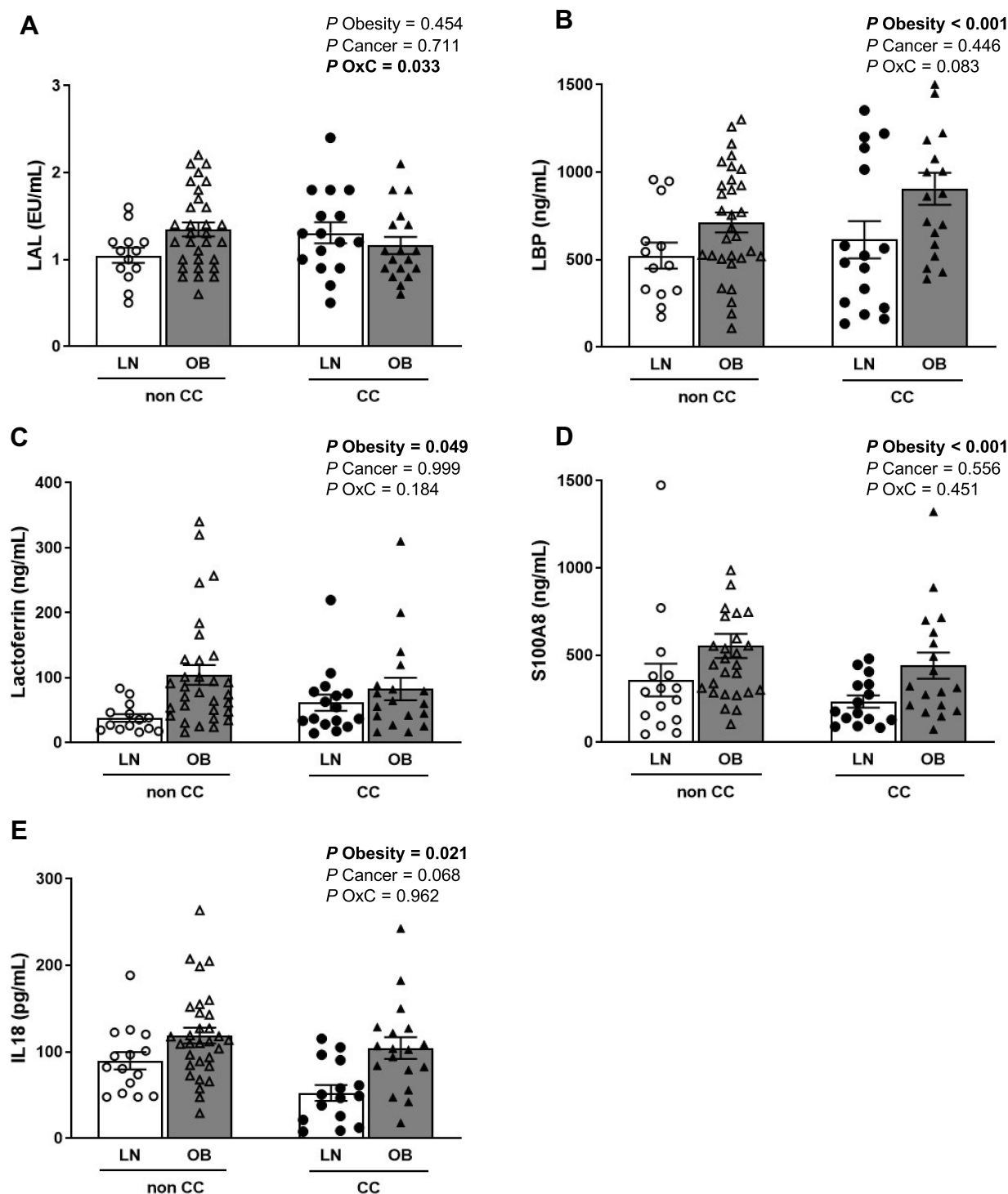
the expression of the inflammasomes in HT-29 cells. LPS and TNF- $\alpha$  treatments induced opposite expression profiles of *NLRP3* being increased ( $P < 0.05$ ) and decreased ( $P < 0.05$ ), respectively (Supplemental Figure 3A and B). Moreover, LPS enhanced ( $P < 0.05$ ) mRNA levels of *NLRP6* and TNF- $\alpha$  upregulated the expression of *NLRP1* (Supplemental Figure 3A and B). No differences on the expression of *NLRPs* in HT-29 cells after the treatment with the anti-inflammatory cytokine IL-13 were observed, but the expression of *NLRP6* decreased after IL-4 stimulation (Supplemental Figure 3C and D). *NLRP1* showed a robust upregulation ( $P < 0.001$ ) under hypoxic conditions mimicked by the divalent transition-metal ion cobalt in contrast with the downregulation observed for *NLRP3* and *NLRP6* (Supplemental Figure 3E). Whereas palmitic acid and leptin did not affect the expression of none of the components of the inflammasome, insulin promoted a decrease ( $P < 0.05$ ) of *NLRP3* (Supplemental Figure 3F-H).

## ACM from Patients with Obesity Influences the Expression Levels of *NLRP3* as Well as Inflammation- and Intestinal-Related Factors in HT-29 and Caco-2 Cells

A dynamic interaction between adipocytes and tumor cells in an obesity context has been proposed. To address this question, we first examined how the ACM from subjects with obesity affects the expression of *NLRPs* and intestinal barrier integrity markers in HT-29 cells. Interestingly, we found a downregulation ( $P < 0.01$ ) in the mRNA of *NLRP3* (Figure 5A). No differences were observed in other inflammasome components neither *IL1B* nor *IL18*. To gain further insight on the potential effects of ACM in tumor cells, we analyzed changes in intestinal integrity-related genes, finding a significant reduction of *MUC2* ( $P < 0.01$ ), *CLDN1* ( $P < 0.05$ ) and *TJP1* ( $P < 0.05$ ) (Figure 5B). Metals constitute vital nutrients for bacterial growth. Importantly, the expression of antimicrobial proteins that chelate iron, zinc or manganese including *TNFAIP9* ( $P < 0.01$ ), *NGAL* ( $P < 0.05$ ) and *S100A9* ( $P < 0.01$ ) were increased after the ACM treatment (Figure 5C). The expression of the widely accepted inflammatory gene *SPPI* ( $P < 0.05$ ) was also increased after the stimulation with the ACM (Figure 5C). No changes were found in *TGFB* and *CCL2* expression levels after exposure to adipocyte-derived factors. Next, we analyzed the effect of ACM from subjects with obesity on the mRNA levels of anti-inflammatory factors, finding a downregulation of *IL33* ( $P < 0.01$ ), *FGF21* ( $P < 0.01$ ) and *KLF4* ( $P < 0.05$ ) (Figure 5D). Changes in the mRNA levels of inflammasome components after ACM treatment were confirmed in Caco-2 cells, another common cell line used to reproduce the features of the tumoral bowel epithelium. We also found a downregulation ( $P < 0.01$ ) in the mRNA of *NLRP3* (Supplemental Figure 4). Interestingly, the expressions of *NLRP6* and *MUC2* were not detected. After the ACM treatment, an upregulation ( $P < 0.05$ ) of *IL1B* and *CLDN1* was also observed.

## *NLRP6* Silencing Results in Increased Expression of *NLRP1* and *NLRP3* in HT-29 Cells

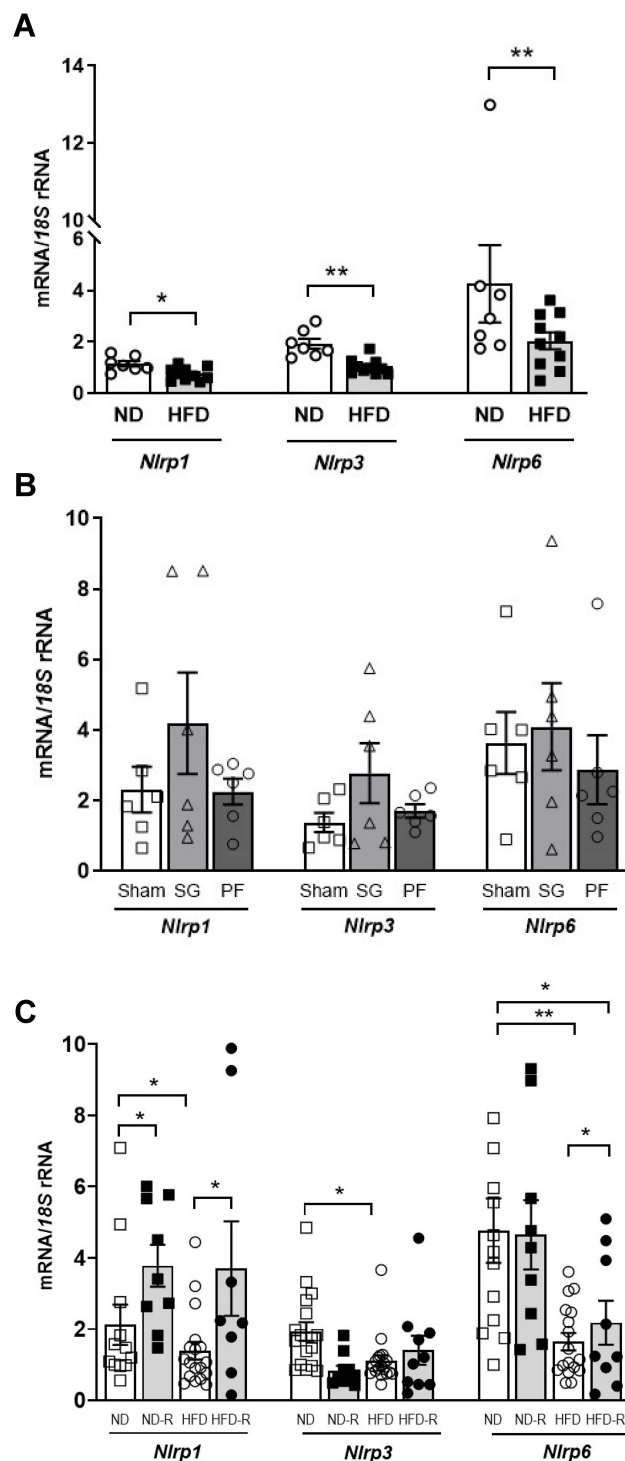
On the basis that *NLRP6* is downregulated in the colon from patients with CC, and given its relevance in maintaining intestinal homeostasis, we reduced its constitutive expression in HT-29 cells using a specific siRNA. As shown in



**Figure 3** Circulating concentrations of factors affecting intestinal barrier integrity in obesity and colon cancer (CC). Circulating concentrations of (A) limulus amebocyte lysate (LAL), (B) LPS-binding protein (LBP), (C) lactoferrin, (D) calgranulin A and (E) interleukin (IL)-18 of lean (LN) and obese (OB) volunteers classified according the presence or absence of CC. Differences between groups were analyzed by two-way ANOVA. Bold values denote statistically significant  $P$  values. LN non CC:  $n=13-15$ ; OB non CC:  $n=31$ ; LN CC:  $n=16$ ; OB CC:  $n=18$ .

Figure 6, siNLRP6 significantly increased ( $P<0.05$ ) the expression of NLRP1 and NLRP3 compared to the control group, whereas the stimulation with LPS upregulated the

expression of NLRP3 and IL1B. No differences were observed regarding the expression levels of IL18. Importantly, the inhibition of NLRP6 significantly reduced



**Figure 4** Impact of weight loss achieved by bariatric surgery and caloric restriction on the inflammasome components. **(A)** Gene expression levels of *Nlrp1*, *Nlrp3* and *Nlrp6* in the colon from rats fed a normal diet (ND) or a high-fat diet (HFD). **(B)** mRNA levels of *Nlrp1*, *Nlrp3* and *Nlrp6* in the colon from rats submitted to sleeve gastrectomy (SG) surgery and fed a ND. **(C)** Gene expression levels of *Nlrp1*, *Nlrp3* and *Nlrp6* in the colon from rats fed a normal diet (ND) or a high-fat diet (HFD) and a 25% of caloric restriction (ND-R and HFD-R). Bars represent the mean  $\pm$  SEM. Unpaired Student's *t*-test was used to observe differences between ND vs HFD control groups while the effect of the SG and diet was evaluated by a two-way ANOVA followed by Tukey's tests. \* $P < 0.05$  and \*\* $P < 0.01$ .

**Abbreviation:** *Nlrp*, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin.

( $P < 0.05$ ) the expression levels of *MUC2* whereas no changes in other intestinal-integrity markers were observed.

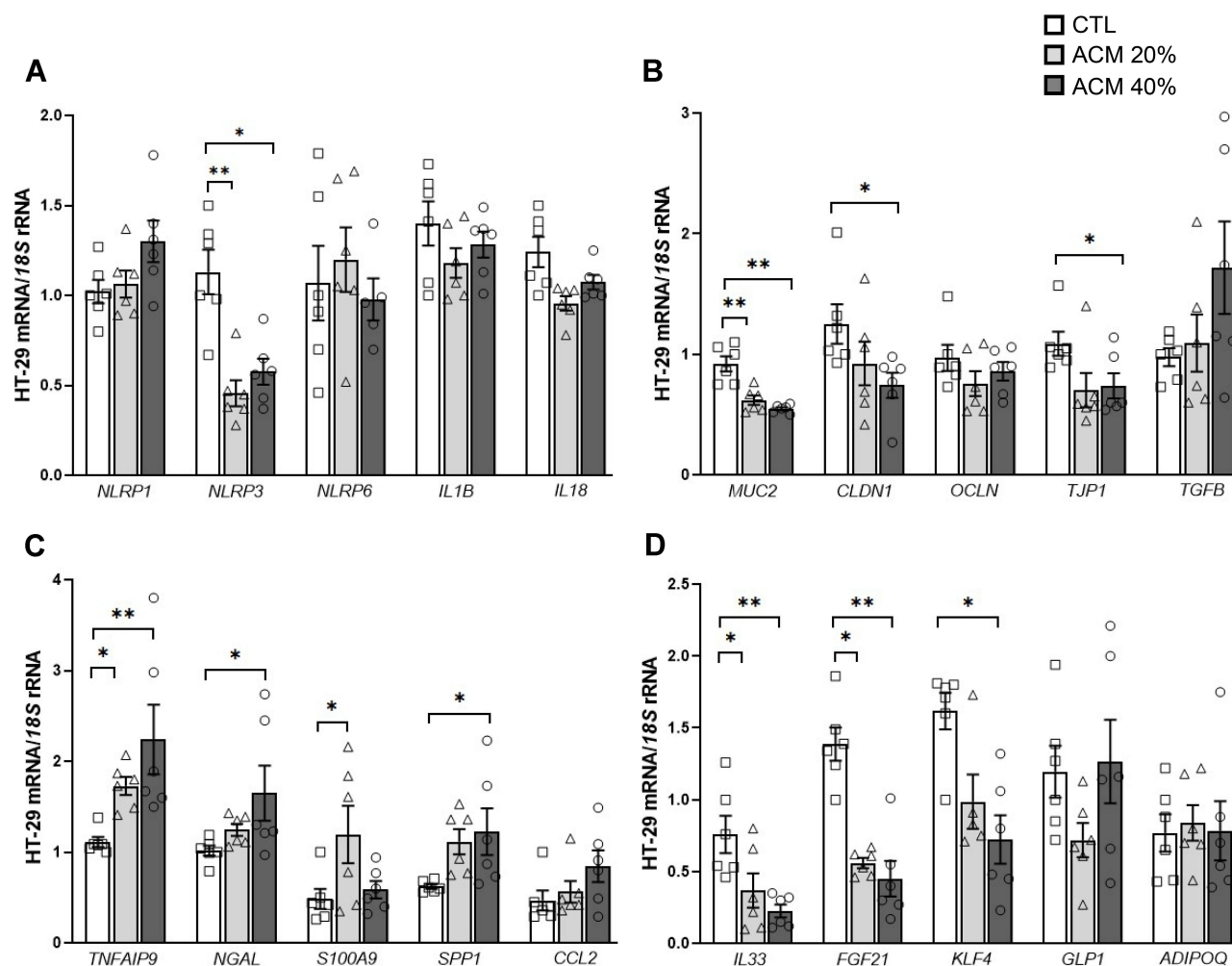
### *Akkermansia muciniphila* Improved the Inflammasome Expression Profile as Well as Intestinal Integrity-Related Genes and Aberrant Inflammation in HT-29 Cells

To explore the proposed antitumoral properties of *A. muciniphila*,<sup>24</sup> HT-29 cells were cocultured with the bacteria alive, heat-inactivated and also with the bacterial-conditioned medium (BCM). As shown in [Supplemental Table 4](#), gene expression levels of *NLRP3* ( $P < 0.001$ ), *ASC* ( $P < 0.001$ ), *IL1B* ( $P < 0.01$ ) and *HMGB1* ( $P < 0.01$ ) were increased in HT-29 cells treated with *A. muciniphila* alive. Importantly, we revealed an upregulation ( $P < 0.05$ ) in the mRNA of the intestinal integrity-related genes *CLDN1* and *MUC2*. Heat-inactivated bacteria did not exert significant changes but the BCM upregulated *NLRP1* ( $P < 0.001$ ), *NLRP6* ( $P < 0.001$ ), *CLDN1* ( $P < 0.01$ ) and *MUC2* ( $P < 0.01$ ) together with a downregulation of *NLRP3* ( $P < 0.01$ ) and *HMGB1* ( $P < 0.001$ ). We also measured the secretion levels of MUC-2 into the culture medium after *A. muciniphila* treatment, finding an increased ( $P < 0.01$ ) secretion into the medium ([Supplemental Figure 5](#)).

### Discussion

A huge body of knowledge supports the multifaceted roles of inflammasomes in obesity and its associated risk for CC development displaying both tumor-suppressive and pro-tumorigenic functions.<sup>9,25</sup> The main finding of our investigation is that patients with CC show increased expression of different inflammasome components in VAT in the context of obesity together with downregulated levels of *NLRP6* and *IL18* in the colon.

Important studies have proposed that AT expansion in obesity is a key first step in promoting an enhanced state of inflammation that confers an additional risk for cancer development.<sup>26</sup> Our experiments confirmed increased expression of *NLRP3*, *NLRP6*, *ASC* and *NOD2* in VAT due to both, obesity and CC suggesting that obesity-associated VAT inflammation creates a microenvironment favourable for CC development. We also showed upregulated levels of *IL1B* and *IL18* in the VAT from patients with obesity and CC, strengthening that the inflammasome-dependent production of these cytokines may influence colon tumorigenesis. In this line, we previously

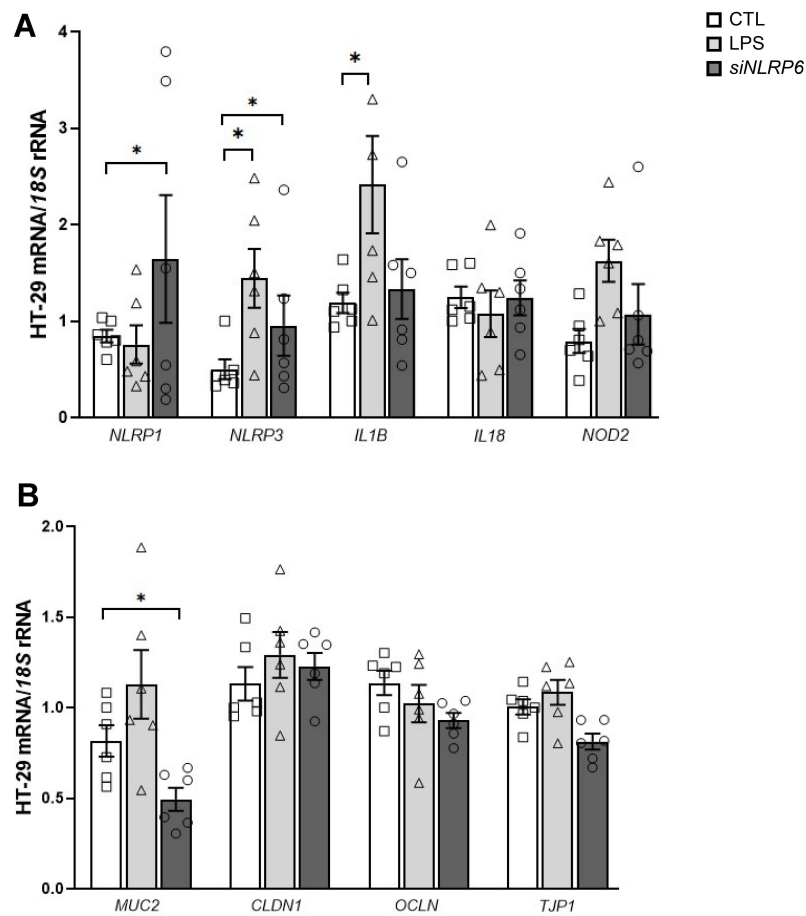


**Figure 5** Effect of adipocyte-conditioned media (ACM) on the gene expression levels of the inflammasome as well as on intestinal integrity- and inflammation-associated markers in HT-29 cells. Bar graphs show the effect of ACM (20 and 40%) from obese subjects incubated for 24 h on the transcript levels of (A) inflammasome-, (B) intestinal integrity-, (C) inflammation- and (D) anti-inflammatory-associated genes in HT-29 cells. Values are the mean  $\pm$  SEM (n=6 per group). Differences between groups were analyzed by one-way ANOVA followed by Tukey's tests. \* $P<0.05$  and \*\* $P<0.01$ . (n=6 per group).

**Abbreviations:** ADIPOQ, adiponectin; ASC, apoptosis-associated speck-like protein containing a CARD; CCL2, monocyte chemoattractant protein-1; CLDN1, claudin 1; FGF21, fibroblast growth factor 21; GLP1, glucagon-like peptide-1; GSDMD, gasdermin D; HMGB1, high mobility group box 1; IL, interleukin; KLF4, kruppel-like factor 4; MUC2, mucin 2; NGAL, lipocalin 2; NLRP, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin; NOD2, nucleotide-binding oligomerization domain containing 2; OCLN, occludin; S100A9, S100 calcium-binding A9; SPP1, osteopontin; TGFB1, transforming growth factor- $\beta$ ; TLR4, toll-like receptor-4; TJP1, tight junction protein 1; TNFAIP9, tumor necrosis factor- $\alpha$ -induced protein 9.

reported that the blocking of the expression of *NLRP3* reduces the inflammation of the AT together with an important attenuation in fibrosis controlling the development of obesity-associated comorbidities, including T2D or NAFLD.<sup>5</sup> Moreover, a pathogenic role of IL-1 $\beta$  as an important player in colon carcinogenesis has been previously proposed.<sup>26</sup> Low levels of adiponectin constitute a risk factor for obesity-associated cancers including CC and, accordingly, we found decreased levels of adiponectin in the VAT from subjects with obesity and CC. Results obtained regarding the expression levels of inflammasomes in the colon indicate that *NLRP6* and *IL18* were downregulated in patients with CC, identifying opposed

functions for both molecules in VAT and colon, probably due to their highly tissue-specific expression. Certainly, the characteristics and functions of the inflammasome complexes depend on the simultaneous expression of their components in the same cell type of inflamed tissues at the same time.<sup>12,27</sup> In this line, ASC, an inflammasome component, acts as a tumor promoter in the myeloid cells infiltrating the tumor being required for the synthesis of IL-1 $\beta$  and also functions as a tumor suppressor in the epithelial skin tumor cells.<sup>28</sup> The *NLRP-6* protein is mainly expressed in the intestine<sup>29</sup> and thus, the homeostasis of the intestinal epithelium is extremely dependent on *NLRP-6* with its uncontrolled expression critically



**Figure 6** Effect of blocking *NLRP6* expression in the gene expression levels of (A) inflammasome- and (B) intestinal integrity-related factors in colon cancer cells. The downregulation of *NLRP6* increased the mRNA levels of *NLRP1* and *NLRP3*. HT-29 cells were incubated with LPS (1000 ng/mL) and also were transfected with or without 100 pmol/L *NLRP6* siRNA/2×10<sup>5</sup> cells/well for 24 h. Values are the mean ± SEM (n=6 per group). Differences between groups were analyzed by one-way ANOVA followed by Tukey's tests. \**P*<0.05 vs scrambled siRNA. (n=6 per group).

**Abbreviations:** ASC, apoptosis-associated speck-like protein containing a CARD; *CLDN1*, claudin 1; IL, interleukin; OCLN, occludin; *NLRP*, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin; *NOD2*, nucleotide-binding oligomerization domain containing 2; *TJP1*, tight junction protein 1.

affecting the susceptibility to intestinal bowel disease (IBD). Importantly, the protein expression levels of *NLRP-1*, *-3* and *-6* are reduced in patients with CC and the loss of *NLRP-6* and *IL-18* have been associated with an increased mortality.<sup>30</sup> The expression of *NLRP6* and its effector *IL18* are closely linked. *Nlrp6*-knockout mice exhibit reduced circulating levels of *IL-18* in physiological conditions and after chemical-induced colitis and colitis-associated tumorigenesis.<sup>15,31</sup> *NLRP-6* signaling protects against colitis-associated tumor development by decreasing the epithelial barrier permeability and by inducing immune responses against different microbes.<sup>15,29,31</sup> In this regard, the outer mucosal layer of the colonic epithelium that prevents the direct exposure to invading commensals is covered by mucin and, *NLRP-6* is critical for an appropriate mucin secretion.<sup>32</sup> *Muc2*-knockout mice showed impaired secretion of mucus promoting bacterial

colonization of the crypts, inflammation and carcinogenesis.<sup>33</sup> Accordingly, we found decreased expression of *MUC2* in the colon from subjects with CC accompanied with increased expression of *CLDN1*. The complex role of *CLDN1* expression in CC has been exhaustively reviewed,<sup>34</sup> with some studies showing upregulated and others decreased expression levels. The higher levels of *CLDN1* found in our study may reflect an invasive and metastatic behavior of tumor cells as well as a poor prognosis.<sup>35</sup>

A pioneering study by Elinav et al<sup>15</sup> revealed a significant alteration in the microbiota composition from *Nlrp6*-, *Asc*- and *Il18*-knockout mice compared to that of wild-type mice. The lack of *Nlrp6* together with environmental microbial diversity have been shown to promote not only dysbiosis but also a diminished intestinal-barrier integrity that triggered inflammation,

intimately linked to tumor growth.<sup>36</sup> Indeed, metabolic endotoxemia is strongly related with the development of metabolic alterations including obesity, insulin resistance and liver steatosis.<sup>37,38</sup> In this line, although no effects of CC were observed in our cohort, patients with obesity exhibited increased circulating levels of factors of loss of the intestinal integrity barrier, including LPB, S100A8 and lactoferrin. These gut-derived endotoxins may function as potential priming and activating danger signals for the inflammasomes in VAT, driving a vicious cycle and the perpetuation of inflammasome-mediated chronic inflammation and insulin resistance and prompting CC development.

We observed that the expression of *Nlrp1*, 3 and 6 was decreased in rats fed a HFD. Although weight loss achieved by SG tended to increase the expression of the inflammasome components, differences were not statistically significant. Previous studies had demonstrated that the reduced intestinal *Nlrp6* levels in obesity may be efficiently reversed by RYGB.<sup>39</sup> The lack of differences found in our study may be explained by the inherent variations between both bariatric surgery techniques as well as by the specific changes in gut microbiota after surgery since without microbiota changes, the induction of *Nlrp6* was diminished.<sup>39</sup> In addition, bariatric surgery has been described to enhance glucose tolerance via decreasing *Nlrp3* inflammasome signaling in the AT from obese rats.<sup>40</sup> Similar phenotypes have been reported in caloric restriction studies<sup>41</sup> and, in our study, an increased expression of *Nlrp1* and *Nlrp6* in the colon after food intake restriction was also observed. An increased gastrointestinal permeability together with higher circulating concentrations of LPS have been previously reported in rats with HFD-induced obesity.<sup>42,43</sup> Furthermore, rats undergoing Roux-en-Y gastric bypass exhibited lower LPS and inflammatory cytokine concentrations than rats in the control group, suggesting that RYGB decrease the degree of endotoxemia, being also associated with strong intestinal tight junctions and an improved intestinal barrier integrity.<sup>44</sup>

To decipher the inflammasome activation pathways in CC, HT-29 cells were treated with different endogenous and exogenous pathogenic insults. While *NLRP3* and *NLRP6* showed the same trend in their expression levels after the treatment with LPS, TNF- $\alpha$ , CoCl<sub>2</sub> and insulin, *NLRP1* displayed an opposite tendency. The complex and differential regulatory pathways to promote not only a balanced but also an efficient immune response mediated

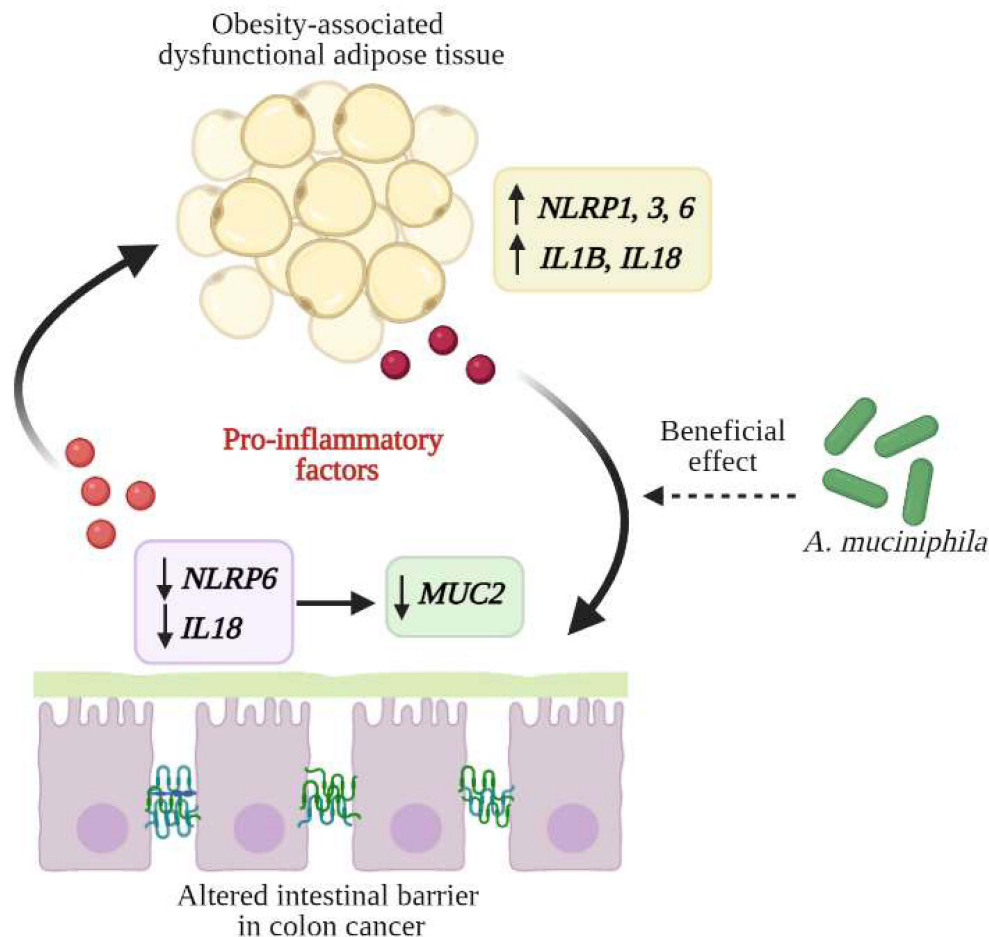
by the inflammasomes have been extensively reviewed by Latz et al.<sup>45</sup> The identification of upstream mechanisms is a very interesting field to develop novel strategies for the activation or inhibition of individual inflammasomes.

AT dynamically participates in cancer development not only as an endocrine organ releasing adipokines, proinflammatory cytokines, and extracellular matrix factors but also acting as a reservoir of energy.<sup>2</sup> We found an imbalance in inflammation and intestinal barrier integrity markers after the treatment of HT-29 cells with the adipocyte-derived factors from patients with obesity. The decreased levels of *NLRP3* found in the in vitro study suggest a dysregulation of immunosurveillance in HT-29 cells favouring immune escape and limiting the recruitment of antitumor cells.<sup>30</sup> Additionally, we found a reduction in the expression of crucial genes for the maintenance of epithelial barrier integrity, including *MUC2*, *CLDN1* and *TJPI*. An imbalance in the levels of tight junction proteins has been proposed as an initial event in the pathogenesis of IBD.<sup>46</sup> In line, ACM stimulates a proinflammatory profile increasing the levels of the inflammatory molecules *TNFAIP9* and *SPPI* as well as a reduction of the anti-inflammatory markers *IL33*, *FGF21* and *KLF4*. Iron and zinc constitute nutrients for bacterial growth and the increased levels of *NGAL* and *S100A9* observed in our study may reflect a strategy to prevent bacterial growth by limiting the availability of iron and zinc, respectively as a response to inflammation.<sup>47</sup> Defining the mechanisms that underline this active interaction, especially in the context of obesity, may help to identify novel therapeutic strategies. The downregulation of *NLRP3* after the treatment with ACM was confirmed in Caco-2, another colon cancer cell line. On the contrary, gene expression levels of *NLRP6* and *MUC2* were not detected and *IL1B* and *CLDN1* were increased. Differences exist between Caco-2 and HT-29, the most widely human intestinal cell lines used for in vitro attachment and mechanistic studies. While Caco-2 cells form polarized cells with high homology to enterocytes, HT-29 cells are more heterogeneous, containing a small proportion of mucus-secreting cells and columnar absorptive cells.<sup>48</sup> The development of co-cultures comprising both Caco-2 and HT29 cells has been proposed as an optimal strategy to find an in vitro model to mimic the intestinal epithelium.<sup>49</sup>

Although the maturation and activation of IL-1 $\beta$  and IL-18 via the *NLRP6* inflammasome has been described in macrophages,<sup>50</sup> gene expression levels of both interleukins remain unaltered after the inhibition of *NLRP6* in our in vitro experiments. Similarly, *Nlrp6*-deficient mice did not exhibit changes in their circulating levels of IL-1 $\beta$ .<sup>31</sup>

Of note, we found that *NLRP1* and *NLRP3* were upregulated after the treatment with si*NLRP6* in HT-29 cells. In this line, the expression of *Nlrp3* in the liver from *Nlrp6*-knockout mice was upregulated, suggesting a compensatory mechanism of *Nlrp3* activation upon *Nlrp6* deficiency.<sup>29,51</sup> The finding of increased levels of *NLRP1* and *NLRP3* potentially explains the unaltered levels of *IL1B* and *IL18* found in HT-29 cells. Importantly, a downregulation of *MUC2* was observed after si*NLRP6* treatment reinforcing the role of *NLRP6* in mucin release. The exocytosis of mucin 2 from sentinel goblet cells by *NLRP6* activation, and therefore, the protection of the colonic crypt from bacterial intruders has been elegantly described by Birchenough et al.<sup>52</sup> In accordance, *Nlrp6*<sup>-/-</sup> mice exhibited a failure of exocytose mucin granules due to impaired autophagy and hyperplasia of goblet cells.<sup>32</sup>

The drastic shift in the gut microbiome of patients with obesity and T2D influences the impaired maintenance of the intestinal barrier integrity.<sup>53</sup> Among these, *A. muciniphila* is a beneficial bacterial species with reduced levels of obesity and its related comorbidities.<sup>54</sup> The administration of *A. muciniphila* and its derived components has demonstrated to improve the metabolic profile and diet-induced obesity in terms of body weight gain and body composition in both animal models and humans,<sup>18,19</sup> as well as the symptoms of DSS-induced colitis in mice.<sup>55,56</sup> Lee et al demonstrated that the administration of *Lactobacillus paracasei* induced changes in the gut microbiota composition in HFD-fed rats being associated with improvements in gut barrier functions as well as in local and systemic inflammation.<sup>57</sup> Bariatric surgery-induced weight loss has also been associated with an enrichment in the abundance of *A. muciniphila*.<sup>43</sup> Our results showed that the stimulation of HT-29 with alive



**Figure 7** Proposed mechanism in the crosstalk between visceral adipose tissue (VAT) and colon in the development of colon cancer (CC) in an obesity context. The downregulated levels of *NLRP6* and *IL18* in colon cancer (CC) may be responsible of a diminished intestinal-barrier integrity, triggering local inflammation which in turn acts on dysfunctional adipocytes increasing the expression of *NLRP3*, *NLRP6* and its pro-inflammatory mediators *IL1B* and *IL18* promoting a vicious cycle of uncontrolled inflammatory cascades that favours a pro-tumorigenic microenvironment for CC development.

**Abbreviations:** IL, interleukin; *MUC2*, mucin 2; *NLRP*, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin.

*A. muciniphila* had a stronger effect compared with the heat-inactivated bacteria, increasing the expression levels of *NLRP3* and *ASC*. On the other hand, the bacteria-derived medium induced a strong upregulation of the expression of *NLRP1*, *NLRP6* and *MUC2* together with a decrease of the alarmin *HMGB1*. In addition, increased levels of secreted mucin-2 were detected after the treatment with the bacteria-derived medium. In this regard, Amuc\_1100, a protein isolated from *A. muciniphila* enhanced the intestinal barrier.<sup>18</sup> The increase in *NLRP* expression after *A. muciniphila* may reflect a better mechanism to recognize derived PAMPs and DAMPs to inhibit persistent and undesirable inflammatory responses.

This study has some limitations. Although the number of subjects in the groups may appear somewhat limited, the detailed clinical, biochemical and metabolic characterization of our subjects has to be stressed. Patients were carefully selected to avoid confounding factors and the groups encompassed highly homogeneous patients that allowed them to perform a robust analysis. Further prospective studies to assess the involvement of inflammasomes in the obesity-associated colon cancer may contribute to better elucidate the role of this protein complex in the context of obesity.

The downregulated levels of *NLRP6* and *IL18* in the colon from patients with CC may be responsible for a diminished intestinal-barrier integrity, triggering local inflammation, which in turn acts on dysfunctional adipocytes in obesity, increasing the expression of different inflammasome components and flaring up a vicious cycle of uncontrollable inflammatory cascades that favour a pro-tumorigenic microenvironment (Figure 7). Since the colon is the predominant site for host-microbiome interactions, a reduction in metabolic endotoxemia through the improvement of the gut barrier permeability combined with strategies to restore the functions of immunosurveillance of inflammasome components could represent an interesting target to identify and treat patients with obesity at increased risk for CC development.

## Ethics Approval

The experimental design was approved, from an ethical and scientific standpoint, by the Clínica Universidad de Navarra's Ethics Committee responsible for research (2018.094). This study was conducted in accordance with the Declaration of Helsinki.

All experimental protocols were approved by the Ethics Committee for Animal Experimentation of the University of Navarra (049/10) and conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU).

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## Disclosure

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