Final Degree Project

Biochemistry and Molecular Biology

UNIVERSITAT ROVIRA i VIRGILI

CIGARETTE SMOKING ALTERS ADIPOSE-DERIVED STEM CELLS IN CROHN'S DISEASE. IS NICOTINE THE GREATEST METHOD FOR QUITTING SMOKING?

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This project shows the result obtained during my internship at the **Inflammatory Bowel Disease Research Group** (IBODI), which belongs to '**Institut d'Investigació Sanitaria Pere i Virgili**' (IISPV), **Joan XXIII University Hospital** (Tarragona, Spain), under the supervision of Dr. **Carolina Serena Perelló** and PhD. Student **Diandra Monfort Ferré**.

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CONFIDENTIAL VERSION PROJECT

INDEX

ABBREVIATIONS

Table 1. Abbreviations used in the project.

Adipose-derived Stem Cells	ASCs
BCL2 associated X, apoptosis regulator	*BAX*
B-cell lymphoma-2	*BCL-2*
Bovine Serum Albumin	BSA
C-reactive protein	PCR
Chemokine CCL2	*CCL2*
Cigarette Smoking	CS
Creeping Fat	CrF
Chron's Disease	CD
Crohn's Disease Activity Index	CDAI
Dimethyl sulfoxide	DMSO
Ethylenediaminetetraacetic Acid	EDTA
Fetal bovine serum	FBS
Inflammatory Bowel Disease	IBD
Interleukin 1β	*IL–1β*
Interleukin 6	*IL-6*
Macrophages	MØ
Mesenchymal Stem Cells	MSCs
Nicotine Replacement Therapies	NRT
Phenotype 1 macrophages	M1
Phenotype 2 macrophages	M2
Phosphate-buffered saline	PBS
Subcutaneous Adipose Tissue	SAT
Stromal Vascular Fraction	SVF
T Helper 1 Cells	TH1
Tumor Necrosis Factor alpha	*TNF-α*

1. ABSTRACT

Background: Chron's Disease (CD) is a chronic inflammatory bowel disease for which there is currently no widespread effective treatment. Cigarette smoking (CS) is one of the environmental factors with the greatest impact on CD, worsening symptomatology and favoring an aggressive course. For this reason, to quit smoking with the help of nicotine replacement therapies (NRT) such as nicotine patches, is a currently standard clinical recommendation. Intriguingly, our group proved recently that adipose-derived stem cells (ASCs) from smoking CD patients have switched their immunomodulatory phenotype to pro-inflammatory. ASCs play a crucial role in maintaining the integrity and function of adipose tissue and their dysregulation could contribute to the observed inflammatory processes in CD. However, the specific effects of nicotine, which is the main component of tobacco cigarettes and most of NRT remains largely unexplored in ASCs from CD patients.

Hypothesis: ASCs obtained from heavy smoking CD patients would show an aggravated pro–inflammatory profile when compared to light smoking CD patients. In addition, nicotine *per se* can be the cause of the enhanced pro-inflammatory phenotype in ASCs from smoking CD patients.

Aim: To study how the different levels of cigarette consumption (light smokers versus heavy smokers) impact on the inflammatory phenotype of ASCs from smoking CD patients. We also wondered whether nicotine could induce alterations *per se* in ASCs from non-smoking CD patients.

Materials and methods: Adipose tissue biopsies from different levels of smoking (light and heavy) and non-smoking CD patients and healthy subjects were processed and ASCs were isolated and culture. Gene expression analysis by Real-time PCR was performed. In addition, an *in vitro* experiment with ASCs and different dosages of nicotine was carried out.

Results: Pro-inflammatory genes (*CCL2*, *IL1β* and *IL6*) were overexpressed in ASCs from heavy CD smokers when compared to a light CD smoker, in addition, both smoking groups presented a higher expression of those genes when compared to a control non-smoker CD patient. Nicotine promoted pro-inflammatory gene expression in ASCs from non-smokers with CD but had no direct effect in ASCs apoptosis.

Conclusion: Heavy CD smokers showed higher expression of pro-inflammatory cytokines in ASCs compared to light smokers and non-smokers. To note, nicotine *per se* also promoted the gene expression of pro-inflammatory cytokines which means that nicotine replacement therapies may not be appropriate in these patients. However, to ascertain that further experiments should be done.

Keywords: Crohn's Disease, inflammatory bowel diseases, chronic inflammatory diseases, adipose tissue-derived stem cells, cigarette smoking, nicotine, nicotine replacement therapies.

2. INTRODUCTION

Crohn's Disease (CD) is known to be one of the major forms of idiopathic inflammatory bowel diseases (IBD) along with ulcerative colitis. CD is a chronic, persistent, and destructive inflammatory pathology associated with an altered immune response that involves different sites along the gastrointestinal tract emphasizing the distal small intestine and the proximal colon [1]. CD, characterized depending on the affected location by diarrhea, abdominal pain, cramping, weight loss and perianal fistulas among other symptoms, is associated with significant morbidity and poor quality of life, alternating remission periods with phases of activity [2, 3].

2.1 *Incidence and prevalence of Crohn's Disease*

IBD prevalence and incidence are increasing through the years in different countries all over the world (*Figure 1*), which makes those pathologies a global disease [4]. Despite the variation in the incidence considering geographic regions, CD impact has gradually increased over the course of the twentieth century [4]. Recent data, depending on the geographical location, reveals that the incidence of CD in Europe ranges between 0,4 to 22,8 per 100.000 person-years, while the prevalence ranges between 1,5 to 331 per 100.000 [5]. CD seems to be a life-long disorder that can became clinically evident from childhood to late adulthood, nevertheless, there is a peak from 20 to 40 years, and currently, over 80% CD patients are diagnosed before the age of 40 [6].

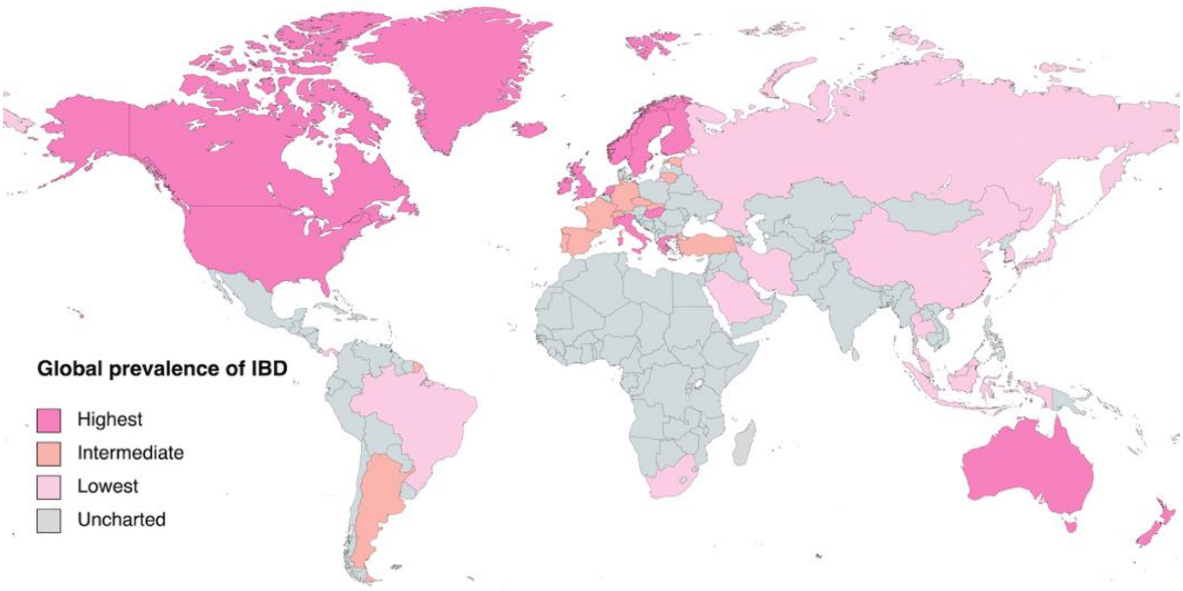

Figure 1. Global prevalence of inflammatory bowel diseases (IBD) from 2015 to 2025. *Adapted from [4].*

When it comes to how to detect CD, endoscopy is still the main diagnostic modality in IBD. Nevertheless, a comprehensive diagnosis includes clinical findings alongside endoscopic testing. Biopsies from the disease segments are also needed to help in the determination of the histologic extent by comparing with normal-appearing tissue biopsies [2, 7]. Being biomarker defined as a naturally occurring molecule, characteristic or gene that can be associated with a pathological or physiological process, some CD related biomarkers are C-reactive protein (PCR>40mg/L) and proinflammatory cytokines [8].

2.2 *Etiology and pathogenesis of Crohn's Disease*

CD is considered a complex heterogeneous disorder with multifactorial interactions. Although the etiology and pathogenesis of CD is not fully understood, according to several epidemiological and clinical studies; genetic susceptibility, dysregulation of the innate and adaptative immune system, infectious agents, and environmental factors (such us lifestyle) are believed to play an important role in the development of the disease (*Figure 2*) [1]. One of the main theories, although there are still unanswered questions related to the etiology of CD, holds that the disease is consequence of an aggressive and uncontrolled immune response to microbiota microorganisms in genetically predisposed individuals [1].

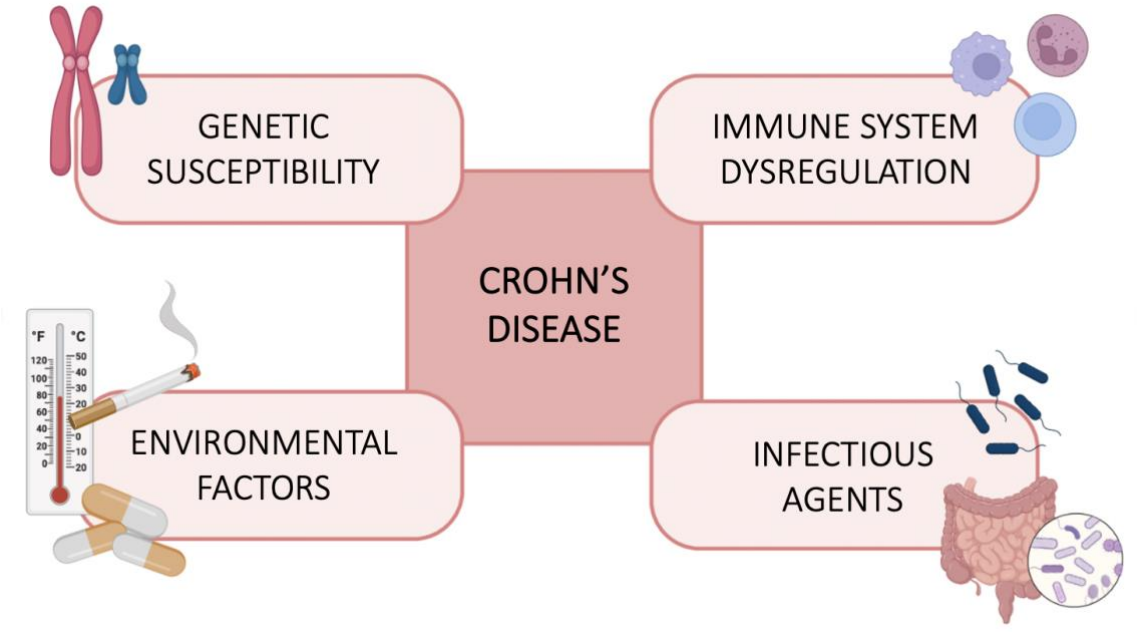

Figure 2. CD is believed to be the result of a multifactorial interaction among the immune system, infectious agents, genetic predisposition, and lifestyle habits. *Created in BioRender.com*.

Despite of being diseases with a complex genetic architecture, Genome Wide Association Studies (GWAS) have been reported as remarkably successful when it comes to evaluate the pathogenetic pathways and the genetic background of IBD [9]. When it comes to an inheritable component, IBD family aggregation has now long been recognized and, it seems to be stronger in CD than in ulcerative colitis [10].

Both innate and adaptative immune cells come into play in the development of IBD pathogenesis by initiating or propagating bowel inflammation [11]. Mucus layer and epithelial barrier act as the first physical line to fight in front of bacteria invasion, studies have demonstrated an affected barrier integrity in CD patients, showing a permeability increase which enables bacteria translocation, leading to an immune system hyperactivation and uncontrolled chronic inflammation, which is recognized by some experts as a key point in the development of CD [12]. Moreover, interactions between host and microbial agents may be adverse in CD patients due to the release of damaging metabolites and changes in the beneficial-harmful bacteria proportion [12].

In addition, a hallmark of CD patients that plays a role in the progression of the disease is the creeping fat (CrF), also known as fat wrapping, a wrapping arising from mesenteric adipose tissue (a peri-intestinal component of visceral adipose tissue) which seems to have a natural protective role wrapping the inflamed zone that ends up acting as a source of soluble factors, both pro and anti-inflammatory cytokines, and non-immune and immune cells (*Figure 3*), which plays an extensive role in the regulation of both immunity and inflammation [13]. Thus, confirms how adipose tissue contribute to the inflammatory process observed in CD with an endocrine role [14].

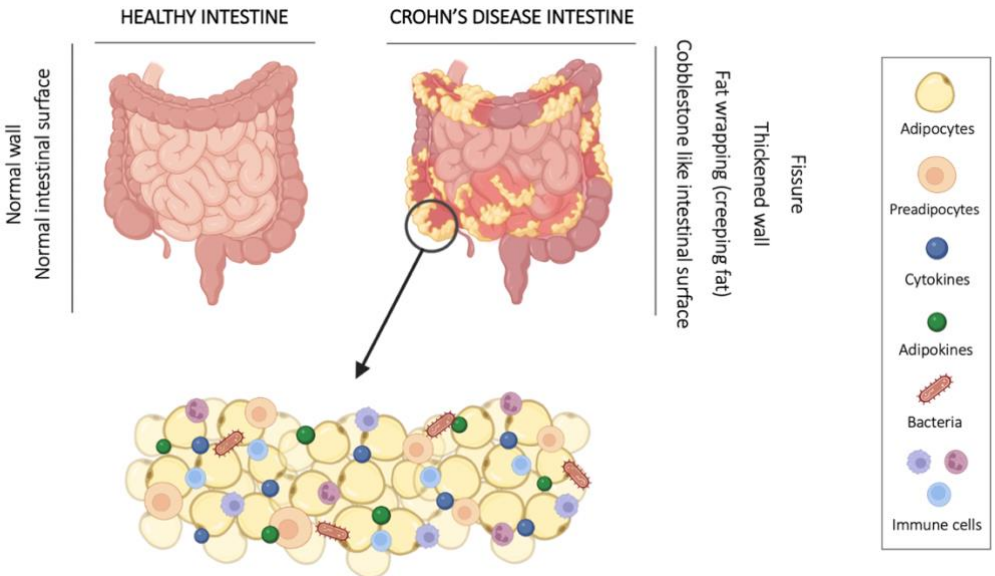

Figure 3. Healthy intestine is characterized by a normal wall and intestinal surface, while CD intestine is typable by cobblestone like intestinal surface, thickened wall, fissure, and fat wrapping, who works as a source of immune and non-immune cells. *Created in BioRender.com.*

At the same time, several studies [2, 15, 16] blame environmental factors shown in *Figure 4* for increasing CD incidence and prevalence, which confirms environmental features playing a key role in the development and severity of the disease. Despite the existing evidence of those factors being implicated in the pathogenesis of IBD, some mechanism, for example cigarette smoking action, remain still unanswered [16].

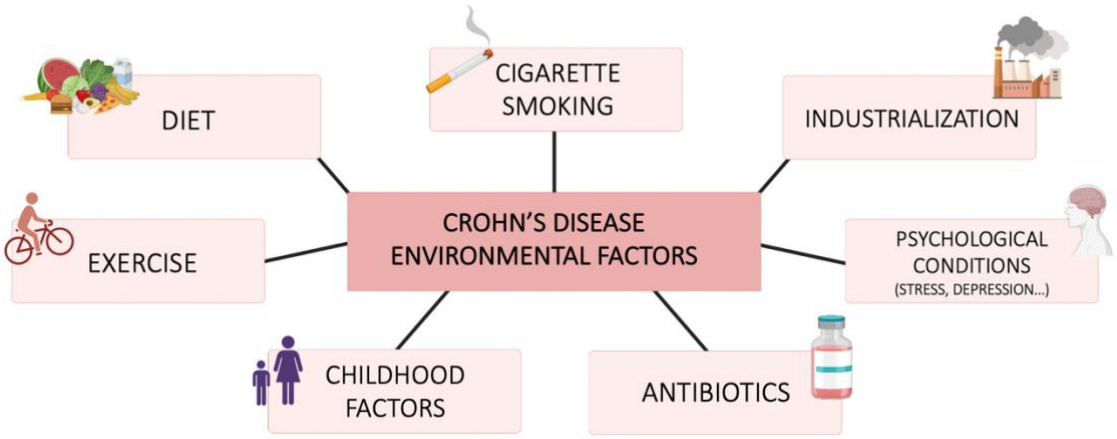

Figure 4. Environmental factors related to CD. *Created in BioRender.com.*

Cigarette smoking (CS) is a lifestyle habit practiced by about 21% of the population [17] and associated not only with a huge variety of health problems including digestive and respiratory system infections, but also with damage in organs along the body and even in the gut microbiota [18]. Cigarettes are believed to be a complex mixed of approximately 7.000 compounds, including nicotine, tar, hydrocyanic acid, or carbon monoxide, of which about 250 turn out to be toxic or carcinogenic [19]. Studies have reported before how CS, or even exposure to nicotine, increases the risk of various systemic diseases and cancers although mechanisms are not still full understood [19]. Nicotine, a naturally alkaloid in plants that works as a highly addictive drug and is behind the continuous used of cigarettes, is known to be the main psychoactive compound finding in tobacco, nevertheless, it is not believed to be the only responsible when it comes to the associated cigarettes damaged produced by tobacco smoking [20]. An unanswered question about nicotine action remains its influence on apoptosis, some results aim to a dose-dependent anti-apoptotic

effect while others defend the adverse, a pro-apoptotic action [21]. In addition to this controversial, an ambiguous data is point towards in other publications [21, 22].

Although CS being one of the well-documented risk factors in the incidence and progression of CD, mechanisms are still unclear due to how complex tobacco smoke composition is [23]. In a CD context, CS has largely been associated not only with aggravated prognosis but also with more inflammatory relapses, higher postoperative recurrence, worse symptomatology, and an aggressive disease course. When comparing smoking to non-smoking CD patients, data reveals smokers being more likely to develop perianal fistulas [23].

Those perianal fistulas are one of the most common complications in CD suffer by up to 50% of CD patients within 20 years after the diagnosis, which lead to a drastically decrease in patient's quality of life. [24]. Medical treatments for perianal fistulas in CD patients are not always as effective as expected, indeed, they are specially challenging. Nevertheless, novel therapies based on Adipose tissue-derived Mesenchymal Stem Cells (ASCs) have shown to be a promising treatment approach for CD complex fistulas in terms of their capacity to attenuate the immune system response, their self-renew potential, and their ability to differentiate into multiple cell linages [25].

2.3 *Immuno-regulatory properties of Adipose-derived Stem Cells*

Studies have shown how stem cells are able to regulate the immune system by two mechanism (*Figure 5*), on the one hand, *direct cell-cell communication*, involves ASCs interaction with different cell types, while on the other hand, *indirect cell-cell communication,* represents the secretion of soluble mediators, growth factors and extravascular vesicles [26]. In addition to their immunoregulatory properties, in a CD context, ASCs also play a crucial role in maintaining adipose tissue function and integrity [27].

- Direct cell-cell communication

According to several studies reported before, MSCs use their multipotency as a mediator of immunomodulation. Significant effects have been found with both innate and adaptative immune cells, but also with for example endothelial cells and pre-adipocytes [28]. When it comes to ASCs-

immune cells interaction, effects of ASCs in modulating T and B cells and macrophages (MØ) among others, have been well documented lately [26]. Coculture of ASCs with MØ have demonstrated MØ polarization into M2 type, whose characteristics involves secretion of anti-inflammatory cytokines and trophic factors, in addition pro-inflammatory cytokines (such as *IL-1ß*) levels are downregulated [26]. Talking about T cells, ASCs have demonstrated to downregulated TH1 cells proliferation, which helps inhibiting the immune response. In addition, MSCs can secrete immunosuppressive factors, chemokines and adhesion molecules involve in effective T-cell suppression. As regards B cells, human MSCs have demonstrated to be involved in the inhibition and activation of those adaptative immune cells in addition to playing a role in the differentiation suppression [26].

- Indirect cell-cell communication

ASCs have been shown to harness the release of soluble factors and extravascular vesicles (exosomes and microvesicles) to modulate the immune system leading to a regenerative medicine application [26]. On the one hand, those soluble factors include cytokines, growth factors, chemokines, and hormones which all together are capable of predetermine immune cells functions getting to an immunology activity suppression [28]. On the other hand, extravascular vesicles, recognized as a heterogeneous population of membrane vesicles, have been shown to have therapeutic potential by allowing proteins, lipids, and genetic material exchanges between cells [29].

Another remarkable property of ASCs consists of their tissue-renewal and proliferation through tissue homeostasis regulation. Through changes in the microenvironment based on paracrine signals and on the secretion of soluble molecules and extravascular vesicles, ASCs have capacity to regulate oxidative stress, apoptosis, or angiogenesis [30].

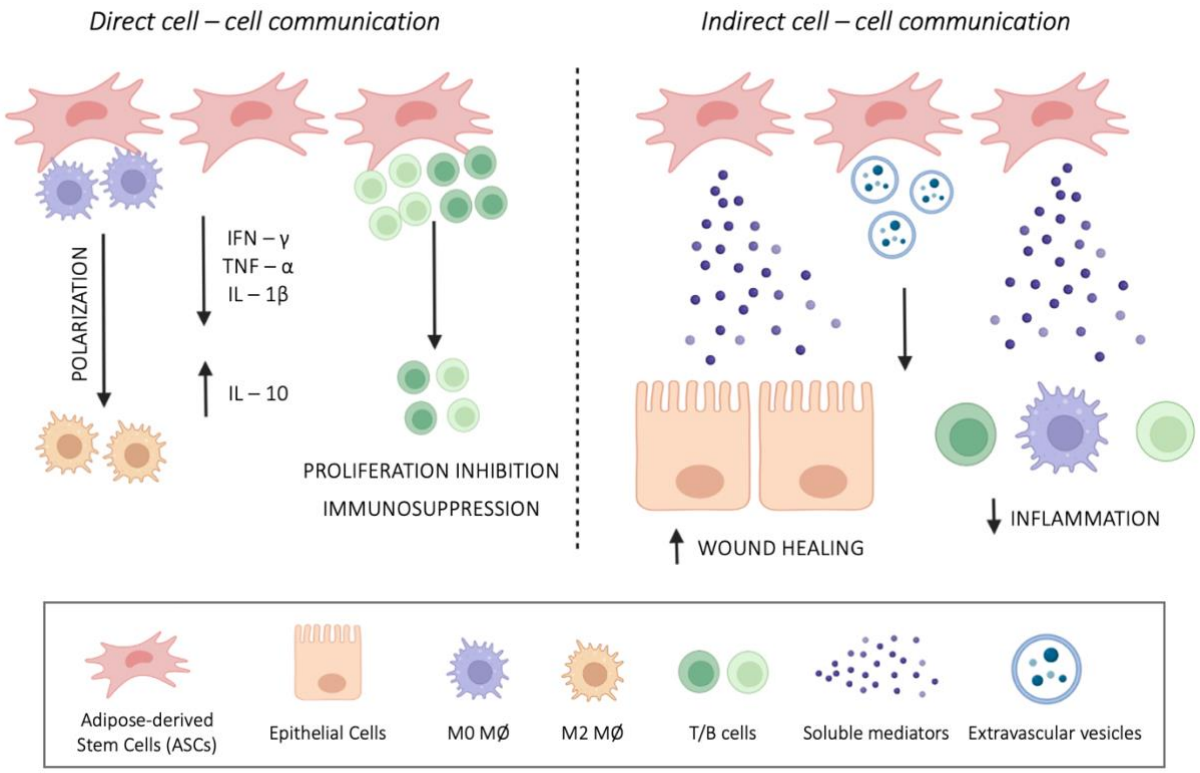

Figure 5. Mechanisms involve in the immune system regulation by ASCs. Direct cell-cell mechanism (left panel). Indirect cell-cell mechanism (right panel). *Created in BioRender.com.*

2.4 *Smoking suppresses the therapeutical properties of Adipose-derived Stem Cells in Crohn's Disease patients*

Damage in metabolic and cell signaling pathways can disturb ASCs phenotype and functions. Such damage may result not only from the disease concerned but also from lifestyle habits or behavioral risk factors including, as mentioned before, cigarette exposure [23]. ASCs play a crucial role in maintaining the integrity and function of adipose tissue and their dysregulation have been reported to play a role in the observed inflammatory processes in CD [27].

When comparing ASCs from CD patients and healthy subjects, some studies by our group reported significant differences [27]. A higher proliferation was revealed in CD ASCs, corresponding to a higher cell ratio when isolating from the same amount of adipose tissue. Results also revealed how CD modifies ASCs plasticity promoting a decreased in typical adipogenic markers expression when comparing to healthy ASCs. In addition, immune response was reported as activated in CD

ASCs by showing a pro-inflammatory profile expressing higher typical inflammatory markers (such as *IL6* and *IL1β*). CD ASCs also revealed an exacerbated macrophage-like phenotype, with a lower M2 MØ polarization rate [27]. Furthermore, protective immunosuppressive properties associated to healthy ASCs, including inhibition of T and B cells, are damaged in CD ASCs and invasion capacity and bacterial phagocytic activity, which can lead to bacteria participation in the intestinal inflammation, is activated [27].

According to some previous epigenomics studies and methylation assays by our group, distinct DNA methylation patterns were found in ASCs isolated from CD patients when comparing to a non-CD control group. Differentially methylation regions are mainly associated to genes involved in immune system, cell differentiation, and metabolic and development processes [31].

Based on a recently published study by our group where smoking status (smokers, non-smokers, and ex-smokers) was compared in CD patients, immune regulatory properties of ASCs were affected in smoking CD patients [23]. Results indicated that smoking plays a key role disturbing ASCs property to modulate the immune system, specially by inducing inflammatory cytokines production and secretion, facilitating M1 pro-inflammatory phenotype polarization, and not being able to inhibit T and B cells proliferation [23]. This mentioned study revealed the damage found in ASCs properties from CD smokers may be due to the fact of smoking inducing changes in the methylation pattern of CD ASCs. Analysis showed smoking playing a role when it comes to explain epigenetic variations in ASCs, what means that smoking induces epigenetic changes which disturb ASCs therapeutic potential, including anti-inflammatory and regenerative properties [23].

Considering that a dysregulation in ASCs properties could enhanced the chronic inflammatory process observed in CD [30], to quit CS is a standard clinical recommendation for CD patients. Helping in the transition to quit CS, nicotine replacement therapies (NRT) consist of different medical formulations who aim to reduce nicotine addiction. NRT enables nicotine absorption via the skin (transdermal patches) and the oral (sublingual tablets, chewing gums, etc.) and nasal (spray) mucosa [32]. For instance, widely used nicotine patches, containing non-medical ingredients and different nicotine doses, including 7, 14 and 21mg/day, are placed gradually over the skin with a low nicotine release for 24h and used depending on the nicotine addiction the patient has [33].

3. HYPOTHESIS AND OBJECTIVES

In this pilot study we hypothesize that the affected pro-inflammatory phenotype of SAT-derived ASCs from smoking CD patients could be more aggravated depending on the level of cigarettes consumed per day. Moreover, we presume that nicotine could be one of the cigarette components blame for enhance the observed pro-inflammatory phenotype switch in ASCs isolated from smoking CD patients.

According to the established hypothesis, in this study we aimed to elucidate 1) whether ASCs obtained from CD patients (light smokers and heavy smokers) show a different gene expression of pro-inflammatory markers (*CCL2*, *IL1β* and *IL6*) depending on their daily cigarettes' consumption compared to a non-smoker CD and a healthy patient as a control. And 2) to analyze, whether different dosages of nicotine (0, 0.1, 0.5 and 1µM) induce pro-inflammatory (*CCL2*, *IL1β* and *IL6*) and apoptotic (*BAX* and *BLC2*) gene expression in ASCs from non-smokers CD patients. In this sense, confirming whether nicotine directly affects ASCs or not, this study would help to start clarifying if NRT, focusing on widely used nicotine patches, which include nicotine as the medical ingredient, are an accurate therapy to help CD patients to quit smoking.

4. MATERIALS AND METHODS

4.1 Study subjects

Subjects were recruited at the Vall D'Hebrón University Hospital (Barcelona, Spain), Santa Tecla Hospital (Tarragona, Spain) and the Joan XXIII University Hospital (Tarragona, Spain). The study was developed according to the values of the Helsinki Declaration (CEIM 177/2018) and was approved by the ethics committees of each hospital (PR[CS]383/2021). All participants signed an informed consent. Patients were classified as active (in relapse) or inactive (in remission) following criteria of the Crohn's Disease Activity Index (CDAI) and based on clinical and biological parameters such as high-sensitivity C-reactive protein (hsCRP). Patients with active CD were recruited from those undergoing surgery for symptomatic complications, whereas inactive CD patients and non-IBD (healthy control) were recruited from subjects undergoing non-acute surgical interventions such hernias or cholecystectomies, scheduled as routinary surgery.

Subcutaneous adipose tissue (SAT) samples were aseptically collected from non-IBD patients (healthy control), and CD patients classified as smokers (light smoker; less than 5 cigarettes/day, and heavy smokers; more than 12 cigarettes/day) and non-smokers. Clinical data, anthropometric, demographic, and biochemical variables of cohort are shown in Supplementary Table S1.

4.2 Adipose tissue-derived Stem Cells isolation and culture

Human subcutaneous adipose tissue (SAT) biopsies were initially washed with Phosphate-buffered saline (PBS) to remove blood traces. Damaged because of the extraction was removed with the help of a scalpel. The tissue was then cut into small pieces and treated with PBS and 1% Bovine Serum Albumin (BSA) and 1% collagenase for 90 minutes at 37ºC under gentle agitation. The digested samples were then centrifugated at 1250rpm at 24ºC for 10 minutes. A phase separation was obtained, with the mature adipocytes at the top and the Stromal Vascular Fraction cells (SVF) in the pellet. The SVF consists mainly of stem, macrophage, endothelial and blood cells. A wash with PBS was perform before another centrifugation to isolate ASCs. The resulting pellet from the last centrifugation was then resuspended in 1 mL of DG maintenance medium (DMEM/F12 in 10% fetal bovine serum (FBS) and 1% antibiotics and antimycotics) and added to a T25 flask (ThermoFisher Scientific) with 4 mL of DG maintenance medium. 24 hours later, 4 washes with PBS were done to ensure that only ASCs remain in the T25 flask and remove non-adherent cells.

After the washes, cells were allowed to grow for approximately seven days in a humidified incubator with 5% CO_2, 95% O_2 and 37ºC up to 90% confluence. After this period, cells were harvested with trypsin (ThermoFisher Scientific) and named as Passage 0 (P0) in a T25 flask (T75 flasks were used form P1 onwards). DG maintenance medium was replaced every 2-3 days to ensure optimal nutrient supplementation. When achieving 90% confluence, ASCs were treated with trypsin-Ethylenediaminetetraacetic Acid (EDTA) for new passages to prevent spontaneous differentiation [23] until getting to P3, where cells were harvested in 100mm culture plates (ThermoFisher Scientific). All the experiments were performed between passages 3 to 5.

4.3 Adipose-derived Stem Cells culture with different nicotine dosages

Cells at a concentration between 1,25 and 1,45 · 10^5 were harvested in a 12-well cell culture plate (Costar 12-well Clear TC-treated plate; Corning). The following day, making sure cells had adherent to the plate and a confluence of 90% was achieved, different dosages of nicotine (REF: N3876, Sigma-Aldrich; Merck); 0, 0.1, 0.5 and 1µM (*Figure 6*) as reported in others studies before [34,35], diluted in dimethyl sulfoxide (DMSO) [36] and DG maintenance medium, were used to treat the cells with an exposure time of 24 hours and collected then to analyze gene expression. 0µM condition was treated with the corresponding DMSO and DG maintenance medium to ensure changes were caused by nicotine.

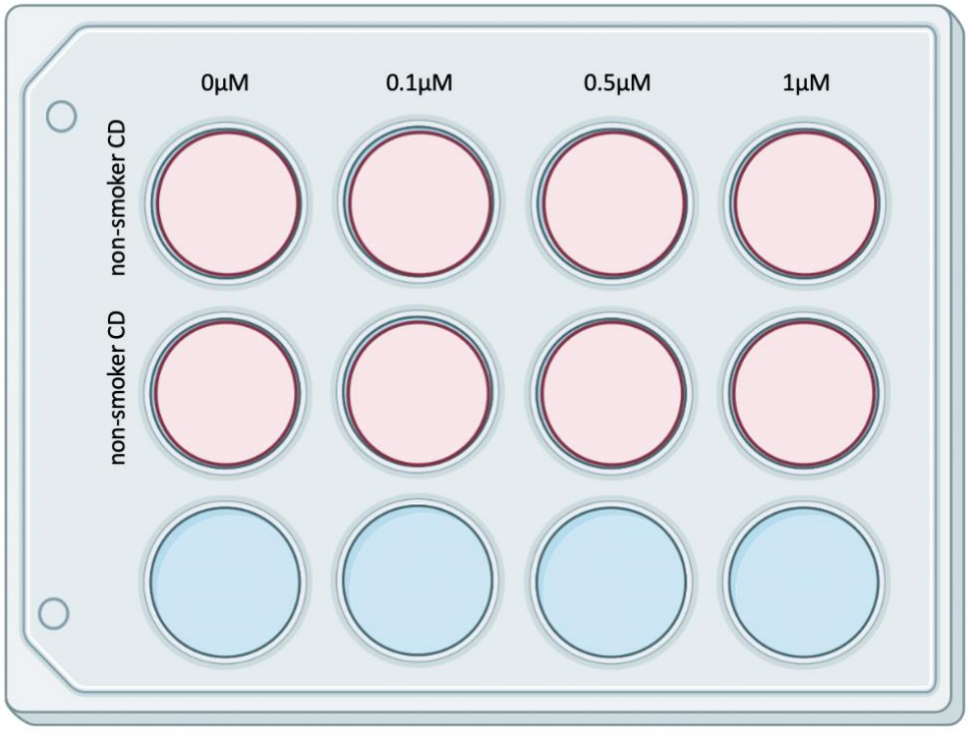

Figure 6. 12-well culture plate representation. *Created in BioRender.com.*

4.4 Gene expression analysis

4.4.1 RNA extraction and quantification

Total RNA was isolate from ASCs by washing with 1mL 1X PBS following a cell resuspension and lysis with 1mL of TRIzol® RNA isolation reagent (REF. 15596026), supplied by Invitrogen (Carlbad) for 5 minutes at room temperature (RT). Subsequently, 200µl of chloroform were added before being centrifugated at 12000g for 15 minutes at 4ºC. Each aqueous phase was then transferred into a new Eppendorf tube were 500µl of isopropanol were added and centrifugated at same conditions but for 10 minutes. In the next step, the aqueous fraction was discarded, and the resulting pellet was then washed twice with 1mL of ethanol 75% (v/v) followed by a spin and centrifugated between and after the washes at 7500g for 5 minutes at 4ºC. Subsequently, the aqueous phase was discarded and the resulting pellet, containing the RNA fraction, was dried at room temperature for a maximum time of 15 minutes. Afterwards, to resuspend the pellet, from 10 to 30µl of RNase-free water (Qiagen GmbH-Hilden) depending on the size of the pellet, were added, and incubated during 10 minutes in a Thermobloc at 65ºC. Afterwards, 1µl of the samples was then used to automatically quantified using the Varioskan LUX multimode microplate reader (ThermoFisher Scientific) following the manufacturer's instruction. The purity of each extraction was determined by the OD260/OD280 ratio. Later, RNA samples were handled on ice and preserved at -80ºC until cDNA retrotranscription.

4.4.2 cDNA retrotranscription

1µg of RNA from each sample was reverse-transcripted throughout the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems-Van Allen Way, CA, USA), using random primers for cDNA synthesis. In this process, RNA samples were primed for 10 minutes at 25ºC followed by the reverse transcription polymerase chain reaction (RT-PCR) for 120 minutes at 37ºC. Subsequently, the reverse transcriptase was inhibited by its inactivation for 5 minutes at 85ºC. Samples were then stored at -20ºC until the next step. This process was carried out in the ProFlex 3 x 32-well PCR System (Applied Biosystems).

4.4.3 Real Time qPCR

cDNA samples at concentration of 50ng/reaction were intended for real-time PCR using TaqMan Gene Expression Assay in 96-well plates (PCR plate, 96-well, non-skirted; Thermo Scientific) to analyze the selected genes expression. Each plate contained predefined assays of genes, which

use TaqMan probes as detectors (referenced in **Supplementary table S2**) obtained from ThermoFisher Scientific. This process was carried out in a QuantStudio™ 7 Pro Real-Time PCR System, 96-well, 0.1mL (Applied Biosystems). The comparative method $2^{-\Delta\Delta Ct}$ was used to analyze data obtained, using values at arbitrary units resulted from 18S rRNA as housekeeping gene (endogenous control) and expressed relative to the control condition set to 1.

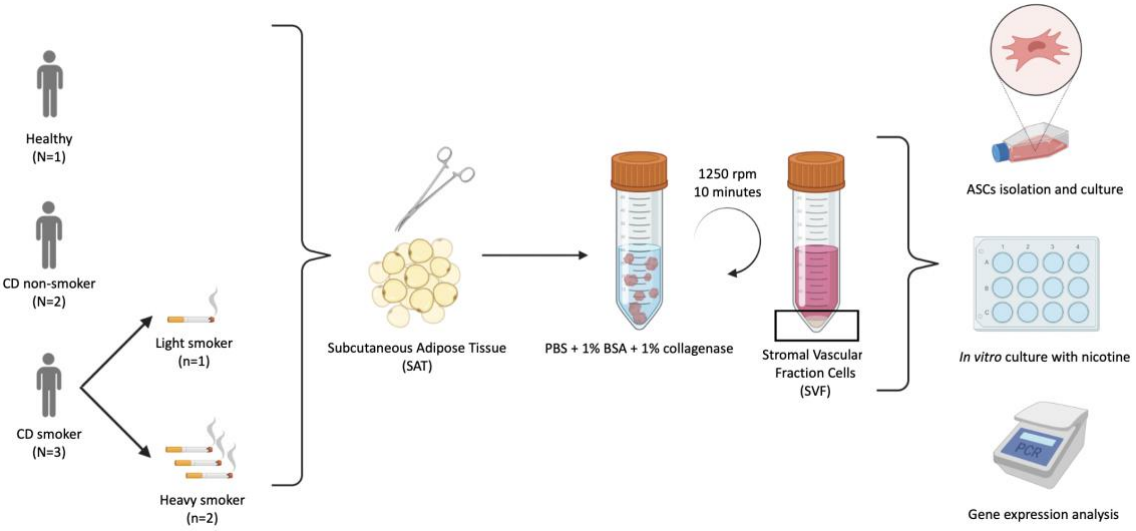

Figure 7. Schematic representation of the followed methodology. *Created in BioRender.com.*

5. RESULTS

Pro-inflammatory genes (*CCL2*, *IL1β* and *IL6*) were overexpressed in ASCs from heavy CD smokers when compared to a light CD smoker, in addition, both smoking groups presented a higher expression of those genes when compared to a control non-smoker CD patient. Nicotine promoted pro-inflammatory gene expression in ASCs from non-smokers with CD but had no direct effect in ASCs apoptosis.

6. DISCUSSION

As mentioned before, CD in an heterogenous disease where multifactorial interactions lead to an uncontrolled and continuous immune system response against microbiota microorganisms in genetically predisposed individuals, where environmental factors play a key role in the etiology and pathogenesis of the disease [1]. According to a recent study where CD patient's perspective on smoking was analysed, 63,1% of the CD participants were identified as currently smokers [37]. CS, defined as a prejudicial environmental factor and which action has not yet been clearly answered, has been reported as fatal in CD patients, from aggravated inflammatory relapses to a higher likelihood of developing perianal fistulas, one of the major CD associated complexities [23]. Perianal fistulas, as mentioned before mostly viewed in CD smokers, are a debilitating CD complication which have lately been treated with ASCs in terms of their anti-inflammatory phenotype and self-renew capacity [25].

Unfortunately, previous studies with SAT-derived ASCs from CD patients have reported affected therapeutical properties in those cells, including in patients in remission (although an aggravated pro-inflammatory environment has been seen in active patients), when compared to healthy ASCs [27]. This disrupted therapeutical properties include an increase in pro-inflammatory genes expression [27], which can be explain through a distinctive DNA methylation pattern [31]. In our findings, on the contrary, similar pro-inflammatory gene expression in CD patients and in the healthy subject may be due to the fact of being a CD patient in remission, who was under the effect of biological Anti-TNF treatments, being this treatment capable of decreasing pro-inflammatory cytokines expression such as *IL1β* and *IL6* [38]. As seen in this study and in previous ones by our group [23], smoking enhances a pro-inflammatory phenotype in SAT-derived ASCs isolated from CD patients by increasing pro-inflammatory genes expression, which has been suggested to be consequence of epigenetics changes [23]. In agreement with our findings, where smoking level of cigarettes consumption per day affected the inflammatory phenotype of ASCs, previous results reported a longer process of intestinal inflammatory activity in heavy smokers [39] and a dose-depending negative effect of smoking in CD patients [40]. In line with this idea, high levels of *CCL2* expression, which function consist of attracting monocytes to the lesion area and facilitating macrophage infiltration, could be leading to an uncontrolled immune system activation [41].

Considering the given information, a pro-inflammatory environment added to the fact of how dysfunctional ASCs contribute to the inflammatory processes observed in CD [27], patients with an aggravated ASCs pro-inflammatory phenotype, in fact those with a heavy smoking habit, should be extremely aware of how smoking detrimental effects could increase the chronic inflammation observed in CD. Thereby, ASCs derived from heavy smoking CD patients could be ruled out as a possible therapeutic approach to treat perianal fistulas due to the loss of their anti-inflammatory profile. On this account, a decrease in the number of cigarettes smoked per day could be considered as a first step to fight against CS complications. In addition, treatments to quit smoking should be given more importance to help CD patients in the transition to quit smoking. The primary shortcoming of this study is the necessity for additional research including more patients. The lack of statistical analysis was caused by the minimal number of samples. Thus, the next step will be incrementing the cohort and matching patients to confirm our results as well as studying how CS affect other molecular aspects in ASCs such as macrophage polarization, and T and B cell proliferation among others.

CS mechanisms are still unclear because of cigarette smoke composition complexity [23], where about 7.000 compounds can be find [19]. As being related to inflammatory relapses and with post-operative recurrence in addition to a worse prognosis and a less effective response to treatments [23], to quit smoking is a clinical standard medical recommendation [33], however, evidence reported a high rate of smoking along CD patients [37]. NRT, focusing on widely prescribed nicotine patches, consist of medical formulations which contains nicotine absorbed via the skin as the medical ingredient [33]. Although some studies have reported a negative effect of nicotine in MSCs, mechanisms by which this addictive compound acts are not still full understood [42]. In agreement to our study, overexpression of pro-inflammatory genes (especially *IL1β* and *IL6*) has been seen before in studies where other types of cells [43] and even other stem cells [44] were treated with nicotine, nevertheless, is the first time to our knowledge, the effect of nicotine in SAT-derived ASCs from CD patients have been reported. We suggest that nicotine could be one of the compounds involve in the epigenetics changes observed in ASCs from smoking CD patients, however, there is not a clear knowledge to indicate nicotine effect by itself on those patients [45] and further studies should be carried out to elucidate this hypothesis and to reach an agreement on the role of nicotine in ASCs. On the contrary to our findings, where we point forward to a pro-inflammatory role, some authors suggested that nicotine could have a therapeutical effect influence by its complexity pharmacology [45], that may be used to explain the slight decrease of pro-inflammatory genes expression observed at high nicotine concentrations, consequently,

further studies should focus on finding the optical nicotine concentration to elucidate this effect in ASCs.

Given our results, where we emphasise the observed higher pro-inflammatory genes expression at any concentration when compared to the control without exposure to nicotine, it seemed that nicotine could have a detrimental effect in ASCs promoting the expression of pro-inflammatory genes which, in fact, can contribute to the maintenance of the pro-inflammatory microenvironment generated nearby the intestinal affected region in these patients. Thus, although further experiments are required with a larger and better characterized cohort, it is reasonable to start considering NRT as not the best approach to help smoking CD patients quitting this toxic habit. Instead, if our results are confirmed, other therapeutical compounds, such as *Varenicline*, a non-nicotinic smoking cessation pharmacotherapy [46], should be considered.

When it comes to apoptosis, defined as a normal and controlled cell process of death, no previous information to our knowledge tested the effect of nicotine in SAT-derived ASCs from CD patients, consequently, this project carried out an in vitro experiment to elucidate the direct relation of nicotine and apoptosis in those mentioned cells. On the contrary to our results, most studies have reported before the idea of nicotine promoting an anti-apoptotic effect in different cell line types which could be explain through the activation and phosphorylation of anti-apoptotic proteins such as *BCL2* [47, 48], controversially, some authors revealed a dose-depending apoptotic effect in stem cells which could be explain by the induction of oxidative stress [49] via the mitochondrial pathway [50]. In agreement to our findings, despite being in other cell line types, previous studies revealed neither a pro-apoptotic nor anti-apoptotic direct effect of nicotine in various human cell lines [22] which could be due to fact of a dual role. This can be supported with no differences neither in the ARN quantification nor in ASCs morphology of the treated cells. Considering our results, in this study nicotine *per se* had not a direct effect on SAT-derived ASCs apoptosis, however, investigating nicotine direct effect on apoptosis is not an easy job due to the different reported effects depending on the tested concentrations and the exposure time. Thereby, further studies should be carried out with the aim of elucidating the relation between nicotine and ASCs from CD patients' apoptosis.

To conclude, it should be noted that our study had some limitations. First, it is important to highlight that the present study has a small number of participants due to the reliance on biopsies arising from the mentioned hospitals (see *4.1 Study Subjects*) and the limited period in which cells needed to be cultured. Furthermore, there is some intrinsic variability between participants which

can influence the study (see *Supplementary Table S1*). Moreover, the exposure time of nicotine could be affecting its action as there is neither consensus time of application nor used nicotine concentrations. Therefore, further studies improving the mentioned limitations are needed to corroborate the obtained results in this project.

7. CONCLUSION

CS is one of the environmental factors with the greatest impact on CD pathogenesis. Findings here suggested that a higher number of cigarettes smoked per day promotes an aggravated pro-inflammatory phenotype in ASCs, which is involve in the loss of their therapeutical properties and contribute to the chronic inflammatory process observed in CD patients. Reducing the number of cigarettes per day could be a first approach to avoid detrimental CS effects, but actually, smoking CD patients should be encouraged to quit this toxic habit. In the transition to quit smoking, NRT, which use nicotine as the medical ingredient, are widely prescribed. According to our findings, nicotine *per se* seemed to have direct prejudicial effect on ASCs promoting an overexpression of pro-inflammatory genes, which boost the inflammatory environment associated to CD, thus, nicotine could be one of the cigarette compounds who is behind the fatal smoking impact on CD.

Thereby, NRT could be questioned as a safe approach when it comes to CD patients due to having nicotine as the main ingredient, and other non-nicotinic options should be considered for CD patients. Anyway, further studies would be necessary to elucidate the proposed hypothesis.

SUPPLEMENTARY MATERIALS

Supplementary table S1. Clinical data, anthropometric, demographic, and biochemical variables of cohort.

	Healthy	Crohn's Disease				
	Non-smoker	Smokers			Non-smokers	
		Light smoker (<5 cigarettes/day)	**Heavy smokers** (>12 cigarettes/day)			
n	1	1	2		2	
CDAI	-	Inactive	Inactive	Inactive	Inactive	Active
Hospital	Santa Tecla	Joan XXIII	Vall D' Hebrón	Santa Tecla	Vall D'Hebrón	Joan XXIII
Age	38	40	42	36	22	21
Gender (female/male)	female	female	male	female	female	male
BMI (kg/m²)	20,3	22,3	24,9	20,8	24,5	22,5
Biological treatment (Anti-TNF)	-	Infliximab (Yes)	-	Ustekinumab (Yes)	Adalimumab (Yes)	Adalimumab (Yes)
Montreal classification (Supplementary table S3)	-	A2L1B2	A2L1B2	A1L1B2	A2L3B3	A2L1B2
PCR (mg/dL)	-	1,1	0,4	23,6	0,15	0,7

Abbreviations: Crohn's Disease Activity Index (CDAI), Body Mass Index (BMI), C-reactive protein (PCR).

Supplementary table S2. TaqMan probes used to perform qPCR.

Gene symbol	Description	TaqMan Probes
Pro-inflammatory TaqMan probes		
CCL2	Monocyte Chemoattractant Protein 1	Hs00234140_m1
IL1β	Cytokine Interleukin-1β	Hs01555410_m1
IL6	Interleukin 6	Hs00985639_m1
Pro-apoptotic TaqMan probe		
BAX	BCL2 associated X, apoptosis regulator	Hs00180269_m1
Anti-apoptotic TaqMan probe		
BCL2	B-cell lymphoma-2	Hs00608023_m1
Housekeeping TaqMan probe		
18S	18S ribosomal RNA	Hs99999901_s1

Supplementary table S3. Montreal classification of Crohn's Disease.

Table 1. Montreal classification of Crohn's disease

Age of diagnosis	A1: below 16 years A2: between 17 and 40 years A3: above 40 years
Disease location	L1: ileal L2: colonic L3: ileocolonic L4: isolated upper disease (Added to L1/L3 when concomitant upper gastrointestinal exists)
Disease behaviour	B1: non – structuring, non – penetrating B2: structuring B3: penetrating p: perianal disease modifier (Added to B1/B3 when concomitant perianal disease exists)

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