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‘Therapeutic approach with mesenchymal stem cells secretome on a cell model of inflammation and fibrosis.’

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Τίτλος Διπλωματικής Διατριβής: Therapeutic approach with mesenchymal stem cells secretome on a cell model of inflammation and fibrosis

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Thesis title: Therapeutic approach with mesenchymal stem cells secretome on a cell model of inflammation and fibrosis

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I. Περίληψη

Οι ιδιοπαθείς φλεγμονώδεις νόσοι του εντέρου (IBD), η νόσος του Crohn (CD) και η ελκώδης κολίτιδα (UC), είναι χρόνιες φλεγμονώδεις ασθένειες με σημαντική αύξηση των περιστατικών τα τελευταία χρόνια. Η ακριβής αιτιολογία του IBD παραμένει άγνωστη, ενώ ένας μεγάλος αριθμός παραγόντων, όπως το περιβάλλον, η ανοσοαπόκριση και η γενετική προδιάθεση, συμβάλλουν στην παθογένεση αυτών των ασθενειών. Τα επιδημιολογικά δεδομένα υποδηλώνουν ότι ο δυτικοποιημένος τρόπος ζωής είναι ο λόγος της αυξημένης συχνότητας εμφάνισης IBD και παρέχει στοιχεία για το ρόλο που παίζει το περιβάλλον στην εμφάνιση αυτών των ασθενειών. Η θεραπευτική πρακτική που συνήθως χρησιμοποιείται είναι η σταδιακή χρήση ανοσοκατασταλτικών και αντιφλεγμονωδών παραγόντων που ακολουθούνται από τη χρήση βιολογικών παραγόντων όπως το anti-TNF-α. Οι υπάρχουσες θεραπείες, εκτός από την αποτυχία εξάλειψης της ανάγκης για χειρουργική επέμβαση, προκαλούν σημαντικές παρενέργειες, μερικές από τις οποίες είναι πολύ σοβαρές και μπορούν να θέσουν σε κίνδυνο τη ζωή των ασθενών εάν δεν υπάρχει σωστή παρακολούθηση. Για το λόγο αυτό, είναι επιτακτική η αναζήτηση νέων θεραπειών.

Η αναζήτηση πιο αποτελεσματικών και ασφαλέστερων θεραπειών μελετά τη χρήση ανθρώπινων βλαστοκύτταρων από διάφορες πηγές, καθώς και το ρυθμισμένο θρεπτικό μέσον τους (conditioned medium) που μελετόναι ως μορφές θεραπείας για ασθένειες που σχετίζονται με φλεγμονή, όπως η IBD. Ανθρώπινο αμνιακό υγρό- μεσεγχυματικών βλαστικών κυττάρων που ¨έχει ρυθμιστεί¨ έχει ήδη χρησιμοποιηθεί στο εργαστήριο για τη θεραπεία ενός IBD σε πειραματικό μοντέλο ποντικών για επαγώμενη απο DSS ελκώδη κολίτιδα. Η αξιολόγηση της αντιφλεγμονώδους διαδικασίας πραγματοποιήθηκε με ιστοπαθολογική παρατήρηση του ιστού του παχέος εντέρου και ανοσοϊστοχημικές μεθόδους με πραγματικά ενθαρρυντικά αποτελέσματα.

Στην παρούσα σειρά πειραμάτων ανθρώπινοι εντερικοί υποεπιθηλιακοί μυοϊνοβλάστες που φαίνεται να εμπλέκονται στην εντερική φλεγμονή και ίνωση απομονώθηκαν από ασθενή με νόσο του Crohn και καλλιεργήθηκαν στο εργαστήριο. Στη συνέχεια υποβλήθηκαν σε επώαση με LPS (λιποπολυσακχαρίτη: ένα μικροβιακό μόριο) που χρησιμοποιήθηκε για την πρόκληση φλεγμονώδους απόκρισης, χορηγούμενο με ρυθμισμένο μέσο hAF-MSC (conditioned medium) . Στα αποτελέσματά μας παρατηρήθηκε μείωση της έκφρασης των δοκιμασμένων προ-φλεγμονωδών κυτοκινών TNF-α, IL-6 και IL-8, οδηγώντας στο συμπέρασμα ότι το προσαρμοσμένο μέσο μπορεί να έχει θεραπευτικό δυναμικό και την επιβεβαίωση αυτών των αποτελεσμάτων και τη βελτιστοποίηση του ιατρικού τους η χορήγηση θα υποδείξει μια νέα θεραπευτική προσέγγιση για τις φλεγμονώδεις νόσους του εντέρου.

II. Abstract

Idiopathic inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory diseases with a significant increase of incidence in recent years. The exact etiology of IBD remains unknown, while a large number of factors, such as the environment, immune response and genetic predisposition, contribute to the pathogenesis of these diseases. The epidemiological data suggests that westernised lifestyle is the reason of increased incidence of IBD and provides evidence of the role that the environment plays in the appearance of these diseases. Therapeutic practice that is usually used is the gradual use of immunosuppressive and anti-inflammatory agents which are followed by the use of biological agents such as anti-TNF-α. Existing treatments, apart from the failure to eliminate the need for surgery, provoke significant side-effects, some of which are very serious and can endanger patients' life if there is no proper monitoring. For this reason, it is imperative to search for new therapies.

Seeking more effective and safer treatments targeting various sources of human stem cells and their conditioned medium are being studied as forms of treatment for inflammation related diseases such as IBD. Human amniotic fluid- mesenchymal stem cells conditioned medium have already used in the laboratory for the treatment of an IBD in mice model using chemical induced colitis. Evaluation of anti-inflammatory procedure was realized by histopathological observation of the colonic tissue and immunohistochemical methods with really encourage results.

In the present series of experiments human intestinal subepithelial myofibroblasts which are shown to be implicated in intestinal inflammation and fibrosis were isolated by patient with Crohn's disease and were cultivated in the laboratory. Then they were treated with LPS, lipopolysaccharide, a microbial molecule used for the induction of inflammatory response, administrated with conditioned medium of hAF-MSC. In our results was observed reduction in the expression of the tested pro-inflammatory cytokines TNF-α, IL-6 and IL-8, leading to the inference that conditioned medium may have therapeutic potential and the confirmation of these result and optimization of their medical administration will indicate a new therapeutic approach for Inflammatory Bowel Diseases.

III. Introduction

Inflammatory bowel disease (IBD) is worldwide disorder. Its main characteristic is the chronic recurrent inflammation of the intestinal tract, where its origin remains unknown [(1)]. IBDs are a heterogeneous group of diseases with multifactorial etiology that manifest in a genetically predisposed individual, as a result of an excessive immune response to an unknown antigenic stimulus. They present with a variety of symptoms such as chronic or recurrent diarrhea, often with blood clots, abdominal pain, fever, anemia, and cachexia. These symptoms are combined with histological evidence, such as dense infiltration by immunocytes and architectural distortion, laboratory tests that prove the accretion of inflammatory biomarkers, CRP, ESR, PLTs, and fecal calprotectin and endoscopical analyses in which is obvious the loss of normal characteristics of the mucosa, the development of edema, erythema, ulceration, or friability in order to get a distinct diagnosis. In some cases, IBD patients suffer from extra-intestinal manifestations like inflammatory disorders of the skin, eyes, or joints [(1)]. The best understood IBDs are Crohn's disease (CD) and ulcerative colitis (UC), and with higher frequency in developed countries. Crohn's disease (CD) tends to create transmural inflamed patches through the alimentary tract, presents granulomas and it shows relapsing inflammation [(2)]. On the other hand, ulcerative colitis (UC) performs continuous inflammation and ulceration in the mucosa and submucosa, presents micro-abscesses and it usually affects the rectum and the colon [(1)]. Moreover, these two diseases are idiopathic; and there are many other diseases such as chronic granulomatous disease and Behcet disease which can cause immune inflammation of gastrointestinal tract. The long-lasting, progressive, and destructive nature of these disorders in patients, frequently lead the development of complications severe for life safety. These complications can be associated with the appearance of colitis-associated colon cancer, thus many patients must undergo surgery, so the urge of new, more effective, not-invasive therapeutic approaches is highlighted [(20)].

IV. Causes and Pathology

A. Genetic Susceptibility.

As already mentioned, inflammatory bowel disease is a multifactorial disorder, and it is considered polygenic and familial in 5-10% of individuals and sporadic in the remainder [(2)]. Heritability is more important in Chron's disease than to ulcerative colitis. Genome-wide association studies (GWASs) have shown risk loci in 12 chromosomes. More specific, one hundred and sixty-three genetic sites have been identified to be related with the onset of the disease. Additionally, one hundred and ten genetic loci are common in both diseases (CD & UC), while seventy-one are seemed to be specific for the Chron's disease and forty-seven for

the ulcerative colitis. However, these recognized loci represent up to 10-20% of the total range of risk for the disease [2, 4].

These genetic loci seem to be implicated in several crucial pathways for intestinal homeostasis, counting among others barrier function, epithelial restitution, microbial defense, innate and adaptive immune regulation, reactive oxygen species (ROS) generation and autophagy. An interesting fact is that multidisease comparative analysis have revealed that more than the 50% of IBD loci have also been linked with other inflammatory and autoimmune diseases and these overlapping genes can cause different effects in different diseases [5]. As an example, the *PTPN22* (R620W) loci is a risk factor for type 1 diabetes and rheumatoid arthritis in contrast to Crohn's disease which seem to play a protective role. Moreover, genes like *MST1, IL2, CARD9* and *REL* can cause malfunctions in both ulcerative colitis and primary sclerosing cholangitis (PSC). In parallel, *NOD2, C13orf31, LRRRK2, CARD9 LTA, ITLN1* and *IRGM* are some common risk loci to both Crohn's disease and the response of a patient in mycobacterial infections [5].

A wide category of these loci is usually associated with genes that encode transcription factors, especially those that regulate cytokine expression and function and are implicated in T-cell differentiation. For example, some of them are related to the interleukin-23 receptor pathway (*IL23, JAK2, STA3, TYK2, IL12B* and *PTPN2*) and are involved in the maintenance and stimulation of Th-17 cells emphasizing the significance of IL-23-related pathways in human IBD and the important role that they play in many diseases. Several IL23R polymorphisms have been accused of higher risk for IBDs and other diseases [4,5]. The transcription factor STAT3 and the kinase proteins TYK2 and JAK2 are activated responding to IL-6, IL-22, IL-10, IL-21, IL-26 etc. stimulation [4,5]. Additionally, TNF-signaling genes (*TNFRSF9, TNFRSF14* and *TNFRSF15*) encode proteins with a variety of immune effects including systemic inflammation and activation of inflammatory transcription factor NF-κB. Genetic polymorphisms in specific genes, such as *ATG16L1* gene and human *defensing* gene, are able to change the immune response and tolerance to normal lumbar bacteria. This means they can cause an excessive immune response, leading to chronic intestinal inflammation in susceptible individuals [4].

B. Crohn's Disease

Genetic risk sites for Crohn's disease present an overlap in the susceptibility loci of Mycobacteriym Leprae infections, including *NOD2, C13orf31* and *LRRK2* genes [2,4]. Other CD- related genes are associated with the response to mycobacterial infections, such as *CARD9, LTA, ITLN1*, and *IRGM1*. The main high-risk gene for Crohn's disease is NOD2/CARD15 which encodes an intracellular receptor, and it is mainly expressed in Paneth

cells of the intestinal crypt. This receptor recognizes the muramyl-dipeptide (MDP), a component of the bacterial cell wall, and regulates the immune response through NF-κB activation. NOD2/CARD15 was the first gene that was recognized to be implicated in bacterial recognition by the innate immunity and malfunctions of this process are mainly associated with the development of ileal Crohn's disease.

NOD2 consists of two amino-terminal caspase uptake regions (CARDS), a central nucleotide binding region and multiple leucine-rich repeats (LRRs) at its carboxyl-terminus. The most common mutations of NOD2 gene are Arg702Trp, Gly908Arg and Leu1007incC and they are found in 25%-45% of Caucasian CD patients and only in 15%-20% of healthy individuals. It is reasonable to think that the more mutant alleles are carried by an individual, the higher susceptibility and the risk for developing Crohn's disease is accelerated. In addition to increasing sensitivity to Crohn's disease, NOD2 gene variants can also affect the pathology of the patient and the need for surgery [2,4,5].

SMAD3, ERAP2, IL10, IL2RA, TYK2, FUT2, DNMT3A, DENNDIB, BACH2 and *TAGAP* are some more genes that are found to be involved in the onset of Crohn's disease. Most of these genes are implicated in the recognition of intestinal microflora by the innate immune system, or IL-17/IL-23 pathway, or autophagy and they are able to conjure up differences in the manifestation of the disease [2, 4, 5].

In parallel, there many environmental factors which are implicated in the manifestation of the disease. The best studied factors are smoking and intestinal dysbiosis which its result is the raised numbers of intramucosal bacteria [2]. The disorder resembles infectious granulomatous ileitis conditions including intestinal tuberculosis and Johne's disease, caused by mycobacteria and shares similar immune responses to Crohn's disease. Mycobacteria have been identified in tissues and blood of adult and paediatric patients and still remain an important differential diagnosis. Anti-tuberculous drugs have failed to cure the disorder.

C. Ulcerative Colitis

It is known that there is a genetic overlap between the two forms of IBD, with some genes to get involved in the development of both Crohn's disease and ulcerative colitis. However, there are recognized more than 20 genetic loci to be exclusively accused of the breakthrough of the ulcerative colitis, including IL-10, ARPC2, ECM1, HERC2, STAT3 and PTPN2. Also, some resent studies have found some genes, such as HNF4A, LAMB1, CDH1 and GNA12, which are important for the function of the epithelial barrier and which are implicated in the developing of ulcerative colitis and yet the genes with the most important correlation are again IL23R, HLA, CARD9 and MST1 [4,5].

D. Immunobiology

Crohn's disease seems to occur from a diminished interaction of the intestinal commensal microbiota that has a symbiotic relationship with the human host (immune system); still this relation is not fully understood [(2)]. Metagenomic analyses suggest that up to four major bacterial phyla (*Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria*) colonize the human gut with a steep stomach-acid driven, proximal distal gradient. The diversity of these microorganisms usually is influenced by temporal, individual, dietary and drug induced factors [(2)]. Comparative studies showed clustering and reduced diversity especially within the *Firmicutes* and *Bacteroidetes* phyla in patients with Crohn's disease. Especially, the reduction of *Faecalibacterium prausnitzii* (a *Firmicute*), was associated with an increased risk of postoperative recurrence of ileal Crohn's and its experimental restitution had anti-inflammatory effects [(2)]. The diminished commensal diversity is not able to cause Crohn's disease by itself, but it is needed the collaboration of genetic susceptibility [(2)].

V. Normal Intestinal Structure and Function.

The intestinal tract is following the stomach and is composed of the small and large intestine. These two parts have profound functional differences, but they share some structural similarities. The first part of the intestinal tract is the small intestine which is consisted of the duodenum, jejunum, and the ileum. The ileum connects the small with the large intestine via the cecum. The large intestine is consisted of the ascending colon, transverse colon, descending colon, and rectum [(6)]. The whole length of the intestine is covered by a single layer of epithelial cells. Beneath this line of cells is the lamina propria (LP) which is compound of connective tissue full of blood vessels, lymphatic vessels, mesenchymal cells, and enteric nerves, and it supplies the whole intestine with blood, lymphatic cells and innervates through the submucosal plexus. In lamina propria (LP) are maintained many immune cells of both the innate and adaptive immune system.

The enteric enervation is located in the thin layer of smooth muscle which is called muscularis mucosa. The role of muscularis mucosa is to separate the lamina propria (LP) from the underling submucosa. Underneath the submucosa the muscularis, a thick muscle layer consisted of an inner circular layer and outer longitudinal layer. In the middle of these two layers is the myenteric plexus, a crucial element for the enteric nervous system which controls the intestinal peristalsis; the outermost of the intestine is covered by serosa [(6)].

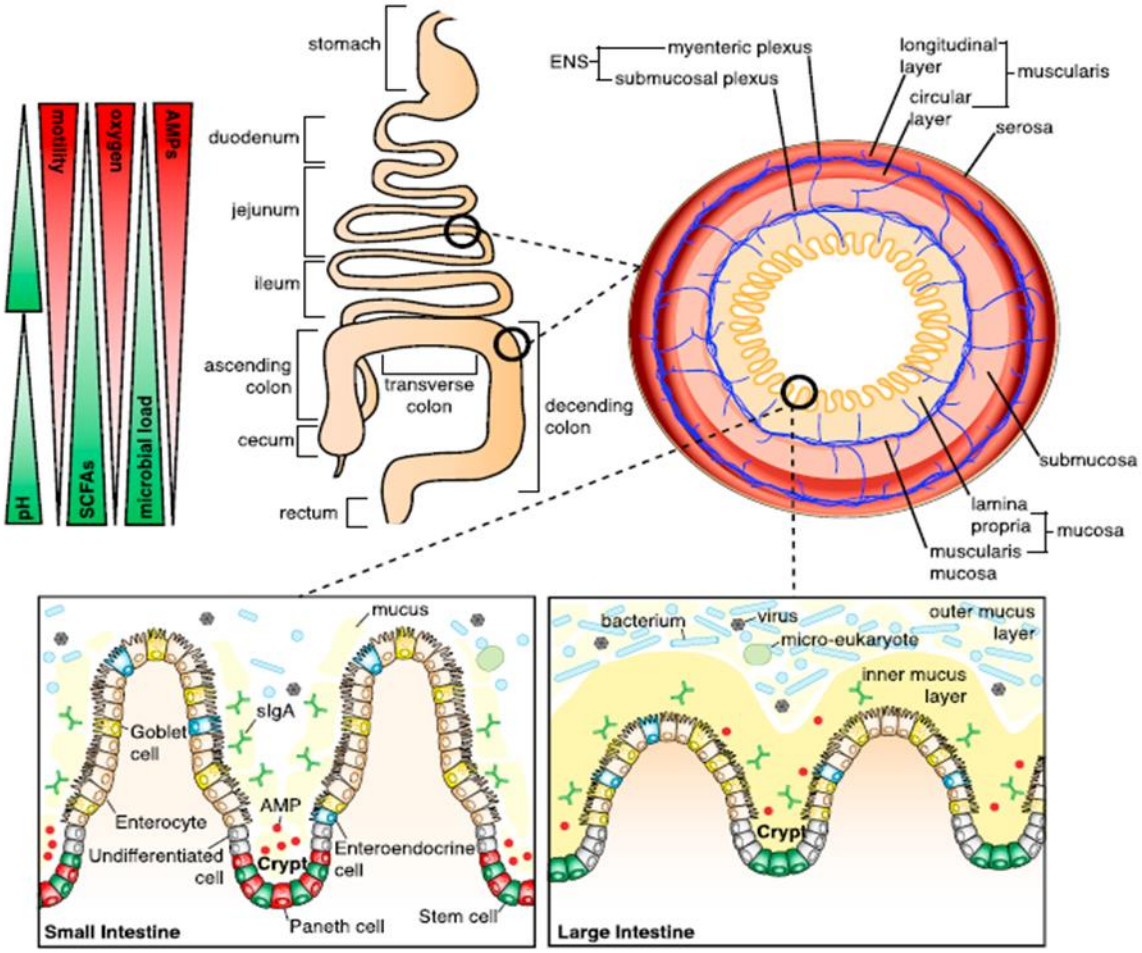


Fig 1. Differentiating Features of the Small and Large Intestinal Landscape.

As it is referred, small intestine is the first part of the intestinal tract. It shows a characteristic structure of long "finger like" villi, which are protruded into the lumen. At the bottom of the villi, the crypts are resided. They are consisted of stem cells, AMP-producing Paneth cells and undifferentiated cells compared to the villi which contain the differentiated enterocytes, enteroendocrine cells and goblet cells. Paneth cells produce α-defensins, C-type lectins, lysozyme and phospholipase A2; this secretion is regulated by autophagic genes. The goblet cells are responsible for the mucus secretion, which is consisted of mucin 2, a single highly O-glycosylated protein and it is secreted into the lumen. In the small intestine the microbiota triggers the enterocytes leading to the secretion of meprin β which cleaves the MUC2 on the surface of epithelium and this fact causes the formation of an untied, non-adherent mucus layer.

The small intestine is compounded of three distinct segments. Each segment expresses specific transcription factors responsible for the regulation of the epithelium. As an example, epithelial cells in the duodenum and jejunum express GATA4. Reduction of GATA4

expression leads these cells to absorb bile acid which is usually limited to epithelial cells of the ileum. The digestive and absorptive function of the intestine takes place in the duodenum and jejunum and is promoted by long villi, as well as microvilli, which is full of digestive enzymes and enzymes proper for the transport of the nutrients. In the small intestine there are higher oxygen levels and antimicrobial peptide (AMP) production, and escalated motility.

One common enzyme in the intestine is the alkaline phosphatase (IAP) which is mostly found in the duodenum. Its function is the hydrolyzation of monophosphate esters, leading to detoxification of microbial ligands such as LPS and it is crucial for maintaining intestinal homeostasis. It is common in patients with Chron's disease and ulcerative colitis to be found a reduced IAP production in the inflamed mucosal. Inflammatory diseases that affect the small intestine often result in blunting of villi, driving in malabsorption and malnutrition as seen in celiac disease and environmental enteropathy.

The large intestine is the following part of the intestinal tract, next to the small intestine. In the large intestine is also found villi, without the strict "finger like" construction. Here, the crypts do not contain Paneth cells; however, there are resided stem cells and undifferentiated cells as long as differentiated cells include enterocytes, enteroendocrine cells, and goblet cells. As Paneth cells are absent, the enterocytes are implicated in the production of AMPs such as β-defensins, C-type lectins, cathelicidins, galectins and lipocalin [(8)]. Interestingly genes that provide resistance in specific classes of AMPs are expressed in the large intestine. In contrast to small intestine, the goblet cells invest the tract with a bilayer mucosal structure with the inner and outer mucus layers due to the absence of meprin β. The inner layer is firmly attached to the epithelium and is considered sterile, in contrast to outer layer which is a looser matrix and hosts microrganisms. This bilayer structure is probably necessary due to the decreased production of AMP, in parallel with elevated bacterial load of the large intestine and the slower transit of feces.

Moreover, the large intestine is responsible for the reabsorption of water and the vitamin uptake (vitamin K, vitamin B12, thiamine, riboflavin etc.). As it is noticed there is a higher microbial load compared to the small intestine and it is the part of the tract that takes place the enzymatic degradation of indigestible fiber by the microbiota, leading to the assembling of short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate. These acids are abundant and have a protective effect on epithelial cells and cause fluid absorption. For example, SCFAs are able to bind to G protein-coupled receptor 43 (GPR43) and play a profound role in various inflammatory conditions, such as colitis, arthritis and asthma. More specific, it can work as sensor for excessive dietary energy and cause the reduction of sensitivity in the insulin stimulation and the fat accumulation in adipose tissue [(6)].

VI. The Barrier Function of the Intestinal Epithelium

As Maynard refers [7], the intestinal tract is the largest barrier in the human body (300 m^3) making it the most extensive portal for entry of commensal or pathogenic microbes. The microbiota of the intestine is constricted by extremely specialized barrier defenses; thus, its main function of nutrient uptake is maintained. The stratified mucosal of the intestine, leads to a layered defense strategy including a highly responsive but no-arrant impenetrable epithelium, and a lamina propria colonized by innate and adaptive immune cells that actively regulate the homeostasis and they restrain the microbiota without undue inflammation [2,7].

However, some species are able to invade the host's tissues and cause diseases. Also, it is possible, changes in the composition of the microbiota, caused by dietary changes, antibiotic treatment or invasive pathogens can disturb the intestinal homeostasis leading to the production of potentially pathogenic constituents. This situation is called dysbiosis and it is able to trigger immune regulatory networks, usually restrained in intestinal inflammation which disrupts the barrier of the intestinal epithelium. The excessive activation of these networks, combined with the absent of down-regulatory mechanisms where they would control the long-term inflammation, are usually associated with the appearance of immune-mediated diseases, especially inflammatory bowel diseases, including Crohn's disease and ulcerative colitis [7].

Gut epithelium is an active sensor and promotes the communication between the host and microbiota. It consists of different cell types, absorptive enterocytes, goblet cells, Paneth cells, M cells and enteroendocrine cells which have a common stem cell ancestor located near the base of the intestinal crypts. Each cell type is both responsive to the microbiota and conditioned by it, performing particular roles in intestinal homeostasis [7]. The epithelial barrier has a polarized structure which is pivotal to its function [2]. The firm junctions of the intestinal epithelial cells (IECs) separate the epithelium into an apical, lumen-exposed surface and a basolateral surface anchored to the basement membrane. The regulation of junctional integrity and paracellular permeability is extremely crucial for the immune system homeostasis [8].

Determined by whether the membrane-associated factors PRRs are expressed on the apical or basolateral surface of the IECs and on which side of the epithelial barrier MAMPs are found, cells of epithelium initiate responses that either induce the release of protective factors that are directed luminally (secreted mucins and antimicrobial peptides) to directly restrain the microbiota, or internally (cytokines and chemokines) to either promote immune quiescence at homeostasis or activate inflammatory immune responses when the epithelial barrier has been breached [7].

The mucus of the intestinal epithelium is the first line of defense against microbes. As it is referred above, it is constantly produced by goblet cells and is consisted of heavily glycosylated mucin proteins and other protective molecules, such as trefoil factor, that are conducive to epithelial restitution and repair. Mutations in MUC2 gene, causes the creation of misfolded multimers that leads to stress conditions in the endoplasmic reticulum (ER) and initiation of the unfolded protein response (UPR) [8]. Microbial products regulate the mucosal production. This is obvious in germ-free mice where the colonic mucous layer is extremely attenuated, despite normal numbers of mucin-laden goblet cells. The stimulation of MAMP lipopolysaccharide or peptidoglycan and benign constituents like butyrate, causes the secretion of mucin and quick reconstruction of the colonic inner mucous layer leading to the maintenance of the mucous barrier. Deficiency of the mucous barrier have shown increased translocation of commensal and pathogenic bacteria which results in the development of spontaneous colitis.

As it is also said, the mucus is separated regionally and presents differences in its thickness and continuity. More detailed, in the proximal small intestine has a slender and discontinuous manifestation, compared to the distal small and large intestine which shows to have higher density. In addition, mucus is stratified into two functionally distinct layers: the inner layer which has a compact solidly adherent structure that is sparsely colonized by bacteria and the outer looser layer which is at least tenfold more densely populated by microbiota.

Even though, there is a Muc2-dominated configuration in both mucosal layers, the proteolytic cleavage of Muc2s' polypeptide backbone that takes place in the outer layer permits the accessibility to colonization by commensals. The resided bacteria enrich this mucous layer with biofilms that exclude pathogens. Furthermore, dietary glycans and mucin glycans are metabolized by these commensal bacteria producing short-chain fatty acids (SCAFs) (like acetate and lactate) which are toxic to some pathogens; other metabolites are also produced (such as proprionate and butyrate) that are the main nutrient source for colonic IECs. SCAFs are able to modulate the host inflammatory responses through the G-protein-coupled receptors on IECs.

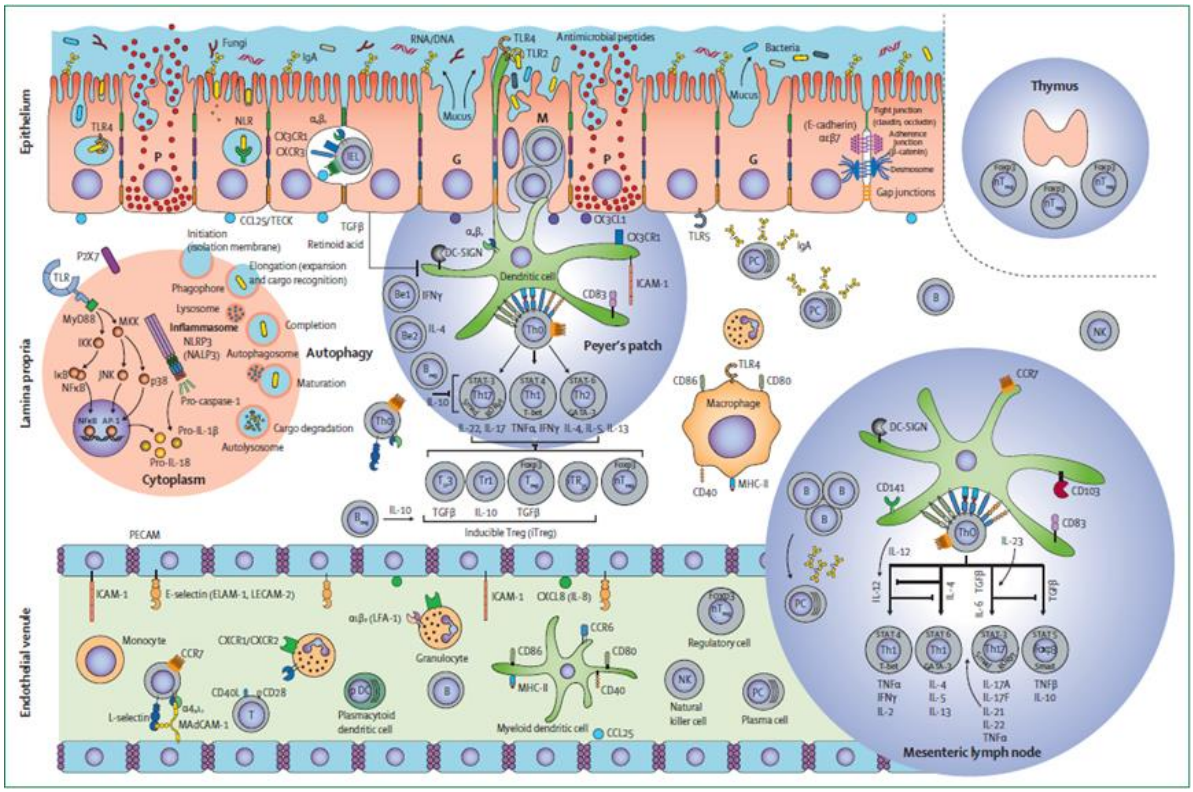


Fig 2. A graphical representation of Intestinal Barrier.

Conversely to the outer mucous layer, the inner mucous layer invests the intestinal tract with a relatively impermeable barrier against microbiota. Its main function is the storage and the maintenance of microbicidal products of the epithelium, including antimicrobial peptides specialized to kill different classes of microbes and sIgA which is shuttled across the epithelium by polymeric immunoglobulin receptor (pIgR) and then is retained in the mucous layer. As a result, the inner mucous layer is a 'killing field' that few pathogens and commensals have developed strategies to pierce. In parallel, the concentration of the antibacterial lectin Reg-IIIγ, which is particularly bactericidal for Gram-positive bacteria, converts this layer to a microbe-sparse zone. A similar spatial segregation of bacteria from the epithelium has been identified in the colon, in which a thicker inner mucous layer excludes most bacteria even in the face of a substantially bacterial load.

The more deficient coverage of protective mucous is found in the proximal small intestine which also has the greatest exposed epithelial surface area to its prominent villous structure. This region of the intestine favors the digestion and absorption of nutrients, but it is also vulnerable to the entrance of microbiota and pathogens. Nevertheless, the bacterial load retains the lowest of the whole intestine. This is, in part, owing to the more vigorous peristaltic motility of the proximal small intestine, which rapidly clears materials (and microbes) from the lumen. The lumen of the proximal small intestine is also the entry point of

the contents of the gall bladder and stomach, which contain high levels of bile salts and acid that have antimicrobial effects.

As it is previously referred, many Paneth cells are resided in the bases of the intestinal crypts and they express zinc, lysozyme, and a wide range of PRRs, they are specialized for the production and release of plentiful antimicrobial peptides, including α-defensins [2,7,8]. Paneth cells sense the microbiota and control their production of antimicrobial peptides via cell-autonomous MyD88-dependent activation of Toll-like receptors (TLRs), thereby limiting barrier breaches by commensals and pathogens [8]. In contrast with other antimicrobial peptides, the production of α-defensins does not need the stimulation from the microbiota, though it is synthesized and stored in Paneth-cell granules. However, the release of the granules is induced by MAMPs stimulation and the active α-defensins seems to influence the composition of the microbiota. Individuals with Crohn's disease usually carry a 300T→A mutation in the ATG16L autophagic gene, which provokes the dysmorphic and malfunctioned granules. *NOD2*, encodes NLR and is crucial in sensing the microbiota and controls the release of antimicrobial peptides by Paneth cells. Deficiencies of antimicrobial peptide production, such as occur in NOD2 mutants, are considered to come out in altered microbial consistency and density in the small intestine, leading to acceleration of susceptibility to intestinal inflammation, especially in the terminal ileum where the vast majority of Paneth cells are resided [2,7].

In Crohn's disease, it is noticed decreased expression of the mucin gene *MUC1* in the inflamed terminal ileum and the paracellular route of fluxes, which are normally blocked by tight junctions, are become leaky, concluding in increased permeability and access of luminal antigens to lamina propria, which is densely populated with immune cells causing acute inflammation [2]. Immune cells in inflammatory intestinal mucosa of IBD patients produce inflammatory factors such as tumor necrosis factor (TNF-) α and interferon-γ. These factors induce the expression of epithelial cell apoptosis related proteins, such as Caspase-1 and inhibit the expression of anti-apoptotic proteins, such as Bcl-2, leading to epithelial cell apoptosis, weakened function of epithelial cells resisting pathogens and increased permeability of IECs [2,9].

VII. Colonic Subepithelial Myofibroblasts (cSEMFs)

Colonic subepithelial myofibroblasts (cSEMFs) are mesenchymal cells with a pivotal role in the pathophysiology of Crohn's disease (CD). They are located immediately subjacent to the basement membrane in the normal intestinal mucosa, juxtaposed to the base of the epithelial cells [31]. They are positioned between the epithelium and immunocytes of the

lamina propria, interacting with both, either directly or via an intricate network of soluble mediators [33]. It seems to play a key role in fibrogenesis process as it has been proven the vast production of ECM components. They are characterized by specific cellular markers of both fibroblast and smooth muscle cells, such as vimentin and alpha-smooth muscle actin (α-SMA) and they are implicated in wound healing [30, 31]. Under physiological conditions, acute inflammation provokes their activation that increase the production of extracellular matrix components in order to heal the wound. Chronic accumulation of immune cells and constant release of inflammatory mediators lead to persistent tissue damage and excessive production of ECM [32, 33].

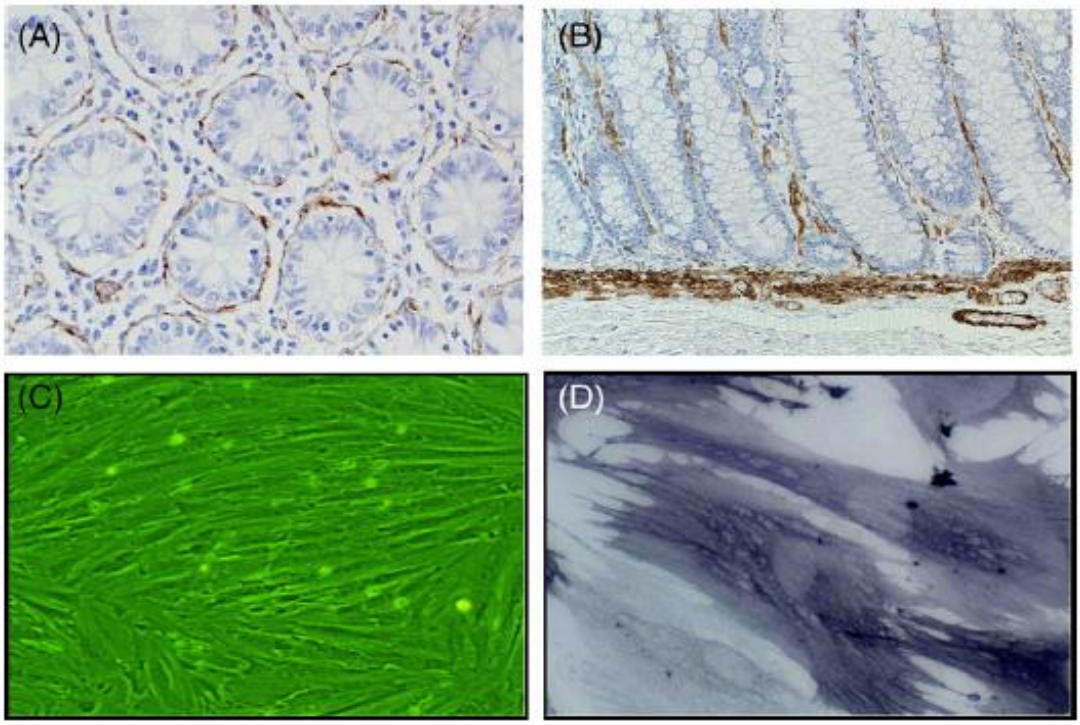


Fig 3. Colonic subepithelial myofibroblasts (ISEMF). (A and B) Immunostaining for α-SMA in the human colon. (C) Isolated human colonic ISEMF. (D) Immunostaining for α-SMA in isolated ISEMF. (35)

Accumulating evidence incriminate colonic SEMFs in autoimmune inflammation and post-inflammatory fibrosis. More specific, SEMFs respond to pro-inflammatory cytokines by upregulating the expression of interleukin (IL)-8, IL-6, monocyte chemotactic protein-1 (MCP-1/CCL-2), granulocyte macrophage colony-stimulating factor and macrophage colony-stimulating factor. They also express Toll-like receptors (TLR)-2 and TLR-4 and other molecules that are stimulated by lipopolysaccharide [32, 34]. Moreover, they have been found to constitutively express class II major histocompatibility complex, and possibly act as non-professional antigen-presenting cells to promote $CD4^+$ T cell differentiation. It has been shown their response to pro-inflammatory and profibrotic cytokines, such as IL-17A and TGF-β, through the increasing production of collagen and MMPs [32].

Epithelial injury and pathogen entry release DAMPS and PAMPS, resulting in activation of TLPs, NOD-like receptors, c-type lectin receptors and inflammasomes on resident macrophages, leading to secretion of pro-inflammatory cytokines and chemokines. These cytokines and chemokines are produced by immune and non-immune cells, are exhibited gradients with main purpose the recruitment of innate immune cells. Platelet adhesion to the damaged endothelium and subsequent PDGF production drives MCP-1/CCL-2 production by endothelial cells, mesenchymal cells, and monocytes, which in turn induces monocyte migration and infiltration at the site of injury. This initial phase of tissue injury is characterized by local infiltration by classically activated (M1) macrophages that secrete IL-1, IL-2, IL-12, 1L-23, TNF-α and reactive oxygen and nitrogen species. All these products participate in myofibroblast activation as part of the normal wound healing, but also link persistent inflammation to fibrosis [32].

VIII. Innate and Adaptive Cellular Immunity in Crohn's Disease.

Microbe associated molecular patterns such as lipopolysaccharide, peptidoglycan-derived muramyl dipeptide, lipoteichoic acid, single and double stranded RNA and methylated DNA (CpG) are recognized by different innate immune-cell populations of the intraepithelial and lamina propria mucosal spaces through pattern recognition receptors such as Toll-like receptors (TLR) and nucleotide binding domain (NOD) like receptors (NLR) [1, 2]. A wide range of pattern recognition receptors are expressed by dendritic cells which interpret microbial patterns and regulate the stimulation of other immune cells leading either tolerance or immunity. They directly sample luminal antigens using transepithelial dendrites [1, 2]. Finally, DCs transport to lymphoid tissues and cause the activation of T cells to secrete cytokines such as IFN-γ and IL-6 inducing immune response. In addition, there is a responsive loop between DCs and Treg cells. Tregs maintain a normal amount of DCs, whilst the reduction of DCs leads to a decrease of Treg cells and aggravation of the intestinal inflammation [9].

Macrophages (also antigen presenting cells) are tremendously increased in intestinal inflamed mucosa. They express a great number of T cell co-stimulating molecules (CD40, CD80, CD86 etc.) and triggering receptor expressed on myeloid cells-1 (TREM-1) which promotes secretion of pro-inflammatory factors [9]. Furthermore, NK cells receive signals from CD1d which is expressed by IECs and perform cytotoxic damage functions devoid of participation of T cell surface receptor or immunoglobulin signals. NK and NKT population are also found increased in IBD patients and their activation is depended on the concentration

of the cytokines. More specific, stimulation of IL-21 can strengthen their cytotoxic killing activity and increase secretion of pro-inflammatory factors (TNF-α and IFN-γ) [6,9].

The adaptive immune system in Crohn's disease is generally considered to mediate and perpetuate, but not initiate intestinal inflammation. The main characteristic of the disorder is an imbalance in the amount of effector T cells versus naturally regulatory T cells. Predominantly T helper (Th1 or Th17) cells protect the mucosa against bacteria, fungi and against viruses through secretion of interferon-γ, TNF-α and interleukins 17 /22 opposite to Tregs which secrete IL-10 and transforming growth factor (TGF) β or IL-35 and repair the inflamed mucosa. These two main opposing cell types (Th17 and Treg) have a common ancestor and which phenotypes is going to be expressed is controlled by RORγ/FOXP3 transcription factor and it shows a reversible regulation [1,2,9]. Stimulation of IL-23, and IL-6 concludes in differentiation of original Th cells into Th1/Th17 cells. These cells secrete IL-21 and IL-22 leading to activation of NF-κB and promotion of a variety of pro-inflammatory factors. Contrarily, Tregs inhibit the activation of Th1/Th17 cells via direct contact and release of IL-10 and high concentration of TGF-β in order to maintain immune tolerance. Tregs are also response for the suppression of occurrence autoimmune diseases. The imbalance model seems to be influenced by genetic loci crucial for the differentiation of effector T cells in Crohn's disease. More specific individuals homozygous to *IL10R* gene mutations derange the strictly regulated cytokine-T-cell balance leading to the consolidation of the Th1 and Th17 phenotype which is linked with the early onset and retention of the disease [1,2,9]. It is needed to refer that to date studies about the resided cellular subpopulations are trying to clarify special cellular patterns for better discrimination among CD and UC as long as the analogization of the disease behavior with the excitingcell types [12,13]. For example, *Natalia Jaeger* performed in a single cell analysis from CD patients, notice, among others, an increased number of $CD39^+$ Th17 cells, subpopulation which is able to induce immunity and tolerance by maintaining Tfh cells [13].

Notably, in inflammatory bowel disease (IBD) has been observed a reduction of the tolerance to self-antigens indicating the production of several serological autoantibodies in distinct disorder subtypes which could be used to predict the course of the disease and the impact of treatment outcomes [10,11]. Research classifies autoantibodies in two groups autoantibodies specified to intestinal and non-intestinal self-constituents and microbial antibodies [10,11]. Indicatively as self-constituent is referred the ANCA (anti-neutrophil cytoplasmic antibodies and perform a perinuclear distribution) and the ASCA (anti-Saccharomyces cerevisiae antibodies) which are used to distinguish CD and UC patients (UC: P-ANCA+/ASCA-, CD: P-ANCA-/ASCA+) [10,11,12]. In addition, anti-OmpC (specific for the

outer membrane porin C transport protein of Escherichia coli) and Anti-I2 (antibody IgA for fragment of bacterial DNA) have been shown to be implicated in disease behaviors and progress (stricruting and/or penetrating) [10,11,12]. To conclude, the utilization of these autoantibodies in clinical practice may offer new perspectives in disease diagnosis and the cure [10, 11, 12].

IX. Immune Driver Components of the Disease

IBD, in line with the most widely accepted hypothesis, is developed in genetically predisposed individuals, because of dysregulated immune response against constituents of the commensal flora, under the influence of undefined environmental triggers. Particular intestinal cytokines have been accused of being mediators in the malfunctions of the innate and adaptive immune responses in disorder pathogenesis [14, 15, 16]. The main mediators are IL-23, IL-6 and TNF-α which are produced by $CD14^+$ intestinal M1 macrophages, even though CD is also related with excessive production of IL-12p40 and IFN-γ/ IL-17 which assails the small bowel and colon [14, 15,16]. In addition, inflammation in CD is influenced by the impairments in the recognition of anti-inflammatory cytokines such as IL-10 and TGF-β which are produced by M2 macrophages, with main role the orchestration and reduction of inflammation. More specific, mucosal immune cells (T cells and macrophages) are maintained in the mucosal. Stimulation caused by these cytokines enhances proliferation, brings resistance against apoptosis leading to accumulation of pro-inflammatory cells and excessive production of pro-inflammatory cytokines resulting in the perpetuation of the inflammatory condition and tissue damage [14, 15, 16].

Nowadays, a wide range of antibodies, special for these cytokines, have been produced in order to control and ameliorate the excessive inflammation and avoid the relapse and remission of the disease. Despite the tremendous advances made in recent years in IBD therapeutics, unfortunately 30% of patients are primarily unresponsive to anti- “cytokine” and even among responders up to 10% will lose their response to the drug every year [17].

X. Epidemiology and Risk Factors

Until the late 1900s IBD was considered as a rare disorder, but in the industrialized of countries in Northern Europe and America has been noticed a steady rise in the incidence of CD after World War II. The geographic region, environment, immigrant population and ethnic groups exhibit a lot of variation in the incidence and prevalence of CD. In the past UC was thought to be more spread, but the increasing number of CD patients in the past few decades makes them both equally prevalent. 201 individuals per 100,000 population are

affected by IBDs. The frequency of IBD's appearance has been stabilized. The incidence of CD is higher in Ashkenazi Jews, urban population, and northern latitudes [39].

The peak of CD manifestation, which is similar between males and females, is among the 2nd-4th decade of life. Crohn's disease started to be appeared to Asia in which previously was not so frequent but remains rare in Africa and South America [39].

Except from genetics factors, which have already been referred, some other factors that can influence the onset and the progress of CD is smoking which is associated with a two-fold increased risk and early onset for active and passive smokers. It has been shown to alter smooth muscle tone, disrupt the endothelial function through nitric oxide production, and influence gut mucosal integrity [39].

Other proven factors are diet and lifestyle. It has been shown that a diet with plenty fibers reduces the risk of CD, with greater score for fibers derived from fruits and not from cereals. These fibers are metabolized by the intestinal bacteria producing short chain fatty acids (SCFA) that do not permit the expression of pro-inflammatory mediators. Furthermore fibers influence the maintenance of the epithelial barrier and reduces the translocation of *E.coli* across Peyer's patches in vitro. In parallel with diet, a good lifestyle including well-sleep, low-stress and exercise has been shown association with lower risk of CD [39].

Moreover, appendectomy, microbiome medication and vitamin D are the rest of the main that can influence the manifestation of the disease. To begin with, it seems that appendectomy has no clear association with the development of the disease, and it is probably correlated with the causes of the operation. For example, appendectomy done for perforating appendix is highly associated with developing CD, but on the other hand appendectomy which is performed for other causes exhibits lower association in disease development. To continue, it is observed that CD patients have reduced diversity in gut microflora compared to healthy individuals with adherent-invasive *E.coli* AEIC, which has the ability to replicate in macrophages, has been presented higher frequency in CD patients. Opposite microorganism like *Faecalibacterium praunitzii*, which is responsible for the production of butyrate, offers protection against CD. The *phyla* and their diversion are depended on dietary patterns with Enterotype 1 (Bacteroides) to be correlated with western-rich in animal protein and saturated fat diet, while Enterotype 2 (*Prevotella*) to be correlated with rich in carbohydrate and fiber diet [39].

Medication can also influence the gut microflora especially if the antibiotics, aspirin, nonsteroidal anti-inflammatory drugs (NSAID) and oral contraceptives are consumed in early childhood. This happens because the gut microbiota is unstable in this period of life, and

disruptions caused by antibiotics could influence the gut immune response and increase the susceptibility to IBD. Especially there is a strong association between NSAIDs and CD, because NSAID might trigger relapse in up to one-third of the users. Finally, it seems that is more frequent for IBD patients to exhibit vitamin D deficiencies compared to healthy individuals. Lower levels of vitamin D have been associated with higher risk of CD related hospitalization, surgery and restored levels attenuate the risk [39].

XI. Diagnosis and Current Therapeutics in IBD

Crohn's disease diagnosis still depends on the combination of endoscopic, radiographic, and pathological findings showing focal, asymmetric, transmural, or granulomatous features [39]. However, there are other diseases such as non-infectious (irritable bowel syndrome or Behcet's disease) and infectious diagnoses (Yersinia or enteroviruses) that mimic Crohn's pathology and makes a blur scenery for proper diagnosis [2, 11, 18, 39]. Following the diagnosis, patients should be phenotyped according to the Montreal classification regarding age of onset, disease localization and behavior; and checked for extraintestinal malfunctions and associated autoimmune diseases [2, 11, 18].

Differential diagnosis for Crohn disease.

1.	Infectious colitis
2.	Ischemic colitis
3.	Radiation colitis
4.	Medication-induced colitis, particularly non-steroidal anti-inflammatory drugs
5.	Ulcerative colitis
6.	Celiac disease
7.	Microscopic colitis
8.	Irritable bowel syndrome

Fig 4. Differential diagnosis for Crohn disease. (39)

Laboratory and serologic markers are used for CD's diagnosis. Initially blood studies, basic metabolic panel, erythrocyte sedimentationrate (ESR), liver function tests, iron studies, vitamin B12 and D levels are examined in patients, in order to detect anemia and vitamin deficiencies [39]. Furthermore, stool studies can prove the existence of fecal neutrophil-derived biomarkers (calprotectin and lactoferrin) and prove the manifestation of inflammation. Although these biomarkers are widely used for indicating active disease or predicting relapse, they are not specific (ie. CRP, lactoferrin and calprotectin) [18, 39]. However, clinicians often use ASCA (antibodies against mannan of *Saccharomyces cerevisiae*) and ANCA

(antineutrophil-cytoplasmic antibodies) which are proved really useful for the discrimination among CD and UC [18]. In parallel, new immunological markers such as autoantibodies against GP2 and CUZD1 targeted by PAB (pancreatic autoantibodies) and anti-microbial antibodies specified for glycans, outer membrane porin C, and Cbir1 flagellin are under evaluation with the prospect of an early and precise diagnosis and prognosis [18].

Endoscopy is routinely used for the diagnosis of CD, assessment its location and obtain tissue for pathological evaluation [39]. The CD's activity and duration can vary the endoscopic appearance. For example, in early mild CD, the mucosa seems normal or small punched-out aphthous ulcers are often observed which are produced by submucosal lymphoid follicle expansion. In moderate disease, the aphthous are coalesced into larger ulcers. As the disease get more severe submucosal edema and injury can lead in cobble-stoning of mucosa, which has been more usual in CD colitis. Individuals with sever disease may have large, linear, and deep serpiginous ulcers (**Fig 5**.).

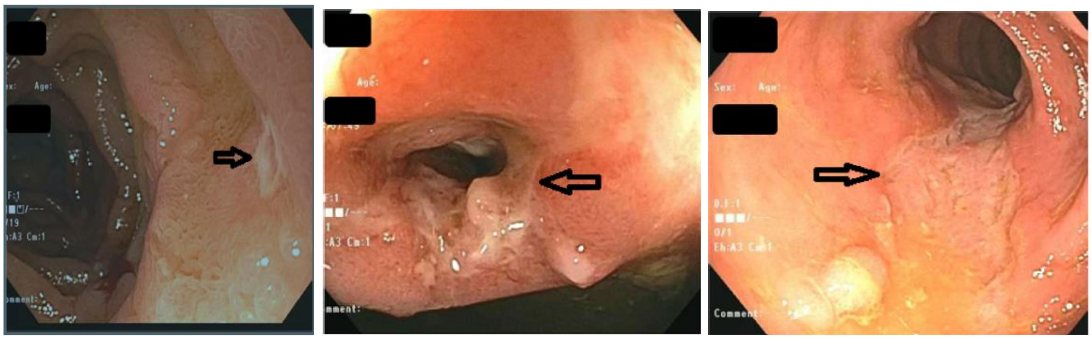


Fig 5. CD's activity Diagnosis using endoscopical evaluation. The first picture represents early mild CD with punched-out aphthous. The second picture represent moderated stage of the disease in which aphthous are coalesced into larger ulcers. The third picture represents severe stage with cobble-stoning of mucosa.

More methods that are used for the diagnosis of CD are, contrast radiography (air contrast barium enema, small bowel follow through and enteroclysis. Computed Tomography (CT) or Magnetic Resonance Imaging (MRI) scans can also be used for confirming the disease location and intestinal complications [39].

Recommended therapies for CD patients are settled according to disease location, disease severity, disease associated complications and future disease prognosis [19]. Also, therapeutic approaches are personalized based on the symptomatic response and tolerance to medical intervention [19]. Cross-sectional imaging (CTE or MRE) and endoscopy (e.g., colonoscopy), are currently used for the evaluation of "maintain response/remission". The main categories of medical therapy that are used to treat CD are 5-aminosalicylates (5-ASA), antibiotics, corticosteroids, immunomodulators, and biologics (the anti-TNF agents' infliximab,

adalimumab, certolizumab pegol; agents targeting leukocyte trafficking, including vedolizumab, natalizumab; and the anti-p40 (anti-IL-12/23) antibody, ustekinumab) [19].

However, it is observed medical therapy failures which are classified in three scenarios, mechanistic failure (is referred to individuals with therapeutic drug levels and no antibodies with the presence of active mucosal ulceration), non-immune-mediated drug failure (is observed in patients who have subtherapeutic trough concentrations and absent antidrug antibodies because of rapid drug clearance) and immune-mediated drug failure (is noticed in patients who have low or undetectable trough concentration and high titers of antidrug antibodies) [19]. Unfortunately, in enteric complications of Crohn's disease surgery remains the main treat. Patients with intractable hemorrhage, perforation, persisting or recurrent obstruction, abscess, dysplasia or cancer or medically refractory disease are gone through an intestinal resection, but there can be recurrence which is defined by endoscopic lesions at the anastomotic site [19]. Ultimately, in spite of the various medical and surgical options, only one-third of patients achieve a long-term remission and a symptom relief [20]. These poor treatment results obvert interest in developing novel treatment options, safe with improved efficacy [20].

XII. Mesenchymal Stem Cell Secretome

Use of mesenchymal stem cells and mesenchymal stem cells secretome is investigated as novel regenerative therapies. They are noticed for their paracrine work fashion and their immunomodulatory properties [20]. Especially in Crohn's disease, evidence indicate the production of anti-inflammatory molecules such as IL-10, TGF-β and indole-amine 2,3-dioxygenase which signal close to immune cells including lymphocytes, dendritic cells and macrophages leading in maintenance of a local anti-inflammatory environment. More interesting is the observation that MSCs are able to upregulate Tregs cells which probably could restore the balance among Tregs and T effector cells [20]. However, there are several notable limitations such as the great number of cells that are required for the transplantation, the ensuring that MSCs reach damaged tissue, the donor variance and senescence before culture expansion, the immunogenicity induced during culture and cryopreservation, as well as all the sort of apparatus that is required makes the use of MSCs difficult in daily clinical practice [20, 21].

These limitations have turned the research interest to the development of acellular regenerative products which perform highly potent immunosuppressive properties [20]. Cell "communication" and regulation is taken place via their secreted substances, the secretome [22, 24], which contains a set of factors/molecules secreted to the extracellular space. These bioactive molecules contain, among others, soluble components, proteins (enzymes, signal

and signaling transduction proteins), immunomodulatory and growth factors (VEGF, SCF, HGF, IGF-1, IGF-2, and SDF-1 [25]), free nucleic acids (DNA, RNA fragments, miRNAs) and lipids that can be enveloped in extracellular vehicles (EVs). These can be subdivided into apoptotic bodies, microparticles and exosomes and they are consisted of a lipid bilayer and proteins (tetraspanins, integrins, ligands for cell surface receptors) [22, 23,24]. Also, these molecules can be found free and soluble in concentrated conditioned medium.

Nowadays, the use of a secretome product that contains both EV and soluble proteins is an attractive physiological approach to maintain tissue homeostasis. Considering the diverse range of proteins that are secreted and the heterogeneity of vesicles that are released from MSCs, it becomes impossible for a unique factor to be isolated. Nonetheless, a secretome therapeutic product permits the enrichment of therapeutic factors and thereby maximizes its effect [38].

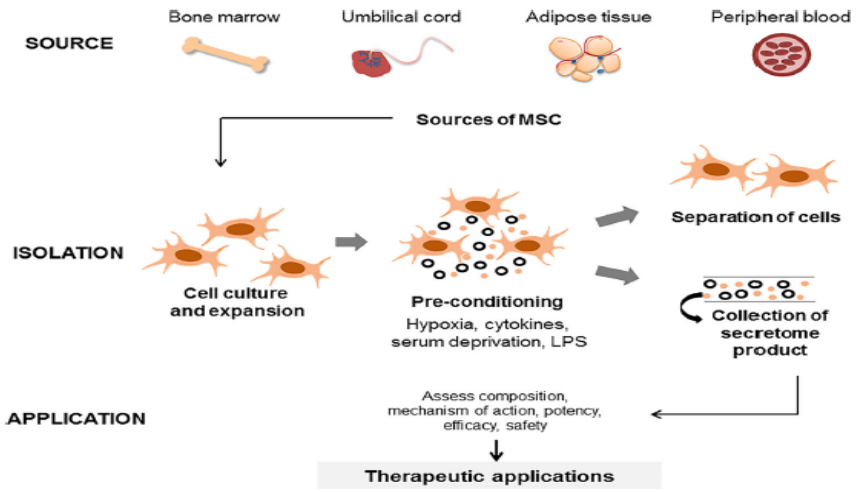


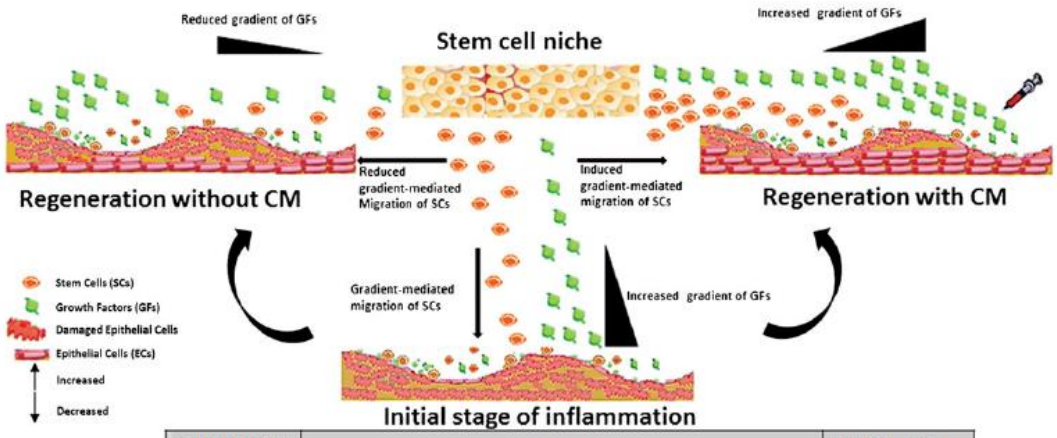
Fig 6. Approaches to developing an MSC secretome based therapeutic agents. An MSC secretome-based therapeutic product can be generated from autologous stem cells obtained from common sources such as bone marrow, or adipose tissue. The cells are expanded in culture, followed by conditioning to enhance release of soluble proteins or EV and resulting in an enhanced secretome product. The conditioned media is subsequently collected and further isolation procedures to remove the cellular components, such as using ultracentrifugation or tangential flow filtration, can be performed to isolate the acellular secretome product.

For succeeding this, the protein and vesicular content of the MSC secretome could be manipulated in several ways to meliorate favored functional effects. Different procedures have been described for stem cells conditioning and finally manipulation of secretome and EVs contents. These could be culture conditions such as modulation of oxygen tension, variations in matrix, serum deprivation, fluid shear or compression, or 3-dimensional culturing. For example, hypoxic pre-conditioning of MSCs can enhance the angiogenic potential of the secretome product. Also, hypoxia conditioning can raise the production of chemokines and several immune mediators such as IL-6, IL-5, and IL-1b.

Moreover, induction of differentiation can also alter the secreted product. This becomes obvious in the secretome product from MSC of umbilical cord origin, which when the cells are differentiated into hepatocyte-like cells, the secretome contains higher amount of glycoprotein MFGE8 and has superior anti-fibrotic effects [38].

These soluble factors and EVs are founded in MSC-derived conditioned medium (MSC-CM) with the option to be collected by centrifugation, filtration, chromatography etc. or act via the direct administration of the conditioned medium itself in the region of interest [24]. Crucial for using the MSC-CM is to understand which substances are the most useful for the observed effects. Research have shown that conditioned medium treated by trypsin and RNase had diminished its therapeutic potency. Also, the cellular source of CM can influence its protein content. For example, experiments have noticed that conditioned medium collected from ASCs 3D spheroid culture showed a 20-fold increased expression to pro-angiogenic factors (VEGF, bFGF, HGF and CXCL12) compared to the CM collected from ASCs monolayer culture. This probably explains the superiority of spheroid-collected CM in its ability to facilitate wound healing in a model of hind limb ischemia. Moreover, the cellular origin influences the constitution of the concentrated CM. Membrane-bound proteins are another class of CM factors with potential clinical relevance. For example, neprilysin is a membrane-bound enzyme with the ability to degrade β-amyloid peptide, a hallmark of Alzheimer disease. ASCs exosomes are characterized by a higher concentration of neprilysin compared to BM-MSCs exosomes and it has been shown that ASCs exosomes but not BM-MSCs exosomes, can reduce β-amyloid peptide levels in mouse neuroblastoma cells [36].

High-throughput RNA sequencing analysis showed that the majority of RNA molecules that are found in CM and EVs are non-coding RNAs, including long non-coding RNAs, ribosomal RNA etc. Some experiments shown that MSCs with knockdown of Dorsa, an enzyme with pivotal role for miRNA generation, produced EVs which had presented impaired protective effects in mouse acute kidney injury (AKI) compared to normal EVs. [36, 37]. Also, different studies have shown a correlation between upregulated miRNA expression in EVs and improved EV protective effects [36]. Among others, EVs also carry long no-coding RNAs acting as regulators and mRNAs. The proof the EVs carry messenger RNA has come by the observation of mouse proteins which were expressed by human cells after treatment of the latter with mouse EVs [36]. Nonetheless, because of the great diversity of RNA molecules and our limited knowledge about each of their function, more thorough research in necessary for choosing the most relevant CM product to the intended clinical application [36].



WITHOUT CM	EVENT	WITH CM
↑	Inflammation	↓
↑	Apoptosis	↓
↓	GF gradient towards niche	↑
↓	Migration of SCs towards site of inflammation	↑
↓	Proliferation of ECs	↑
↓	Vascularisation and engraftment of cells	↑

Fig 6. A graphical representation of the potential role of conditioned medium obtained from stem cell cultures and the events that occur during tissue generation.

In many experimental research MSC-sourced secretomes exhibited immunoregulatory, angiomodulatory and anti-apoptotic effects that led in enhanced tissue repair and regeneration [22, 23, 24, 25]. Returning to previous statement, several immunomodulatory factors such as TGF-β, hepatic growth factor (HGF), indolamine 2,3-dioxygenase-1 (IDO-1), IL-10, IL-1Ra and are concentrated in MSC-CM and MSC-EVs. Administration of MSC-CM manage to inhibit generation of inflammatory Th1 and Th17 cell by arresting cell cycle of naïve T-bet and RORγT-expressing T cells in G1 phase and by inducing tolerogenic and regulatory phenotype in DCs [22, 23, 24, 25].

In patients with Crohn's disease MSCs have been engrafted in their gut and two scenarios were observed. In the first scenario, where the patient presented dominant Th1 and Th17 immune response and elevated concentrations of IFN-γ, TNF-α and IL-17, MSCs developed an anti-inflammatory phenotype by producing IL-10 and KYN leading to inhibition of proliferation, activation, and effector function of inflammatory M1 macrophages, Th1 and Th17 cells, resulting in CD alleviation. Conversely, in the scenario of no abundance in Th1/Th17 inflammatory cytokines, the engrafted MSCs adopted a pro-inflammatory phenotype, produced large amounts of inflammatory mediators which promoted migration and activation of neutrophils and effector T cells resulting in aggravation of CD [22, 23, 24, 25].

Aiming to avoid unwanted effects of MSC-based therapies, several research groups, investigate therapeutic potential of MSC-sourced secretome as cell-free therapy for IBD. Much experimental evidence has been accumulated using DSS-induced chronic colitis mouse models. Seunghun Lee et al., Maryam Heidari et al. indicated the amelioration of clinical symptoms, the restoration of weight loss and repair of damaged tissue in MSC-CM treated mice via histologic examination [21, 27]. Also, in agreement with Legaki's et al. results it was shown a reduced expression of a wide range of inflammatory cytokines and chemokines specialized in the modulation of systemic immune response [21, 26].

Furthermore, investigation in TNBS-induced colitis in mice, have shown neuroprotective effects in ENS after the initiation of intestinal inflammation [28]. In parallel, CM-MSC treatment seems to reduce cartilage destruction in antigen-induced arthritis (AIA) in mice and prevention of high levels TNF-α production [29]. More research in the field will determine which specific factors are responsible for these immunomodulatory effects for CM to be used as a cell-free therapy tool not only in idiopathic, but also in autoimmune diseases [25,28].

XIII. Aim of this thesis

The aim of this study is to discriminate whether there are changes in subepithelial myofiblasts inflammatory expression profile after their treatment with conditioned medium. Cells were isolated from Crohn's Disease patients and were cultured in LPS in order to induct inflammation. Following they were cultured in SS-AF-MSC conditioned medium and cell pellets were collected and used for the evaluation of inflammatory genes expression levels, TNF-α, IL-6 and IL-8 via Real Time PCR. The expression differences could provide evidence for the reduction of inflammation and for efficiency of CM-MSC as a new and innovative approach for IBD cell-free therapy in humans.

XIV. Materials and methods

A. SS-AF-MSCs Conditioned Medium

For CM preparation, 1.5 x 10^6 SS-AF-MSCs were cultured until 80 % confluency and further allowed to grow in DMEM (Sigma-Aldrich) media, containing 0.5 % FBS (Gibco-BRL) for 48 h at 37 °C in a 5 % (vol/vol) humidified CO_2 chamber. At 48 hr of culture, percentage of dead SS-AF-MSCs was estimated by trypan blue staining (Gibco-BRL) at 9.96 ± 1.29 %. Conditioned media were collected and the total number of living (0 %) or dead cells (0 %, trypan blue positive cells) was estimated. CM was then centrifuged at 1200 rpm for 10 min and the supernatants were then filtered. The supernatant was moved in a new falcon. CM was centrifuged at 2.500G for 30min using a suitable ultra-filtration device (Millipore, Bedford, Massachusetts, USA) which is suitable for concentration (10–25 fold) of the original volume and for the collection of protein with molecular weight higher to 3 kDa (21). The total protein amount was applied in 6-wells plates contained 80% confluency of Subepithelial Myofibroblast (SEMF's). There performed two different conditions, one with 100μl of CM and one with 500μl.

B. Colonic Subepithelial Myofribroblas (cSEMF) thawing

Prior to starting the procedure, the water bath was turned on at 37^{O}C and the culture medium was heated. Heated medium was placed inside the hood and 10ml was transferred to a 15ml centrifuge tube. The cryovial was removed from -80^{O}C and it was placed inside the hood for 1min incubation at room temperature. Following the cryovial was incubated in the water bath and was swirled gently until thawed. The cells were resuspended by pipetting up and down and they were moved to the prepared 15ml centrifuge tube. They were centrifuged at 3000G for 7min, and the media was discarded. 2 ml of fresh medium was added, and the cells were resuspended and transferred to a 25cm^2 flask containing 4ml media. The flask was moved back and forth and side to side to evenly distribute the cells. The flask was placed into the incubator and after 24hr was observed in an inverted microscope and changes the medium on order to discard dead cells and debris.

C. cSEMF passaging

When cell population was confluent to a 90-100% in a 75cm^2 flask, the old media was removed from the flask, and it washed 3 times with D-PBS without Ca^{2+} and Mg^{2+}. 3ml Trypsine/EDTA was added and swirled gently for the Trypsine to cover the entire flask surface. 1,5ml Trypsine/EDTA was removed, and the flask was incubated for 3 min in 37^{O}C. Then, the flask was observed in the microscope for detaching cells. 10ml of fresh media was added in the flask in order to collect as many cells as possible and they were transferred to a

15ml falcon. Then, they were centrifuged at 300G for 7min. The old media was discarded, and 1 ml of fresh media was added, the cells were resuspended and transferred to new 75cm^2 flask which was contained 11ml media. The flask was moved back and forth and side to side to evenly distribute the cells and was placed into the incubator.

D. cSEMF Culture

Cells were isolated by CD patients. Colonic subepithelial myofibroblast at passages 2-5 were used in our studies. The cells were cultured in complete medium (10%vlv FBS, 1% v/v Penicillin-Strepromycin, 1ml Amphotericin B and 5ml MEM non-Essential Amino Acids 100X). All experiments were performed with FBS-free media (the rest of the ingredients were included) 95% culture confluence with a stable ratio of supernatant volume-to-surface available for cell adhesion (1,5mL:9.6cm^2).

There performed several conditions for our study. In the initial set of experiments the cells were cultured in 6-wells plates (10^4 cells per well), and they were untreated or treated with LPS (100ng/ml) and the incubation duration were 1hr, 4hr and overnight. In the second set of experiments, cells were incubated in conditioned medium (CM) (100µl and 500µl) or conditioned medium with 100ng LPS. The duration of incubation was the same (1hr, 4hr and O/N).

Following to incubations serum from the wells was collected and stored, and cell pellets were washed with cold D-PBS, lysed with NucleoZol Reagent and kept at -80^{O}C until the RNA Isolation/Purification.

E. RNA Extraction and Quantitative Real Time PCR

For the RNA extraction 500µl NucleoZol were added in each well of the 6-well plate. The cell pellets were detached and transferred to appropriate named tubes. 200µl RNase-free water was added to each tube, the samples were shaken vigorously, and they were incubated at room temperature for 5 minutes. The samples were centrifuged at 12000G for 15 min at room temperature. 500µl of the supernatants were moved to properly named fresh tubes. 500µl isopropanol were added to each tube in order to precipitate RNA. The samples were incubated on the bench at room temperature for 10 minutes. Then they were centrifuged for 10 minutes at 12000G. The supernatant was discarded. Following 500µl 75% Ethanol was used in each tube. The tubes were centrifuge for 3 minutes at 8000G. Then the ethanol was removed and the pellet dried. The RNA pellets were dissolved in 35 µl RNase-free water.

For the cDNA synthesis, Takara PrimeScript 1st strand cDNA Synthesis Kit was used, and the purpose is the production 0,8µg/ml cDNA. For each reaction was used 2µl Buffer, 0,5µl Enzyme, 0,5µl Oligo dT, 0,5µl Random 6-mere. The volume of water and RNA were

depended on RNA samples consecration. Each reaction was initially incubated at 37^{O}C for 30 min. Following the samples remained at 85 ^{O}C for 5'min and they are stored at -20^{O}C.

Assessment of the TNFα, IL6, IL8, mRNA levels was performed by employing the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels as a reference gene. Quantitation was performed using SYBR-Green PCR master mix (Applied Biosystem, Foster City, CA, United States) on an ABI Prism 7700 apparatus (Applied Biosystem). The thermal profile consisted of 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 1 min, and elongation with optics on for fluorescence monitoring at 72 °C for 1 min. For each individual reaction, a final melting curve analysis from 72 to 95 °C was performed to ensure the homogeneity of PCR products.

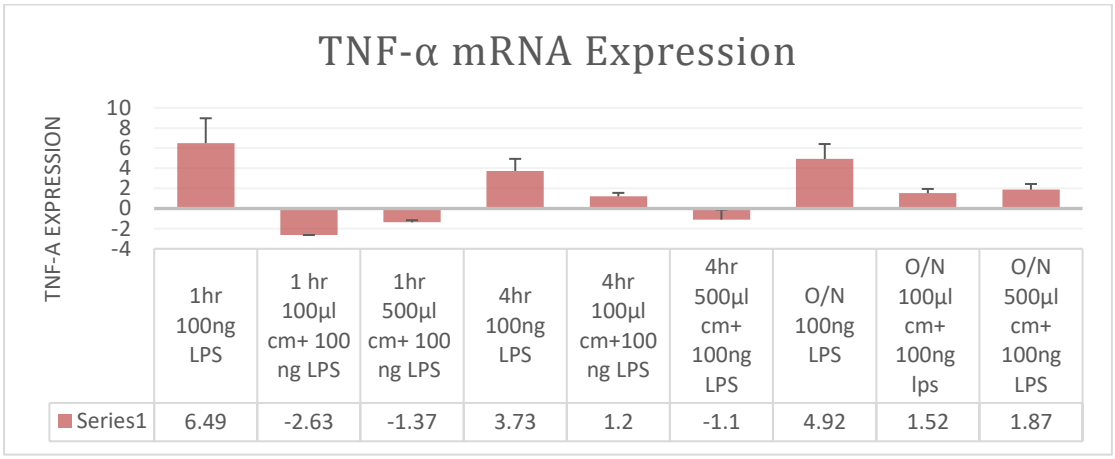
The primers' sequences were as follows:

TNFα-(Fw)	5′-CACGTCGTAGCAAACCACCAAGTGG-3′
TNFα-(Rv)	5'-GATAGCAAATCGGCTGACGGTGTGG-3′
IL-8-(Fw)	5'-ACTGAGAGTGATTGAGAGTGGAC-3'
IL-8-(Rv)	5'-AACCCTCTGCACCCAGTTTTC-3'
IL-6-(Fw)	5'-CCAGCTATGAACTCCTTCT-3'
IL-6(Rv)	5'-GCTTGTTCCTCACATCTCT-3'
GAPDH-(Rv)	5′-TTCACCACCATGGAGAAGGC-3′
GAPDH-(Fw)	5′-GGCATGGACTGTGGTCATGA-3'

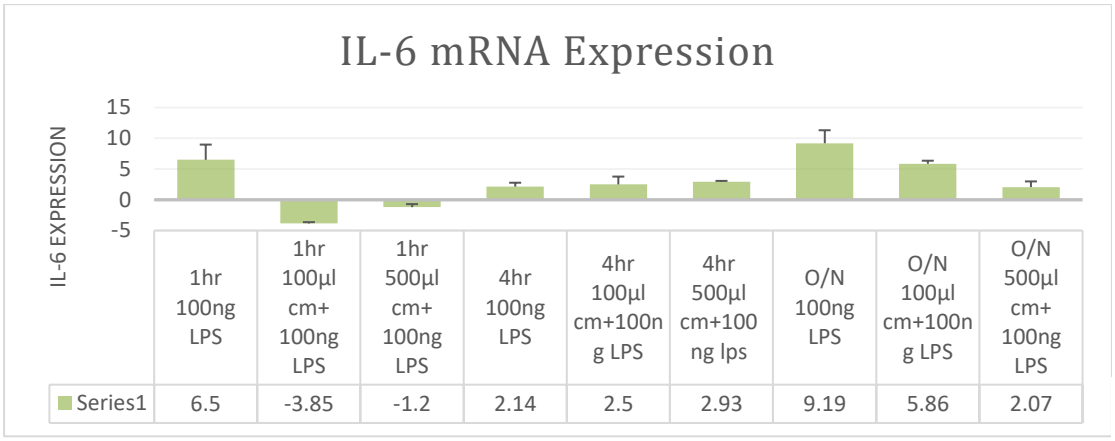
Table 1. Each used primer's sequence.

XV. RESULTS

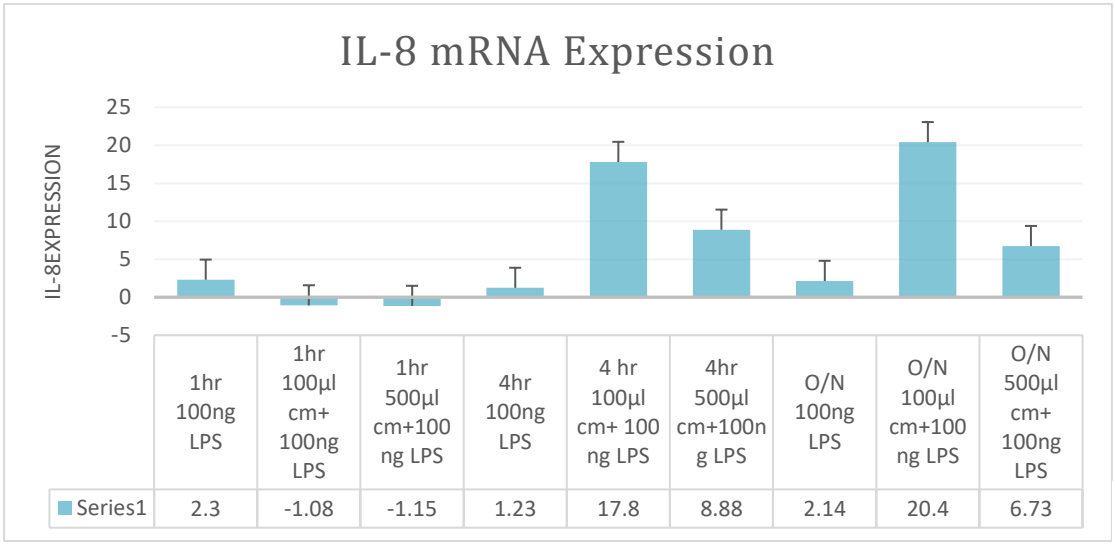
A. CM–SS-AF-MSC Treatment Reduced the Expression of TNF-α, IL-6 and IL-8 in human Subepithelial Myofibroblast of a patient with Crohn's disease.



a.



b.



c.

Fig. The relative expression of TNF-a, IL-6 and IL-8 as it was found from real time RT-PCR.
a. TNF-α. Each condition was performed as duplicate (p=0,0008).
b. IL-6. Each condition was performed as duplicate ((p=0,0008).
c. IL-8. Each condition was performed as duplicate (p=0,0206).

It has been reported previously that SS-AF-MSC CM treatment exhibits an anti-inflammatory role in mouse models related to inflammatory diseases [21]. More specifically, recent evidence support that CM treatment can cause the downregulation of pro-inflammatory and upregulation of anti-inflammatory cytokine levels [21]. Moreover, in our lab it has been shown that in mice colonic sections in the distal and proximal tracts, CM treatment significantly decreased the extension and severity of the inflammation in the DSS-treated mice compared to control once.

In order to investigate the changes in the inflammatory profile of human cells we examined the mRNA levels of proinflammatory (TNF-α, IL-6, IL-8) cytokines after the treatment with LPS and CM. We cultured human subepithelial myofibroblast in different time frames using 100ng of LPS and 100μl or 500μl conditioned medium respectively. Notably, we found that CM treatment decreased the inflammatory response as it is noticed from the relative expression levels of TNF-α, IL-6 and IL-8 which performed 3-fold reduction in their expression (with $p_{value}<0.1$) and which are related in systemic inflammation and were significantly reduced. More specific conditioned medium downregulates the expression of TNF-α in all treatment conditions and durations. Whereas 'this effect is more tense in the 1st hour of our treatments for IL-6 and IL-8, while it is presented declined in the 4 hours and overnight treatment.

XVI. Discussion

Crohn's disease is characterized by multitude of presentations, extra-intestinal manifestations, and complications [39]. More specific, Crohn's disease is characterized by ongoing mucosal inflammation in which a dysfunction of the host immunologic response against dietary factors and bacteria are involved. The chronic inflammatory process leads to the disruption of the epithelial barrier and formation of epithelial ulceration. Resolution of inflammatory activity is associated with repair processes that facilitate tissue remodeling, which restores normal intestinal architecture. In Crohn's disease patients often occur stricture formation associated with excess fibrosis. Whilst, the etiology of the disease is undefined, it is acknowledged that epithelial cells are able secrete and respond to a wide pallet of immunological mediators, thus presuming that these cells may play an important role in IBD pathogenesis. Especially colonic subepithelial myofibroblast are known for their implication to fibrosis in extensive response to inflammatory factors [30].

Intestinal subepithelial myofibroblast (ISEMFs), as it is referred above, are mesenchymal cells with features from both fibroblast and smooth muscle cells, localized in the mucosa immediately subjacent to the basement membrane, at the interface between enterocytes and lamina propria [43]. In IBD mucosa, there is a relative increase in the number of a-SMA-positive cells as compared with normal mucosa. The increase in a-SMA-positive cells is remarkable at the margin of deep ulceration in UC patients. a-SMA-positive cells are also relatively increased in inflamed mucosa of CD patients. Mckaig et al. [47] previously demonstrated that proliferation of SEMFs isolated from CD patients is significantly greater than that of SEMFs derived from normal or UC patients. Also, it has been proved that IGF-I and procollagen mRNA levels are elevated in the involved mucosa of CD patients.

It is known that they mediate information among epithelium and the mesenchymal elements of the lamina propria by secreting various cytokines, growth factors, and inflammatory mediators. Characteristic cytokines that are produced by ISEMFs are IL-6, IL-8 and MCP-1 in response to IL-17, lipopolysaccharide stimuli etc, leading to the recruitment and activation of immune cells. Unregulated cytokine production may lead to excessive recruitment of leukocytes into the intestine concluding in uncontrolled inflammatory states and development of chronic pathologic conditions. Patients with Crohn's disease exhibit an increased number of myofibroblast. As a result, is the excessive collagen production and in abnormal immune response concluding in fibrosis. Deregulation of IL-6 synthesis has severe effects on immune response and is associated with various immune mediators of inflammatory disease [44].

In our study we investigate the way that conditioned medium might influence the expression levels of TNF-α, IL-6 and IL-8 inflammatory factors which are produced by intestinal subepithelial myofibroblast. It is known that among others, TNF-α is the main mediator produced by $CD14^+$ intestinal macrophages which are infiltrated into the inflamed intestine CD patients. The epithelial barrier function which comprises fencing properties against small ions as well as larger molecules as antigens has been seemed to be severely disrupted in CD. TNF-α which is found in elevated levels in patients with CD is thought to play a central role in this barrier defect. Studies have been indicated that TNF-α reduced epithelial barrier function by affecting both induction of single cell apoptosis and the epithelial tight junction [15, 41].

In CD, mucosal immune cells such as macrophages and T cells have been accused of being active leading in the maintenance of intestinal inflammation. The main characteristics of the involved T cells are the excessive proliferation, resistance against apoptosis, and massive production of pro-inflammatory cytokines [15]. Resistance against apoptosis causes the accumulation of pro-inflammatory cells that are involved in the initiation and perpetuation of inflammatory reaction. In these conditions, TNF-α signaling can drive into two different pathways, the first includes the induction of survival and proliferation of cells via the NF-kB signaling and the second includes the initiation of apoptosis via regulation of caspases [15,41].

Initially is expressed as a membrane bound form (mTNF) by immune cells like macrophages. mTNF can be processed by the TNF-α converting enzyme (TACE) into a soluble form (sTNF) which is able to activate both TNFR1 and TNFR2, whereas sTNF mainly activates TNFR1 on immune cells like CD4+ T cells. The binding of mTNF or sTNF to TNFR1 or TNFR2 can lead to apoptosis, proliferation, and different cytokine production [15].

Anti-TNF agents are commonly used as a highly efficient therapy in Crohn's Disease, underling the pivotal importance of cytokines like TNF in the immunopathogenesis of the disease. Even though, they are often effective in the resolution of CD, leading to endoscopic remission, they however fail in a relevant subgroup of patient, resulting in ongoing inflammation with signs of endoscopic disease activity [15]. One third of individuals with CD do not demonstrate therapeutic efficacy upon initiated anti-TNF therapy (primary non-response); additionally, 30%-50% lose response to therapy in the course of treatment (secondary non-response) [15].

Especially, secondary non-response to anti-TNF therapy has initially been associated with immunogenicity. Interestingly, the application of another anti-TNF antibody in these patients often also fails to show therapeutic efficacy. Except of neutralizing antibodies against the

anti-TNF agents, insufficient levels of the drug in the serum have also been thought to interpret inefficient therapeutic response [15]. Opposite, a recent study proved that a large cohort of patients who lose response to anti-TNF therapy show sufficient levels of anti-TNF antibody in the serum and do not develop relevant levels of neutralizing antibodies against TNF. This leading to the thought of existed TNF-independent forms of CD that confound resolution of inflammation leading to failure of anti-TNF agent therapy. All these findings indicate that immune cells and the interplay of cytokines in the inflamed mucosa of CD patients must be regarded as critical components for the resolution of inflammation process [15].

The second inflammatory cytokine that we examined is Interleukin-6 which is mainly involved in inflammation by controlling differentiation, proliferation, migration, and apoptosis of target cells. A malfunction in the complex regulatory cytokine network might lead to acute and chronic inflammation, autoimmune diseases, or neoplastic disorders [42]. It has been shown that IL-6 modulates the intestinal immune system by increasing the expression of adhesion molecules on endothelial cells enabling transmigration of phagocytes and lymphocytes to inflammatory regions. IL-6 is a pleotropic mediator and plays an important role in pathogenesis of IBD. It exerts its biological effect via signaling through its membrane-bound IL-6 receptor or via trans-signaling by binding to a soluble form of IL-6 receptor and subsequently to the membrane-bound glycoprotein 130 [43].

IL-6 exhibits both pro-and anti-inflammatory properties. In several animal models of human diseases, it was shown that on target cells IL-6 classic signaling mediates the activation of anti-inflammatory and regenerative pathways. In a murine colon cancer model IL-6 classic pathway is essential for the activation of STA3-mediated signaling pathways which include the regeneration of intestinal epithelial cells after DSS induced damage [42]. In a pancreatitis model it was shown that the absence of IL-6 leads to a more severe inflammation response. In contrast, IL-6 trans-signaling is observed in chronic inflammatory disorders such as Crohn's disease, atherosclerosis, and rheumatoid arthritis. Trans-signaling was responsible for anti-apoptosis of lamina propria T cell in chronic intestinal inflammation leading to activation of the immune system by the recruitment of monocytes to the inflamed area via activation of endothelial cells and smooth muscle cells. Stimulation of T-cells by IL-6 trans-signaling leads to inhibition of apoptosis, inhibition of Treg differentiation, and differentiation of T_H17 cells [42].

Patients with inflammatory bowel disease (IBD), have increased risk to develop colorectal cancer, and IL-6 has emerged as potential therapeutic target. Experiments in IL-6 deficient mice exhibited decreased tumor load in the murine dextran sodium sulfate-azoxymethane

(DSS-AOM) colitis-associated carcinogenesis model, proposing that IL-6 was necessary for tumor development and growth [42]. Also, IL-6 $^{-/-}$ were more inflamed indicating that IL-6 plays a role in regenerative response of the intestinal epithelium. Nevertheless, mice which did not express STAT3 in the intestinal epithelial cells demonstrated complete lack of regeneration in response to the irritant dextran sodium sulfate.

In parallel, IL-6 is accused of the appearance of colon cancer. Inactivating mutations in the TGFbeta receptor II are frequently observed in patients with this type of cancer. In mice, the expression of dominant-negative TGFbeta receptor II variant in T cells led to higher frequency and size of tumors in the AOM-DSS colitis-associated carcinogenesis model which was dependent on IL-6 trans-signaling. Whilst, IL-6 was expressed by intestinal epithelial cells, lamina propria $CD4^+$ T cells, $CD11c^+$ dendritic cells, sIL-6R was shed by tumor cells directly [42].

High levels of IL-6 and soluble form of IL-6 receptor (IL-6r) are present in serum and in the intestinal tissue of CD patients. The IL-6 signaling is crucial to the pathogenesis and physiopathology of CD. Excessive IL-6 production in CD patients is related to etiology of this pathology, leading to chronic inflammatory response [44]. A 1995 study reported that the high levels of IL-6 in the serum are correlated with C-reactive protein levels [45]. Base to these observations, tocilizumab, an anti-IL-6 antibody, was evaluated in a placebo-controlled phase II randomized controlled trial (RCT). Despite the encourage results of the trial, the development of tocilicumab for Crohn's disease did not procced due to rare reports of gastrointestinal perforations observed in concurrent clinical trials in arthritis and because of an increased understanding of the homeostatic role of IL-6 in intestinal epithelium. Combined this evidence proposed that patients with Crohn's disease might be at increased risk of potential detrimental effects of IL-6 inhibition [45].

The last factor that we examined is IL-8 or CXCL8 [46]. It was firstly recognized as a small molecular weight protein secreted by stimulated human mononuclear cells that induce polymorphonuclear cell (PMN) chemotaxis, degranulation, and superoxide and hydrogen peroxide (H_2O_2) production for body's antimicrobial defense mechanisms [46]. It is expressed by a variety of resident and invading cell types within the gastrointestinal (GI) tract, including monocytes, macrophages, endothelial cells, epithelial cells, fibroblasts, and neutrophils [46].

The expression of IL-8 occurs acutely during gastrointestinal (GI) infection caused by bacteria, viruses, and parasites, while elevated levels of this factor are also found in patients with chronic GI inflammation. Dysregulation of IL-8 has been accused of contributing to the pathophysiology of various disease states and it has been proposed to be targeted for therapeutic prospects [46].

To begin with, its expression is regulated by NF-κB signaling which response to pro-inflammatory stimuli. The activation of NF-κB can be occurred by a variety of host- or pathogen-derived proinflammatory stimuli that bind different receptor families, including Toll-like receptors (TLRs), nucleotide oligomerization domain-containing protein 1 and 2, interleukin-1 receptor (IL-1R) and tumor necrosis factor α (TNFα) receptor [46]. It has been shown that NF-κB p65 accumulation in the nucleus leads in subsequent transcription and translation of IL-8 in GI cell lines. In parallel, inhibition of NF-κB pathway in in vitro intestinal epithelial monolayers reduces the IL-8 mRNA production. It is necessary to refer, that in inflamed mucosal biopsy specimens collected from IBD patients there were noticed increased expression of both activated NF-κB p65 and IL-8 [46].

Numerous research findings have stablished that IL-8 regulates the recruitment of PMNs into GI tissues. During an acute inflammatory response, granulocyte colony-stimulating factors are released, and this leads to CXCL 12 (a factor responsible for the retention of PMNs in the bone marrow) expression, in parallel with acceleration of CXCR2 ligands, including IL-8 by endothelial cells concluding in promotion of PMN bone marrow egression [46]. Following this, PMNs leave the vasculature and enter tissues via postcapillary venules in a series of highly characterized steps [46].

Endothelial cells can transcytoses IL-8, which is produced by various cell types, to the luminal surface where it is subsequently bound to heparan sulfate. As a result, the secreted chemokines are immobilized and able to induce firm adhesion and migration of the circulated PMNs. Binding of IL-8 to its receptor triggers signaling cascade that enhances the binding affinity and avidity of PMN integrin molecules (CD11a/CD18 etc.). This increases the affinity to endothelial surface ligands such as intercellular adhesion molecules and undergo firm adhesion and crawling of PMNs into the inflamed tissue [46].

The aim of this migration to an inflammatory or infectious site is the host cleaning of invading or translocated microbes using the production of oxidants. Also, PMNs carry a wide range of granules, organelles full of noxious antimicrobial compounds which are divided into three subsets based on their contents, order production and release within host tissues. Especially in IBD patients, mucosal biopsy tissues collect from areas of active inflammation have exhibited excess PMN-derived oxidants. Also, ROS production has been shown to contribute to intestinal epithelial damage in patients with IBD [46].

IL-8 has been characterized as a PMN-activating compound which induced PMN degranulation. Among others, proteolytic enzymes are contained in the granules. As an example, PMN elastase is increased in fecal samples collected from patients with CD, while separate reports have indicated that its antagonists, elafin and secretory leukocyte protease

inhibitor (SLPI) are significantly decreased. To date it has been shown that administration of elafin in in vivo models of colitis, reduced the PMN tissue infiltration and the IL-8 expression. In parallel, some pathogens have the ability to release proteases that proteolytically degrade IL-8 leading to a constant infection. Moreover, this ongoing damage during chronic inflammatory states triggers a multitude of changes within host tissues that can result in the development of cancer [46]. To conclude, research has demonstrated that IL-8 is elevated in a variety of diseases states, including GI infection, IBD exacerbations and GI malignancy, can be used as predict clinical marker and is responsible for proinflammatory PMN events. However, the accurate mechanism that IL-8 orchestrate the induction of inflammation remain unclear [46].

As it is referred above, there is a great effort for the alleviation of patients with Crohn's disease, but conventional medical therapy fails to this purpose, with failure to be presented by three different scenarios, mechanistic failure, non-immune-mediated drug failure, and immune-mediated drug failure [19]. Unfortunately, in enteric complications of Crohn's disease surgery remains the main treat. Patients with intractable hemorrhage, perforation, persisting or recurrent obstruction, abscess, dysplasia or cancer or medically refractory disease are gone through an intestinal resection, but there can be recurrence which is defined by endoscopic lesions at the anastomotic site [19]. Ultimately, despite the various medical and surgical options, only one-third of patients achieve a long-term remission and a symptom relief [20]. These poor treatment results obvert interest in developing novel treatment options, safe with improved efficacy [20].

It has been shown that MSC treatment can cause the reduction of a wide range of inflammatory cytokines and chemokines that inflect the systemic immune response. Hence, many research have been investigated the use of MSC therapy to treat inflammation disorders, including IBD [21].

Cell therapy protocols generally require hundreds of millions of MSCs per treatment, therefore, cell expansion in vitro is needed for about 10 weeks before implantation. The patient's age and clinical characteristics influence the optimal culture conditions for clinical scale production of human MSCs. The cellular senescence of MSCs in vitro contributes to aging and age-related diseases [22]. Research have proved that implantation time of MSCs can influence their therapeutic impact, indeed, it has been reported that <1% MSCs survive for more than one week after systemic administration. Also, MSCs typically express major histocompatibility complex (MHC)-I, but lack expression of MHC-II CD40, CD80 and CD86 on the surface of the cell, leads MSCs to escape T-cell recognition and the engraftment fails to orchestrate the immune response in the transplant area [22].

Regardless, the accurate mechanisms that cause MSCs effects must be elucidated. It has been proposed that the effective results of MSCs are appeared either via engraftment and differentiation by replacing damaged tissue and/or as tropic suppliers by stimulating tissue repair through the secretion of paracrine factors [21,22]. Particularly it has been shown that their therapeutic effects are not prompted by engraftment and differentiation, but instead, depends on the paracrine and/or endocrine factors that MSCs secret favoring wound healing and restoring the disrupted balance [21].

It has already mentioned that the product of the MSCs is the MSC secretome and conditioned media. This product is defined as the set of factors/molecules secreted to the extracellular space. These factors which are included in the secretome are specific and change in response to fluctuation in physiological states or pathological conditions and it can influence the proliferation, activation, and function of immune cells. Pre-clinical studies on animal models have shown a suppressive effect on both innate and adaptive immunity [22], thus we hypothesized that there could be similar effects in human cell types such as intestinal subepithelial myofibroblasts which are accused of implanting in immune response.

Generally, it is well established that the anti-inflammatory effect of MSC-CM is at least in part mediated by soluble immunoregulatory molecules, such as tumor necrosis facto β1 (TGFβ1), IL-13, IL-18 etc., as well as pro-inflammatory cytokines such as IL-1b, IL-6, Il-8 and IL-9. It is possible that the balanced concentration of all types of cytokines may determine the final effect. Recent studies in an experimental model of uveitis in rats, indicated that hUCESC-CM treatment significantly reduced mRNA expression of IL-6, IL-8 TNF-α and MIP-1a pro-inflammatory cytokines and increased the mRNA expression of the IL-10 anti-inflammatory cytokine [22]. Also, it was found that hUCESC-CM reduced the infiltration of leucocytes in ocular tissues. All these experimental evidences, have turned the interest in the investigation of conditioned medium effects in human inflammatory conditions.

In our lab's previous studies, AF-MSCs were found to secret numerous anti-inflammatory molecules such as interleukins IL-10, IL-1ra, IL-13 and IL-27, as well as angiogenic factors such as angiopoietin-1, PD-ECGF uPA and endostatin/collagen XVIII. Also, it has been investigated the anti-inflammatory effects of SS-AF-MSCs secretome in DSS colitis mouse model. An intraperitoneal injection of CM at the onset of the DSS-colitis improved the clinical and histopathologic severity of colitis. Furthermore, data collected by real-time RT-PCR analysis support that the CM-treatment significantly downregulated the expression of inflammation markers such as TNF-α and IL-1b at mRNA level, while was noticed upregulation of anti-inflammatory cytokines such as IL-10 [21]. In addition, it is known the higher concentration of active immune cells in the intestine in IBD which overproduce pro-

inflammatory cytokines such as TNF-α etc. and is implicated for the long-term immune response [21, 40].

In the present study we concentrated to examine if the SS-AF-MSC secretome can induce anti-inflammatory effects in subepithelial myofibroblast. Based on previous studies, SS-AF-MSCs represent an advantageous cell type due to their inherently low immunogenic profile and high proliferative ratio compared to adult MSCs. The collected CM was used for the treatment of our cells collected from a CD patient. These cells were stimulated by LPS which is known for the induction of inflammation in these cells and then they were incubated in condition medium.

In agreement with our previous research, data collected by real-time RT-PCR analysis support that the CM-treatment significantly downregulated the expression of inflammatory markers such as TNF-α, IL-6 and IL-8. at mRNA level in human cells for first time. As it is referred above every one of these proteins cause inflammatory stimulation. TNF-α is the best-known inflammatory conductor, overexpression of IL-8 could lead in impairments of adaptive immunity and an interesting function of IL-6 is the differentiation of original Th cells into Th1/Th17 cells which are the dominant cell type for the persisted inflammation. Downregulation of all these factors in the same moment could probably lead reduction of Th1-Th17-driven inflammatory responses conclude in an inflammatory remission. Of course, further functional in vivo and in vitro studies are necessary to uncover the exact mechanism by which the conditioned medium presented anti-inflammatory effects on intestinal subepithelial myofibroblast.

Nowadays, only a limited number of human clinical studies are already available on the use of secretome products from MSCs. For example, application of allograft ADSC-CM, after the treatment of fractional carbon dioxide laser resurfacing on human skin, enhances wound healing by the reduction of adverse effects such as hyperpigmentation, erythema and increased trans-epidermal water loss. Likewise, intradermal injection of ADSC-CM into the scalp of alopecia patients using a split-scalp study design significantly promoted hair growth in both male and female patients. Similarly, a retrospective and observational study of female pattern hair loss treated with ADSC-CM showed efficacy after 12 weeks, significantly increasing both hair density and thickness. None of the patients reported severe adverse reactions. Finally, BMMSC-CM has also been used safely to improve alveolar bone regeneration [22].

In parallel, clinical applications of MSC-derived exosomes have been reported. A preliminary study demonstrated that increasing dosage of MSC-derived exosomes in a patient with severe treatment-refractory graft-versus-host grade IV disease, affecting skin and

intestinal tract, was well tolerated and showed a significant and sustainable improvement of symptoms, which remained stable for five months. Another clinical trial applied MSC-derived exosomes for improving-cell mass in type 1 diabetes mellitus patients. Many more studies are expected to be initiated shortly [22].

As described earlier, MSC derived CM preparations and EVs have demonstrated significant potential to treat diseases with high unmet medical needs, as well as safety and product handling advantages over MSCs. To progress this new class of therapies to clinical trials and ultimately to commercialization and widespread use, it must be established processes compliant with the GMP that can be scaled to levels necessary for commercial production and quality control criteria that would be both practical for implementation and linked to the product potency. CM and EVs therapeutics can be produced by multiple MSC lineages which influence and the constitution of its product and so their specificity [36].

Observations from our laboratory propose that paracrine effects and secreted molecules from SS-AF-MSCs might present an attractive tool for cell-free therapy for IBD. Generally, MSC-sourced secretome bypassed cell therapy limitations including unwanted differentiation, potential activation of allogeneic immune response or tumor formation. Secretome-based approaches using CM or exosomes may present considerable potential advantages over living cells in terms of manufacturing, storage, handling, product shelf life and their potential as a ready-to-go biological therapeutic agent. Multiple experimental studies demonstrate that secretome-derived products are sufficient to significantly improve multiple biomarkers of pathophysiology in many animal models of different diseases. Also, MSC-conditioned medium is able to be massively produced by the commercially available cell lines exempting from invasive cell collection procedure and it could be immediately available for an individual's treatment. Moreover, it is a low-cost and convenient source of bioactive factors which can be evaluated in manners analogous to conventional pharmaceutical agents [22, 23, 24, 25]. However, proteomic analysis of secretomes has revealed some challenges such as the low amounts of secretions produced by cells and high solubility of some proteins [48]. Non-secretory proteins released by the lysis of dead cells and contamination in the culture medium are some limitations of secretome applications [48].

Although secretome application as a cell-free method can be a promising alternative to stem cell therapy, various problems arise to be dealt with before clinical translation. Since there are complex interactions among secretome molecules during tissue repair, the determination of a therapeutic and clinical schedule is difficult. Another limitation of secretome is related to its hard collection and preparation and analysis workflow. In addition, some secreted factors have very low concentrations because of culture media dilution.

Moreover, dead cells or apoptotic cells secreted proteins contaminate the secretome. Therefore, collection of MSC-conditioned medium can be done carefully without interruption of any other contaminant molecules. Finally, proteomic analysis interferes with salt and other compounds that exist in the culture media, which makes specific protein precipitation almost necessary for a proper proteomic analysis [48].

However, thorough validation of the key molecular determinants and mechanism (s) mediated by CM [21,22], standardization of isolation procedures, conditioning and characterization of potency of secretome products is really high need before their systematic use as therapeutic agents not only for inflammatory bowel disease, but also in much more other disorders [38].

Much research underlines the diverse nature of the CM-proteins and EV contents and the functional variation of the MSC secretome. Also, the anatomical origin of the MSC can influence our choice for using them. For example, the hUCESCs conditioned medium, produced by hUCESCs are obtained by Pap cervical smear, which is much easier to get, because is the less invasive and painful method than those used for obtaining other MSCs, such as bone marrow or adipose tissue. These may give chances for bespoke functional applications based on detailed understanding of cell behavior, state of differentiation and response to external milieu (22).

Considering that multiple clinical trials involving MSCs have been approved by national agencies, it is perfectly reasonable to expect approval of secretome-derived products from MSCs. Although the composition of secretome-derived products from MSCs is complex, this itself should not be an impediment for regulatory approval of a regenerative product. For example, platelet-rich plasma or amniotic fluid, which are highly complex and include numerous growth factors and exosomes that remain poorly characterized, are routinely used as a regenerative therapy for multiple applications in wound healing and orthopedics.

Exosomes sourced from dendritic cells have already reached the clinical-trial stage for immunotherapy of certain cancers. Nevertheless, regulatory requirements for manufacturing and quality control will be necessary to establish the safety and efficacy profile of these products [22]. Further, secretome-based approaches reduce biological variations and enable precise dosing, which contributes to the development of safe and efficient therapeutic systems with potentially predictable results. However, further research is needed to optimize the isolation and evaluate the efficacy of MSC-derived secretome and identify suitable indications for therapeutic applications [48]. We hope that this thesis will encourage such researchers to investigate the therapeutic potential of MSC-derived secretome.

To conclude, our experiments showed that conditioned medium can cause the reduction of inflammatory cytokine production in intestinal subepithelial myofibroblast. This evidence agrees with many experimental data from animal models. We have to consider that this was the first time for conditioned medium to be administrated in actual human cells, isolated from individual with a certain disorder and lead in decreased expression of the mediators of inflammatory response. This may propose a new therapeutic approach for the cure and remission of Crohn's disease.

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