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PhD Program in Biomedicine and Pharmacy

CHARACTERIZATION OF INTESTINAL MICROBIOTA AND METABOLITE-SENSING GPCRs IN INFLAMMATORY BOWEL DISEASES: RELEVANCE OF GPR91 AND GPR109A

Doctoral Thesis

CRISTINA BAUSET PASTOR

Directors:

Dr. Mª Dolores Barrachina Sancho

Dr. Jesús Cosín Roger

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Dña. **Mª Dolores Barrachina Sancho,** Catedrática de la Universitat de València y D. **Jesús Cosín Roger,** Profesor Ayudante Doctor de la Universitat de València,

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Que el trabajo presentado **CRISTINA BAUSET PASTOR,** titulado *"Characterization of intestinal microbiota and metabolite-sensing GPCRs in Inflammatory Bowel Diseases: Relevance of GPR91 and GPR109A"*, para obtener el grado de Doctor, ha sido realizado bajo nuestra dirección y asesoramiento.

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(Cançó de l'aire – Oques grasses)

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ABBREVIATIONS & ACRONYMS

^{1}H-NMR	Proton Nuclear Magnetic Resonance
5-ASA	5-Aminosalicylic Acid
ABC	Avidin-Biotinylated Peroxidase Complex
AIM	Absent in Melanoma
AJs	Adherent Junctions
AMPs	Antimicrobial Peptides
APS	Ammonium Persulfate
ASCA	Anti-Saccharomyces Cerevisiae Antibodies
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CCA	Constrained Correspondence Analysis
CD	Crohn's Disease
cDNA	Complementary DNA
CRP	C-Reactive Protein
CTGF	Connective Tissue Growth Factor
DAB	Diaminobenzidine Tetrahydrochloride
DC	Dendritic Cells
DSS	Dextran Sulphate Sodium
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal-Regulated Kinase
ET	Endothelins
FA	Fatty Acids
FBS	Fetal Bovine Serum
FCp	Fecal Calprotectin
FFAR	Free Fatty Acid Receptor
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GC-MS	Gas-Chromatography Mass Spectrometry
GLP1	Glucagon-Like Peptide 1
GPCRs	G-protein Coupled Receptors

GWAS	Genome Wide Association Studies
HCAR	Hydroxyl Carboxylic Receptor
HDAC	Histone Deacetylase
HIER	Heat-induced Epitope Retrieval
HRP	Horseradish Peroxidase
HSIFs	Human Small Intestine Fibroblasts
IBD	Inflammatory Bowel Disease
ICAM	Intracellular Adhesion Molecule
IECs	Intestinal Epithelial Cells
IFN	Interferon
IFX	Infliximab
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
IL	Interleukin
IL-1R	IL-1 Receptor
JAK	Janus Kinase
LCFAs	Long-Chain Fatty Acids
LC-MC	Liquid-Chromatography Mass Spectrometry
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MCFAs	Medium-Chain Fatty Acids
MCT	Monocarboxylate Transporter
MEK	MAPK/ERK Kinase
MHC	Major Histocompatibility Complex
MMPs	Matrix Metalloproteases
mTOR	Mammalian Target of Rapamycin
NFκB	Nuclear Factor Kappa Light Chain Enhancer of Activated B cells
NLRC	NLR Family Card
NLRPs	NOD-like receptors
OGR	Ovarian Cancer GPCR
PAB	Antibodies Against Exocrine Pancreas
PAGE	Polyacrylamide Gel Electrophoresis
PAMPs	Pathogen-Associated Molecular Patterns

pANCA	Perinuclear Anti-Neutrophil Cytoplasmic Antibodies
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor
PMA	Phorbol Myrstate Acetate
PPAR	Peroxisome Proliferator-Activated Receptor
PUFAs	Polyunsaturated Fatty Acids
qPCR	Quantitative Polymerase Chain Reaction
RAS	Renin-Angiotensin System
RT-qPCR	Real-Time Quantitative PCR
S1P	Sphingosine-1-Phosphate
SCFAs	Short-Chain Fatty Acids
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
sIgA	Secretory Immunoglobulin A
SMCs	Smooth Muscle Cells
SMCT	Sodium Monocarboxylate Transporter
SNPs	Small Nuclear Polymorphisms
STAT	Signal Transducer and Activator of Transcription
TBS-T	Tris-Buffered Saline Tween 20
TCA	Tricarboxylic Acid
TDAG	T Cell Death-Associated Gene
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFF3	Trefoil Factor 3
TGF	Transforming Growth Factor
Th0	CD4+ Th cells
TIMP-1	Tissue Inhibitors of Metalloproteinase
TJs	Tight Junctions
TLRs	Toll-Like Receptors
TNBS	2,4,6-Trinitrobenzenesulfonic Acid
TNF	Tumor Necrosis Factor
Treg	T Regulatory Cells
UC	Ulcerative Colitis
UPLC-MS	Ultra-Performance Liquid-Chromatography Mass-Spectrometry

VGF	Vascular Growth Factor
WT	Wild Type
α-SMA	α-Smooth Muscle Actin

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RESUM

INTRODUCCIÓ

La Malaltia Inflamatòria Intestinal (MII), que inclou patologies com la Colitis Ulcerosa (CU) i la malaltia de Crohn (MC), és un trastorn del tracte gastrointestinal que es caracteritza per la inflamació crònica i l'alteració del sistema immunitari. Durant les darreres dècades, la incidència i prevalença d'aquesta malaltia han augmentat contínuament convertint-la en un problema seriós de salut pública mundial. Hui dia, es desconeix l'etiologia exacta d'aquest trastorn, però està àmpliament considerada com una malaltia multifactorial caracteritzada per la complexa interacció de la predisposició genètica, els factors ambientals, el paper de la microbiota intestinal i la resposta immunitària. Com a resultat, les persones afectades d'aquesta malaltia poden arribar a patir una sèrie de complicacions greus, tals com la fibrosi intestinal o les fístules, que influeixen de manera significativa en la seua qualitat de vida. Habitualment, les persones afectades de MII també pateixen complicacions extra-intestinals tals com artritis, colangitis o infeccions del tracte urinari, així com problemes psicològics tals com ansietat, fatiga o depressió. És per això que aquesta malaltia requereix d'un abordatge multidisciplinari i específic. Actualment, no existeix cap teràpia farmacològica que revertisca per complet aquestes malalties, o que previnga les complicacions associades. És per això que conèixer les seues causes, complicacions i la relació entre tots els factors que participen en la seua fisiopatologia són crucials per tal de millorar el diagnòstic, l'abordatge i l'enfocament terapèutic.

La CU és la forma més comú de MII i es caracteritza per una inflamació de la mucosa que sol començar al recte i pot estendre's o no a altres parts del còlon. Depenent de la localització de la part afectada i l'extensió de la inflamació, es distingeixen els següents subtipus d'acord amb la classificació de Montreal: proctitis, colitis esquerra o pancolitis, quan afecta a tot el còlon. Les principals manifestacions clíniques d'aquesta patologia inclouen diarrea amb sang, i depenent del grau d'extensió i severitat de la malaltia també presència de moc, així com altres símptomes tals com febre, dolor i còlics abdominal, pèrdua de pes, anèmia i

disminució de la gana. Quan la inflamació és severa, sagnat excessiu, megacòlon tòxic o càncer poden també arribar a desenvolupar-se.

La MC és el subtipus de MII que pot afectar qualsevol part de l'aparell digestiu, tot i que l'ili i el còlon solen ser les parts més afectades. Aquesta patologia es caracteritza principalment per una inflamació transmural i asimètrica que pot afectar a qualsevol part de la paret intestinal incloent la mucosa, submucosa, muscular o serosa. A més, aproximadament la meitat de persones afectades de MC desenvolupen complicacions en forma d'estenosi, fístules i abscessos, que poden requerir cirurgia. Els diferents subtipus de MC que reconeix la classificació de Montreal d'acord amb el comportament de la malaltia són: B1 o fenotip inflamatori, B2 o fenotip estenosat i B3 o fenotip fistulitzant. Les manifestacions clíniques més comuns són dolor abdominal, diarrea crònica, pèrdua de pes i fatiga. A més, depenent de la localització i la severitat de la inflamació, també poden anar acompanyades de sagnat rectal, i diarrea amb sang.

Per diagnosticar la MII, s'avalua la clínica de la persona afectada i es realitzen una sèrie de proves complementaries com ara l'anàlisi de sang, on es quantifiquen els nivells de certs biomarcadors, tot i que aquests no solen proporcionar un diagnòstic específic. És per això que altres tècniques d'imatge com ara la colonoscòpia o ileoscòpia, i la confirmació histològica mitjançant biòpsies, solen ser també necessàries per a determinar el diagnòstic.

Tal i com s'ha comentat anteriorment, actualment no existeix cap teràpia capaç de revertir per complet aquesta malaltia i per tant, l' actual abordatge terapèutic té com a objectiu principal el tractament simptomatològic i el control de la inflamació per previndre els brots actius. Els principals tractaments consisteixen en teràpies biològiques basades en la immunosupressió que exerceixen també un efecte anti-inflamatori. L'infliximab ha estat el tractament per excel·lència però al llarg del temps, deixa de tindre resposta en les persones afectades, per tant, s'han desenvolupat noves teràpies biològiques més eficaços i amb perfils més segurs.

Durant els últims anys, s'han dut a terme grans esforços per tal de millorar el coneixement sobre aquesta malaltia i caracteritzar més profundament els factors que contribueixen al seu desenvolupament. En aquest aspecte, la microbiota intestinal juga un paper fonamental preservant la salut intestinal. Una de les característiques de les persones afectades de MII és la disbiosi intestinal, una alteració desequilibrada en la composició i funcions dels microorganismes residents al tracte gastrointestinal. S'ha observat que aquesta disbiosi agreuja la inflamació i contribueix a la progressió de la malaltia. A més, diferents estudis han demostrat en biòpsies intestinals de persones afectades per MII una disminució del nombre total d'espècies bacterianes a l'intestí, acompanyada d'una reducció de la biodiversitat associada al grau de la malaltia.

D'una banda, microorganismes pro-inflamatoris del fílum Proteobacteria com ara *Actinomycetes, Aspergillus, Ruminococcus gnavus* o *Escherichia coli* s'han trobat incrementats en la microbiota de les persones amb MII. Aquests microorganismes contribueixen a la inflamació atacant i danyant la barrera epitelial, incrementant la secreció de citocines pro-inflamatòries, i perpetuant l'activació de cèl·lules del sistema immunitari i de la resposta inflamatòria. D'altra banda, els microorganismes productors de butirat que pertanyen als fílums Firmicutes i Bacteroidetes com ara *Lachnospiraceae, Faecalibacterium prausnitzii, Bacteroides unioformis, Roseburia inulinivorans, Ruminococcus torques, Clostriudium lavalense* o *Clostridium difficile*, entre d'altres, s'han trobat disminuïts en les persones afectades de MII. Aquests microorganismes sintetitzen compostos amb propietats anti-inflamatòries i poden modular la producció de citocines anti-inflamatòries i també de substancies que contribueixen a preservar la barrera intestinal.

En aquest context, com a malaltia inflamatòria crònica, el sistema immunitari juga un paper important en la fisiopatologia de la MII. Un dels principals signes d'afectació de la mucosa intestinal és la disrupció de la barrera epitelial a l'intestí. L'epiteli intestinal està organitzat de manera que constitueix una barrera física que

separa l'interior de l'organisme de tot allò que circula pel lumen intestinal, permetent de manera selectiva, el pas de substancies i responent a estímuls perjudicials. Com a conseqüència de la inflamació intestinal, aquesta barrera queda afectada, augmentant la seua permeabilitat i comprometent les funcions reguladores que exerceix. Per exemple, la composició de la capa mucosa que és la més externa, es pot veure afectada deixant que agents perjudicials entren en contacte més directe amb les cèl·lules de l'epiteli intestinal i del sistema immunitari. Les cèl·lules de l'epiteli intestinal expressen en la seua superfície una sèrie de receptors que reconeixen patrons moleculars associats a patògens i al entrar en contacte amb les cèl·lules, activen vies de senyalització que tenen com a resultat l'activació de la resposta inflamatòria. A més, com a resposta al dany epitelial, una sèrie de mediadors cel·lulars del sistema immunitari, com ara cèl·lules dendrítiques, neutròfils, macròfags o limfòcits, acudeixen a la zona afectada iniciant la resposta immunitària i activant la secreció de substancies pro-inflamatòries per fer front a les agressions patogèniques. Per tant, si aquestes vies de senyalització es troben en activació contínua per la constant interacció dels agents patògens amb cèl·lules epitelials i/o immunitàries, té lloc una cronificació disfuncional de la resposta inflamatòria.

Per tal de fer front a les lesions tissulars que apareixen com a conseqüència de la resposta inflamatòria, existeixen mecanismes que duen a terme un procés reconegut com a reparació tissular. En aquest procés, participen una sèrie de cèl·lules, classificades com a mesènquimes, que tenen la capacitat de migrar a la localització afectada, proliferar i diferenciar-se, secretant citocines com ara el factor de creixement transformant beta (TGF-β). Quan existeix una inflamació perpetuada, la constant activació d'aquests mecanismes acaba ocorrent d'una forma patològica. Aquest procés és el que es coneix com a fibrosi intestinal, que es caracteritza per una acumulació excessiva de matriu extracel·lular, principalment col·lagen I d'entre altres fibres, i que causa alteracions funcionals i estructurals en el teixit. La fibrosi intestinal és una de les complicacions més habituals en les persones afectades per MII. Els fibroblasts són els principals protagonistes d'aquest procés, ja que quan

s'activen pels estímuls prèviament citats, inicien la secreció de nombrosos factors pro-fibròtics com el TGF-β, que contribueixen a la producció de més matriu extracel·lular i que alhora activen més mediadors cel·lulars, contribuint a la fibrogènesi. Per exemple, cèl·lules de l'epiteli intestinal poden activar-se i dur a terme un procés de transformació conegut com a transició epiteli-mesènquima (EMT), en el qual aquestes cèl·lules perden els marcadors pròpiament epitelials, com ara l'expressió de Caderina-1, i adquireixen les característiques pròpies de les cèl·lules mesènquimes, expressant marcadors com la Vimentina. S'ha vist que aquest procés pot ser un dels mecanismes responsables de la formació de la fístula, una de les complicacions dels pacients i les pacients de MII, que consisteix en la formació d'un teixit anormal que connecta el tracte intestinal amb altres estructures properes. Actualment, els mecanismes exactes que participen en la formació de la fístula no es coneixen i per tant, no existeix un tractament farmacològic capaç de previndre o revertir aquesta complicació.

D'altra banda, com a resultat de les pertorbacions prèviament descrites, també s'han observat alteracions en el perfil metabolòmic de les persones afectades de MII. Degut als canvis en la població de microorganismes intestinals i a la inflamació present en la patologia, els nivells de metabòlits relacionats amb el metabolisme energètic, d'àcids grassos i d'aminoàcids, entre d'altres, s'han vist afectats. Com a conseqüència del paper d'aquests compostos en el metabolisme i en processos fisiològics, aquestes alteracions tenen un impacte en mecanismes cel·lulars i moleculars que sovint juguen un paper en el sistema immunitari i la inflamació. Per tant, identificar i caracteritzar l'origen i les conseqüències d'aquestes alteracions té una gran importància per tal d'entendre millor les afectacions que tenen lloc en aquesta patologia i com a conseqüència, millorar les aproximacions terapèutiques i la qualitat de vida de les persones afectades.

En aquest context, els receptors acoblats a proteïnes G (GPCRs) sensibles a metabòlits, han sorgit com a protagonistes de la fisiopatologia de la MII. Els GPCRs són una àmplia família de receptors de membrana encarregats de diverses accions

fisiològiques tals com el reclutament de cèl·lules immunitàries, la producció de citocines i el manteniment de la barrera intestinal entre altres. Per tant, la desregulació d'alguna de les seues funcions pot afectar negativament la funció inflamatòria i per consegüent, contribuir a la progressió de la MII.

D'una banda, el GPR91 o receptor del succinat, es localitza a la membrana plasmàtica de diferents tipus cel·lulars com ara cèl·lules hepàtiques estrellades, megacariòcits, plaquetes, cèl·lules de la macula densa, adipòcits blancs o neurones retinals. També s'expressa en cèl·lules del sistema immunitari com macròfags, cèl·lules dendrítiques immadures i també cèl·lules epitelials i fibroblasts, totes elles involucrades en la fisiopatologia de la MII. El seu lligand principal és el succinat, un metabòlit que a nivells fisiològics es troba a la mitocòndria però com a resposta a condicions patològiques, s'acumula i es secreta al medi extracel·lular activant el seu receptor. El GPR91 es troba acoblat a diferents vies de senyalització depenent de la localització tissular específica.

D'altra banda, el GPR109A es localitza a gran varietat de tipus cel·lulars del sistema nerviós, a adipòcits, hepatòcits, queratinòcits o cèl·lules de l'epiteli intestinal. També s'expressa a cèl·lules del sistema immunitari com monòcits, macròfags, cèl·lules dendrítiques i neutròfils. Estudis realitzats en models murins lliures de microorganismes (germ-free), han demostrat que l'expressió d'aquest receptor es depenent de la presència de microbiota intestinal, ja que en aquestes condicions, l'expressió del receptor era molt baixa i tornava a valors normals quan el tracte intestinal es recolonitzava amb microorganismes. Existeixen diversos lligands que activen el receptor desencadenant l'activació de diferents vies de senyalització. Entre ells trobem la niacina, el butirat o el β-hidroxibutirat. Com que la concentració d'aquests dos últims pot estar influenciada per la microbiota intestinal, la seua presència té efectes directes sobre les cèl·lules de l'epiteli intestinal.

OBJECTIUS

Els objectius generals d'aquesta tesi doctoral són caracteritzar l'eix composició de la microbiota intestinal, metabòlits i els GPCRs sensibles a metabòlits en reseccions intestinals quirúrgiques de pacients d'ambdues malalties, CU i MC, així com investigar el paper específic dels receptors GPR91 i GPR109A en la patogènesi de la MII.

Per tal de dur a terme aquests objectius, es desenvoluparan els següents objectius específics:

1. Caracteritzar en reseccions intestinals quirúrgiques de CU i MC:
    a. La microbiota intestinal.
    b. El perfil metabolòmic dels metabòlits que activen GPCRs.
    c. L'expressió gènica del GPCRs sensibles a metabòlits.
2. Determinar la relació entre marcadors pro-inflamatoris i pro-fibròtics amb l'expressió dels GPCRs sensibles a metabòlics en pacients de MII.
3. Analitzar el paper del GPR91 en la transició epitelial-mesenquimàtica (EMT) i la seua rellevància en la formació de la fístula.
4. Estudiar la rellevància del GPR91 en l'activació de l'inflamasoma i el seu paper en un model murí de colitis crònica.
5. Analitzar l'expressió tissular del GPR109A en pacients amb MII.
6. Determinar els efectes del β-hidroxibutirat, agonista del GPR109A, en l'expressió de marcadors M1 i l'expressió de citocines en els macròfags.
7. Estudiar el paper del GPR109A en la polarització dels macròfags cap al fenotip M1, l'expressió de citocines, i el seu efecte en fibroblasts intestinals.

METODOLOGIA I RESULTATS

Per dur a terme els estudis d'aquesta tesi doctoral, han participat persones afectades per CU i MC en estat refractari greu que van estar intervingudes per cirurgia. A més, com a controls, es van utilitzar reseccions intestinals quirúrgiques tant ileals com colòniques, de la part no afecta de pacients amb càncer colorectal. El fet que s'utilitzen reseccions intestinals com a punt de partida per a estudiar les afectacions estructurals, moleculars i funcionals de la patologia, té un gran valor biològic ja que front a altres mostres, aquestes constitueixen una mostra representativa de la part afectada.

A més, per als estudis d'aquesta tesi, també es van dur a terme dos models animals en ratolins wild type (WT) i GPR91$^{-/-}$. D'una banda, en el model de colitis crònica, la colitis s'indueix en ratolins amb 4 cicles de percentatges creixents (1%, 1%, 1,5% i 1,5%) de DSS en l'aigua de beguda durant 7 dies, intercalada per 10 dies amb solament aigua. D'altra banda, es va induir fibrosis intestinal mitjançant el model de trasplantament heterotòpic del coll, que consisteix en el trasplantament subcutani de reseccions d'1 cm de còlon a partir d'un ratolí donant, a la regió dorsal del coll d'un ratolí receptor. Després de 7 dies, els ratolins van ser sacrificats i la resecció de còlon es va recollir per analitzar. Com a controls s'utilitzaren segments adjacent del còlon dels ratolins donants a dia 0.

Finalment, també es van realitzar estudis *in vitro* en els quals s'utilitzaren tres línies cel·lulars. La línia de cèl·lules epitelials HT-29, la línia de monòcits humans U937, que es polaritzaven a macròfags amb forbol-12-miristat-13-acetat (PMA), i la línia de fibroblasts intestinals HSIFs. Aquestes cèl·lules es van sotmetre a distints tractaments per simular models d'inflamació o fibrosi intestinal. A més, es van utilitzar sondes de siRNA específic del GPR91 i del GPR109A i Lipofectamina per a silenciar aquests receptors en les diferents línies cel·lulars.

Caracterització de la microbiota intestinal

Mitjançant l'extracció de DNA i la seqüenciació del gen 16S rRNA de les reseccions intestinals quirúrgiques de CU i MC, es va caracteritzar la microbiota intestinal. Es van trobar diferencies significatives en la composició de la microbiota entre CU i els seus controls. A més, també es va observar una menor riquesa i càrrega bacteriana en les mostres de CU respecte als controls, mentre que en els teixits de MC no es van obtindre diferències significatives. En quant a gèneres, es va identificar 10 vegades més abundància de *Cellulosimicrobium* en mostres de CU en comparació amb els controls, metre que en MC es va observar una disminució de 20 vegades del gènere *Enteroccocus* front als controls. A més, l'espècie *Faecalibacterium prausnitzii*, associada amb efectes beneficiosos en la salut intestinal, també es va observar reduïda en MC.

Quantificació de metabòlits agonistes de GPCRs

Per tal de quantificar els metabòlits presents en les reseccions intestinals MII, s'utilitzaren les tècniques de ressonància magnètica nuclear (NMR) i la cromatografia líquida d'alt rendiment acoblada a l'espectrometria de masses (UPLC-MS). En comparar els nivells de metabòlits obtinguts en mostres de persones afectades i controls, es van trobar alteracions significatives en la majoria dels grups analitzats. Per exemple, els àcids succínic, β-hidroxibutíric i làctic estaven significativament incrementats en reseccions de MC al comparar en els controls. Els àcids grassos de cadena llarga com els àcids docosahexaenoic, α-linolènic i mirístic, així com els aminoàcids fenilalanina, àcid aspàrtic i àcid glutàmic, estaven incrementats en CU respecte als controls. També es van trobar diferencies entre MC i CU, com ara en els nivells dels àcids butíric i acètic, la fenilalanina o l'àcid aspàrtic, que estaven significativament incrementats en MC respecte a CU.

Expressió gènica dels GPCRs sensibles a metabòlits

Mitjançant la tècnica de la qPCR es va analitzar l'expressió gènica dels receptors GPCRs sensibles a metabòlits a partir de cDNA obtingut de mRNA de les

mostres de reseccions intestinals de CU, MC i els respectius controls. Es van obtindre diferencies significatives en la majoria dels receptors analitzats, destacant nivells incrementats en la majoria d'ells en les malalties respecte als controls, especialment en la MC.

Relació entre l'expressió dels GPCRs sensibles a metabòlits i la inflamació i fibrosi

A més de quantificar l'expressió gènica dels receptors, també es va analitzar l'expressió de marcadors pro-inflamatoris com la *IL1B* i la *IL8*, el marcador de macròfags M1 *CD86*, i els marcadors pro-fibròtics *COL1A1, COL3A1* i *COL4A1*. L'expressió de tots els marcador pro-inflamatoris i el *CD86* va ser significativament més elevada tant en CU com MC en comparació als seus respectius controls, metre que l'expressió dels col·làgens solament era significativament més elevada en MC respecte als controls ileals. Al relacionar aquests marcadors i els GPCRs sensibles a metabòlits, es van obtindre correlacions significatives i positives per a la majoria de receptors amb els marcadors pro-inflamatoris, tant en CU com MC. A més, els marcadors pro-fibròtics van correlacionar positivament amb els GPCRs sensibles a metabòlits majoritàriament en MC, especialment en el cas del *COL4A1*.

El paper del GPR91 en la EMT

Basats en estudis previs realitzats pel nostre grup, el receptor GPR91 es va trobar incrementat en reseccions intestinals de MC, i es va descriure un paper pro-inflamatori i pro-fibròtic d'aquest en un model murí d'inflamació i fibrosi intestinal. Per tant, en aquesta Tesi Doctoral s'ha analitzat el paper del receptor en una de les complicacions greus que pateixen les persones afectades de MC, la fístula. Un dels processos associats a la formació de les fístules és la EMT prèviament descrita. Per tant, es van analitzar en reseccions intestinals de MC tant del fenotip B2 (estenosant), com del fenotip B3 (fistulitzant), l'expressió gènica del GPR91 i de marcadors de la EMT com els factors de transcripció *SNAI1* i *SNAI2*. Tant l'expressió del receptor, com dels marcadors de la EMT es va trobar més elevada en reseccions intestinals de MC-B3 en comparació amb els controls. A més, també es van obtindre correlacions

positives entre l'expressió del receptor i aquests marcadors. Mitjançant la tècnica de la immunohistoquímica, es va caracteritzar l'expressió del receptor en mostres tissulars de fístula i es va observar l'expressió del receptor en regions adjacents a la fístula, així com l'expressió del receptor en fibroblasts Vimentina+ i macròfags CD206+.

Per a confirmar el paper del GPR91 en l'activació de la EMT, es va utilitzar el model murí de fibrosi del transplantament heterotòpic del coll amb ratolins WT i GPR91$^{-/-}$ i es va observar com l'increment de l'expressió gènica dels marcadors d'EMT induïda pel model de fibrosis, es reduïa significativament en els ratolins GPR91$^{-/-}$. A més, es va induir també l'EMT amb TGF-β durant 48 h en la línia de cèl·lules epitelials HT-29 i es va observar com al silenciar el GPR91, l'expressió gènica dels marcadors d'EMT es reduïen significativament respecte a les cèl·lules siCtrl tractades amb TGF-β.

El paper del GPR91 en l'activació de l'inflamasoma

També es va analitzar l'expressió del receptor GPR91 en reseccions intestinals de CU i el seu paper en l'activació de l'inflamasoma, un mecanisme molecular homeostàticament de defensa que en condicions patològiques pot contribuir a la inflamació. Es van observar l'expressió gènica, mitjançant qPCR, i proteica mitjançant Western Blot, i es van trobar nivells més elevats del receptor i dels components de l'inflamasoma en mostres de CU i també es van obtindre correlacions positives entre aquests marcadors i el receptor. A més, es va analitzar el paper del GPR91 en un model de colitis crònica induïda per DSS i es va observar mitjançant qPCR com en els ratolins GPR91$^{-/-}$ tractats amb DSS no s'incrementava l'expressió gènica dels marcadors pro-inflamatoris, pro-fibròtics ni els components de l'inflamasoma com sí que va ocórrer en els ratolins WT tractats amb DSS. Finalment, es va analitzar el paper d'aquest receptor en cèl·lules de l'epiteli intestinal, concretament amb la línia cel·lular HT-29, i es va veure com l'increment en

l'expressió dels components de l'inflamasoma induïda per un còctel inflamatori, no tenia lloc en absència d'aquest receptor.

El paper del GPR109A en la fisiopatogènesi de la MII

L'expressió proteica del receptor GPR109A va ser analitzada per Western Blot i es van trobar nivells més elevats en les mostres de MC en comparació amb els controls. A més, mitjançant la tècnica de la immunohistoquímica, es va caracteritzar la localització tissular d'aquest receptor en reseccions intestinals de CU, MC i els seus respectius controls, i es van trobar cèl·lules positives a l'epiteli i a la làmina pròpia. Considerant la morfologia d'aquestes cèl·lules, podrien tractar-se de macròfags, leucòcits, neutròfils o fibroblasts. Per tant, es va decidir analitzar el paper del receptor en macròfags. Primerament, la línia cel·lular de monòcits U937 es va polaritzar a macròfags i aquests es van tractar amb β-hidroxibutirat, un agonista del receptor GPR109A, que va resultar en un augment en l'expressió gènica del receptor, així com del marcador de macròfags M1 *CD86* i dels marcadors pro-inflamatoris *IL1B* i *NOS2*. A més, també es va analitzar el paper del GPR109A en macròfags M1, polaritzats amb LPS i IFN-γ, i es va observar que l'augment de l'expressió i secreció de les citocines pro-inflamatòries IL-1β, IL-6 i TNF-α dels macròfags M1, no tenia lloc en absència del receptor. Finalment, es va analitzar els efectes del secretoma d'aquests macròfags en fibroblasts intestinals, concretament en la línia cel·lular HSIFs. En tractar les HSIFs amb el secretoma de macròfags M1, es va observar un increment de marcadors pro-fibròtics en els fibroblasts, tot i que no es va obtindre significació estadística. En tractar les HSIFs amb secretoma de macròfags M1 amb el GPR109A silenciat, es va veure que els nivells de marcadors pro-fibròtics *COL1A1* i *COL3A1* es reduïen considerablement.

CONCLUSIONS

1. Les reseccions intestinals quirúrgiques de persones afectades de MII exhibeixen:
    a. En CU, canvis en la composició i riquesa de la microbiota intestinal amb un increment en la proporció del *Cellulosimicrobium*. En MC no es s'observen canvis en la composició de la microbiota, tot i que s'observa una tendència a la baixa en la riquesa i càrrega bacteriana, a més d'una disminució significativa en l'abundància d'*Enterococcus*.
    b. Alteracions en el perfil metabolòmic de metabòlits agonistes de GPCRs.
    c. Expressió gènica elevada en la majoria de GPCRs sensibles a metabòlits, especialment en MC.
2. L'expressió gènica elevada de la majoria de GPCRs sensibles a metabòlits es correlaciona positivament amb una elevada expressió gènica de citocines pro-inflamatòries com la *IL1B* i la *IL8*, el marcador de macròfags M1 *CD86*, i els col·làgens *COL1A1*, *COL3A1* i *COL4A1*.
3. Les persones afectades de MC fistulitzant exhibeixen una elevada expressió del GPR91 i dels marcadors d'EMT *SNAI1* i *SNAI2*, que correlacionen positivament.
4. L'absència del GPR91 impedeix l'activació de l'EMT tant en cèl·lules de l'epiteli intestinal com en un model murí de fibrosi intestinal.
5. Les persones afectades de CU exhibeixen una elevada expressió del GPR91 i dels components de l'inflamasoma Caspasa-1, NLRP3 i IL-1β que correlacionen positivament.
6. L'absència del GPR91 impedeix la inflamació i l'activació de l'inflamasoma tant en cèl·lules de l'epiteli intestinal, com en un model murí d'inflamació i fibrosi crònica.

7. Les persones afectades de MC exhibeixen nivells elevats de β-hidroxibutirat i del seu receptor GPR109A, que s'expressa en cèl·lules de l'epiteli intestinal i cèl·lules de la làmina pròpia.

8. El GPR109A és responsable de l'expressió de citocines pro-inflamatòries IL-1β, IL-6 i TNF-α en macròfags M1 i modula la seua habilitat per activar fibroblasts.

ABSTRACT

Inflammatory Bowel Diseases (IBD), which comprise Ulcerative Colitis (UC) and Crohn's Disease (CD), are disorders of the gastrointestinal tract characterized by chronic inflammation and abnormal immune system function. Exact etiology is still unknown, but it is considered a multifactorial disease with a complex interplay of genetic predisposition, environmental factors, gut microbiota, and immune response. As a result, several complications such as fibrosis or fistulas can arise, significantly impacting the quality of life of affected individuals and requiring appropriate management and monitoring. Nowadays, there are not effective pharmacological treatments which completely reverse the disease or fully prevents associated complications. Thus, understanding the causes, complications and relationships between all factors involved in its pathogenesis is crucial for improving diagnosis, management, and therapeutic approaches.

In recent years, significant progress has been made to better characterize the factors contributing to IBD pathogenesis. The gut microbiota plays a crucial role in maintaining gut health. Changes in the composition and function of gut microbiota, including dysbiosis, are hallmarks of IBD. This dysbiosis is believed to exacerbate inflammation and contribute to the progression of the disease. As a result, metabolomic profile alterations, including metabolites related to energy metabolism, fatty acids, and amino acids, amongst others, also arise.

In this scenario, metabolite-sensing G-protein coupled receptors (GPCRs) have emerged as pivotal players in the pathophysiology of IBD. GPCRs are a large family of cell membrane receptors involved in various signalling pathways, including immune cells recruitment, cytokines production and mucosal barrier maintenance. Dysregulation of their functions contributes to exacerbation of inflammation and IBD progression.

The aim of this study was to characterize the axis intestinal microbiota composition, metabolites, and metabolite-sensing GPCRs in intestinal surgical resections from IBD patients. First, we detected lower microbiota richness and

bacterial load in UC patients characterized by a higher proportion of genus *Cellulosimicrobium*. In CD patients, decreased abundance of *F. prausnitzii* was also observed. In addition, alterations in the levels of energy metabolism compounds, fatty acids, and amino acids were also observed in both UC and CD tissue samples. Finally, upregulated expression of most of the metabolite-sensing GPCRs analysed, associated with enhanced expression of pro-inflammatory and/or pro-fibrotic markers, was also observed in both UC and CD patients. With these results, we decided to analyse in depth the role of GPR91 and GPR109A in IBD pathogenesis.

On the one hand, GPR91 expression was higher in fistulizing B3-CD patients. In addition, epithelial-to-mesenchymal transition (EMT) markers, were also upregulated in these patients and positively correlated with the receptor. The absence of the receptor prevented the EMT induced by an experimental murine model of fibrosis, the heterotopic transplant model, and also in intestinal epithelial cells (IECs) treated with TGF-β after stimulation with the GPR91 agonist succinate. On the other hand, GPR91 expression was also higher in UC patients and positively correlated with the expression of inflammasome components. Furthermore, the deletion of the receptor impaired the increase in pro-inflammatory, pro-fibrotic, and inflammasome markers in a chronic DSS-induced colitis model, and the inflammasome priming IECs.

On the other hand, expression of GPR109A was higher in UC and CD patients, and was detected in epithelial cells and cells from lamina propria, so its role in macrophages was further analysed. The treatment with its agonist β-hydroxybutyrate increased gene expression of *GPR109A*, *CD86*, *IL1B* and *NOS2* in MØ macrophages. In addition, increased mRNA expression and secretion of IL-1β, IL-6 and TNF-α was impaired in M1 macrophages when GPR109A was transiently silenced. Finally, the secretome from M1 macrophages lacking the receptor reduced the expression of *COL1A1* and *COL3A1* in intestinal fibroblasts.

While the precise molecular mechanisms require further clarification, GPR91 and GPR109A appear to play a role in specific complications associated with IBD. A better understanding of metabolite-sensing GPCRs could facilitate the development of more efficacious treatment approaches and determine their potential as therapeutic targets for IBD management.

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I. INTRODUCTION

1 Inflammatory Bowel Disease (IBD)

Inflammatory Bowel Disease (IBD) is a relapsing chronic inflammatory disorder of the gastrointestinal tract characterized by a disrupted function of the immune system. Within the last years, IBD has become a public health challenge worldwide due to the associated morbidity and its increasing prevalence and incidence (1). The two major clinical subtypes of IBD are Ulcerative Colitis (UC) and Crohn's disease (CD), which differ in the disease manifestations but share pathological aspects. There is a third subset of patients which are classified as IBD-Undetermined because their assessment does not categorize to neither UC nor CD (2). The exact etiology of IBD remains poorly understood, but increasing evidence points alterations in intestinal microbiome, environmental and genetic factors, and dysfunction of immune system as responsible of the disease onset (3). Nowadays, complete remission of IBD is still a research challenge and due to the relapsing and chronic immune activation, several complications such as intestinal fibrosis, fistula, perforation, and even colorectal cancer arise, and then, surgery is frequently required (4). Apart from intestinal inflammation, associated symptoms to IBD have also been related with extra-intestinal complications such as arthritis, cholangitis, or urinary tract infections. As a result, this disease also negatively impacts patients' quality of life causing stress and psychological issues which need also to be addressed. Several co-morbidities such as anxiety, fatigue, depression, or sexual dysfunction may also arise, especially in active disease periods (5, 6).

UC was the first IBD subtype identified and is the most common form of IBD. Mucosal inflammation, that usually starts in the rectum and may or not spread to other parts of the colon, characterize the disease. According to the location of the injury and extent of inflammation, the different subtypes of UC based on Montreal's classification are proctitis, left-sided colitis and pancolitis (7). This classification also comprises the disease's severity level, as outlined in Table I.1. Regarding histologic evaluation, UC patients exhibit low density and even disrupted crypts, and

the presence of infiltrates containing lymphocytes, plasma cells and granulocytes. The main clinical feature of UC is bloody diarrhoea with or without mucus and depending on the disease extent and severity, other symptoms such as rectal bleeding, tenesmus, abdominal pain, fever, weight loss and malaise may also arise. In the case of severe inflammation, acute complications such as excessive bleeding, toxic megacolon, epithelial dysplasia, or cancer may appear (8).

***Table I. 1. Montreal's classification of UC** (7).*

Extent		**Severity**	
E1	Proctitis (rectum)	**S0**	Clinical remission
E2	Left-sided colitis (distal UC)	**S1**	Mild
E3	Pancolitis (extensive UC)	**S2**	Moderate
		S3	Severe

CD is the IBD subtype that can affect any segment of the gastrointestinal tract, being the terminal ileum and colon the most commonly affected parts. This pathology is characterized by segmental, asymmetrical, and transmural inflammation, which can affect any layer of the gut wall (mucosa, submucosa, muscularis or serosa). In addition, half of CD patients develop complications such as fistulas, strictures, or abscesses, being surgery commonly required. According to the age at diagnosis, disease location and behaviour, different subtypes of CD are identified following Montreal's classification (7), as summarized in Table I.2. Regarding histological features, chronic focal, discontinuous, and transmural inflammatory infiltrates, and lymphoid aggregates, as well as preservation of goblet cells, are found in CD patients (9). Clinical manifestations of this disease are usually abdominal pain, chronic diarrhoea, weight loss and fatigue. Depending on the location and severity of inflammation, rectal bleeding and bloody diarrhoea are also observed in these patients. In addition, almost half of CD patients, show extra-intestinal manifestations in skin, joints or eyes (10).

***Table I. 2. Montreal's classification of CD** (7).*

Age at diagnosis	
A1	Below 16 years
A2	Between 17 and 40 years
A3	Above 40 years
Location	
L1	Ileal
L2	Colonic
L3	Ileocolonic
L4	Upper tract (added to L1-L3 when concomitant)
Behaviour	
B1	Inflammatory
B2	Stricturing
B3	Penetrating
p	Perianal disease modifier (added to B1-B3 when concomitant)

Although the clinical classification represented in Table I.2 regarding CD behaviour is the most widely used (7), other parameters should also be included, since symptoms, disease and injury progression are not fully correlated. In addition, degree of intestinal fibrosis and associated injuries which derive in structuring and/or penetrating complications, should be also considered in this classification. Indeed, in more than 85% of penetrating CD patients there is a coexistence of strictures (11). Besides, the newly discovered serological markers and genetic and epigenetic alterations, could also modify this classification to better address IBD diagnosis and as a consequence, its management and treatment (7). To achieve an improved classification, a better understanding in cellular and molecular mechanisms involved in pathogenesis is highly required (11).

I.1.1 IBD epidemiology

Firstly, in the second half of the 20th century, IBD was characterized as a disease of the Western world, mainly affecting Europe, North America, Australia and New Zealand. However, recent studies suggest that the incidence in developing countries

of Asia, Africa and South America is significantly increasing as they become newly industrialized areas, following an analogous trend previously seen in western countries (1). Current worldwide IBD incidence is shown in Figure I.1. Indeed, an increase of 85.1% of IBD cases was registered worldwide from 1990 to 2017, going from 3.7 million to more than 6.8 million. In 2017, approximately 3.9 million females and nearly 3.0 million males were living with IBD. The country with highest global prevalence rate is USA with approximately a fourth of the total worldwide patients with IBD. In Europe, UK is the country with highest prevalence, with a ratio of 373 per 100,000 population reported (12). In Spain, a study revealed that the 0.78% of the population suffers from IBD, what means approximately 300,000 IBD patients (13). Although IBD mostly affects young adults, over the last 25 years, a huge increase in the incidence and prevalence of IBD in pediatric population has been noticed worldwide, predominating the number of cases of CD versus UC (14). In conclusion, both high prevalence and rising incidence are crucially accounting in global worldwide burden of IBD for society (1).

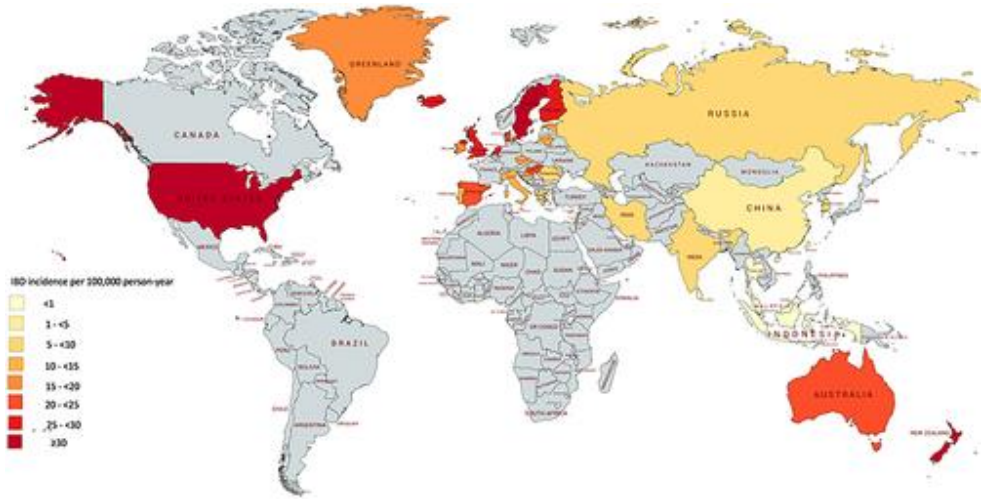


Figure I. 1. Incidence of IBD according to population-based studies from 2010 to 2019 (15).

I.1.2 IBD diagnosis

Early therapeutic intervention seems crucial to improve management of IBD. For that, appropriate diagnostic tools are fundamental (14). Despite the huge efforts made up to now in order to stablish a "gold standard" test to diagnose IBD, it is still difficult to differentiate between CD, UC and other gastrointestinal diseases. In the field of biomarkers, some serological antibodies such as perinuclear anti-neutrophil cytoplasmic antibodies (pANCA), anti-saccharomyces cerevisiae antibodies (ASCA), antibodies against exocrine pancreas (PAB), fecal calprotectin (FCp), circulating non-coding RNAs including mi-RNAs and lncRNA, C-reactive protein (CRP), cathelicidin, and trefoil factor 3 (TFF3), can be found among others (16). Recently, cytokine-based diagnosis is also starting to be applied since a wide variety of inflammatory cytokines are increased in serum from IBD patients (17).

Furthermore, since many of the above-mentioned biomarkers can be altered in other intestinal and inflammatory conditions, imaging and histological confirmation are usually required. To better characterize macroscopic alterations in the bowel, sigmoidoscopy and colonoscopy are commonly used (18). In addition, when possible, abdominal computed tomography is the imaging tool of choice for both diagnosis and monitoring of disease (19). Of interest, imaging has been improved and techniques such as small bowel ultrasound, which allows gut wall thickness measurement, as well as video capsule endoscopy, are being applied (14). Finally, biopsies from different parts of the gastrointestinal tract depending on patient's clinical presentation and suspected location, are also examined by pathologists. They are acquired to diagnose, define the type of IBD, identify and grade dysplasia, assess cellular and structural alterations, and determine whether there is coexistence of other complications (20, 21).

I.1.3 IBD treatment

As previously reported, combined periods of active disease with exacerbation of the symptoms and remission periods, characterize the disease behaviour of IBD (22). Hence, pharmacological management of IBD has been mostly based on controlling and preventing the symptoms of active inflammatory episodes, prolonging periods of remission to prevent associated complications. Within the last decades, IBD therapeutic approach has considerably changed since novel treatments have emerged or are in advanced stages of clinical trials, with improved efficacy and safety profiles. Nowadays, IBD therapeutic strategies can be classified in three groups: i) conventional therapies, ii) novel small molecule drugs and iii) novel biologics (23).

Regarding conventional therapies based on biological and immunomodulatory or anti-inflammatory drugs, infliximab (IFX), an antagonist of tumor necrosis factor (TNF)-α, was used for the first time to treat CD in 1992 and it has been the biological therapeutic approach in IBD per excellence within the last decades (24). Indeed, it was approved to treat moderately to severely active CD and UC patients who failed to respond to other therapies. The reduction in the symptoms of inflammation and the induction and maintenance of remission, were key actions that made IFX so attractive. Other anti-TNF-α drugs such as adalimumab, certolizumab for CD and golimumab for UC have also been widely used. In addition, immunosuppressant drugs such budesonide, azathioprine and 5-aminosalicylic acid (5-ASA), are also included in this group and they have been mainly used to induce remission of inflammation, but failed to maintain long term remission. Hence, the wide variety of serious side effects including immunogenicity and treatment failure, makes necessary the development of other strategies (23, 25).

Next, small molecule drugs have been developed within last years and represent advantages regarding administration method, non-immunogenicity, and low cost. Oral JAK inhibitors, such as tofacitinib, upadacitinib or filgotinib, reduce

notoriously inflammation since they block JAK-STAT signalling pathway which is involved in several steps of the inflammatory response. On the other hand, sphingosine-1-phosphate (S1P) modulators, including ozanimod, have also been recently approved for moderate to severe active UC. However, although these small molecule drugs are very promising, they still show serious side effects such as infections and cardiovascular events, which need to be better addressed (23, 25).

Finally, novel biologics are emerging, and they seem to show a better safety profile, with lower incidence of adverse reactions and improved efficacy. IL-12/IL-23 inhibitors such as ustekinumab and risankizumab were approved in 2019 and 2022 to treat moderate to severe active UC and CD. Integrin inhibitors such as natalizumab or vedolizumab can also inhibit migration of lymphocytes from blood to the tissue preventing intestinal inflammation (23, 25).

Taking all together, a wide variety of therapies can help to reduce clinical symptoms of IBD and improve histological and transmural healing in patients. Apart from pharmacological treatment, non-pharmacological approaches, such as dietary modulation, have also been considered, not only to improve malnutrition associated with IBD, but also to relieve inflammation. However, although within the last years the need of surgery in IBD patients has been reduced (26), in most cases patients stop responding to the different therapies and it does not exist a gold-standard therapy which fully reverts IBD pathology and prevents complications yet, what makes this pathology considerably difficult to manage. Among these complications, in susceptible patients, fibrosis is not prevented with none of the therapies currently available becoming surgical intervention the only option for most of the cases. Hence, an important goal of IBD treatment must also be the reduction or reversal of intestinal fibrosis (11).

I.1.4 IBD pathogenesis

Although within the last decades IBD understanding has improved notoriously, the exact etiology of the disease remains poorly characterized. One of the main reasons is that it is considered a multifactorial disease in which different factors are involved, including genetics and epigenetics, environment, microbiota and immunological responses. Whether these factors are responsible of triggering the disease or are consequences of them is still an opened question for the scientific community (27).

I.1.4.1 Genetic factors

Genome-wide association studies (GWAS) have identified over 50 immune response-related genes that are associated with an increased risk of developing IBD (18), including genes involved in gut microbiota disruption (28). The *NOD2/CARD15* gene, which is a cytoplasmic receptor recognizing bacterial cell wall peptidoglycan, has been found mutated in 10% to 30% of Caucasian CD patients. Hence, carrying a mutation in NOD2 receptor which results in a loss of its functions, implies a three-fold risk of developing CD among heterozygotes, and nearly a 20-fold increased risk for homozygotes (29). Indeed, studies performed on murine models demonstrated that the lack of NOD2 is related to altered gut microbiota composition (28). Similar mutations have been identified in other genes such as *interleukin (IL)23R, Janus kinase (JAK), signal transducer and activator of transcription (STAT), IL12B, IL10, NLRP3* or *CCR6*, as well as other autophagy-related genes such as *IRGM, ATG16L1* and gut embryogenesis *NKX2.3* and *GLI1* (18). Family history of IBD has also been described, hence, the pathology also has a hereditary component (29).

I.1.4.2 Environmental factors

Although genetic susceptibility accounts in the risk of developing IBD, the rapid and huge increase in incidence over the last 50 years in both western and newly

industrialized countries, cannot only be explained by genetic alterations (29). In fact, westernization of the diet, including a high consumption of fast food, saturated fats, red meats, and refined sugars play a role in intestinal inflammation development and microbiome alteration, which account for IBD pathogenesis (30). For instance, studies point a decreased risk on developing CD and UC in individuals consuming high amounts of fibre, mainly fruits, and omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) respectively (31, 32). Interestingly, a positive correlation between smoking and developing CD has been established, although in the case of UC it has been paradoxically reported a protective effect (33, 34). In parallel, stress and depression have also been pointed as risk factors for developing IBD (35). Moreover, the likelihood of developing IBD has been positively correlated with the socioeconomic status. Greater hygiene habits contribute to a higher risk of developing autoimmune and chronic inflammatory disorders, since these habits imply reduced exposure to common infections due to improved household hygiene, antibiotic use and vaccination (11). Then, the immune response to environmental antigens may be increased and have severe consequences in genetically susceptible populations (36). Finally, pathogenic transmission of IBD has also been considered in some cases of chronic and persistent infections by microorganisms, which may trigger an abnormal activation of the mucosal immune system, contributing to CD development in genetically predisposed individuals (37, 38).

I.1.4.3 Gut microbiota

Gut microbiota plays an important role in intestinal maintenance, metabolism balance and immune homeostasis. The host immune system is continuously exposed and interacting with gut microbiota. Bacterial load is estimated to represent about 10^{14} colony forming units/g of colonic content (39). Although genetics can stablish part of microbiome composition, it is also highly influenced by the environment, and adapts to the hosts' needs. Indeed, the composition widely varies among healthy individuals and disturbances in both, the composition and abundance of host

intestinal microbiota commonly result in several pathologies such as IBD (40). In fact, dietary habits strongly influence gut microbiome composition and as a result, byproducts of their metabolism. For instance, it has been reported that microbiome composition can be altered due to high-fat and low fibre diets (41). It is widely known that IBD is associated with microbiota dysbiosis characterized by an imbalance in the composition and diversity between harmful and beneficial bacteria in the gut, which triggers immune responses, alteration of intestinal epithelial barrier and as a consequence, inflammation (28, 29, 42-44). In this scenario, gut microbiota interaction with host immune system seems to be disrupted (45). Several studies have evidenced in intestinal biopsies of IBD patients a decrease in total number of species, which results in significant reduction of bacterial biodiversity associated with disease degree (46-50).

On the one hand, pro-inflammatory microorganisms from the Proteobacteria phylum including *Actinomycetes, Aspergillus, Ruminococcus gnavus* or *Escherichia coli* are increased in IBD patients' microbiota (39, 40, 43, 44, 51, 52). For instance, *R. gnavus* contributes to mucosal epithelial barrier disruption by secreting mucin-degrading compounds and mediates through TLR4, the release of the proinflammatory cytokine TNF-α, resulting in inflammation (53). In addition, other species can compromise epithelial barrier triggering inflammation in IECs such as enterotoxigenic *Bacteroides fragilis* and adherent/invasive *Escherichia coli* (AIEC), by inducing E-cadherin cleavage and by secreting colibactin, respectively (40).

On the other hand, anti-inflammatory microorganisms including butyrate-producing bacteria from the Firmicutes and Bacteroidetes phyla including *Lachnospiraceae, Faecalibacterium prausnitzii, Bacteroides unioformis, Roseburia inulinivorans, Ruminococcus torques, Clostriudium lavalense* or *Clostridium difficile*, among others, are reduced (39, 40, 43, 44, 51, 52). For instance, lactic acid bacteria, such as *Lactobacillus*, metabolize carbohydrates into lactate, and also produce cyclic peptides with wound healing and anti-inflammatory properties (54,

55). Moreover, it has been reported that *F. prausnitzii* produces an anti-inflammatory protein, named Microbial Anti-inflammatory Molecule, which can inhibit NFκB pathway (56), and induces IL-10 synthesis modulating production of pro-inflammatory cytokines by dendritic cells (57). In parallel, *Roseburia* regulates antimicrobial peptides (AMPs) production and contributes to intestinal barrier function (58). In addition, certain microorganisms, such as *Akkermansia muciniphila,* exert a protective role enhancing mucus layer by producing bacteriocin and modulating goblet cells (59, 60).

Additionally, intestinal fibrosis, the most common complication in IBD, can also be induced by gut microbiota (28). Indeed, murine models have demonstrated that intestinal fibrosis does not develop in the absence of microbiota. Intestinal fibroblasts can respond to bacterial substances and become pro-inflammatory and/or pro-fibrotic triggering proliferation, promotion, and contraction of fibroblasts, which may result in luminal narrowing and stricture formation (61). Recent studies have identified the fibrotic role of specific microorganisms. For instance, AIEC, which is increased in the terminal ileum of IBD patients, has been positively correlated with fibrotic stricture formation in CD (62), whereas *F. prausnitzii*, *Coprococcus*, *Bacteroides* and *Oscillospira* have been negatively associated with fibrosis (63). Finally, circulating antibodies against microbial antigens from *Saccharomyces cerevisiae* or *Pseudomonas fluorescens* correlated with intestinal fibrotic stenosis and surgical interventions (64), and most of them have been associated with a higher disease severity (65). Nowadays, whether these disturbances are cause or consequence of IBD is not fully established. Nevertheless, it is necessary to better elucidate: a) which are the most common microbiota associated alterations in IBD, b) which mechanisms trigger them and are modified as a result, and c) how could these alterations be addressed.

The characterization of gut microbiota is intended not only to identify species composition alteration, but to use these variations to predict disease state and

improve assessment and therapy. In line with this, fecal transplantation to replace "harmful" microbiome of a patient by gut microbiota from a healthy donor, as well as the use of probiotics with anti-inflammatory properties, have been considered (66, 67). For instance, fecal flora enemas from healthy donors diluted 1:2 in normal saline were administered to six UC patients suffering severe and recurrent symptoms, and complete remission of symptoms and histological evidence were achieved in all patients (68). Moreover, a study reported the safety and effectiveness of a high-potency probiotic, named VSL#3, in inducing clinical responses and remissions in patients with mild-to-moderately active UC (69). Next, it is also important to consider that bacterial species have also been genetically modified to use them in order to release *in situ* therapeutic molecules. For instance, *Lactococcus lactis* has been transfected with a plasmid producing the Microbial Anti-inflammatory Molecule produced by *F. prausnitzii*, anti-TNF antibodies, IL-10, or IL-27 but studies are still in the laboratory stage (39).

Furthermore, correlations between species abundance and risk of relapse or complications have also been identified, although in most cases the impact of this data on risk prediction has not yet been assessed (39). For instance, low levels of *Bacteroides* and *F. prausnitzii* can be indicators of clinical relapse (57, 70). In addition, risk of endoscopic relapses has been positively correlated with a group of species including *R. gnavus*, *Gammaporteobacteria* and *Corynebacterium* (71). Besides, a pediatric study of CD patients stablished a correlation between the abundance of *Rothia* and *Ruminococcus* with stenosis formation, and *Collinsella* with fistula development (72). Certain studies have demonstrated that bacterial populations are associated with the effectiveness of treatments becoming microbiota as an indicator of the efficacy of the pharmacological treatment used. In this case, a study performed by Lewis et al. in pediatric CD patients showed a good response to enteral feeding treatment or anti-TNF therapy with increased *Roseburia* and *Lactococcus*, and decreased *Actinomyces* (73). In parallel, Sanchis-Artero and

colleagues described the use of the ratio between *F. prausnitzii/E. coli* and *F. prausnitzii/C. coccoides* as indicators of anti-TNF therapy effectiveness at the beginning and 6 months after treatment (74).

I.1.4.4 Immune response

Next, as a chronic inflammatory disease, the immune system plays a crucial role in IBD pathology. Hence, in this section, alterations of the immune response that occur in IBD and molecular mechanisms and mediators that participate, leading to perpetuated inflammation, are summarized.

I.1.4.4.1 Disruption of intestinal epithelial barrier

Intestinal epithelium constitutes a physical barrier separating the inside of the organism from everything that circulates in the intestinal lumen, allowing the selective passage of substances and responding to harmful stimuli. As a result of intestinal inflammation, this barrier is disrupted, and regulatory functions are compromised (75).

The structure of the intestinal barrier is crucial for its physical and immunological defence function. Distribution of the main components are outlined in Figure I.2. The first line of defence is constituted by the mucus layer, which contains mainly Mucin2 secreted by goblet cells and it is in close contact with gut microbiota. This mucus also contains nutrients, AMPs and secretory immunoglobulin A (sIgA) which prevents the invasion of pathogenic microorganisms. Next, there is the physical intestinal barrier itself with specialized epithelial cells that are continuously renewed. All these cells are organized forming invaginations, known as crypts, and protrusions, known as villi (75). In the base of the crypts, epithelial stem cells $LGR5^+$ are found, which can proliferate and differentiate to form a variety of cell types with different phenotypes and functions. For instance, absorptive enterocytes undergo nutrient absorption; enteroendocrine cells secrete different gastrointestinal hormones; microfold cells participate in the

immune system maturation bringing antigens to resident immune cells in gut-associated lymphoid tissue; goblet cells secrete mucins, mainly Mucin2 and trefoil factors, and Paneth cells secrete AMPs, mainly defensins. All these cells are connected by tight junctions (TJs), adherent junctions (AJs) and desmosomes, contributing to the epithelial barrier structure and regulating paracellular transport among them. Finally, innate and adaptative immune cells such as macrophages, dendritic cells and lymphoid cells reside in the inner part of the intestinal barrier (76).

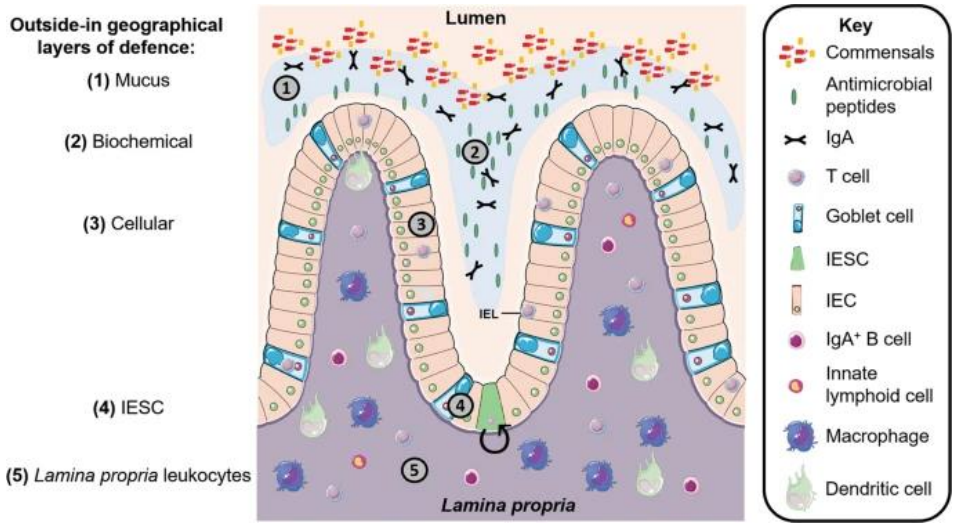


Figure I. 2. Distribution and main cells constituting the intestinal epithelial barrier *(77). The intestinal epithelium constitutes a line of defence separating the interior of the organism from the outside. 1) It is the outer part, which is mainly formed by commensal bacteria and mucus, mainly mucins. 2) Inner mucus layer containing antimicrobial peptides and immunoglobulin A. 3) Physical intestinal barrier itself with single-cell layer of different epithelial cell types and intraepithelial lymphocytes. 4) In the base of intestinal crypts, intestinal epithelial stem cells are found which proliferate and differentiate to form a variety of cell types. 5) At the other part of the barrier, lamina propria is found populated with immune cells such as macrophages, dendritic cells and lymphoid cells. IgA, Immunoglobulin A; IESC, intestinal epithelial stem cell; IEC, intestinal epithelial cells; IEL intraepithelial lymphocytes.*

Several factors occur in IBD pathogenesis that result in the disruption of the abovementioned structure and function. First, as observed in Figure I.3, the disruption of the epithelial barrier leads to diminished and inadequate mucosal layer, enabling the penetration of pathogens and initiating their interaction with the intestinal epithelial cells (IECs), and the immune system (40). For instance, a decrease in defensins secreted by Paneth cells has been reported in CD patients (78). Of interest, several polymorphic genes associated to dysfunction of Paneth cells have been correlated to increased risk of CD (61). In addition, studies performed on Mucin2-defficient mice showed worsening of experimental colitis symptoms (79).

Next, TJs alterations also occur in IBD pathogenesis since certain pro-inflammatory cytokines such as TNF-α α and interferon (IFN)-γ can increase TJs permeability. As a result, ions, and water diffusion from blood to lumen is favoured and may trigger diarrhoea in IBD patients. Such alterations allow pathogens and inflammatory agents to enter easier in the epithelial barrier contributing to mucosa damage (75). Finally, IECs express different receptors such as Toll-like receptors (TLR) and NOD-like receptors (NLRPs), which identify pathogen-associated molecular patterns (PAMPs) that stimulate different intracellular signalling pathways including nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and mitogen-activated protein kinase (MAPKs), triggering the activation of the immune response. For instance, disturbances in TLR3 and TLR4 have been reported in IBD patients, which result in an altered sensing that perpetuates the disease (80). Taking all together, the disruption of the intestinal epithelial barrier strongly contributes to continuous immune activation and perpetuation of the inflammatory response (75).

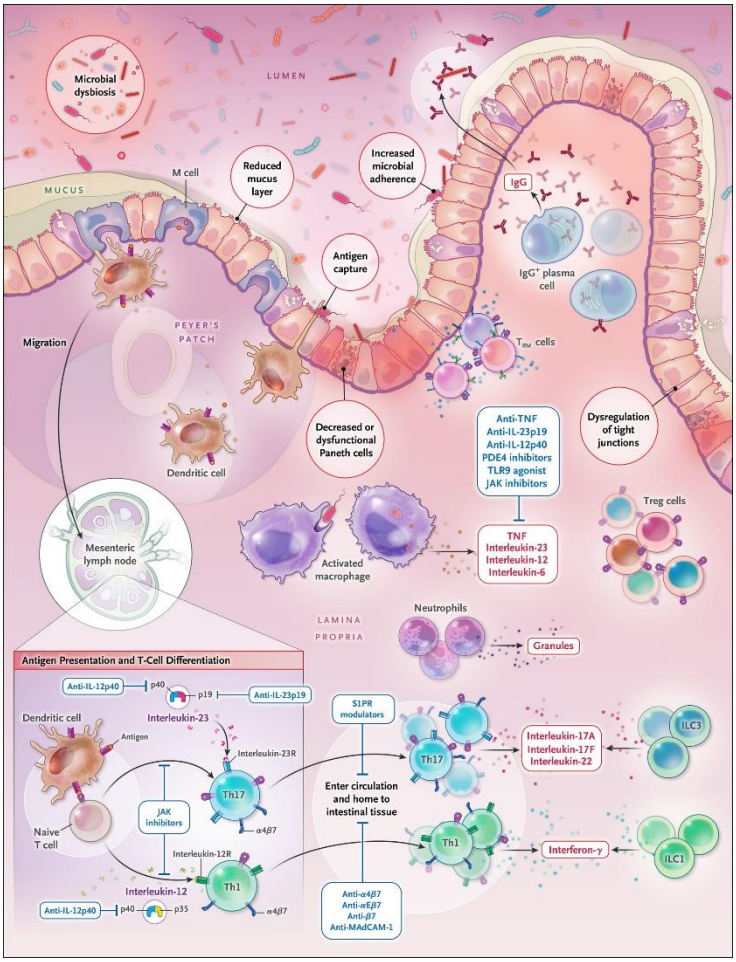


Figure I. 3. Immune response in Inflammatory Bowel Disease (IBD) *(81). As a result of harmful stimuli, alterations of the mucus layer and tight junctions, defects in Paneth cells and increased intestinal permeability, result in intestinal epithelial barrier disfunction with increased exposure of microorganisms. Macrophages become activated to remove bacterial substances increasing the production of TNF, interleukin-23, interleukin-12, and interleukin-6, which contribute to inflammation. Dendritic cells present antigen to naïve T cells. CD4+ T cells proliferate and differentiate into type 1 helper T (Th1) and type 17 helper T (Th17) cells. Th17 cells contribute to inflammation through production of interleukin-17A, interleukin-17F, and interleukin-23, whereas Th1 cells produce interferon-γ. Plasma B cells secreting IgG are also activated. IgG, Immunoglobulin G.*

I.1.4.4.2 Inflammatory response

The loss of epithelium and the increase in intestinal permeability allow a stronger immune system–microbiota interaction. In this scenario, several cell types and mediators play a crucial role in their perpetuation (80).

I.1.4.4.2.1 Innate immunity

As a response to injury, the innate immune system is the rapid and immediate response that is activated. One of the first signs of inflammation is neutrophils' infiltration in intestinal mucosa. Their presence disrupts epithelial barrier function and contributes to tissue damage through continuous secretion of pro-inflammatory factors (82), as illustrated in Figure I.3. Next, dendritic cells (DC) have also a key role initiating innate immune response and providing initial protection and defence, but they also participate in initiating adaptive immune response, providing an important crosstalk between innate and adaptive immunities. Given their ability to migrate from resident tissue to lymphoid tissue, they present antigens to other immune cells including T cells from other parts. In IBD, DCs show an increased expression of TLR2 and TLR4, CD40, and the chemokine receptor CCR7, whose activation enhances the production of pro-inflammatory cytokines such as IL-12 and IL-6 (83, 84).

On the other hand, macrophages are also key protagonists of innate immunity. In the mucosal layer, resident macrophages interact with microbiota maintaining gut homeostasis (85). Commonly, they come from peripheral blood monocytes, and due to their plasticity, they can acquire a wide variety of phenotypes depending on the microenvironment. Under homeostatic conditions, they exhibit a non-inflammatory phenotype with low expression of innate immune receptors and production of pro-inflammatory cytokines (86).

Although macrophage classification involves high heterogeneity, traditionally they have been classified as pro-inflammatory or classically activated (M1) phenotype and pro-resolving or alternatively activated (M2) phenotype. On the one

hand, polarization towards M1 phenotype occurs through lipopolysaccharide (LPS), IFN-γ or TNF-α stimulation, and results in upregulation of NFκB pathways and increased production of pro-inflammatory mediators such as IL-1β, IL-6, IL-12, IL-23, TNF-α, CCL2, as well as reactive oxygen species (ROS). Such activation is essential to eliminate pathogenic agents, although pro-inflammatory mediators produced upon activation also contribute to IBD pathogenesis. On the other hand, cytokines such as IL-4, IL-10 and IL-13 promote M2 macrophage polarization which, in turn, is divided into M2a, M2b, M2c and M2d subclasses. First, IL-4 and IL-13 polarize towards M2a macrophages, which express CD206, IL-1 receptor (IL-1R) and CCL17, which are involved in tissue repair and wound healing. Next, TLR and IL-1R stimulation polarizes macrophages towards M2b phenotype, which exerts a regulatory function secreting both pro- and anti-inflammatory cytokines. Upon IL-10 stimulation, macrophages polarize towards M2c phenotype, an anti-inflammatory subset characterized by phagocyting apoptotic cells. Recently, a tumour-associated subtype has been identified, the M2d phenotype, which stimulates angiogenesis and cancer metastasis (87).

Intestinal macrophages undergo a high phagocytic activity and remove apoptotic and senescent cells. They reside in the lamina propria and protect the epithelial barrier eliminating circulating pathogenic microorganisms without migrating to lymphoid tissue. Such macrophages are recognized by the expression of several molecules such as CD64, major histocompatibility complex (MHC) class II and F4/80 (88). Recently, CD11c has also been identified in a population of macrophages in intestinal mucosa (89). Certain alterations in macrophage phenotypes and functions have been identified in IBD patients. For instance, in CD patients, increased number of $CD14^+$ macrophages expressing pro-inflammatory cytokines such as IL-23 and TNF-α have been identified compared with UC and control subjects (86). Of interest, M1 macrophages, through secretion of TNF-α, can contribute to impairment of TJs in the intestinal epithelial barrier triggering its

disruption (90). In addition, an increased abundance of M2 $CD206^+$ macrophages has been found in CD compared to non-IBD patients, and levels of these macrophages are even higher in chronic stages of the disease compared to newly diagnosed patients (91, 92). Indeed, a functional role of M2a macrophages in wound healing via STAT6 has been reported since the administration of IL-4 treated macrophages could revert acute colitis and improve intestinal regeneration (93). Moreover, an anti-fibrotic role of M2a macrophages was also demonstrated since their administration reduced intestinal fibrosis (94).

On the other hand, due to the damage of the epithelial barrier, immune cells from deeper layers are also exposed to harmful agents nearby, triggering an immune response as illustrated in Figure I.3. The release of the microbe-derived ligand LPS by gram negative bacteria activates macrophages towards a pro-inflammatory phenotype. Therefore, a change in gut microbiota composition can induce macrophage dysfunction increasing the number of pro-inflammatory subsets versus anti-inflammatory ones, resulting in increased susceptibility to IBD. In this scenario, macrophages activate inflammatory pathways such as NFκB, JAK-STAT and PI3-Akt via releasing pro-inflammatory mediators such as IL-1, IL-6, IL-12, IL-23, TNF-α and producing ROS (40).

I.1.4.4.2.2 Adaptive immunity

Adaptive immunity constitutes the more specific response and the one that provides long-term immunity, known as memory. In this scenario, T and B cells represent the main effectors of adaptive immunity. Once DCs and macrophages recognize antigens, they present them to CD4+ Th cells (Th0), inducing the activation of T regulatory (Treg) cells. Under inflammatory conditions, Th0 are stimulated by different cytokines which induce their differentiation into T helper (Th) cells, such as Th1, and Th17, as depicted in Figure I.3, and also towards Th2 (95).

First, Th1 cells contribute to IECs apoptosis and differentiation of mesenchymal cells to myofibroblasts through TNF-α and IFN-γ secretion. Next, Th2 cells also contribute to IECs apoptosis and increase intestinal barrier permeability by releasing IL-4, IL-5, and IL-13. Both Th1 and Th2 differentiation are induced by IL-12. Classically, CD and UC have been classified according to different subtypes of pro-inflammatory immune responses, considering CD a Th1 response, and UC a Th2 response (80). Several studies contributed to such classification. For instance, in CD patients, an abnormal Th1 immune response promoted by increased levels of IL-12 and IL-18 has been identified to cause inflammation (96, 97). Moreover, higher levels of IL-2 and IFN-γ have been found in T cells from CD patients, in comparison with UC patients or controls, whilst UC exhibited increased levels of IL-5 and IL-13 (98-101). However, recent findings have reconsidered such classification since the levels of cytokines in each pathology do not always follow these classically established patterns. Finally, Th17 responses have also been identified as important mediators of IBD pathogenesis. Their differentiation is mediated by IL-6, IL-23 and TGF-β, and they participate in the recruitment of neutrophiles through STAT3 activation and producing IL-17A, IL-17F, IL-21, and IL-22. Several studies have identified increased levels and overexpression of IL-17A in the mucosa of IBD patients. Of interest, another subtype of T cells classified as Th1/Th17 cells, characterized by production of both IFN-γ and IL-17A, has been identified. Indeed, inflamed mucosa of IBD patients shows higher levels of Th17 and Th1/Th17 subsets compared with healthy controls. In turn, IL-21 produced by Th17 induces Th1 and Th17 responses contributing to inflammation maintenance. In fact, IL-21-deficient mice were prevented from Th1/Th17 cells mediated colitis. Finally, Treg cells contribute to maintain intestinal homeostasis regulating abnormal immune responses activation by secretion of IL-10 and TGF-β. When activated, they promote tolerance and control inflammation, preventing activation of other T cells. They express Foxp3 and it has been demonstrated that IL-6 stimulation reduces Foxp3 expression, whereas induces IL-17 expression, which is characteristic of Th17 subtype,

contributing then to persistent intestinal inflammation. In fact, impaired function of Treg cells has been identified in IBD patients (95).

Last but not least, B cells are also part of adaptive immunity. They are responsible of antibodies production, but they express also different types of cytokines according to the signals that they perceive. For instance, there is a subset of B regulatory cells known as Breg which secretes IL-10 and it is reduced in CD patients (102). In addition, IBD patients exhibit increased production and secretion of immunoglobulins (Ig)M, IgG and IgA, as well as disturbed proportions of Igs classes which appear due to gut inflammation (80).

I.1.4.4.3 Molecular mechanisms involved: inflammasomes

Among the molecular mechanisms involved in IBD pathogenesis, we focus on inflammasomes, a group of cytosolic protein complexes which also recognize pathogens and danger-associated endogenous components. When these complexes are activated, pro-Caspase-1 is proteolytically cleaved inducing the production of IL-1β and IL-18, which in turn, activate and perpetuate inflammation and immune responses. Different types of inflammasomes have been described such as NOD-like receptor (NLRP)1, NLRP2, NLRP3, NLR family CARD (NLRC)4, or absent in melanoma (AIM)2, although the most intensively studied has been NLRP3. Inflammasome activation consists of two steps. Firstly, the inflammasome priming consists of an induction of the transcriptional expression of main inflammasome components including NLRP3, ASC, IL1B and IL18. Next, the activation takes place when pro-Caspase-1 is split into Caspase-1 and IL-1β is released (103).

NLRP3 inflammasome has been associated with a wide range of chronic pathologies including diabetes, atherosclerosis and even IBD (104). In fact, in NLRP3 gene, some small nuclear polymorphisms (SNPs) such as rs10754558 and rs10925019 have been found to increase risk of suffering UC (105). In addition, CD clinical severity has been related with increased caspase-1 activity and production of

IL-1β and IL-18 in isolated intestinal immune cells (106). A study reported that AIM2 deletion resulted in the inhibition of IL-18 and IL-22 secretion in IECs, but also resulted in loss of Reg3 family antimicrobial peptides via STAT3, which produced dysbiosis and colitis in mice (107). Nonetheless, controversial results have been obtained in different studies. In some studies, $NLRP3^{-/-}$ mice exhibited a protective effect on experimental colitis models such as oxazolone, TNBS or DSS (108-110), whilst others have reported worse disease features in the absence of NLRP3 (111-113). Given the previously described function of epithelial cells maintaining homeostasis of the epithelial barrier, NLRP3 inflammasome exerts a dual role that depends on the cell type and the duration of the stimulus. Initially, it provides protection but become damaging under constant activation (114).

I.1.5 IBD complications

As stated above, IBD pathology is related with many complications that commonly require surgical intervention. Most of IBD complications such as strictures, bowel penetration, obstruction and fistulas start as a result of intestinal fibrosis. The increasing incidence in IBD and IBD-associated complications, together with the limited treatment strategies available, evidence the huge necessity of better understanding the molecular mechanisms involved in intestinal fibrosis development associated to IBD.

I.1.5.1 Fibrosis

Intestinal fibrosis represents one of the most common complications associated to IBD, characterized by an excessive accumulation of extracellular matrix (ECM), including collagen type I, which causes structural and functional tissue alterations. At the time of diagnosis most patients present purely inflammatory phenotype, but within years, almost 30-50% of CD patients and around 1-12% of UC patients, suffer from fibrosis-related complications (62, 115, 116). In CD, as transmural inflammation affects all layers, the excessive accumulation of ECM is accompanied

of mesenchymal cell expansion of muscularis mucosae and inner muscularis propria, as well as muscularization of submucosa. Of interest, although there exist differences in the degree of transmural inflammation, it has been demonstrated that both stricturing (B2) and penetrating (B3) phenotypes show similar degree of fibrosis. Whilst in the case of UC, the accumulation of ECM is limited to the submucosa layer, and there is also a thickening of muscularis mucosa which progresses as inflammation becomes chronic and more sever. As a result, there is an increased stiffness of gut wall which drives motility abnormalities in these patients (11).

At first instance, fibrosis arises as a homeostatic mechanism which contributes to tissue repair after injury. Under these conditions, IECs can be activated to regenerate tissue damage, since they have the ability to lose their polarity and migrate to the site of damage. Once there, they proliferate and differentiate in an attempt to restore mucosal epithelium, a process known as mucosal healing (75). Several cytokines such as transforming growth factor (TGF)-β, IL-1β, IFN-γ, epidermal growth factor (EGF), or fibroblast growth factor (FGF), participate in this process and activate molecular pathways such as NFκB (117). IBD pathology also comprises impairment and alterations of such mechanisms making difficult the epithelial restoring. Two signalling pathways, Wnt and Notch, regulate intestinal epithelial stem cells proliferation and are crucial to epithelium repair after injury (75). For instance, it has been reported that IECs in inflamed tissues, have an upregulated expression of Notch1 receptor and their ligands (118). Besides, in 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-treated mice, a macrophage phenotype which is dependent on STAT6 triggers mucosal repair via the Wnt pathway and when this healing process is disrupted, fibrosis arises (93).

Central protagonists in fibrogenesis are mesenchymal cells including fibroblasts (α-smooth muscle actin [α-SMA$^-$]), myofibroblasts (α-SMA$^+$), and smooth muscle cells (SMCs) (α-SMA$^+$ and desmin$^+$), since they proliferate, expand and differentiate into active myofibroblasts contributing to ECM deposition. In homeostatic

conditions, when the healing process is finished, fibroblasts become apoptotic or senescent and ECM is degraded by matrix metalloproteases (MMPs). However, under chronic inflammation, as happens in the case of IBD, the continuous stimulation of fibroblasts impairs this last step and fibrogenesis is then sustained leading to the formation of complex scar tissue, stricture formation and further bowel obstruction (61), as observed in Figure I.4. Indeed, enhanced MMPs activity can result in immune-mediated tissue damage (119). Besides, fibroblasts also potentially interact with immune cells to perpetuate the development of fibrosis (11).

Whether fibrosis is dependent on inflammation or not is still an open question and recent theories suggest the existence of both inflammation-dependent and inflammation-independent fibrogenesis. It is widely assumed that the inflammatory process, through the secretion of pro-inflammatory mediators by immune cells, leads to the activation of mesenchymal cells that synthetize exacerbated ECM. In addition, inflammation also activates SMCs, which contribute to the thickening of muscular layers by hypertrophy and/or hyperplasia, as depicted in Figure I.4. Apart from that, the presence of a stiff ECM itself also contributes to fibrogenesis in an inflammation-independent process. ECM components and fibroblasts themselves exert a regulatory process by secreting mediators which further contribute to integrin-mediated activation, resulting in abnormal ECM deposition with pathological architecture (11, 120-122).

Among the mediators that participate in fibrogenesis, TGF-β1 is the major cytokine involved in intestinal fibrosis (123), since it undergoes several pro-fibrotic actions, such as inducing differentiation of fibroblasts into myofibroblasts, and therefore, production of ECM and tissue inhibitors of metalloproteinase (TIMP)-1 and prevents myofibroblasts apoptosis. Apart from the previously mentioned mediators, other mediators such as TNF-α, IL-23, IL-36, activins, vascular endothelial growth factor (VEGF), products of oxidative stress, mammalian target of rapamycin (mTOR), endothelins (ET)-1, -2 and -3, galectin-3 and components of

the renin-angiotensin system (RAS) among others, also contribute to fibrosis development (11). Besides, Th17 and Th2 cells secrete a wide variety of ILs such as IL-17, IL-21, IL-4, or IL-13 which induce ECM synthesis in fibroblasts and activate macrophages. In turn, macrophages secrete pro-fibrotic mediators such as TGF-β1, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), connective tissue growth factor (CTGF) and also MMP-9 (116).

Although immune cells activate the main contributors to fibrogenesis, there are also other cell types which differentiate and undergo this activity. In fact, they can originate from epithelial cells, in a process known as epithelial-to-mesenchymal transition (EMT), which is part of the wound healing mechanism, but, under dysregulated and pathological conditions, it contributes to fibrosis development. In parallel, mesenchymal cells can also originate from endothelial cells in a process known as endothelial-to-mesenchymal transition (EndoMT), in which capillary endothelial cells, upon persistent inflammatory stimuli, become spindle-shaped granulation tissue cells (124). Fibrocytes, pericytes and bone-marrow-derived stem cells can also differentiate and undergo fibrogenesis (116).

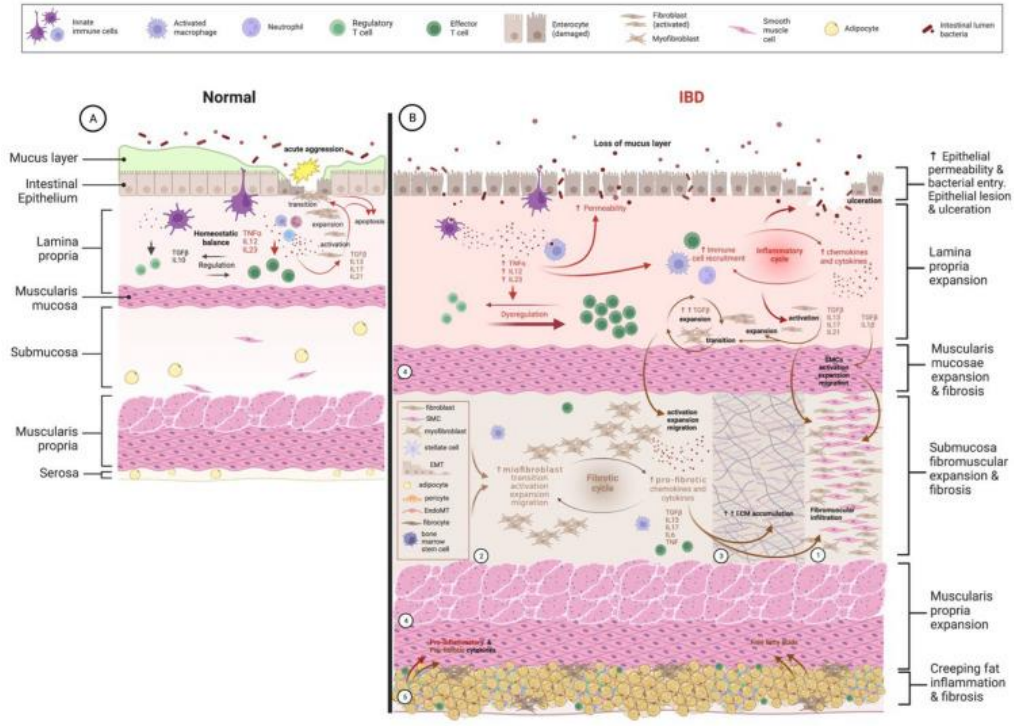


Figure I. 4 Molecular mechanisms occurring in inflammation-mediated fibrogenesis *(11).* ***(A)*** *Normal homeostatic conditions with a balanced immune response in the intestinal lamina propria. When the intestinal epithelium is injured, the inflammatory process is activated to eliminate the causative agent and promote tissue repair by local fibroblasts. Once tissue is repaired, fibroblasts participating suffer apoptosis.* ***(B)*** *Dysregulated chronic inflammation produces increased permeability and disrupted intestinal epithelial barrier which allows the entrance of microorganisms and activate inflammatory cascades and cause tissue injury. Perpetuated inflammation activates fibroblasts in the lamina propria. Activated fibroblasts, smooth muscle cells (SMCs) and myofibroblast expand contributing to thickening of muscular layers, hyperplasia and/or hypertrophy. These activated cells secrete pro-fibrotic mediators increasing the number of activated cells enhancing fibrogenesis. Creeping fat, as a source of pro-inflammatory and pro-fibrotic mediators, can also contribute to fibrogenesis. EMT, Epithelial-to-mesenchymal transition; EndoMT, Endothelial-to-mesenchymal transition; TNF, Tumour necrosis factor, TGF, Transforming growth factor, IL, Interleukin; ECM, Extracellular matrix.*

I.1.5.2 Fistula

An important IBD complication, especially in CD patients, is the penetrating or fistulizing disease behaviour. A fistula is an abnormal tissue formation which connects the intestinal tract with another structure nearby such as rectum, intestinal loops, genital structures, or abdominal wall, although the most common is the perianal fistula (54% of the cases). Among 17% to 50% of CD patients develop fistula and nowadays, pharmacological treatment does not prevent its formation nor reversion. Hence, these patients commonly require surgical interventions which not always succeed (125).

The histology of fistula is characterized by a lumen filled with cell debris, inflammatory cells and erythrocytes, covered by squamous or flattened epithelial cells. In some cases, instead of epithelial cells, fistulas are lined by myofibroblast-like cells known as transitional cells. As a result of chronic inflammation and intestinal epithelial barrier disruption, IECs migrate to inner gut layers and contribute to fistula formation. The process associated to fistula formation is not well established, but evidence suggests that EMT is one of the mechanisms involved (126, 127). In this process, IECs lose their features, specifically the expression of Cadherin-1, an epithelial cell-cell adhesion molecule, cell to cell junctions and apical-basal polarity. Hence, they acquire mesenchymal cell morphology, expressing markers such as Vimentin, fibroblast-specific protein-1 (FSP1), α-SMA and producing the pro-fibrotic cytokine TGF-β (125, 128). Other molecular mediators such as TNF-α and IL-13 are also increased and contribute to EMT process. Among the molecular pathways involved in EMT, TGF-β/Smad, Wnt, Notch and Hedgehog are the most widely studied and in all of them, the activation of SNAIL transcription factors such as SNAI1 and SNAI2 has been reported (129). Furthermore, upregulated MMP-3 and MMP-9 expression is found in CD fistulas, whilst TIMP-1, TIMP-2 and TIMP-3 are downregulated, resulting in an imbalanced ECM which contributes to inflammation and tissue injury, inducing EMT and fistula formation (125, 130).

I.2 Metabolomics in IBD

Metabolomics consists in the large-scale study of a huge variety of small molecules, known as metabolites, comprising from molecular intermediates to metabolism-end products. Within the last years, metabolomics has emerged as a promising technique to deeply study and better understand a wide range of diseases such as cancer, cardiovascular ischemia, and many autoimmune diseases. Therefore, this analysis can be performed to characterize the metabolomic profile of biological samples, including plasma, urine, stool, biopsies, and surgical resections. According to the type of sample and chemical features of the metabolites to identify, different techniques can be performed. The most widely used include proton nuclear magnetic resonance (1H-NMR) spectroscopy, gas-chromatography mass spectrometry (GC-MS) and liquid-chromatography mass spectrometry (LC-MS). Hence, metabolomic analysis allows the characterization of specific biomarkers that may improve disease diagnosis and assessment, and the identification of alterations in pathology-related molecular pathways, especially those related with the gut microbial metabolism (131).

In the context of IBD, the lack of appropriate biomarkers to diagnose and assess disease status, makes metabolomics a useful technique to identify specific metabolic signatures. Besides, assuming that intestinal inflammation alters several metabolic pathways, such as tricarboxylic acids (TCA) cycle, β-oxidation and urea cycle among others, the identification of altered metabolites in IBD patients constitutes a starting point to identify those pathways and their role in IBD pathogenesis (Sugihara K, 2019). Several studies have pointed dietary metabolites and microbiota dysbiosis responsible of metabolomic disturbances and molecular and cellular pathways disruption, since gut microorganisms metabolize dietary products into essential compounds (51, 132-134). In fact, all these molecules commonly play a key role in regulating the immune response and inflammation (135). Hence, altered levels of compounds metabolically interconnected may be associated to disturbances in

cellular metabolism and energy demand due to inflammation associated to IBD (131). In addition, metabolomic studies can also be used to evaluate the efficacy of pharmacological treatments applied to IBD patients. For instance, a study performed by Ding and colleagues has predicted the response to anti-TNF therapy by assessing levels of metabolites involved in lipid, bile acid and amino acid pathways (136).

In this Doctoral Thesis, we have performed a targeted metabolomic analysis quantifying metabolites capable of sensing G protein-coupled receptors. The following sections provide a brief description of the metabolites quantified and their role in IBD pathogenesis. These metabolites include carboxylic acids, fatty acids, and amino acids.

I.2.1 Carboxylic acids

Carboxylic acids are organic molecules characterized by containing a carboxyl group (-COOH), consisting of a carbonyl (C=O) plus a hydroxyl group (-OH), in the same carbon atom. Carboxylic acids are present in a huge range of diverse compounds and exert important roles in biochemical processes (137).

On the one hand, we highlight the role of tricarboxylic acid cycle (TCA) intermediates, such as citrate, isocitrate, succinate, fumarate, or malate among others. These compounds, also known as Krebs cycle intermediates, participate in cell metabolism and are essential to produce energetic compounds (138). Several alterations on TCA intermediates have been reported in different metabolomic analysis in IBD patients (131). For instance, succinate, citrate and aconitate have been mostly found decreased in urine and serum samples of IBD patients when compared to healthy controls, and specifically in CD patients compared to UC (139-143). Regarding TCA intermediates levels on tissue samples two studies reported decreased levels of these compounds in damaged mucosa of IBD and UC patients respectively versus healthy controls (144, 145), whilst another study reported increased levels of succinate in intestinal surgical resections of CD patients (146).

Alterations on these compounds may be also explained due to microbiota dysbiosis associated to IBD. For instance, some bacteria can synthetise succinate, which can be transformed to propionate by *Bacteroidetes* or *Firmicutes* (147). Moreover, it has been reported that IBD patients exhibit decreased abundance of *Phascolarctobacterium*, a succinate-utilizing bacteria, in comparison with healthy controls (148).

On the other hand, another carboxylic acid important in cellular metabolism is lactate, which can be oxidized to pyruvate and enter the TCA cycle (138). Lactate may be synthesized as a by-product of lactic acid bacteria fermentation and can exert a bactericidal and anti-inflammatory effect on the gut. However, several studies reported increased levels of lactate in UC (149-151), and CD patients (140, 151, 152) versus controls. In fact, it has been demonstrated that inflammation and disturbances in metabolism of immune cells contribute to lactate accumulation in tissues (149-151).

Finally, β-hydroxybutyrate, also known as 3-hydroxybutyrate, is a ketone body produced upon branched-chain amino acids degradation. Under fasting conditions and glucose depletion, ketones bodies such as β-hydroxybutyrate are synthetized to provide energy. They are indicators of cellular metabolism disturbances and a high energy demand state of the organism, since they are used as alternative source of energy (140). In addition, increased levels of these compounds have been associated to oxidative stress and cellular damage which contribute to several pathologies (153). Indeed, several studies have found higher concentrations of β-hydroxybutyrate in UC patients than in control subjects (140, 154, 155).

I.2.2 Fatty acids

Fatty acids represent a heterogeneous group of compounds which participate in the regulation of several physiologic and metabolic pathways. Thus, disturbances in their concentration can affect lipid cascades and numerous metabolic signalling

pathways that among others, modulate and constitute the inflammatory response. Regarding inflammation, they seem to exhibit a dual role since depending on their structure they can show pro-inflammatory or anti-inflammatory properties (135).

Depending on the number of carbon atoms in the chain, fatty acids are classified as short-chain (SCFAs), medium-chain (MCFAs) and long-chain fatty acids (LCFAs), including PUFAs. Levels of these fatty acids have been found disturbed in IBD patients versus healthy subjects, comprising different types of biological samples such as serum, feces, and mucosal biopsies (156).

On the one hand, within the last years, a lot of attention has been focused on SCFAs, including butyrate, propionate, and acetate, which are produced by gut microorganisms through fermentation of complex carbohydrates and fibres (51). In the gut, they can enter portal circulation through passive diffusion or active transport through transporters such as monocarboxylate transporter (MCT)-1 or sodium monocarboxylate transporter (SMCT)-1 (157). SCFAs in intestinal mucosa serve as energy source for intestinal epithelial cells and promote their cell growth preserving the epithelial barrier. Besides, they also exert several functions such as: regulation of Treg cells function, protection against induced colitis in mice (158), modulation of intestinal macrophages function (159, 160), and regulation of intestinal B cells activity (161). Among other described functions of SCFAs, they seem to prevent proliferation of carcinogenic cells, modulate appetite promoting peptide YY, control fat accumulation and glucagon-like peptide 1 (GLP1) secretion to regulate energy metabolism and intestinal motility (134). Thus, they have been described as anti-inflammatory compounds. Specifically, butyrate constitutes the energy source for colonocytes, provides protection against colorectal cancer (Kim ER, 2015), and preserves epithelial homeostasis through inflammasome activation and IL-18 synthesis (162). Next, propionate participates in liver metabolism, and acetate constitutes a primary substrate for the synthesis of cholesterol (163). According to several studies, low levels of SCFAs have been reported on feces, urine and serum

from IBD patients (51, 139, 140, 164, 165). Different theories explain this fact. On the one hand, impaired uptake of SCFAs may be due to sodium-potassium pump dysfunction in the mucosal cell membrane, associated to epithelial barrier damage (144). On the other hand, microbiota dysbiosis associated to IBD may also result in decreased abundance of fermentative gut microbiota such as *Faecalibacterium prausnitzii* and *Roseburia hominis* which may result in decreased production of SCFAs (166). In addition, low levels of butyrate in IBD have also been associated with a decreased gene expression of the enzyme responsible of butyrate synthesis butyryl-Coa:acetate CoA-transferase, impairing butyrate transport and oxidation (167). Finally, since anti-inflammatory effects of SCFAs have been observed in the gut, they have been proposed as supplementary therapy for clinical management of IBD patients. Indeed, a clinical trial of SCFAs enemas reported clinical remission in only a subset of UC patients (168, 169).

On the other hand, most of the studies point to a decrease in LCFAs and MCFAs levels in IBD patients (51, 135, 142, 170). IBD patients seem to exhibit an increased β-oxidation of fatty acids associated to a higher energy demand due to inflammation, depleting LCFAs levels. The higher energetic demand combined with the scarce amount of LCFAs result in an aggravated immune response (142). Some MCFAs and LCFAs are agonists of the peroxisome proliferator-activated receptor (PPAR)-γ and PPAR-α receptors which trigger an anti-inflammatory response inhibiting NFκB pathway activation and synthesis of pro-inflammatory cytokines (171, 172). With regard PUFAs, it has been reported that they have an anti-inflammatory role and regulate the inflammatory response by reducing the synthesis of pro-inflammatory cytokines (173). In contrast, some PUFAs, such as arachidonic acid, are involved in leukocyte recruitment by increasing intracellular adhesion molecule (ICAM)-1 (174, 175). They are also involved in the intestinal inflammatory response, synthetizing inflammatory mediators such as prostaglandins, thromboxane, and leukotrienes synthesis (176), and regulating the cell membranes of immune cells (177). Indeed,

increased levels of the prostaglandin PGE_2 have been found in CD patients, which has been linked to the production of IL-23 by dendritic cells, leading to the activation of Th17 lymphocytes (178).

I.2.3 Amino acids

Amino acids are organic substances characterized by containing both amino and acid groups. Multiple types of amino acids have been identified so far, but only 20 constitute building blocks for protein synthesis, which are classified as protein amino acids. In addition, amino acids constitute precursors for several carbon compounds, metabolites, hormones, and neurotransmitters. They participate in anaplerotic reactions providing necessary intermediates for gluconeogenesis, and their metabolism provides cellular energy. Hence, amino acids are key mediators and signalling molecules regulating different physiological functions (179).

In IBD, malabsorption of nutrients, including amino acids, occurs in the gut as a result of intestinal epithelial barrier disruption and inflammation, resulting in their accumulation in feces (164, 165). As a result, reduced levels of amino acids are commonly found in serum samples from IBD patients (135, 142, 152). Regarding intestinal tissue, few studies reported decreased levels of amino acids in mucosa of IBD patients in comparison with non-IBD controls (144, 145, 180), which has been associated to alterations in tissue repair due to inflammation (131). As previously mentioned, altered levels of amino acids may be also associated to microbiota dysbiosis, since several microorganisms metabolize amino acids into other biologically active compounds, and alterations in the abundance and composition of microbiota may also have an effect on amino acids levels (131).

I.3 Metabolite-sensing GPCRs in IBD

G-protein coupled receptors (GPCRs) represent the largest family of plasma membrane receptors regulating thousands of physiological processes in humans and

constitute targets for several drugs prescribed nowadays (181). The most widely described agonists of GPCRs include hormones, neurotransmitters, metabolites, and chemokines (41). Structurally, they have a seven-transmembrane helical domain constituted by G-proteins which are heterotrimeric guanine nucleotide-binding regulatory proteins. These G-proteins consist of three subunits Gα, Gβ and Gγ, although Gβ and Gγ function as a single unit forming a dimer. When an agonist binds to a GPCR, it triggers the exchange of GDP by GTP in the Gα subunit and consequently, it is dissociated from the Gβγ dimer, resulting in two separate functional subunits which activate different cellular pathways (182). Several types of Gα subunits have been identified (Gαi, Gαq and Gαs), which modulate different intracellular signalling pathways involving regulation of cyclic adenosine monophosphate (cAMP) synthesis, activation of MAPK pathway and regulation of Ca^{2+} flux, thus resulting in a wide variety of processes related with cell proliferation, differentiation, survival, and migration (157).

As previously reported, metabolic alterations can influence the immune system and as a consequence have an impact on inflammatory diseases in several ways: acting directly on receptors known as metabolite-sensing GPCRs; undergoing transcriptional and epigenetic modulations such as histone deacetylase (HDAC) inhibition; and acting as transcriptional coactivators. Due to the presence of metabolite-sensing GPCRs in the surface of different intestinal cell types including immune and non-immune cells, modulation of these receptors has a wide variety of effects, including maintenance of epithelial intestinal barrier homeostasis and also metabolism. The most widely described metabolite-sensing GPCRs can be activated by dietary and diet-derived metabolites such as omega-3 fatty acids, MCFAs, tryptophan derivatives and SCFAs. As a result of metabolomic disturbances, local pH may be also altered resulting in pH-sensitive GPCRs activation which are also classified as metabolite-sensing GPCRs. Therefore, metabolite-sensing GPCRs establish a direct link between metabolomic alterations, partially due to diet and microbiota dysbiosis, and inflammatory and metabolic disturbances, observed in

different pathologies including IBD. The most described GPCRs, their agonists and major functions in immunity are summarized in Figure I.5. (41, 157).

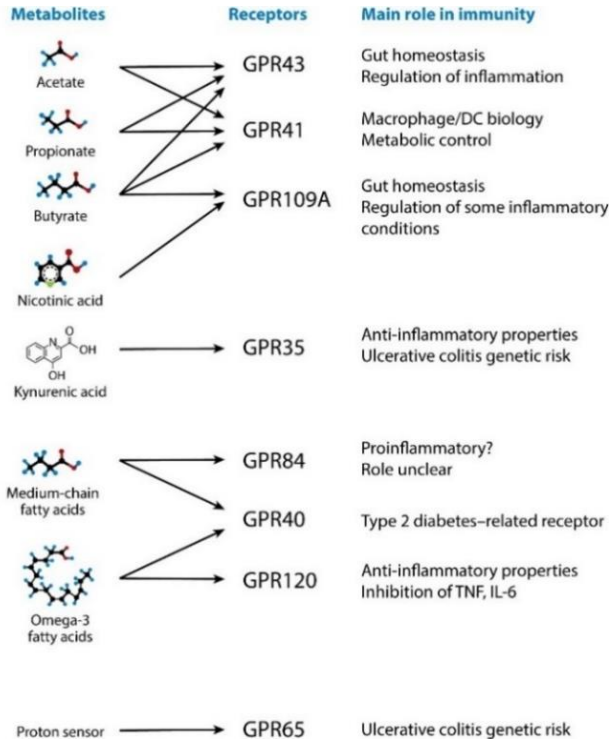


Figure I. 5. Metabolite-sensing GPCRs associated with their metabolites and their roles in immunity. *Adapted from (157). DC, dendritic cells; TNF, Tumor necrosis factor; IL, Interleukin.*

In this Doctoral Thesis, metabolite-sensing GPCRs have been classified according to the type of metabolite acting as ligand.

I.3.1 GPCRs sensing carboxylic acids

Several carboxylic acids-sensing GPCRs have been extensively studied. In this section, GPR91 and GPR109A are deeper described considering that they are key protagonists of this Doctoral Thesis.

I.3.1.1 GPR91

GPR91, also known as SUCNR1, is a receptor which was deorphanized in 2004 (183). It is located on the cellular plasma membrane (184) and expressed in different cell types such as hepatic stellate cells (185), megakaryocytes and platelets (186), macula densa cells (187), white adipocytes (188), retinal ganglion neurons (189), and immune cells such as macrophages and immature dendritic cells (190), among others. The best described endogenous ligand is succinate, a small dicarboxylic acid which is the anionic deprotonated form of succinic acid (184, 190). In physiological conditions, succinate is mostly found in the mitochondria, however, under metabolic alterations due to pathological conditions, succinate is accumulated and secreted to the extracellular medium resulting in GPR91 activation (140, 191-193). This activation triggers different downstream signalling pathways depending on the type of the G-protein coupled as observed in Figure I.6. On the one hand, it can be coupled to Gi, whose activation results in a decrease of cAMP levels. On the other hand, it can also be coupled to Gq and this activation results in PLC-β activation and intracellular calcium mobilization. Finally, this receptor can also activate MAPKs such as extracellular signal-regulated kinase (ERK)1/2 or PI3K-Akt-Src pathway. The specific signalling pathway induced is tissue specific. For instance, in cardiomyocytes, an increase of cAMP levels and PKA activation occurs when α subunit is released from βγ dimer, while a decrease in cAMP levels occurs in adipose tissue. In dendritic cells, βγ dimer phosphorylates ERK1/2 upon intracellular calcium mobilization (194) whereas in platelets, PI3K-Akt-Src pathway is activated (195).

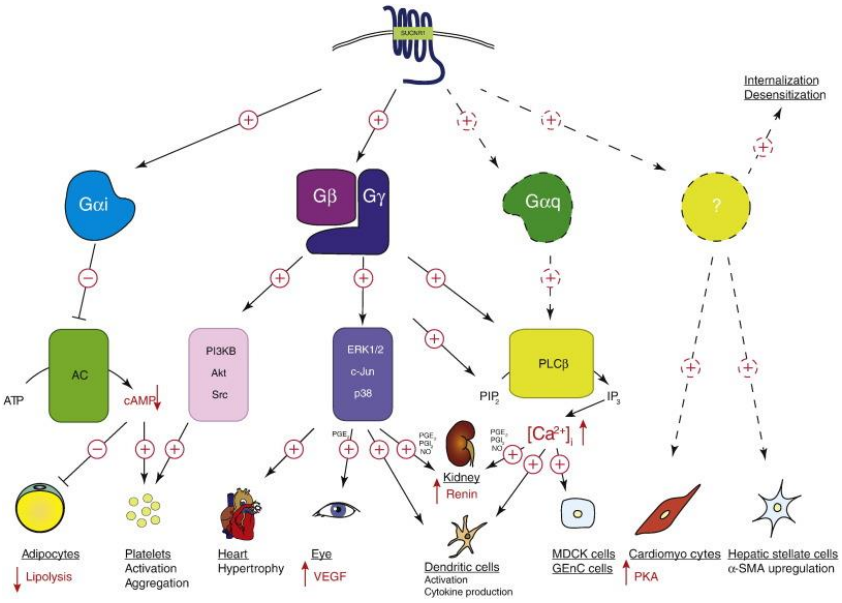


Figure I. 6. Downstream signalling pathways activated upon succinate binding to GPR91. *(196). GPR91 can be coupled to Gi, whose activation results in a decrease of cAMP levels as occurs in adipose tissue. Next, it can also be coupled to Gq and this activation results in PLC-β activation and intracellular calcium mobilization. Finally, this receptor can also activate MAPKs such as ERK1/2, as it occurs in dendritic cells, or PI3K-Akt-Src pathway, which is activated in platelets.*

This receptor has been widely studied in different cell types however, there is some controversy on whether it exerts a pro-inflammatory or an anti-inflammatory role. For instance, activation by this receptor through PKA-CREB-KLF4 pathways in adipose tissue macrophages triggers an anti-inflammatory effect (197). On the other hand, in macrophages, it potentiates the release of IL-1β through HIF-1α stabilization, (198, 199), enhances TNF-α expression, and reduces the expression of IL-10, TLR4 and TLR5 in peripheral blood mononuclear cells (200), while increases chemotaxis in monocyte-derived macrophages from white adipose tissue (201). In addition, in tubular epithelial cells succinate triggers apoptosis via SUCNR1 activation (202). In line with this, different studies point to a pro-inflammatory role

of both succinate and GPR91 in rheumatoid arthritis, in which this metabolite is accumulated in the synovial fluid (192), in diabetes mellitus and kidney injury complications (191), and obesity (203).

In line with this, our group has reported that GPR91 plays a pro-inflammatory and pro-fibrotic role in different murine models of intestinal inflammation and fibrosis, and both succinate levels and GPR91 receptor expression are significantly increased in surgical resections from CD patients (146).

I.3.1.2 GPR109A

GPR109A, also known as hydroxyl carboxylic receptor (HCAR)2, was deorphanized when niacin, also known as nicotinic acid or vitamin B_3, and β-hydroxybutyrate, were identified as ligands (204-207). Several cells exhibit high expression of GPR109A including immune cells such as macrophages, monocytes, dendritic cells and neutrophils (208), different types of cells from the nervous system and others such as adipocytes, intestinal epithelial cells, hepatocytes, retinal cells or keratinocytes (209, 210). Interestingly, studies in mice showed that GPR109A expression is dependent on the presence of gut microbiota and their associated metabolites, as germ-free mice had lower expression of GPR109A, which was reversed to normal levels when the intestinal tract was colonized with bacteria (211).

On the one hand, one of the agonists of GPR109A is niacin, whose union to the receptor triggers antilipolytic and antiatherosclerosis effects and as a result, inhibition of free fatty acids release and triglyceride, VLDL and LDL cholesterol synthesis (206). These effects have been demonstrated *in vivo* since upon niacin treatment, mice enhanced the expression of the cholesterol transporter ABCG1 and as a result, cholesterol efflux was increased. In addition, macrophage recruitment to atherosclerotic plaques through GPR109A was also prevented upon niacin treatment (212). In addition, an anti-inflammatory role of this receptor has also been demonstrated in sepsis, diabetic retinopathy, renal disease, and obesity (209). In fact,

GPR109A activation by niacin inhibits the release of pro-inflammatory cytokines on adipose tissue (212-214).

On the other hand, the other ligand for this receptor is β-hydroxybutyrate, a ketone body which is synthetized under fasting conditions and glucose depletion to provide energy. Under physiological conditions, the concentration of β-hydroxybutyrate is not enough to activate GPR109A, but in periods of short-term starvation, β-hydroxybutyrate synthesis is induced and increased levels of the metabolite can trigger the activation of GPR109A (210). Activation of GPR109A by β-hydroxybutyrate has been deeply studied in neurological disorders. For instance, in a murine model, β-hydroxybutyrate administration resulted in less ischemic brain damage after stroke induction, mediated by the polarization of bone marrow-derived macrophages towards a neuroprotective phenotype (215). Beneficial effects of β-hydroxybutyrate have also been observed in Parkinson's Disease since its administration in mice impairs the activity 1,2,3,6-tetrahydropyridine, a neurotoxin that triggers dopaminergic neurodegeneration and mitochondrial dysfunction (216). Regarding colitis, a study demonstrated that β-hydroxybutyrate decreases colonic inflammation and carcinogenesis in an acute DSS-colitis model (217). Finally, an hepatoprotective effect of β-hydroxybutyrate via GPR109A has also been described, since its supplementation in mice induced polarization towards anti-inflammatory M2 macrophages and an upregulation of the anti-inflammatory cytokine IL-10 (218).

Finally, another described ligand for GPR109A is butyrate, whose interaction with GPR109A has been associated with tumor development prevention (Thangaraju M, 2009). Of note, butyrate can also activate other receptors such as GPR41, GPR43 or PPARγ, and inhibit histone deacetylase activity triggering similar effects. It is important to highlight that since butyrate is a SCFA product of bacterial fermentation, its concentration is much higher in the gut in comparison to other parts of the organism. Hence, the effects of butyrate activating GPR109A have been widely described on intestinal cells regulating intestinal homeostasis, modulating

intestinal epithelial barrier function, and preventing inflammation (52, 168). Indeed, TJs on intestinal epithelial cells and epithelial permeability can also be regulated by butyrate via GPR109A binding, since this metabolite, through AMP-activated protein kinase, modulates the assembly of TJs promoting epithelial intestinal barrier maintenance and development (219). Additionally, activation of GPR109A in IECs by butyrate impairs LPS-induced NFκB activation (220). Regarding experimental colitis, several studies have reported the protective role of GPR109A. First, a reduction in both inflammatory response and epithelial barrier dysfunction has been reported in WT versus GPR109A$^{-/-}$ mice after sodium butyrate treatment in TNBS-induced colitis (221). However, although butyrate activation of GPR109A can mediate AMPs and mucins synthesis, it can also increase IL-18 secretion (222). In turn, IL-18 can modulate IL-22 accumulation and under inflammatory conditions, ILC3 is also accumulated in intestinal mucosa. In fact, both ILC3 and IL-22 contribute to an increase in IL-18 synthesis in IECs, an effect which intensifies gut inflammation and results in intestinal mucosal injury (223, 224).

The G-protein present in this receptor is classified as a Gαi/o-type since upon ligand activation, it decreases cAMP levels exhibiting inhibitory effects (204-206). In addition, recruitment of β-arrestins into the cell membrane also occurs upon its activation (52).

I.3.1.3 Others GPCRs sensing carboxylic acids

Apart from these carboxylic acid-sensing GPCRs, there are others whose role has been less studied. On the one hand GPR109B, also known as HCAR3, shares an elevated homology with GPR109A with only 16 different amino acids (205), 2003). It has less affinity for niacin and higher affinity for other ligands such as essential amino acids including phenylalanine, tryptophan or kynurenine, and their binding seems to trigger chemotaxis on neutrophils (157). On the other hand, GPR81, also known as HCAR1, is the receptor which recognizes lactate and whose activation

seems to have regulatory effects on homeostasis and inflammation in intestinal macrophages and dendritic cells. Indeed, experimental colitis induced in GPR81$^{-/-}$ mice has shown to increase susceptibility to colonic inflammation (225).

I.3.2 GPCRs sensing SCFAs

SCFAs-sensing GPCRs are one of the most widely studied metabolite-sensing GPCRs. In this group we include GPR43 and GPR41. GPR43, also known as free fatty acid receptor (FFAR)2, is the receptor for propionate, acetate, and butyrate. It is highly expressed in immune cells such as neutrophils, dendritic cells, monocytes, mucosal mast cells, eosinophils, basophils, and enterocytes. Among its functions, it has been reported a protective role in gut homeostasis and colon cancer, as well as regulatory effects on the immune system including Treg cells regulation and leukocytes chemotaxis. In addition, it prevents insulin-mediated fat accumulation in the adipose tissue. GPR41, also known as FFAR3, is mainly activated by acetate and propionate and in a less extent by butyrate, valerate or caproate. This receptor plays an important role in metabolic processes, and it is highly expressed in white adipose tissue and pancreas, as well as neural cells. A role for this receptor in allergic diseases has also been proposed since upon activation, it disturbs hematopoiesis resulting in alterations on Th2 cells development and airway inflammation. Both GPR43 and GPR41 share a high proportion of the amino acid sequence and similar effects to those described for GPR109A activated by butyrate are observed with these receptors (157).

I.3.3 GPCRs sensing MCFAs

In this group, the only metabolite-sensing GPCR described to be activated by MCFAs of 9 to 14 carbons chain is GPR84, whose function has been poorly studied. It is highly expressed on macrophages and neutrophils, and it has been reported that inflammation enhances its expression. Upon activation, secretion of pro-

inflammatory cytokines such as IL-8 and TNF-α by neutrophils and macrophages is stimulated (157).

I.3.4 GPCRs sensing LCFAs

Regarding LCFAs-sensing GPCRs, GPR40, also known as FFAR1, and GPR120, also known as FFAR4, are the most widely described. On the one hand, saturated and unsaturated fatty acids of 8 to 22 carbon chain, such as docosahexaenoic, α-linolenic, myristic and oleic acids bind to GPR40. It is highly expressed in the pancreas and the liver, but also in the central nervous system, and in different immune cell types (41). The exact role of this receptor has not been fully characterized given the presence of controversial studies, since it has been reported a reduction of glucose-mediated insulin secretion upon GPR40 deletion in murine models (226), whereas another study demonstrated also in mice increased glucose-mediated insulin secretion when GPR40 was overexpressed (227).

On the other hand, omega-3 fatty acids bind to GPR120. This receptor is highly increased in immune cells such as macrophages, dendritic cells, or eosinophils, but also in colonic epithelial cells, in the adipose tissue and in different enteroendocrine cells. Of interest, the anti-inflammatory role of omega-3 fatty acids seems to be mediated by this receptor, since upon activation, it protects against obesity-associated inflammation (157). Finally, another LCFA-sensing receptor is GPR119, which is also activated by different lipids containing oleic acid on their structure. Its agonists seem to exert a protective role in type 2 diabetes and metabolic disorders (228).

I.3.5 GPCRs sensing amino acids and their derivatives

Although there are several types of GPCRs sensing amino acids, the most widely described is GPR35. This receptor is expressed in the gut including epithelial cells and myeloid cells, such as dendritic cells or macrophages. Among their agonists, the role of kynurenic acid, a product from the catabolism of tryptophan

through the enzyme indoleamine 2,3-dioxygenase, is the most well-studied. GPR35 activation plays a role in intestinal homeostasis inducing Treg cells and controlling effector T cells. Of interest, certain polymorphisms on *GPR35* gene have been associated with increased risk of developing IBD (157). Other receptors included in this group but less studied on inflammation are calcium-sensing receptor (CaSR), which can be activated by L-amino acids, but also by anions, cations, glutamyl peptides or polyamines, playing a role in maintaining cellular homeostasis and reducing intestinal inflammation (229). Next, trace amine associated receptor 1 (TAAR1), whose ligands are trace amines, seems to modulate macrophage-derived inflammation in UC (230), and it is also included in this group. Finally, GPR142 also senses aromatic amino acids, and its role has been more extensively studied in type 2 diabetes (228).

I.3.6 GPCRs sensing pH changes

There exist several receptors which are sensitive to acidic conditions (pH around 6.8), and they are activated when the concentration of protons increases. Their roles have been widely studied in inflammation and fibrosis associated to IBD pathogenesis. First, it has been reported that GPR4 is involved in angiogenesis and extracellular matrix modelling. In addition, its expression is positively correlated with fibrotic markers and its deficiency in mice seems to protect them from intestinal fibrosis (231). Next, GPR65, also known as T cell death-associated gene (TDAG)8, is highly expressed in immune cells such as eosinophils, neutrophils, mast cells and T and B lymphocytes whose viability is increased upon activation of this receptor. Of interest, GWAS studies have identified polymorphisms on this gene associated to increased risk of developing IBD (232, 233). Another receptor sensitive to acidic pH is GPR68, also known as ovarian cancer GPCR 1 (OGR1). It is highly expressed on fibroblasts, macrophages, endothelial cells, and granulocytes. Interestingly, studies performed on this receptor have demonstrated its role in mucosal inflammation and fibrosis associated to IBD (234). Finally, GPR132 is also included

in this family, although it seems be less sensitive to proton changes than the previously described receptors. It is also highly expressed on immune cells, and it is involved in the inflammatory process and lipid metabolism (235).

II. AIMS

The general aims of this Doctoral Thesis are to characterize the axis intestinal microbiota composition, metabolites, and metabolite-sensing GPCRs in intestinal surgical resections from both UC and CD patients, and to investigate the role of both GPR91 and GPR109A in IBD pathogenesis.

The specific aims are:

1. To characterize in intestinal surgical resections from both UC and CD:
    a. Gut microbiota.
    b. The metabolomic profile of GPCR-agonist metabolites.
    c. The gene expression of metabolite-sensing GPCRs.
2. To determine the relationship between pro-inflammatory and pro-fibrotic markers and metabolite-sensing GPCRs expression in IBD patients.
3. To analyse the role of GPR91 in EMT and its relevance in fistula formation.
4. To study the involvement of GPR91 in inflammasome priming and its role in chronic murine colitis.
5. To analyse the tissular expression of GPR109A in IBD patients.
6. To study the role of GPR109A in the secretome of M1 macrophage and its effects on intestinal fibroblasts.

III. MATERIALS AND METHODS

III.1 Reagents

All general analytical grade chemical reagents used to perform experiments were purchased from Sigma-Aldrich (Steinheim, Germany), PanrRac Química S.L.U (Barcelona, Spain), Roche Life Science (Penzberg, Germany) and Merck Millipore (Darmstadt, Germany).

III.2 Human Samples

III.2.1 Intestinal Surgical Resections

Intestinal surgical resections of affected tissue were obtained from UC and/or CD patients with severe refractory disease state who underwent surgery. These patients were not under pharmacological treatment at least three weeks before the procedure. In the case of non-IBD patients, non-damaged mucosa of colonic and ileal resections from patients with colorectal cancer were obtained and used as controls. The Institutional Review Board of the Hospital Universitari i Politècnic La Fe (València, Spain) and Hospital of Manises (València, Spain) approved the study (2021-545-1) (Annex I), following the Helsinki declaration recommendations. Written informed consent was obtained from all participating patients (Annex II).

Depending on the study, different patients were included. Patients which participated in the characterization of microbiota, metabolomic profile and GPCRs expression, including protein expression of GPR109A were UC patients (n=18), colonic controls (n=20), CD patients (n=21) and ileum controls (n=13). Patients which participated in the analysis of the role of GPR91 in EMT were B2-CD (n=19), B3-CD (n=16), and ileal controls (n=10). Finally, patients which participated in the analysis of the role of GPR91 in inflammasome were UC patients (n=25), and non-IBD patients (n=30). The following table (Table III.1) describes the age, gender, location, disease classification and pharmacological treatment of patients participating on these studies.

Table III. 1. Demographic and clinical data from patients who participated in the study.

	UC patients	CD patients	Non-IBD patients
Number of patients	25	36	42
Age			
Median	47	44	57
Interval	[17-69]	[15-77]	[18-89]
Gender			
Male	13 (52%)	17 (47%)	23 (55%)
Female	12 (48%)	19 (53%)	19 (45%)
Location			
Colon	25 (100%)	3 (8%)	30
Ileum		33 (92%)	12
Classification			
	Pancolitis 10 (52%)	B2-Stricturing 20 (56%)	
	Left-sided colitis 5 (20%)	B3-Penetrating 16 (44%)	
	Proctitis 10 (40%)		
Treatments			
Infliximab		6	
Azathioprine	4	6	
Adalimumab		4	
Azathioprine + Adalimumab		3	
Azathioprine + Vedolimumab		1	
Azathioprine + Corticoids + Anti-inflammatory Drugs	3		
Azathioprine + Corticoids	1	3	
Azathioprine + Ustekinumab		1	
Ustekinumab	4	3	
Ustekinumab + Corticoids		3	
Certolizumab		2	
Corticoids	4	2	
Corticoids + Anti-inflammatory Drugs	3	2	
Mesalazine	4		
Mesalazine + Corticoids	1		
Budesonide + Cortocoids	1		

III.3 Animal Studies

III.3.1 Mice

To perform *in vivo* experiments, wild type (WT) C57Bl/6 and GPR91$^{-/-}$ mice bred into a C57Bl/6 background, 9–12 weeks old, 20–25 g weight, kindly provided by Dr. Kenneth McCreath and Dr. Ana Cervera were used (188). Specific pathogen-free conditions were stablished to co-house mice in order to reduce possible differences in microbiota. *Ad libitum* access to water and chow diet were given to animals, which were kept at 21 ± 1ºC under light/darkness cycles of 12 h each. The distribution of animals in the different groups was randomly performed. All experiments and protocols were approved by the institutional animal care and use committees of University of Valencia (authorization code 2019/VSC/PEA/0290) (Annex III) and performed in compliance with the European Animal Research Law (European Communities Council Directives 2010/63/EU, 90/219/EEC, Regulation (EC) No. 1946/2003), and Generalitat Valenciana (Artículo 31, Real Decreto 53/2013).

III.3.2 Induction of Experimental Chronic DSS-Colitis in Mice

Chronic DSS-colitis was induced in WT and GPR91$^{-/-}$ mice with 4 cycles of increasing percentages (1%, 1%, 1.5% and 1.5%) of Dextran Sulphate Sodium (DSS, MP Biomedicals, Illkirch, France) via drinking water solution for 7 days, intercalated by 10 days with water. Control groups received only vehicle and there were the following experimental groups: WT vh, WT DSS, GPR91$^{-/-}$ vh and GPR91$^{-/-}$ DSS (n=10 mice per group). Body weight and clinical signs of disease were monitored daily. Finally, on day 60, mice were properly euthanized to collect colon tissue samples for further analysis.

III.3.3 Induction of Intestinal Fibrosis

To induce intestinal fibrosis *in vivo*, a heterotopic transplant model was performed as previously reported (146, 236, 237). This model consists in the subcutaneous transplant of 1-cm colon resections (Figure III.1A) from a donor mouse in the dorsal neck region of a recipient mouse (Figure III.1B). First, donor mice were sacrificed by neck dislocation and colon resections were obtained, washed with 0.9% NaCl to remove stool and divided into 6 equal 10-mm parts. Then, recipient mice were shaved in a small area in their back, to prevent contamination with hair, and anesthetized with isoflurane to make two incisions perpendicularly to the body axis. Next, colon resections were implanted subcutaneously, and skin was closed with vicryl 5-stiches. After 7 days, recipient WT mice were properly euthanized and intestinal grafts were collected for further analysis (Figure III.1C). Controls were adjacent segments of the colon from donor mice at day 0 (n=7 mice per group).

Figure III. 1. Heterotopic transplant model to induce intestinal fibrosis *(237)*

III.4 Cell Culture

III.4.1 Human Cell Lines

HT-29 cells (American Type Culture Collection American Type Culture Collection (Manassas, VA, USA), a human colorectal adenocarcinoma cell line with epithelial morphology, were cultured with McCoy's Medium Modified (Sigma-Aldrich) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10% inactivated Fetal Bovine Serum (FBS).

Human monocytes U937 (European Collection of Cell Culture, Salisbury, UK) were cultured with RPMI medium, supplemented with 100 U/mL penicillin, 100 µg/ml streptomycin and 10% FBS inactivated.

Human Small Intestine Fibroblasts (HSIFs, Innoprot, Bizkaia, Spain) were cultured with Fibroblast medium (Innoprot) supplemented with 10 ml of FBS inactivated, 5 ml of Fibroblast Growth supplement and 5 ml of Penicillin/Streptomycin solution.

III.4.2 *In vitro* treatments

Depending on the experiment, cells were treated with different compounds. All the treatments are summarized in Table III.2. The effects of two different metabolites, succinate (Sigma-Aldrich) and β-hydroxybutyrate (Sigma-Aldrich) were analysed in the different cell lines. On the one hand, HT-29 cells were treated with succinate at different concentrations from 0.1 to 5 mM during 24 or 48 h depending on the experiment. On the other hand, U937-derived macrophages were treated with β-hydroxybutyrate at 5 and 10 mM during 24 h.

Table III. 2. Stimuli used to treat different cell types including specific concentration, time and suppliers.

Name	Concentration	Treatment duration	Cells treated	Company
Succinate	0.1, 0.5, 1, 5 mM	24 or 48 h	HT-29	Sigma-Aldrich
β-hydroxybutyrate	5, 10 mM	24 h	U937	Sigma-Aldrich
LPS (*E. coli* 0111:B4)	0.1 µg/ml	2 or 24 h	U937, HT-29	Sigma-Aldrich
TNF-α	25 ng/ml	24 h	HT-29	Sigma-Aldrich
IFN-γ	20 ng/ml	24 h	U937, HT-29	Gibco
TGF-β	5 ng/ml	48 h	HT-29	Miltenyi Biotec
U0126	10 µM	2 h	HT-29	Abcam
PMA	0.01 µM	48 h	U937	Sigma-Aldrich

In addition, different pharmacological modulators such as pro-inflammatory cytokines were also used. For instance, HT-29 cells were treated with LPS 0.1 µg/ml for 2 or 24 h depending on the experiment, and with an inflammatory cocktail containing TNF-α 25 ng/ml (Sigma-Aldrich), IFN-γ 20 ng/ml (Gibco™, Thermo Fisher Scientific) and LPS at 0.1 µg/ml (Sigma-Aldrich) from *E. coli* 0111:B4 for 24 h. HT-29 cells were also treated with TGF-β (5 ng/ml) during 48 h to induce EMT.

In addition, to deeply analyse the effect of GPR91 on MAPK/ERK pathway, the MAPK/ERK kinase (MEK) inhibitor U0126 (Abcam) was used at 10 µM for 2 h.

Besides, to differentiate U937 monocytes into macrophages, phorbol-12-myrstate-13-acetate (PMA, Sigma-Aldrich) 0.01 µM was added during 48 h. Then, depending on the experiment, U937-derived macrophages were treated with LPS 0.1 µg/ml plus IFN-γ 20 ng/ml to polarize them towards M1 as previously described (92).

In some cases, to analyse the effects of the secretome from M1 U937-derived macrophages and siGPR109A M1 U937-derived macrophages on HSIFs, supernatants from macrophages were collected and used to treat previously plated HSIFs. Fibroblasts' medium was removed, and supernatants were left for 24 h.

III.4.3 Small Interfering (siRNA) Transfection

HT-29 cells and U937 derived-macrophages were transiently transfected using a control siRNA (siCtrl) and a specific GPR91 siRNA (Invitrogen, Thermo Fisher Scientific) at a concentration of 0.02 nM, and a specific GPR109A siRNA (Invitrogen, Thermo Fisher Scientific) at a concentration of 15 nM during 16 h. Lipofectamine™-RNAiMAX (Invitrogen, Thermo Fisher Scientific) was also used following the manufacturer's instructions. The efficiency of the transfection was confirmed by analysing the gene and protein expression by qPCR and Western Blot respectively.

III.5 Microbiota Analysis

Surgical intestinal resections from IBD and non-IBD patients were provided to the Genomics and Health Department from FISABIO (València, Spain) and the whole microbiota characterization was performed by the Oral Microbiome Laboratory at FISABIO Institute (València, Spain) leaded by Dr. Álex Mira as explained in the following sections.

III.5.1 DNA extraction and sequencing of the 16S rRNA gene

DNA from intestinal resections was extracted using the MagNa Pure LC DNA Isolation kit II in a MagNa Pure Robot (Roche Life Science), following the manufacturer`s protocol with the treatment with a chemical lysis with lysozyme, lysostaphine and mutanolysin (238). Quant-iT™ PicoGreen® dsDNA Assay Kit and a Qubit™ 3 Fluorometer (Thermo Scientific) was used for DNA quantification. The V3-V4 hypervariable region of the 16S rRNA gene was amplified using the universal primers (forward 5'-CCTACGGGNGGCWGCAG-3' and reverse 5'-GACTACHVGGGTATCTAATCC-3'). Metagenomic Sequencing Library Preparation Illumina protocol (Part #15044223 Rev. A) was used for library preparation. Sequencing was performed using 2x300 bp paired-end sequencing with an Illumina MiSeq instrument. Sequencing data have been publicly submitted to the SRA database (Bioproject, PRJNA859102).

III.5.2 Bioinformatic analysis of sequencing data

The software Dada2 v1.16 was used to filter, end-trim, denoise and merge paired reads (239) using default parameters. Adapters and primers were first filtered out from the sequence reads and then end-trimmed in 10 bp windows with quality values <35 and absence of Ns. The remaining reads were merged, clustered and cleaned for host and chimeric reads and finally assigned a taxon at the genus and species level (with Amplified Sequence Variants, or ASVs) using the SILVA non-redundant database v138.1 (240).

R language was used for statistical computing (241) to perform downstream analyses. Genera with an abundance below 0.01% were removed from all samples. For multivariant analysis, an Adonis test (Permutational Multivariate Analysis of Variance Using Distance Matrices), provided by the Vegan library of R (242), was used to compare groups. Considering the minimum number of reads in a sample, rarefaction curves, richness and diversity indexes were performed with 20,000 sequences per sample. To visualize differences among groups, constrained correspondence analysis (CCA) with the Vegan library was used (242). Additionally, to compare species and genera proportions, non-parametric Wilcoxon tests (wilcox.test function of stats library of R) (241) were performed. Adjusted p-values, obtained by the FDR method, were used.

III.6 Targeted Metabolomic Analysis

III.6.1 Metabolite extraction from human intestinal surgical resections

Frozen human intestinal surgical resections (~100 mg) from non-IBD, UC and CD patients were delivered to the Nuclear Magnetic Resonance Service of the Centro de Investigación Principe Felipe from Valencia. Samples were collected in liquid nitrogen and homogenized with a mortar and a pestle until obtaining a powder, adding liquid nitrogen to avoid thawing. The powder was transferred to a pre-weighted eppendorf and weighed. Then, 480 µl MeOH + 240 µl $CHCl_3$ were added, and 3 free-thaw cycles were performed (1 min in liquid N_2 and 2 min on ice). Afterwards, 360 µl $CHCl_3$ and 360 µl H_2O mQ were added, the suspensions were vortexed and centrifuged at 10,000 rcf for 15 min at 4 °C. The resulting phases were separated: the upper phase (polar extract in MeOH/H_2O) was collected with tips, frozen in N_2, lyophilized and store at -80°C till use. The lower phase (organic) was collected and evaporated under nitrogen flux and stored at -80 °C.

III.6.2 Nuclear Magnetic Resonance (NMR) Analysis

Polar metabolites were quantified by NMR. For sample preparation, the extract was allowed to thaw, solubilized in 550 µl of phosphate buffer (0.1 M NaP, pH = 7.4, 0.1 mM TSP, D_2O) and transferred to an NMR tube. Noesy 1D spectra with presaturation were acquired with ns = 256, at a 600MHz spectrometer with cryoprobe, at 27ºC. The following metabolites, acetic acid, propanoic acid, β-hydroxybutyric acid, succinic acid, lactic acid, butyric acid, L-glutamic acid, L-aspartic acid and L-phenylalanine were identified and quantified automatically with an integration template in Mestre Nova in all samples. Results were expressed as µg of metabolite per gram of tissue.

III.6.3 Ultra-Performance Liquid-Chromatography Mass-Spectrometry (UPLC-MS) Analysis

Non-polar metabolites were quantified by UPLC-MS. For that, the organic phase was conserved as dried extract and samples were processed by the Analytical Unit Metabolomics and Bioanalysis of the Instituto de Investigación Sanitaria La Fe (IIS-La Fe) from Valencia. For the preparation of the sample, 1 ml of MEOH:KOH was added to the organic phase of each sample conserved as a dried extract. Next, samples were cooled in ice and 100 µl of formic acid were added to each one. Finally, a liquid-liquid extraction was performed using 1 ml hexane and 1 ml isooctane. After shaking, upper phase was collected and transferred to a glass vial so as to evaporate under nitrogen flow. Once dried, 400 µl of 50% (Mobile phase A, 60:40 H_2O:MeOH + 2.5 mM acetamide) and 50% (Mobile phase B, 95:5 CAN:IPA + 2.5mM acetamide) were added, which had as internal standard (IS) Myristic acid D27. For the quantification of oleic acid, samples were 100-fold diluted.

The metabolomic analysis was performed on an UPLC system coupled to a Q-Exactive Plus (orbirtrap) equipment from Thermo Fisher Scientific. For the chromatographic separation, an UPLC Cortecs C18 (150x2.1 mm, 1.6 µm) column

from Waters (Wexford, Ireland) was used. Column temperature was set to 55ºC with an injection volume of 5µl and a flow rate of 0.8 ml/min, with a total runtime of 21 min. The initial condition was 10% of phase B for 2 min, gradually increasing to 100% over 16.5 min, maintained at 100% B for 2.5 min and then decreased to 10% over 2 min to ensure total column recovery. Then samples were ionized negatively (ESI-) and ion monitoring mode was recorded with an extended dynamic range from 50 to 1500 m/z. Electrospray ionization parameters were the following: gas temperature, 200 °C; drying gas, 14 l min^{-1}; nebulizer, 60 psi; sheath gas temperature, 350 °C; sheath gas flow, 11 l min^{-1}. A pseudo selected reaction monitoring mode (pseudo-SRM) was used for quantification of fatty acids (Table III.3). Data acquisition and analysis were performed using Thermo's Xcalibur software, as previously published (243).

Table III. 3. LC-ESI (−)-MS/MS parameters for the quantification of fatty acids

Analyte	Mass transition	CE (eV)	rt (min)
α-linolenic acid FA(18:3n3)	277.2173	25	7.96
Decanoic acid FA(10:0)	171.1386	25	2.00
Undecanoic acid FA(11:0)	185.1430	25	2.85
Eicosapentaenoic acid FA(20:5n3)	301.2174	25	7.45
Myristic acid FA(14:0)	227.2017	25	8.00
N-oleylethanolamide OEA	384.3122	25	15.90
Docosahexaenoic acid FA(22:6n3)	329.2488	25	22.41
Oleic acid FA(18:1n9)	281.2487	25	7.80

For the calibration and quantification of samples, stock solutions of individual fatty acids FA(18:3n3), FA(10:0), FA(11:0), FA(20:5n3), FA(14:0), OEA, FA(22:6n3), FA(18:1n9) were mixed and diluted in 50:50 (Mobile phase A: Mobile Phase B) to concentration levels ranging between 0.234-15 ppm. For the internal calibration, the peak area ratios (analyte/IS) were plotted against the concentration ratio (analyte/IS).

III.7 Gene Expression Analysis

III.7.1 RNA isolation

Total RNA extraction from cell pellets was performed using Illustra™ RNAspin Mini Kit (Cytiva, Amersham™, Little Chalfont, Buckinghamshire, UK) following manufacturer's instructions. First, cells were mechanically detached and washed using phosphate-buffered saline (PBS). Liquid extracts were centrifuged 1 min at 10,000 rpm to obtain cell pellets. Next, pellets were resuspended in 350 µl lysis buffer plus 3,5 µl β-mercaptoethanol and lysed using a 25 G needle. Then, liquid was transferred to a RNAspin Mini Filter inside collection tube which were centrifuged during 1 min at 11,000 rcf at room temperature. Liquid was collected and 70% ethanol was added and transferred to a RNAspin Mini column inside a collection tube and centrifuged during 30 s at 8,000 rcf. In this case, RNA was retained in the column and flow-through was discarded. Afterwards, desalting, DNA digestion and appropriate washes were performed using buffers provided by the manufacturer. Finally, RNA was eluted with 30 µl RNAse-free H_2O and purity and concentration were determined using the spectrophotometer Multiskan™ SkyHigh (Thermo Labsystems, Beverly, MA, USA). RNA isolation from Tissue

Total RNA from human surgical intestinal resections and mice colon samples was isolated using Direct-zol™ RNA MiniPrep Plus from ZymoResearch (Irvine, CA, USA) according to the manufacturer's instructions. First, small pieces of human intestinal resections or murine samples were homogenized in 300 µl of TRI Reagent™ using gentleMACS™ Dissociator (MACS Miltenyi Biotec). Then, tubes were centrifuged during 5 min at 3,000 rpm at room temperature and supernatants were collected in a new tube. Then, 95-100% ethanol was added, and all liquid was transferred into a Zymo-Spin™ IIICG Column in a collection tube and centrifuged 30 s at 16,000 rcf. Afterwards, flow-through was discarded since RNA was retained in the column. Next, DNA digestion, desalting and appropriate washes were performed using buffers provided by the manufacturer. Finally, RNA was eluted in

30 µl RNAse-free H_2O and purity and concentration were determined as previously described.

III.7.2 Complementary DNA (cDNA) synthesis by Reverse-Transcription

In all cases, cDNA was obtained using the PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa Bio Inc., Otsu, Japan) following manufacturer's instructions by reverse transcription. The amount of RNA used was 1 µg and then, 4 µl of 5X PrimeScript Buffer, 1 µl PrimeScript RT Enzyme Mix I, 1 µl 25 pmol Oligo dT Primer and 1 µl (50 pmol) Random hexamers in a final volume of 20 µl per sample. To perform the reaction, the thermal cycler GeneAmp® PCR System 2400 (PerkinElmer Inc, Waltham, MA, USA) was used with the following settings: 15 min at 37ºC, 5 s at 85ºC and then 4ºC until stored at -20ºC.

III.7.3 Real-Time quantitative PCR (RT-qPCR)

RT-qPCR was performed using TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa Bio Inc.) which contains TaKaRa Ex Taq HS, dNTPs mix, Mg^{2+}, Tli RNAse H and SYBER Green I (the fluorophore which emits fluorescence when bounds to double strain DNA, allowing fluorescence detection and quantification of amplified products). To perform the reaction, 1 µl cDNA, 5 µl TB Green® Premix Ex Taq™, 0.3 µM of reverse and forward primers and RNAse-free H_2O up to a volume of 10 µl were used. Next, the reaction was carried out in the thermal cycler LightCycler® 96 Real-Time PCR System (Roche Life Science) set as follows: 30 s at 95ºC; 5 s at 95ºC, 20 s at 60 ºC (a total of 50 cycles); 1 s at 95ºC, 15 s at 65 ºC, 1 s at 95ºC and 30 s at 40ºC. In all experiments, a negative control using RNAse-free H_2O instead of cDNA was added and all samples were loaded in duplicate. The primers for human and for mice are found in Table III.4 and Table III.5 respectively. Prior to use, primers` specificity was tested by performing a melting curve analysis and standard electrophoresis in 2% agarose gel containing Serva DNA Stain G

(Serva) in TAE 1X buffer (20 mM Tris pH 7.8 0.5 mM EDTA and 10 mM sodium acetate). Bands detection was performed using AMERSHAM ImageQuant 800 (GE lifescience, Cornellà de Llobregat, Spain).

Results were expressed as fold increase using the cycle threshold (C_T) calculated as follows: change in expression (fold change) = $2^{-\Delta(\Delta C_T)}$ where $\Delta CT = C_T$ (target gene) – C_T (housekeeping gene) and $\Delta(\Delta C_T) = \Delta C_T$ (experimental group) - ΔC_T (control), being ACTB/Actb the gene used as housekeeping in human and mice respectively.

Table III. 4. Sequences of human primers used in RT-qPCR

Gene	Sense (5´ - 3´)	Antisense (5´ - 3´)	Size (bp)
ACTB	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	57
PYCARD	CAAACGTTGAGTGGCTGCTG	GAGCTTCCGCATCTTGCTTG	107
CASP1	AGAGAAAAGCCATGGCCGAC	CCTTCACCCATGGAACGGAT	70
CaSR	TCTACGATTGCTGTGGTGGG	TCATCAGCTGCAATTGTGCC	270
CD86	GTCTGTCCACCCCATCAAC	GTATCACCAAAACCCCTCCC	114
COL1A1	GGAGCAGACGGGAGTTTCTC	CCGTTCTGTACGCAGGTGAT	252
COL3A1	CGCCCTCCTAATGGTCAAGG	TTCTGAGGACCAGTAGGGCA	161
COL4A1	CCGGATCACATTGACATGAAACC	TGGAAACCAGTCCATGCTCG	236
GPR109A	CTTATCTGGGCCCAACCTCTC	TTGCAACCAGTCTCCCACTC	198
GPR109B	ATGGACTACTATGTGCGGCG	ATGCACACATTTGCAGTGCC	284
GPR119	ACTCGGAATCCCCATGTTCC	CCATCTTTCGAATCTGCTGGC	188
GPR120	CCTGCCACCTGCTCTTCTAC	ATGGTGGGCCAAATCAGTGT	274
GPR132	GCCCAATGCTACTGAAAAACGG	CTTCGAAGGACACGTTGTTGC	114
GPR142	ACCTCCGAATAAGGCCATCC	GCTGTACCTCTCACCCCTTG	112
GPR35	GTGCTAAGGCCCACAAAAGC	GATCTCGGGCTCCTTGCTAC	146
GPR4	TGCCAGACACCTCCTTTTCC	TGGAGGAGACGAAAGCATCG	285
GPR40	GTAGGACCCTACAACGCCTC	AGCAGTGGCGTTACTTCTGG	205
GPR41	CTAGGTCTGGAGAGACAGCAAG	ACACCGAGAAGACGAACCAG	133
GPR43	TGGTGTGCTTCGGACCTTAC	CCCTGTCCTCATTTGTCCCC	249
GPR65	CTTCTTGACTTGATGCAGGCAC	GCTGACAAACAGCCCACAAG	239
GPR68	GCCCAGCTGTTTGAGGTTTG	AGTGTGGTCTCTGCAACCAG	145
GPR81	CTCAGACGTAGACACTGGGC	TAAGGAACACGATGCTCCCG	94
GPR84	AAGGACTGCTCTTTGGGTGAG	TGAATCGGGTACGGAGCTTG	241

GPR91	GGAGACC CCAACTACAACCTC	AGCAACCTGCCTATTCCTCTG	132
IL18	GCTGAAGATGATGAAAACCTGGA	GAGGCCGATTTCCTTGGTCA	115
IL1B	GCTCGCCAGTGAAATGATGG	TCGTGCACATAAGCCTCGTT	330
IL6	AGTGAGGAAGCCAGAGC	ATTGTGGTTGGGTCAGGGG	143
IL8	CCACCGGAGCACTCCATAAG	GATGGTTCCTTCCGGTGGTT	97
NOS2	ATAATGGACCCCAGGCAAG	TCAGCAAGCAGCAGAATGAG	195
NLRP3	AGAACTGTCATCGGGTGGAG	AACTGGAAGTGAGGTGGCTG	174
GPR91	GGAGACCCCAACTACAACCTC	AGCAACCTGCCTATTCCTCTG	132
TAAR1	GACCACACTCGTTGGCAATC	TAAGGCATGACCAGACACCC	132
TGR5	GACATGGTCCAAACCGCTG	TGTAGTGGTCTTCCAGCCAG	282
TNF	GCTGCACTTTGGAGTGATCG	GGGTTTGCTACAACATGGGC	138
SNAI1	AACTGCAAATACTGCAACAAGG	ATTCGGGAGAAGGTCCGAG	281
SNAI2	ACGCCTCCAAAAAGCCAAAC	ACAGTGATGGGGCTGTATGC	139
VIM	ATGAAGGAGGAAATGGCTCGTC	GGGTATCAACCAGAGGGAGTGAA	196
ITGB6	GTTGGTCTTGTAACGCTGAAGG	AGGTCCAATAAGCAGGCAGTC	182
CDH1	TGGAGGAATTGTTGCTTGC	CGCTCTCCTCCGAAGAAC	103
CD206	CTTTGGACGGATGGACGAGG	CAAGGAAGGGTCGGATCGTG	201
ACTA2	GACCTTTGGCTTGGCTTGTC	AGCTGCTTCACAGGATTCCC	418
FN1	AAACCAATTCTTGGAGCAGG	CCATAAAGGGCAACCAAGAG	142

Table III. 5. Sequences of mouse primers used in RT-qPCR

Gene	Sense (5'-3')	Antisense (3'-5')	Size (bp)
Arg1	GTGGGGAAAGCCAATGAAGAG	TCAGGAGAAAGGACACAGGTTG	232
Asc	TGACTGTGCTTAGAGACATGGG	AACTGCCTGGTACTGTCCTTC	233
Actb	GCCAACCGTGAAAAGATGACC	GAGGCATACAGGGACAGCAC	95
Casp1	CTCGTACACGTCTTGCCCTC	GGTCCCACATATTCCCTCCTG	260
Casr	AGGACGGCTCCATTGTGTTC	GTCTCACCACTGTACTCGCC	235
Ccr7	CTCTCCACCGCCTTTCCTG	ACCTTTCCCCTACCTTTTTATTCCC	125
Fcgr3	GAAGGGGAAACCATCACGCT	GCAAACAGGAGGCACATCAC	293
Cd86	GCACGGACTTGAACAACCAG	CCTTTGTAAATGGGCACGGC	194
Col1a1	CAGGCTGGTGTGATGGGATT	AAACCTCTCTCGCCTCTTGC	317
Col3a1	CTACACCTGCTCCTGTGCTTC	GATAGCCACCCATTCCTCCCA	237
Col4a1	ATTAGCAGGTGTGCGGTTTG	ATTAGCAGGTGTGCGGTTTG	289
Cox-2	CCCGGACTGGATTCTATGGTG	TTCGCAGGAAGGGGATGTTG	153
F4/80	TGTCTGAAGATTCTCAAAACATGGA	TGGAACACCACAAGAAAGTGC	211
Il10	GGACAACATACTGCTAACCGAC	CCTGGGGCATCACTTCTACC	110
Il18	GCTTGCTTTCACTTCTCCCC	TGCCTGGATGCTTGTAAACTTG	262
Il6	GAGTCCTTCAGAGAGATACAGAAAC	TGGTCTTGGTCCTTAGCCAC	150
Nos2	CGCTTGGGTCTTGTTCACTC	GGTCATCTTGTATTGTTGGGCTG	222
Mmp2	GCCAACTACAACTTCTTCCCC	CAAAAGCATCATCCACGGTTTC	112
Nlrp3	GTACCCAAGGCTGCTATCTGG	TGCAACGGACACTCGTCATC	143
GPR91	GACAGAAGCCGACAGCAGAATG	GCAGAAGAGGTAGCCAAACACC	160
Tgfb1	GCGGACTACTATGCTAAAGAGG	TCAAAAGACAGCCACTCAGG	295
Timp1	GGCATCTGGCATCCTCTTGTTG	GTGGTCTCGTTGATTTCTGGGG	147
Tnf	GATCGGTCCCCAAAGGGATG	GGTGGTTTGTGAGTGTGAGGG	86
Vim	GCTCCTACGATTCACAGCCA	CGTGTGGACGTGGTCACATA	190
Mrc1	TGTGGAGCAGATGGAAGGTC	TGTCGTAGTCAGTGGTGGTTC	201

III.8 Protein Expression Analysis

III.8.1 Total Protein extraction

Protein was isolated from HT-29 cells and U937-derived macrophages. Cellular pellets were re-suspended in 35 µl of PhosphoSafe™ Extraction Reagent (Merck Milipore) containing 10x of protease inhibitor cOmplete™, Mini Protease Inhibitor

Cocktail (Roche Life Science). Then, samples were vortexed and incubated 5 min at room temperature. Next, they were vortexed again and finally centrifuged during 5 min at 16,000 rcf at 4ºC. Supernatants containing whole-cell protein extracts were collected and stored at -80ºC.

Protein from human intestinal resections or murine samples was also isolated. Small pieces of tissue were homogenised in 350 µl of PhosphoSafe™ Extraction Reagent (Merck Milipore) containing 10x of protease inhibitor cOmplete™, Mini Protease Inhibitor Cocktail (Roche Life Science) using gentleMACS™ Dissociator (MACS Miltenyi Biotec). Then, tubes were centrifuged during 5 min at 3,000 rpm at 4ºC and supernatants were collected in a new tube. Samples were vortexed and incubated in ice for 10 min. Finally, they were centrifuged during 5 min at 16,000 rcf at 4ºC. Supernatants containing whole-cell protein extracts were collected and stored at -80ºC.

III.8.2 Protein quantification (BCA assay)

To quantify the amount of protein in the extracts, bicinchoninic acid (BCA) Protein Assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) was used. It consists in a colorimetric assay based on Biuret's reaction. First, in alkaline medium containing sodium tartrate, oxidable residues of amino acids such as tyrosine, cysteine, tryptophan or cysteine reduce Cu^{2+} to Cu^{1+} forming a light blue chelate complex. Next, two molecules of BCA react with Cu^{1+} giving a deep purple chelate which exhibits a strong absorbance at 562 nm with increasing protein concentration (Smith PK, 1985).

Following manufacturer's instructions, first, a standard curve with serial dilutions (from 1 to 0,03125 mg/ml) containing bovine serum albumin (BSA) was prepared. Then, protein samples were diluted 1:20 with mili-Q H_2O. In a 96-well plate, 20 µl of samples, standard dilutions, and blanks (mili-Q H_2O) were loaded per well in duplicate. Next, 200 µl of the reagent prepared immediately before use

mixing 1:50 reagent B (4% Cu_2SO_4) in reagent A (BCA, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 M NaOH) were added to each well. Next, the plate was incubated under constant shaking during 30 min at 37ºC and protected from light. Finally, absorbance was measured at 562 nm with Multiskan™ SkyHigh (Thermo Labsystems).

III.8.3 Western Blot

Sample preparation

Proteins, once extracted and quantified, were denaturalized to be ready for the Western Blot analysis. To do that, Laemmli loading buffer (0.5 mM Tris-HCl pH 6.8, 25% glycerol v/V, 10% Sodium dodecyl sulphate (SDS), 0.5% β-mercaptoethanol and 0.5% bromophenol blue) was added to samples at 6x and boiled at 95ºC during 5 min.

SDS-polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels were prepared using a mixed solution of acrylamide/bisacrylamide (37.5:1) (PanReac Química S.L.U). Gels consisted in a resolving part, prepared with different percentage of polyacrylamide (from 8-15%) depending on the molecular weight of analysed proteins, in Tris-HCl 0.375 M pH 8.8 buffer and 0.1% SDS, and a stacking part, always prepared with 3.75% polyacrylamide in 0.125 M Tris-HCl pH 6.8 and 0.1% SDS. To achieve gel polymerization, 0.1% ammonium persulfate (APS) (SERVA, Heidelberg, Germany) and N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma-Aldrich) were used.

Once gels were prepared, equal amounts of proteins were loaded and a molecular weight marker EZ-Run™ Pre-Stained Rec Protein Ladder (Thermo Fisher Scientific) was used to determine the molecular weight of proteins in our samples. The electrophoresis was performed using the Mini-PROTEAN™ Tetra Cell System (Bio-Rad Laboratories, Hercules, CA, USA), where proteins were separated according to their molecular weight. To run out the electrophoresis, the cuvette was

filled with running buffer (25 mM Tris pH 8.3, 192 mM glycine and 0.1% SDS) at 120 V constant voltage during 2 h at room temperature.

Protein transfer to nitrocellulose membrane

Once electrophoresis was finished, proteins were transferred from the gel to a 0.45 μm nitrocellulose membrane (GE Healthcare Life Science). The system used was Mini Trans-Blot ™ Cell (Bio-Rad). In this case, the cuvette was filled with transfer buffer (25 mM Tris pH 8.3, 192 mM glycine and 20% methanol) at 4ºC for 60 min and a constant voltage of 100 V.

Ponceau S staining, blocking and antibodies incubation

Efficiency of the transference was verified using a Ponceau S staining (0.1% Ponceau S, 5% acetic acid solution, Sigma-Aldrich). For that, membranes were incubated in this solution during 1 min and reddish bands appeared in the membrane indicating proteins presence. To remove this reddish die, distilled water was used.

Then, membranes were incubated in a blocking solution (5% BSA [Thermo Fisher Scientific] or 5% non-fat dried milk diluted in Tris-buffered saline and 0.1% Tween 20 [TBS-T], 20 mM Tris-HCl pH 7.2, 150 mM NaCl 0.1% Tween-20 v/v) to prevent non-specific binding of antibodies with continuous gentle shaking during at least 1h at room temperature. Next, membranes were further incubated with specific primary antibodies at 4ºC, with continuous gentle shanking overnight. Primary antibodies were diluted in the blocking solution supplemented with 0.02% sodium azide (NaN_3, Merck Milipore). Primary antibodies used are specified in Table III.6. The next day, membranes were washed with vigorous shanking three times in TBS-T during 10 min at room temperature to remove primary antibody excess. Afterwards, membranes were incubated, depending on the host species of the primary antibody, with the secondary antibody peroxidase-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) or goat anti-mouse IgG (Thermo Fisher Scientific), 1:5000 or 1:2000 diluted respectively, in blocking solution, with

continuous gentle shanking during 1h at room temperature. Finally, membranes were washed again as previously explained.

Table III. 6. Specific primary antibodies employed in Western Blot analysis.

Primary Antibody	Supplier	Dilution
Pro-Caspase-1	2225, Cell Signaling	1:1000
Cleaved Caspase-1 p20 (Asp297)	4199, Cell Signaling	1:1000
NLRP3	13158, Cell Signaling	1:1000
NLRP3 mouse	341865590, MyBioSource	1:1000
GPR91 (GPR91)	IMG-6352a, IMGENEX	1:1000
IkB	SC-371, Santa Cruz Biotechnology	1:1000
pNFkB (pp65)	3033S, Cell Signaling	1:1000
NFkB (p65)	8242S, Cell Signaling	1:1000
Phospho-ERK	9104, Cell Signaling	1:1000
ERK	4695, Cell Signaling	1:1000
GPR109A (HCAR2)	SAB4301084, Sigma-Aldrich	1:1000
Cadherin-1	RA222618/13-1700, ThermoFisher	1:1000
β-actin	A5060, Sigma-Aldrich	1:5000
GAPDH	G9545, Sigma-Aldrich	1:5000

Chemiluminescence detection

Immunolabeling was detected by chemiluminescence using Immobilon® Forte Western horseradish peroxidase (HRP)-conjugated Substrate (Millipore, Burlington, MA, USA) or Immobilon® Crescendo Western HRP Substrate (Millipore) depending on the protein detected. Briefly, secondary antibody HRP-conjugated catalyzes luminol oxidation reaction which in the presence of H_2O_2 generates 3-aminophtalate which emits light at 425 nm. AMERSHAM ImageQuant 800 (GE lifescience) was the digital luminescent image analyzer used to visualize immunolabeling.

To normalize protein bands, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin was used as housekeeping, and Multi Gauge V3.0 software (Fujifilm Life Sciences, Cambridge, MA, USA) was used to quantify the densitometry of the bands.

Stripping method

Once a protein has been detected, it is possible to remove previous primary and secondary antibodies and analyse the presence of other protein in the same nitrocellulose membrane. For that, stripping method was performed using the Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific) following manufacturer's instructions. Briefly, membranes were incubated with this buffer with vigorous shaking during 30 min at room temperature. Next, membranes were blocked again using blocking solution and then incubated with the specific primary antibody as previously described.

III.8.4 Enzyme-linked immunosorbent assay (ELISA)

Secreted protein levels of IL-1β from HT-29 cells supernatants were quantified by Enzyme-linked immunosorbent assay (ELISA) using the human IL-1β ELISA KIT (MyBioSource, San Diego, CA, USA), based in the double antibody sandwich ELISA technique, following manufacturer's instructions. First, in order to remove cell debris, cell supernatants were centrifuged at 4°C for 20 min at 1,000 rcf and diluted 1:10 with PBS. Next, an IL-1β standard curve was prepared with serial dilutions (from 2,000 to 31.2 pg/ml). Then, 100 µl of samples, standards and blanks were loaded in duplicate in the anti-Human IL-1β monoclonal antibody pre-coated 96-well strip plate and incubated during 90 min at 37ºC. Afterwards, liquid was removed and then, 100 µl of Detection Solution A (Biotinylated Antibody) was added during 45 min at 37°C. To remove detection solution A, 300 µl of wash solution 1x was added to each well three times. Afterwards, 100 µl of Detection Solution B (Enzyme Conjugate) were added and the plate was incubated for 45 min

at 37°C. At the end, washing step was repeated as previously defined to ensure antibody removal. Then, to detect the antibody, 90 µl of TMB Substrate Solution was added, and the plate was incubated at 37°C during 15 min protected from light. By this step, a colour change was observed appearing a blueish colour and 50 µl of Stop Solution (acidic) were added to end the enzymatic reaction. Finally, absorbance was immediately measured at 450 nm with the microplate reader SpectaMax Plus 384 (Molecular Devices, San Jose, CA, USA).

III.8.5 Luminex® Multiplex Assay

Levels of several cytokines such as IL-1β, IL-6, IL-8, TNF-α, IL-17a, PDGF-AB/BB and TGF-β1, TGF-β2, TGF-β3 in supernatants from U937-derived macrophages were quantified with a Luminex® 200 analyser system (Luminex Corporation, Austin, TX, US) in collaboration with the Department of Endocrinology and Nutrition in the Hospital Dr. Peset-FISABIO (València, Spain), according to MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A, and MILLIPLEX® MAP TGFβ 1, 2 and 3 Magnetic Bead Panel (Millipore Corporation, Billerica, MA, USA) respectively, following manufacturer's procedure. Briefly, 200 µl of wash buffer were added into each well of the plate and the mix was gently shaken for 10 min at room temperature. Then, wash buffer was decanted and completely removed from the plate. Next, 25 µl of standard/control were added to appropriate wells, 25 µl of assay buffer were added to the sample wells and 25 µl of appropriate matrix solution were added to standards and control wells. Then, 25 µl of each sample were added to the wells. In the case of the assay for TGF-β isoforms, prior to adding them to the plate, samples were centrifuged and 2 µl of 1.0 N HCl were added to each 25 µl of supernatants, making sure that pH was below 3.0. After mixing, samples were moderately shaken for 15 min at room temperature and then, samples were neutralized with 2 µl 1.0 N NaOH. Neutral pH was assessed using colorimetric paper. Finally, 25 µl of beads were added to each well and the

plate was incubated overnight at 4ºC. The next day, liquid from the plate was removed and it was washed three times with 200 µl of wash buffer. After that, 25 µl of detection antibodies solution was added per well and incubated for 1 h at room temperature. Next, 25 µl of Streptavidin-Phycoerythrin were added per well and incubated 30 min at room temperature. Then, the plate was washed three times with 200 µl of wash buffer. Finally, 150 µl of Sheath Fluid PLUS were added per well and the plate was read on Luminex® 200 analyser system. A specialized software convers fluorescence signals into quantitative measurements of the analyte concentrations and calibration curves with known concentrations of analytes are used to determine the concentrations in the sample.

III.9 Histological Analysis

Human intestinal surgical resections and mice colons were also collected for histological analysis. First, tissues were washed with saline solution and fixed with 10% neutral-buffered formalin solution (Histofix™ Preservative, PanReac Química S.L.U) for 48-72 h. Afterwards, dehydration and paraffin embedding were done so as to cut pieces in the microtome Leica RM2245 (Leica) with a thickness of 3 µm. Then, these sections were mounted in glass slides and dried.

III.9.1 Hematoxylin-Eosin Staining

To analyse tissue histology after the induction of chronic DSS-colitis, Hematoxylin-Eosin staining was performed. First, slides were incubated at 55ºC for 30 min to deparaffinate the samples and rehydrated using xylene and decreasing ethanol percentages and finally distilled water. Afterwards, slides were incubated with Hematoxylin 1:25 (Sigma-Aldrich) during 3 min at room temperature. Then, ethanol-HCl 0.5% was added over 30 s and ammonium hydroxide 1% was also added over 30 s. Finally, aqueous eosin Y solution (Sigma-Aldrich) diluted with glacial acetic acid 0.5% was added over 3 min at room temperature and dehydrated

by immersions in increasing ethanol percentages and finally xylene. Slides were mounted using mounting medium DPX™ Mountant for hystolgy (Sigma-Aldrich) and placing a coverslip over the slide. Samples were visualized with a light microscope Leica DMC6200 (Leica DMi8, L'Hospitalet de Llobregat, Spain) at different magnifications using LEICA LAS X software. To analyse the histology of the tissue, the parameters of Obermeier et al. were used. Briefly, as detailed in Table III.7, it consisted of a scale from 0 to 8 which measures the presence of erosion, the depth and surface extension lesions in the epithelium, as well as the degree of inflammatory infiltrate. The total histological score represented the sum of the epithelium and infiltration score (total score = E + I) (244).

Table III. 7. Histological score parameters*. (244)*

	Epithelium (E)		**Infiltration (I)**
0	Normal morphology	**0**	No infiltrate
1	Loss of epithelial cells	**1**	Infiltrate around crypt basis
2	Loss of epithelial cells in large areas	**2**	Infiltrate reaching to L. muscularis mucosae
3	Loss of crypts	**3**	Extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant oedema
4	Loss of crypts in large areas	**4**	Infiltration of the L. submucosa

III.9.2 Sirius Red/Fast Green Staining

To measure the collagen deposition in mice tissues after induction of murine intestinal fibrosis, Sirius Red/Fast Green staining was performed. Briefly, Sirius red molecule contains sulfonic acid groups which react with basic amino groups from collagen molecule, such as lysine and hydroxylysine, staining collagen molecules in red (245).

Slides were deparaffinised and rehydrated as previously described and incubated with 0.01% Fast Green (FCF F7258, Sigma-Aldrich) in a saturated aqueous solution of 1.3% picric acid (Sigma-Aldrich) in water over 15 min at room temperature protected from the light. Afterwards, slides were washed three times with distilled water. Next, 0.1% Sirius Red (Direct red 80 365548, Sigma-Aldrich)

in picric acid/ 0.04% Fast Green solution over 30 min at room temperature. Afterwards, slides were washed again, dehydrated and mounted as previously explained. Slides were observed with a light microscope Leica DMC6200 (Leica DMDMi8) at different magnifications using LEICA LAS X software. Collagen deposition and the thickness of the collagen layer were quantified as mean red intensity per tissue area, using ImageJ (National Institutes of Health, Bethesda, MD, USA). The measurement was performed in a blinded manner by an observer unaware of the corresponding group for each mouse.

III.10 Immunohistochemistry

Immunohistochemistry of human intestinal resections was performed to analyse the presence of GPR91, Vimentin, CD206 or GPR109A depending on the experiment.

Deparaffinisation, and heat-induced epitope retrieval (HIER)

After deparaffinising and dehydrating the samples, to allow antigen recognition, it is necessary to break cross-links made by covalent bonds between formaldehyde and tissue proteins which mask antigens. For that, a citrate buffer solution 1x, Dako Target Retrieval Solution 10x (Agilent, Santa Clara, CA, USA) was used. Depending on the primary antibody, pH was 6.0 or 9.0 as detailed in Table III.8. Slides were immersed in this solution, and then heated in a water bath during 20 min at 95ºC. Afterwards, slides were cooled down in ice for 10 min and then, washed twice with distilled water and twice in TBS-T.

Table III. 8. Specific primary antibodies employed for Immunochemistry.

Primary Antibody	Antigen retrieval	Supplier	Dilution
GPR91	6.0	IMG-6352a, IMGENEX	1;100
Vimentin	6.0	ab92547, Abcam	1:200
CD206	6.0	HPA004114, Sigma-Aldrich	1:200
GPR109A	9.0	SAB4301084, Sigma-Aldrich	1:200

Endogenous peroxidase blocking

Endogenous peroxidases, present in tissues, can react with the chromogenic substrate solution used in this technique, giving unspecific and false positive results. Hence, a peroxidase blocking solution 3% H_2O_2 in distilled water (Sigma-Aldrich) was performed and slides were immersed on it at room temperature for 15 min. Once finished, samples were washed twice in TBS-T.

Blocking and primary antibody incubation

Prior to primary antibody incubation, blocking of unspecific sites is required. For that, blocking buffer, containing 1% of BSA, 5% of horse serum (the specie in which the secondary antibody has been prepared) and 0.5% Triton X - PBS, was added to the slides for 1 h at room temperature. Next, the specific primary antibody was diluted as specified in Table III.8 in blocking solution, and incubated overnight at 4ºC. In all cases, an extra slide incubated only with blocking solution was added to the experiment as a negative control.

Secondary antibody and signal amplification

Next day, slides were washed twice in TBS-T and then incubated with the secondary antibody. In all cases, a universal secondary antibody Horse Anti-Mouse/Rabbit IgG Biotinylated provided in Vectastain™ Universal Elite ABC Kit (Vector Laboratories) diluted 1:100 in blocking solution was used, and slides were incubated during 45 min at room temperature. In this case, the negative control slide was also incubated with the secondary antibody to ensure that signal was not due to

unspecific binding of secondary antibody. Afterwards, slides were washed again twice in TBS-T.

To enhance primary antibody detection, signal amplification was performed using Vectastain™ Universal Elite ABC Kit (Vector Laboratories), which forms avidin-biotinylated peroxidase complexes (ABC) that bind to the biotinylated secondary antibody (246). Thus, following manufacturers` instructions, after washing in order to remove the excess of secondary antibody, ABC solution was added to the slides and incubated during 30 min at room temperature. At the end, slides were again twice washed with PBS.

Chromogenic detection

To detect our protein of interest, a chromogenic substrate which reacts with the peroxidases present in the secondary antibody and ABC, was used. This substrate was 3,3'-diaminobenzidine tetrahydrochloride (DAB) Enhanced Liquid Substrate System (Sigma-Aldrich), which due to peroxidase action, gives rise to an intense brown stain. Following manufacturer's instructions, DAB was added to the slides and left until a brownish stain appeared, no more than 15 min and which varies according to the protein analysed. To stop the reaction, slides were immersed in distilled water.

Mounting and image acquisition

Finally, hematoxylin (Hematoxylin solution, Gill No. 3; Sigma-Aldrich) staining was added during 30 s to stain cells' nucleus. Then, to remove hematoxylin residues, slides were rinsed with distilled water and then immersed in 0.5% Ethanol-HCl for 10 s and finally in distilled water. Then, dehydration and mounting were performed as previously explained. Then, slides were observed, and images were acquired using the light microscope Leica DMC6200 (Leica DMDMi8) at different magnifications using LEICA LAS X software.

III.10.1 Double Immunohistochemistry

In the case of a double immunochemistry, two different proteins can be identified in the same sample using different chromogenic detectors. In this case, double immunohistochemistries of GPR91+Vimentin and GPR91+CD206 were performed.

Briefly, the first steps of the double immunohistochemistry are equal to the previously described in section III.10. Once the chromogenic detection of the first primary antibody, GPR91, with DAB was performed, instead of proceeding to hematoxylin staining, slides were washed with PBS and then, biotin and avidin were blocked with Avidin/Biotin Blocking Kit (Vector Laboratories, Peterborough, UK) for 10 min each. Then slides were washed with PBS, blocked with the blocking buffer for 1 h at room temperature and further incubated overnight at 4ºC with the other primary antibody (Vimentin or CD206). The next day, secondary antibody step was performed, and the chromogenic detection was developed using Vector Purple Kit (Vector Laboratories). Finally, mounting and image acquisition was completed as previously explained.

III.11 Immunofluorescence and Confocal Microscopy

HT-29 cells were fixed with 2% paraformaldehyde in PBS for 20 min and then twice washed with PBS for 5 min. After that, cells were permeabilized with 0.1% Triton-X100 for 10 min and twice washed with PBS for 5 min. Next, cells were incubated with blocking solution (10% serum and 1% BSA in PBS) at room temperature for 1 h and then, primary antibodies diluted in the blocking solution anti-Vimentin (1:100, ab92547 Abcam) or anti-Cadherin-1 (1:100, RA222618 ThermoFisher) were added and incubated at 4ºC overnight. The next day, cells were washed three times with PBS during 5 min and then incubated with the secondary antibodies (anti-mouse-FITC, 1:200, F2761 Invitrogen for Cadherin-1 and anti-rabbit-TexasRed, 1:200, T2767 ThermoFisher for Vimentin) for 45 min at room

temperature protected from light. Next, cells were washed three times with PBS during 5 min and 2µM Hoechst 3342 (Sigma-Aldrich) in PBS was added to stain nuclei for 30 min and then cells were mounted in a slide. Confocal microscope Leica TCS SP8 was used to visualize cells and pictures were taken using the LEICA LASX software.

III.12 Succinate Quantification

Succinate levels were quantified in intestinal surgical resections from IBD and non-IBD patients, intestinal grafts from WT and GPR91$^{-/-}$ and HT-29 cells supernatants using the Succinate Assay Kit (Abcam) according to the manufacturer's instructions. Briefly, frozen tissues were homogenized with Succinate Assay Buffer in the gentleMACS™ Dissociator (MACS Miltenyi Biotec) and then centrifuged. Next, supernatants from tissue or HT-29 cells were filtered with 10 kDa spin columns (Abcam). The Reaction Mix from the kit was used to incubate samples in 96-well plates at 37 °C during 30 min. Finally, the absorbance at 450 nm was measured with the microplate reader SpectraMax Plus 384 (Molecular Devices, San Jose, CA, USA) and the succinate concentration was calculated using the standard curve.

III.13 Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Normality was assessed by Anderson-Darling, D'Agostino and Pearson's, Shapiro-Wilk and Kolmogorov-Smirnov normality tests. Comparisons between two groups were performed with unpaired *t*-test for parametric variables, and with Mann-Whitney test for non-parametric variables. Multiple comparisons were performed with one-way analysis of variance (ANOVA) with Newman-Keuls *post hoc* correction for parametric variables, and with Kruskal-Wallis test for non-parametric variables. Statistical significance was considered when $p < 0.05$.

To assess associations between pairs of variables, Spearman's correlation coefficient (R) was calculated to assess the strength and direction of the linear relationship. Data were analysed using GraphPad Prism® 8.0a (GraphPad Prism® Software Inc.).

IV. RESULTS

CHAPTER I: CHARACTERIZATION OF INTESTINAL MICROBIOTA, METABOLOMIC PROFILE AND METABOLITE-SENSING GPCRS IN HUMAN INTESTINAL SURGICAL RESECTIONS FROM IBD PATIENTS

IV.I.1 Intestinal microbial composition in both UC and CD surgical resections

Firstly, an analysis of the intestinal microbiota composition of surgical resections from IBD and non-IBD patients was performed by the group of Dr. Álex Mira at the Genomics and Health Department from FISABIO (València, Spain), in order to unravel the potential differences in microbiota composition caused by UC and CD. Following the technique previously explained, an average of $86.4x10^3$ reads per sample were obtained from which 78±14% were reached and annotated at the species level. The annotation of these reads reported that microbial composition from all groups was mainly composed of Firmicutes (60%-59%), Proteobacteria (24%-9%), Bacteroidetes (12%-10%) and Actinobacteria (2%-20%).

Next, the four groups of samples, UC, CD, and their respective controls, were compared by a Canonical Component Analysis, and as can be observed in Figure IV.1A, ileum and colon samples clustered separately (ADONIS p=0.001). Significant differences in microbiota composition were found in UC versus control colon, since they formed two separated clusters, whereas CD versus ileum controls showed a higher degree of overlap. Regarding the estimated number of bacterial species, the Chao1 richness index showed a significant lower richness in UC samples compared with colonic control samples, whereas no differences were obtained in CD versus control ileal samples, as shown in Figure IV.1B.

In order to determine the bacterial total amounts of IBD resections, bacterial load was calculated using qPCR with bacterial and human genes (number of bacteria normalized to the number of human cells). As shown in Figure IV.1C, a significant

reduction in bacterial load was seen in UC in comparison to colonic controls, whereas no differences were detected between CD and its respective control.

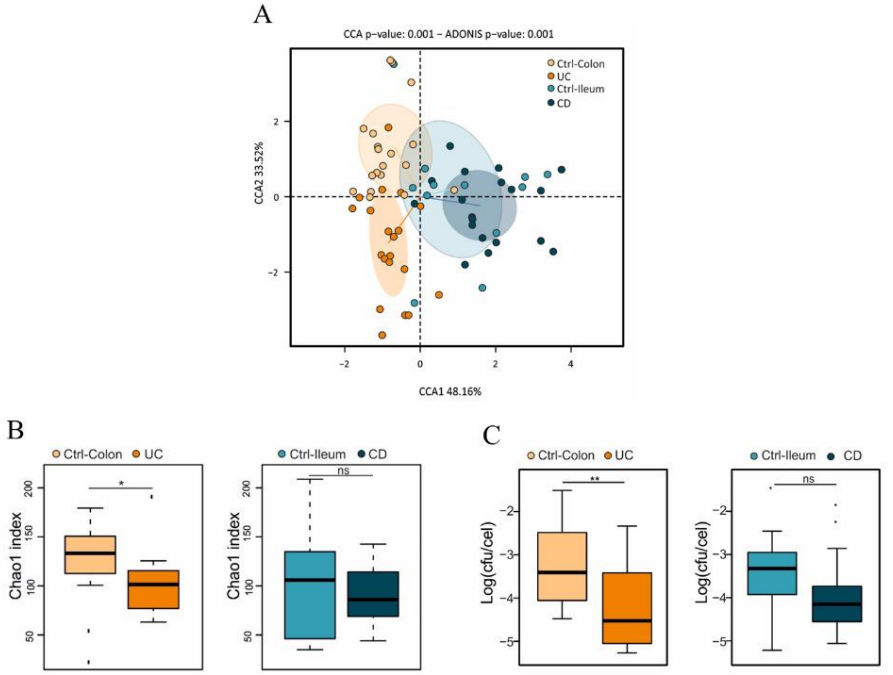


Figure IV. 1. Bacterial distribution, richness, and load in intestinal surgical resections from UC and CD patients. *(**A**) Microbiota composition was compared between groups in a Canonical Component Analysis plot using ADONIS test. (**B**) Graphs show bacterial richness index (Chao1). (**C**) Graphs show bacterial load calculated by qPCR with bacterial and human genes (number of bacteria normalized to number of human cells) in intestinal surgical resections of colonic controls (n=20), UC patients (n=18), ileum controls (n=13) and CD patients (n=21). *p<0.05 and **p<0.01 vs. respective control.*

At the genus level, differences in the abundance of gut microbiota appeared in both UC and CD patients when compared to respective controls. On the one hand, in UC tissue samples, 14 genera exhibited significant differences in abundance, as shown in Table IV.1. However, the vast majority of these genera were present in samples at low abundance (<1%), and only two of them had >1% abundance. Figure IV.2 represents the 20 most abundant genera in all the samples analysed. In this case,

the ones with significant differences were *Escherichia,* which was 3.88 times less abundant in UC samples, and *Cellulosimicrobium,* which was 10 times more abundant in UC samples, as observed in Figure IV.2. On the other hand, only 5 genera were significantly different in CD samples versus ileal controls (Table IV.2), but only *Enterococcus* had >1% abundance, as observed in Figure IV.2, with a 20-fold reduction in tissue from CD patients.

Table IV. 1. Summary of the genera with significant differences in abundance (p<0.05) between colonic controls and UC patients. *Data were analysed with Wilcoxon tests. FC = (reads in UC / reads in ctrl-colon). Colonic controls (n=20) and UC patients (n=18).*

Genus	Log_2FC	p.value
Paraprevotella	-6.99	0.0002
Butyricimonas	-2.5	0.0003
Cellulosimicrobium	3.35	0.0004
Intestinimonas	-2.62	0.0006
Coprobacter	-3.46	0.0013
Pseudomonas	3.6	0.0027
Barnesiella	-2.38	0.0124
Phascolarctobacterium	-2.71	0.0128
Stenotrophomonas	5.24	0.0143
Coprococcus	-1.78	0.0155
Escherichia/Shigella	-1.96	0.0167
Oscillibacter	-1.18	0.0172
Erysipelotrichaceae	-1.53	0.0335
Dialister	0.61	0.0397

Table IV. 2. Summary of the genera with significant differences in abundance (p<0.05) between ileal controls and CD patients. *Data were analysed with Wilcoxon tests. FC = (reads in CD / reads in ctrl-ileum). Ileum controls (n=13) and CD patients (n=21).*

Genus	Log_2FC	p.value
Acinetobacter	0.71	0.0020
Sphingomonas	-0.19	0.0026
Paraprevotella	3.47	0.0156
Staphylococcus	-2.53	0.0163
Enterococcus	4.35	0.0397
Faecalibacterium	1.82	0.0421

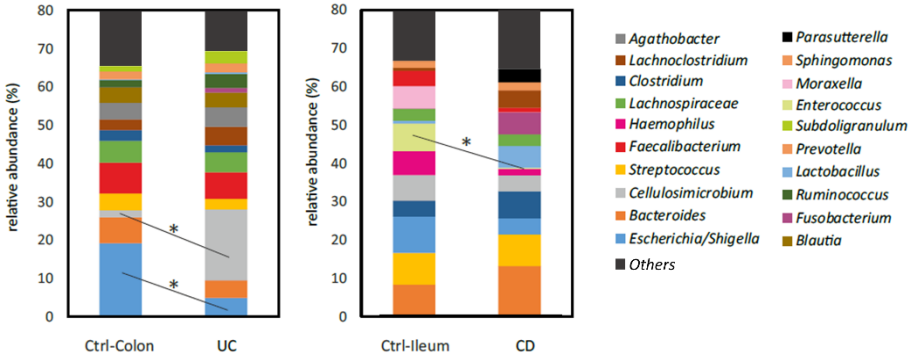


Figure IV. 2. Bacterial composition at genus level in intestinal surgical resections from UC and CD patients*. Bacterial composition at genus level in the four groups of samples. as determined by 16S rRNA gene Illumina sequencing in intestinal surgical resections of colonic controls (n=20), UC patients (n=18), ileal controls (n=13) and CD patients (n=21). * p<0.05 vs. respective control.*

At species taxonomic level, a total of 12 bacterial species were found significantly under-represented and 6 bacterial species over-represented in UC tissue samples when compared to colonic controls, as shown in Figure IV.2B. There were two species whose abundance and prevalence among the disease-associated microbiota deserved special attention. These species included unclassified *Cellulosimicrobium*, which reached 18% of the UC patients' microbial population, and unclassified *Escherichia/Shigella*, which accounted for 19% of the non-IBD colonic.

On the other side, in CD patients, as shown in Figure IV.3, 9 species were significantly under-represented whilst only unclassified *Staphylococcus* and unclassified *Sphingomonas* were significantly over-represented in CD patients.

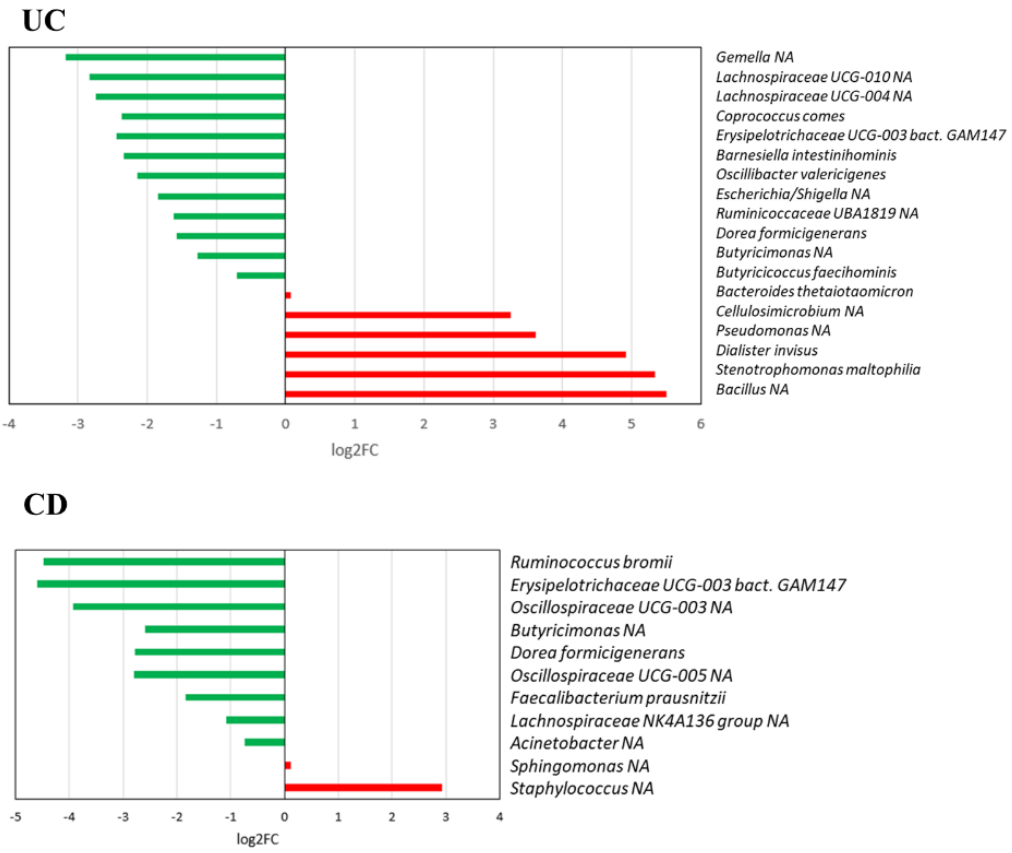


Figure IV. 3. Bacterial composition at species level in intestinal surgical resections from UC and CD patients. *Bacterial composition at species level expressed as log_2FC (FC=reads in UC or CD/ reads in Ctrl-colon or Ctrl-ileum) determined by 16S rRNA gene Illumina sequencing in intestinal surgical resections of colonic controls (n=20), UC patients (n=18), ileal controls (n=13) and CD patients (n=21). Green colour depicts under-represented bacteria vs. control and red colour depicts over-represented bacteria vs. control.*

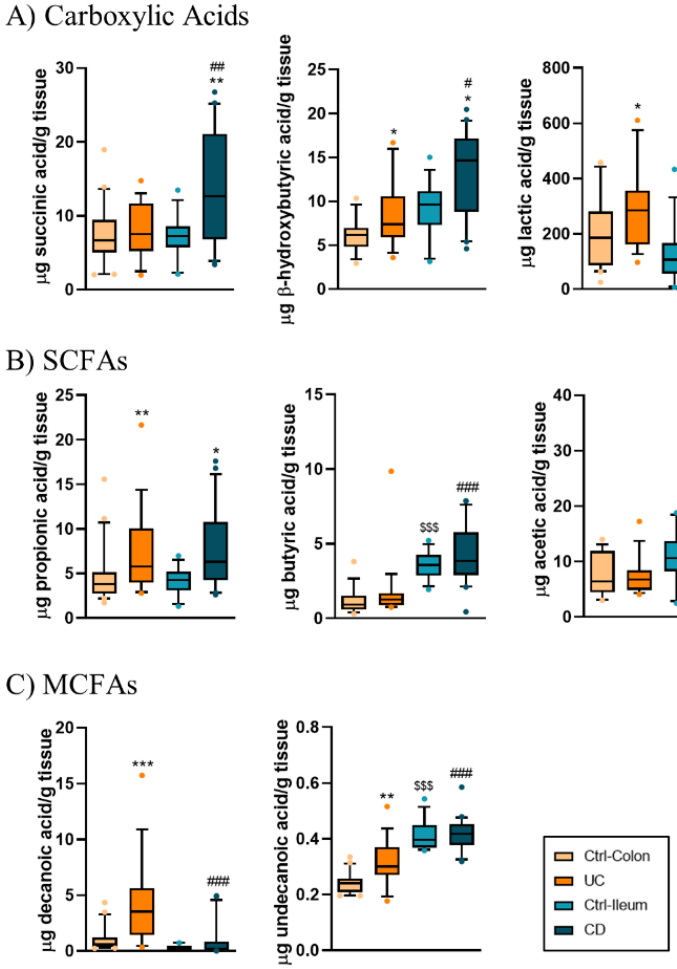
IV.I.2 Levels of GPCRs-agonist metabolites in both UC and CD surgical resections

Once characterized the intestinal microbiota in surgical resections from IBD patients, the metabolite-GPCR axis was also analysed in these samples. For that aim, firstly, levels of those metabolites which can selectively activate GPCRs were

quantified by NMR or UPLC-MS analysis, as previously explained. As shown in Figure IV.4, these metabolites have been classified in 5 different groups: carboxylic acids, short, medium and long chain fatty acids, and amino acids. Regarding carboxylic acids (Figure IV.4A), there were not differences in none of them between colonic and ileal control samples. However, higher levels of all of them were found in both UC and CD versus their respective controls, except succinic acid, whose levels were similar in UC patients and colonic controls. Finally, levels of both succinic and β-hydroxybutyric acids were higher in CD than in UC, as observed in Figure IV.4A.

The quantification of short, medium and long chain fatty acids (Figure IV.4B-D) also exhibited different metabolic profiles between control and affected samples. In this case, as observed in Figure IV.4B, butyric acid was the only SCFA that showed significantly greater levels in ileum than in colon. Next, the amount of propionic acid was higher in both UC and CD patients than in their respective controls, whereas the levels of butyric and acetic acids were significantly higher, only in CD patients. When compared CD versus UC, both butyric and acetic acids amounts were significantly greater in the former (Figure IV.4B). Regarding MCFAs (Figure IV.4C), the amount of undecanoic acid was higher in ileum than in colon, while levels of both, decanoic and undecanoic acids were greater only in UC patients. Besides, as observed in Figure IV.4C, decanoic acid levels were significantly lower in CD versus UC, whereas undecanoic acid levels were significantly higher in CD compared to UC. Next, the analysis of LCFAs revealed, as shown in Figure IV.4D, reduced levels of both eicosapentaenoic and oleic acids in ileum compared with colon. Only UC patients presented higher levels of docosahexaenoic, α-linolenic and myristic acids compared with colonic control samples, while no differences were observed in CD samples versus ileal control samples in none of the LCFAs analysed. Finally, all LCFAs exhibited lower amounts in CD than in UC, except N-oleylethanolamide (Figure IV.4D).

Last, the analysis of amino acids metabolites (Figure IV.4E) revealed greater and significant higher levels of both phenylalanine and aspartic acid in ileal samples compared with colonic samples. In addition, we detected higher amounts of all of them in UC patients than in control samples, and only phenylalanine exhibited greater levels in CD samples vs. ileum. Besides, levels of both phenylalanine and aspartic acid were higher in CD compared to UC, as shown in Figure IV.4E.



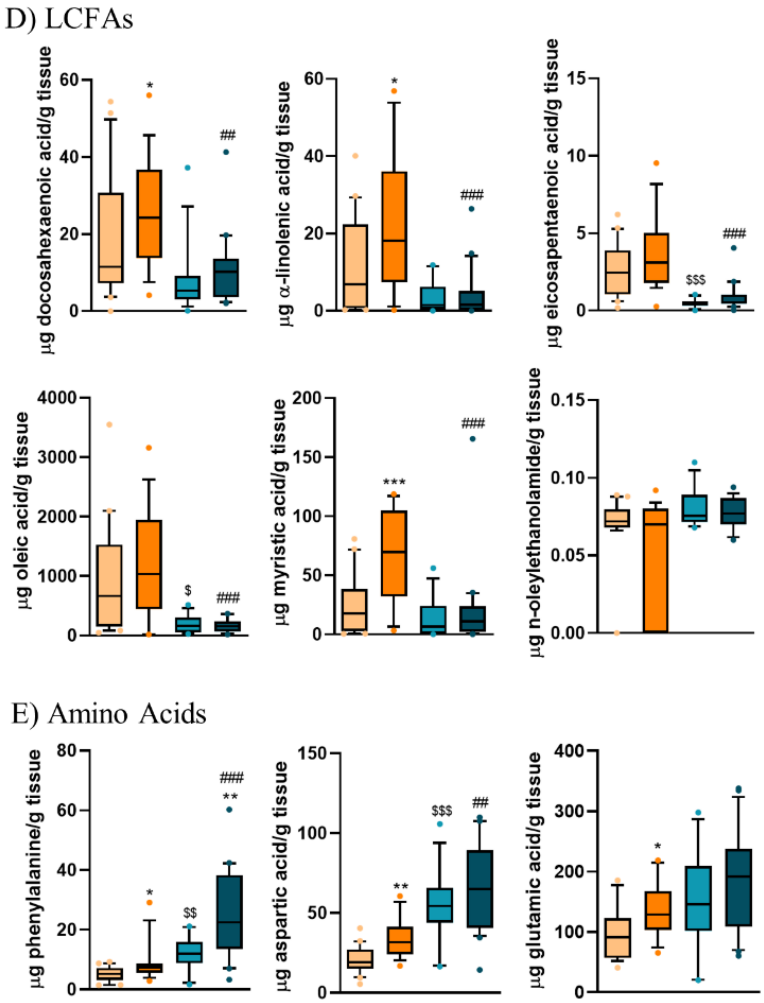


Figure IV. 4. Levels of GPCRs-agonist metabolites in intestinal surgical resections of IBD patients. *Targeted metabolomic analysis from intestinal surgical resections of both UC and CD patients and respective controls was performed by Nuclear Magnetic Resonance for polar metabolites, and Ultra-Performance Liquid-Chromatography Mass Spectrometry for non-polar metabolites. Data are represented in box and whisker diagrams. The line splitting the box in two represents the median value. The upper part of the box represents the upper quartile value, and the lower part the lower quartile value. Horizontal lines in edges represent the maximum and minimum values of data and single points indicate outliers. Data were analysed by one-way ANOVA/Kruskal-Wallis test or unpaired t-test/Mann-Whitney test.*

*Colon controls (n=20), UC patients (n=18), ileum controls (n=13), and CD patients (n=21). *p<0.05, **p<0.01 and ***p<0.001 vs. respective control; #p<0.05, ##p<0.01 and ###p<0.001 vs. UC; $p<0.05, $$p<0.01 and $$$p<0.001 vs. colonic controls.*

IV.I.3 Expression of metabolite-sensing GPCRs in both UC and CD surgical resections

Next, the mRNA expression of the second protagonists of the axis, the metabolite-sensing GPCRs was analysed. Of interest, as shown in Figure IV.5, the expression of most metabolite-sensing GPCRs analysed was similar between the colonic and ileal control samples except *GPR109A* and *GPR41*, whose expression was significantly higher in the ileum than in the colon.

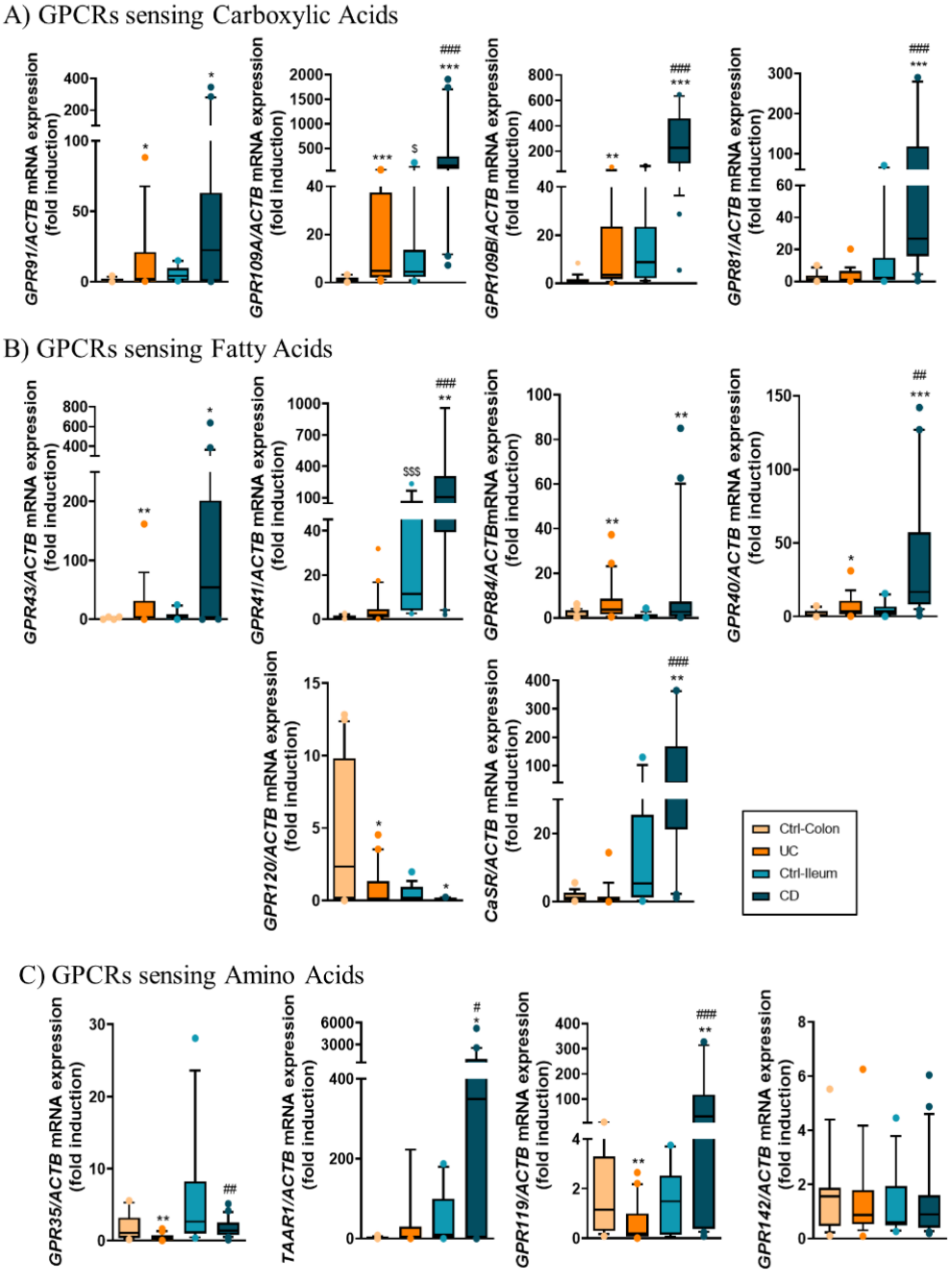
In the case of the GPCRs which can be activated by carboxylic acids (Figure IV.5A), the mRNA expression of *GPR91, GPR109A*, *GPR109B*, and *GPR81* was significantly higher in both UC and CD than in their respective control samples, except *GPR81* that showed similar levels in UC and colon samples. The expression of all of them, except *GPR91*, was significantly higher in CD patients than in UC patients, as detailed in Figure IV.5A.

Regarding GPCRs which can be specifically activated by short, medium and long chain fatty acids, as shown in Figure IV.5B, the expression of *GPR43, GPR41, GPR84* and *GPR40* was significantly higher in both UC and CD patients than in their respective control samples, except *GPR41* in UC patients. In contrast, *GPR120* was significantly downregulated in both UC and CD patients. Conversely, *GPR119* exhibited a differential expression regarding the pathology since it was significantly lower in UC patients and significantly higher in CD patients (Figure IV.5B). Finally, there was a greater expression of *GPR41, GPR40* and *GPR119* in CD compared to UC patients.

On the other hand, regarding the GPCRs which can be activated by amino acids, the expression of *GPR35* was lower in both UC and CD patients than in their

respective controls (Figure IV.5C), although the statistical significance was only reached in the former. On the other hand, the expression of *TAAR1* and *CaSR* were significantly greater in CD patients than in both ileum and UC patients, whereas no differences were observed in the expression of *GPR142* in any of the groups analysed as observed in Figure IV.5C.

Finally, the expression of pH-sensing GPCRs was also analysed and as shown in Figure IV.5D, each GPCR exhibited a different pattern of expression. The expression of *GPR68* was significantly higher in both UC and CD patients than in their respective control samples, as well as in CD than UC patients. In contrast, the *GPR4* expression was significantly lower in UC patients than in controls, but significantly greater in CD than in UC patients, while the expression of *GPR65* was significantly higher in UC patients compared to colonic samples. Finally, the expression of *GPR132* was only greater in CD patients than in ileum (Figure IV.5D).


A) GPCRs sensing Carboxylic Acids
GPR91/ACTB mRNA expression (fold induction)
GPR109A/ACTB mRNA expression (fold induction)
GPR109B/ACTB mRNA expression (fold induction)
GPR81/ACTB mRNA expression (fold induction)
B) GPCRs sensing Fatty Acids
GPR43/ACTB mRNA expression (fold induction)
GPR41/ACTB mRNA expression (fold induction)
GPR84/ACTBmRNA expression (fold induction)
GPR40/ACTB mRNA expression (fold induction)
GPR120/ACTB mRNA expression (fold induction)
CaSR/ACTB mRNA expression (fold induction)
Ctrl-Colon
UC
Ctrl-Ileum
CD
C) GPCRs sensing Amino Acids
GPR35/ACTB mRNA expression (fold induction)
TAAR1/ACTB mRNA expression (fold induction)
GPR119/ACTB mRNA expression (fold induction)
GPR142/ACTB mRNA expression (fold induction)

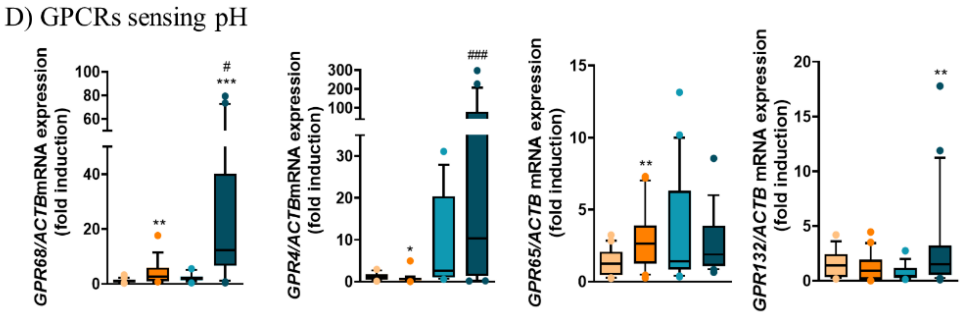


Figure IV. 5. Altered expression of metabolite-sensing GPCRs is found in intestinal surgical resections from UC and CD patients. *Graphs show mRNA expression of (**A**) carboxylic acids, (**B**) fatty acids, (**C**) amino acids and (**D**) pH- sensing GPCRs in intestinal resections from colon controls (n=20), UC patients (n=18), ileum controls (n=13), and CD patients (n=21). Data are represented in box and whisker diagrams. The line splitting the box in two represents the median value. The upper part of the box represents the upper quartile value, and the lower part the lower quartile value. Horizontal lines in edges represent the maximum and minimum values of data and single points indicate outliers. Data were analysed by one-way ANOVA/Kruskal-Wallis test or unpaired t-test/Mann-Whitney test, *p<0.05, **p<0.01, and ***p<0.001 vs. respective control; #p<0.05, ##p<0.01 and ###p<0.001 vs. UC; $p<0.05 and $$$p<0.001 vs. colonic controls.*

IV.I.4 Metabolite-sensing GPCRs are associated with intestinal inflammation and fibrosis in IBD patients

Finally, once detected a differential pattern of expression of metabolite-sensing GPCRs in surgical resections of both UC and CD patients, we sought to analyse whether these receptors were associated with intestinal inflammation and fibrosis (Figure IV.6). To achieve this, we quantified the mRNA expression of the pro-inflammatory cytokines *IL1B* and *IL8*, the M1 macrophage marker *CD86*, and the pro-fibrotic markers *COL1A1*, *COL3A1* and *COL4A1*, in intestinal resections of IBD patients. Both UC and CD patients exhibited a significant higher expression of *IL1B*, *IL8* and *CD86* compared to their respective control samples, as observed in Figure IV.6A. Regarding the pro-fibrotic markers studied, the expression of *COL1A1*,

COL3A1 and *COL4A1* was significantly higher in CD patients than in control ileum, whereas none of the collagen genes analysed were significantly altered in UC patients (Figure IV.6B).

On the one hand, when expression of the pro-inflammatory cytokines was correlated with that of all the metabolite-sensing GPCRs, a positive correlation between the expression of the pro-inflammatory cytokines *IL1B* and *IL8*, and also the M1 macrophage marker *CD86* with most of the GPCRs analysed was detected in UC patients (Figure IV.6C). Only a slight negative correlation was observed between *IL1B* and the expression of *CaSR*. On the other hand, in CD patients' samples most of the receptors failed to correlate with *IL1B* except for *GPR84*, *GPR120, GPR142* and *GPR132*, which correlated positively and *GPR35*, that did it negatively. In contrast, *IL8* and *CD86* strongly and positively correlated with most of the GPCRs except *GPR120,* and *GPR132* which correlated negatively with *IL8,* as observed in Figure IV.6C.

On the other hand, the correlations between the expression of pro-fibrotic markers and the GPCRs revealed in UC patients slight positive correlations between metabolite-sensing GPCRs and *COL1A1,* as detailed in Figure IV.6D. *COL3A1* and *COL4A1* exhibited a similar pattern of correlation with the expression of metabolite-sensing GPCRs, most of them were positive except for *GPR120* and *CaSR* with both *COL3A1* and *COL4A1,* and *GPR81, GPR119, GPR35* and *TAAR1* with *COL4A1,* which showed negative correlations (Figure IV.6D). Next, in CD surgical resections we detected mostly positive correlations with the three collagen markers and all the metabolite-sensing GPCRs except *GPR35*, which exhibited a negative correlation specifically with *COL1A1* and *COL3A1*. The strongest positive correlation for *COL1A1* and GPCRs was observed with *GPR84*. In addition, *COL4A1* showed up the strongest positive correlations with most of the metabolite-sensing GPCRs such as *GPR109A, GPR109B, GPR81, GPR43, GPR40, GPR119, TAAR1, GPR142, GPR68* and *GPR4*, among others (Figure IV.6D).

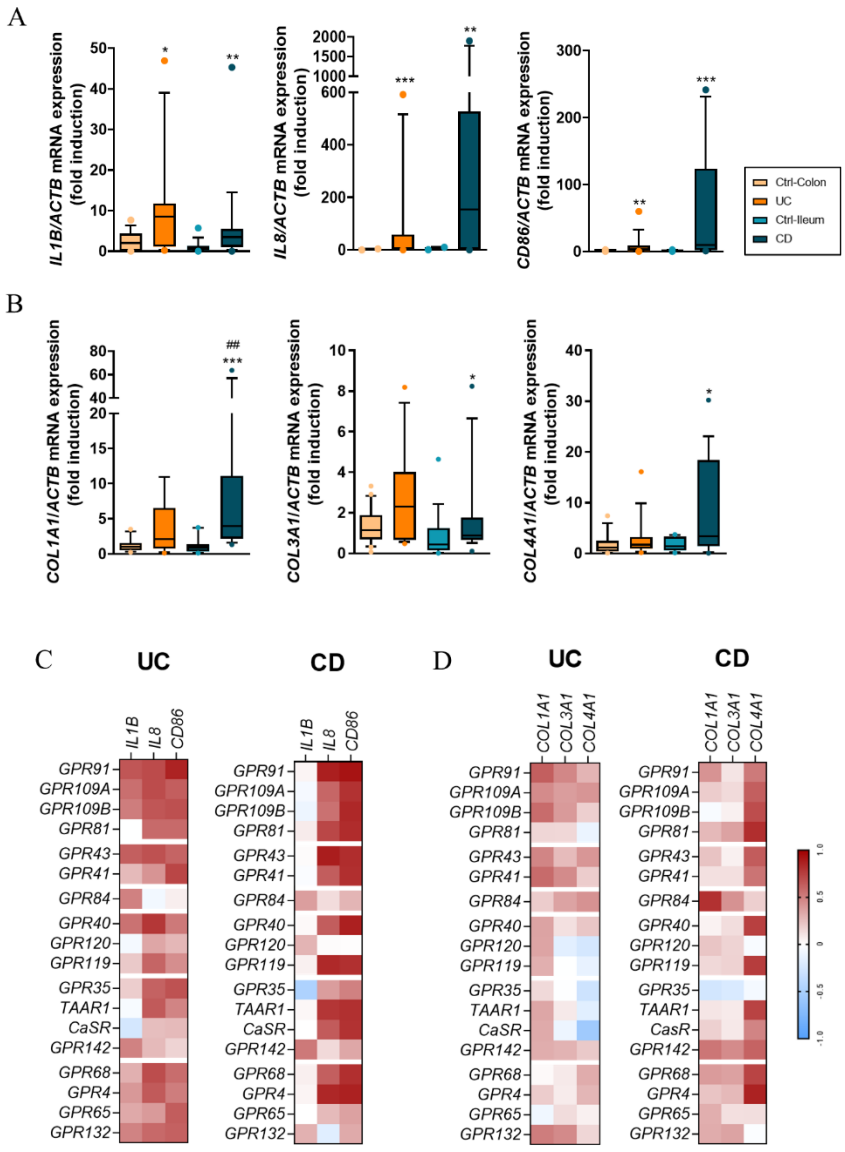


Figure IV. 6. Metabolite-sensing GPCRs are associated with intestinal inflammation and fibrosis from UC and CD patients. *Graphs show mRNA expression of (**A**) pro-inflammatory cytokines IL1B and IL8, and the M1 macrophage marker CD86, and (**B**) pro-fibrotic markers COL1A1, COL3A1 and COL4A1, in intestinal resections of colon controls (n=20), UC patients (n=18), ileum controls (n=13), and CD patients (n=21). Data are represented in box and whisker diagrams. The line splitting the box in two represents the median value. The*

*upper part of the box represents the upper quartile value, and the lower part the lower quartile value. Horizontal lines in edges represent the maximum and minimum values of data and single points indicate outliers. Data were analysed by one-way ANOVA/Kruskal-Wallis test and/or unpaired t-test/Mann-Whitney test, *p<0.05, **p<0.01 and ***p<0.001 vs. respective control; #p<0.05 vs. UC. Heat maps showing Spearman's correlation values between data relative to mRNA expression of metabolite-sensing GPCRs vs. (**C**) mRNA expression of IL1B, IL8, and CD86 (expressed as ΔCt), and (**D**) mRNA expression of COL1A1, COL3A1 and COL4A1 (expressed as ΔCt) on intestinal samples. Red colours indicate positive correlation values, whereas blue colours indicate negative correlation values.*

All these data suggest that metabolite-sensing GPCRs might be involved in inflammatory and fibrotic processes related with IBD. Hence, we decided to analyse in depth the relevance of two different GPCRs in IBD pathogenic mechanisms.

CHAPTER II: GPR91 IN IBD PATHOGENESIS

A previous study performed by our group described a high expression of GPR91 receptor in CD patients, and a pro-inflammatory and pro-fibrotic role of this receptor in an acute murine model of intestinal inflammation and fibrosis (146). Hence, in this thesis, in order to analyse in depth its role in the pathogenesis of IBD, we have studied whether GPR91 is involved in specific molecular processes such as epithelial to mesenchymal transition (EMT), and inflammasome and its relevance in a chronic murine model of colitis.

IV.II.1 GPR91 in EMT

IV.II.1.1 Overexpression of GPR91 is detected in surgical resections from B3-CD patients and it correlates with EMT markers

First, gene and protein expression of GPR91 were analysed in intestinal surgical resections from B2 and B3-CD patients. As observed in Figure IV.7A, intestinal tissue that surrounds the fistula tract in B3-CD patients, exhibited higher gene and protein expression of the receptor than control tissue. In addition, levels of succinate, the main agonist of GPR91, were quantified in the same surgical resections and as shown in Figure IV.7B, they were significantly greater in B3-CD patients than in both B2-CD patients and controls.

Next, the mRNA expression of the transcription factors involved in EMT, *SNAI1* and *SNAI2*, was analysed and results revealed significantly higher expression in samples of B3-CD patients than in samples from both controls or B2-CD patients (Figure IV.7C). To determine whether there was a relationship, correlations between expression of those genes were performed. As shown in Figure IV.7D, both succinate levels and the expression of *GPR91* positively and significantly correlated with the expression of *SNAI1* and *SNAI2*. In addition, succinate levels also positively correlated with the expression of its receptor *GPR91*. All these results suggest that succinate and its receptor GPR91 might be involved in the activation of EMT in CD.

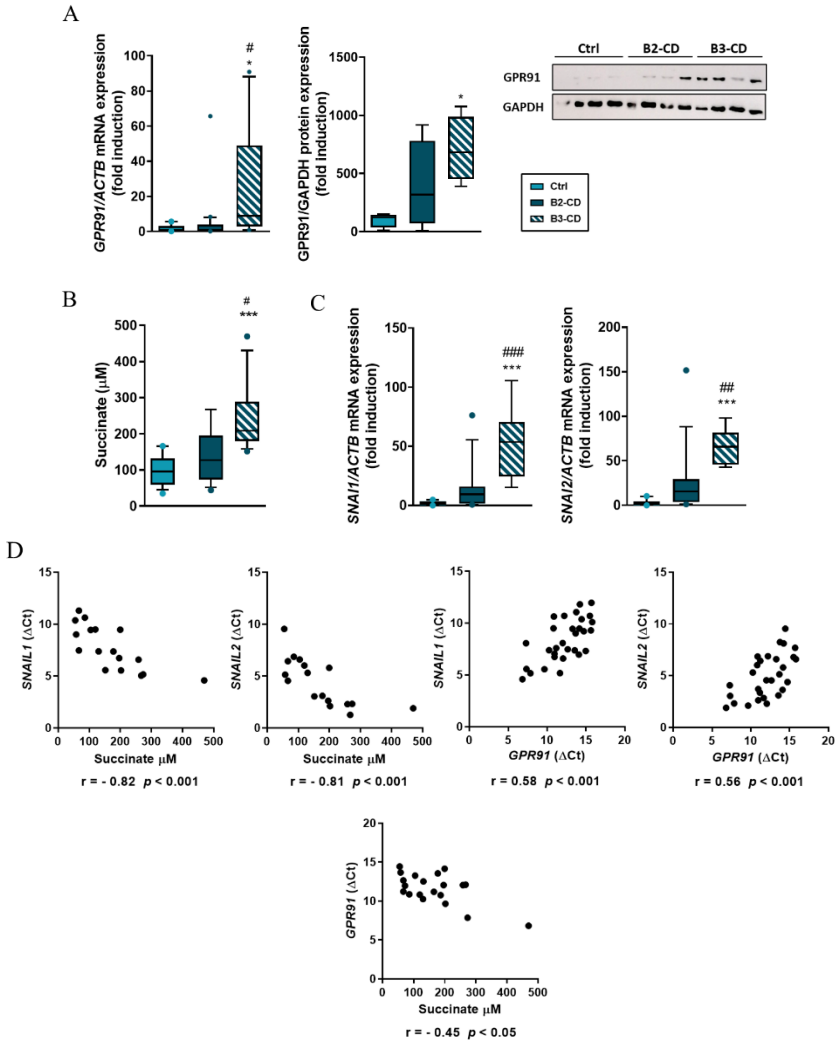


Figure IV. 7. Intestinal succinate levels, GPR91 and EMT markers are higher and positively correlate in B3-CD patients. *Graphs show (**A**) GPR91 mRNA and protein expression, and image of a representative Western Blot, (**B**) succinate levels, and (**C**) mRNA expression of EMT markers SNAI1 and SNAI2 in controls (n=10), B2-CD (n=19), and B3-CD (n=16). Data are represented in box and whisker diagrams. The line splitting the box in two represents the median value. The upper part of the box represents the upper quartile value, and the lower part the lower quartile value. Horizontal lines in edges represent the*

*maximum and minimum values of data and single points indicate outliers. Data were analysed by one-way ANOVA/Kruskal-Wallis test. *p<0.05, and ***p<0.001 vs. control; #p<0.05, ##p<0.01 and ###p<0.001 vs. B2-CD. (**D**) Correlations between data relative to the succinate levels, the mRNA expression of GPR91, and the EMT markers SNAI1 and SNAI2 (expressed as ΔCt). Values of Spearman's correlation coefficient (r) and p values are shown.*

IV.II.1.2 GPR91 is expressed specifically in the fistula tract

A qualitative study of the expression of GPR91 was performed by immunohistochemistry in collaboration with the Department of Gastroenterology and Hepatology from the University Hospital of Zurich, using tissue samples that included the tract of entero-enteric fistulas and adjacent tissue from B3-CD patients (Figure IV.8). As shown in Figure IV.8A, intestinal epithelial cells and cells of the lamina propria, such as fibroblasts (pointed with white arrows), close to the fistula tract expressed GPR91. In addition, a higher intensity of the staining of this receptor was observed in the fistula tract (pointed with a black star) in comparison with non-fistula tract areas. Transitional cells (pointed with red arrows) that surround the fistula also specifically expressed GPR91.

Moreover, in order to analyse which cell type present in the fistula expressed GPR91, a double immunohistochemistry was performed. As shown in Figure IV.8B, Vimentin+ fibroblasts and CD206+ macrophages were also stained with GPR91 antibody.

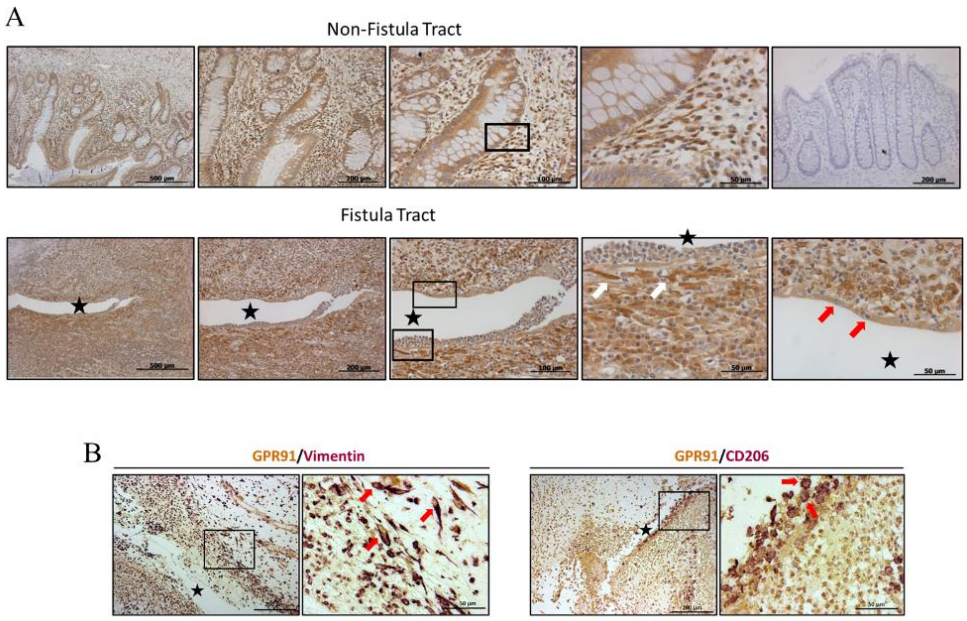


Figure IV. 8. GPR91 is expressed specifically in fibroblasts and macrophages from the fistula tract. *(**A**) Immunostaining of GPR91 was performed in 5-µm sections of paraffin-embedded tissues, and representative pictures of areas without fistula (non-fistula tract) and areas including the tract of entero-enteric fistulas (fistula tract) from 5 B3-CD patients are shown. The black star indicates the fistula lumen. White arrows point to positive cells close to the fistula tract with a fibroblast morphology, and red arrows point to transitional cells (TCs). (**B**) Double immunohistochemistry of Vimentin-GPR91 and CD206-GPR91 was performed in 5-µm sections of paraffin-embedded tissues and a representative picture of each areas including the fistula tract from B3-CD patients are shown (n=5). Red arrows point to cells double positive for GPR91/Vimentin or for GPR91/CD206.*

IV.II.1.3 GPR91 mediates EMT in murine intestinal fibrosis *in vivo*

The relevance of the GPR91 receptor in EMT was analysed *in vivo* with the heterotopic transplant model of intestinal fibrosis (Figure IV.9). First, levels of succinate in intestinal grafts from WT mice 7 days after transplantation were significantly higher than colon samples at day 0, as shown in Figure IV.9A. Of

interest, intestinal grafts from GPR91$^{-/-}$ mice at day 7 exhibited a significant reduction in succinate levels compared with intestinal grafts at day 7 from WT mice.

Moreover, gene expression of EMT markers was analysed and both *Snai1* and *Snai2* were significantly upregulated in WT grafts at day 7, while *Cdh1* was significantly downregulated, as observed in Figure IV.9B. Interestingly, intestinal grafts from GPR91$^{-/-}$ mice 7 days after transplantation exhibited a similar expression of EMT markers to those observed in colon samples at day 0.

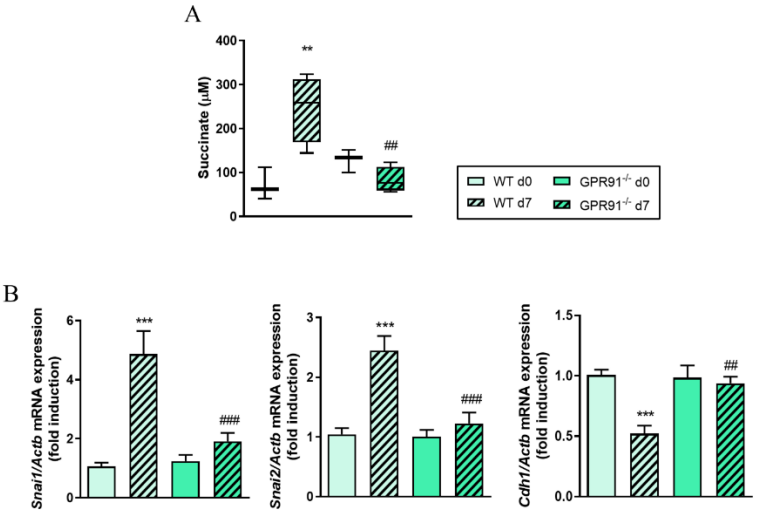
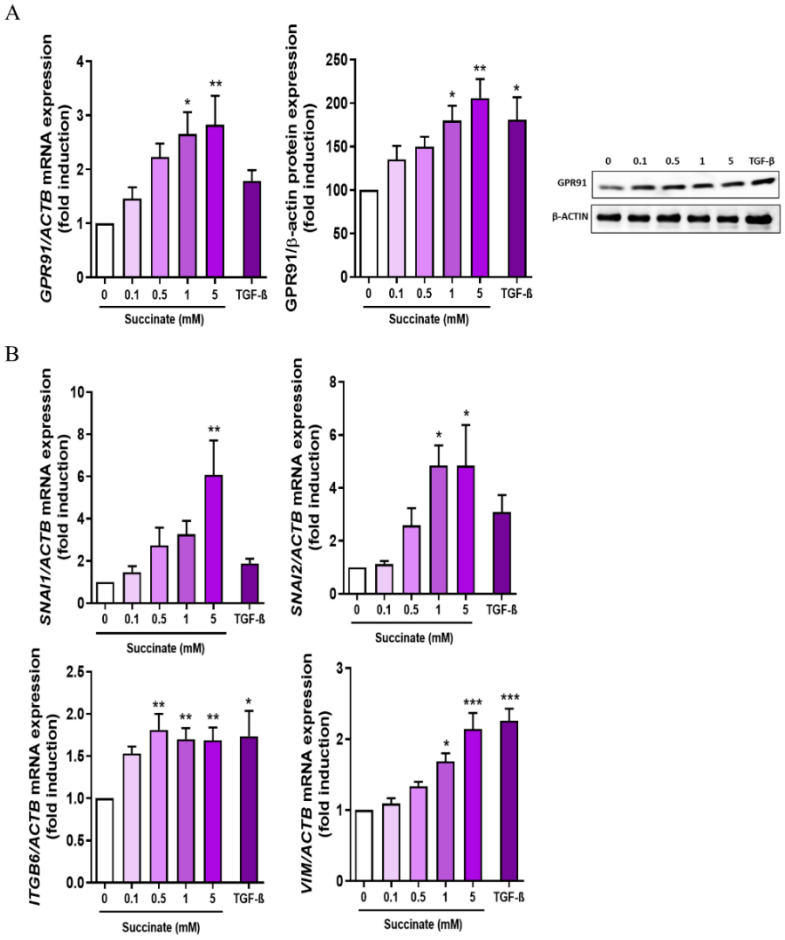


Figure IV. 9. Lack of GPR91 decreases succinate levels and epithelial-to-mesenchymal transition (EMT) activation in vivo. *Intestinal fibrosis was induced in vivo using the heterotopic transplant model. (**A**) Graph shows succinate levels in the intestinal grafts from wild type (WT) and GPR91$^{-/-}$ mice (n = 4). (**B**) Graphs show the expression of Snai1, Snai2 and Cdh1 in intestinal grafts from WT and GPR91$^{-/-}$ mice (n=7). Bars in graphs represent mean ± SEM analysed by one-way ANOVA/Kruskal-Wallis test. **p<0.01 and ***p<0.001 vs. intestinal grafts from WT at day 0; ##p < 0.01 and ###p < 0.001 vs. intestinal grafts from WT at day 7.*

IV.II.1.4 Succinate induces EMT through GPR91 in intestinal epithelial cells

In order to study the effects of succinate in the EMT activation, HT-29 cells were treated with different concentrations of this metabolite (0, 0.1, 0.5, 1 and 5 mM) or its vehicle, for 48 h, and TGF-β (5 ng/ml) was used as a positive control. First, succinate increased both gene and protein expression of GPR91 in a dose dependent manner as shown in Figure IV.10A. In parallel, succinate induced a significant and dose-dependent increase of EMT markers *SNAI1*, *SNAI2* and *ITGB6,* as well as the mesenchymal marker *VIM* (Figure IV.10B), while it decreased both gene and protein expression of the epithelial marker Cadherin-1.



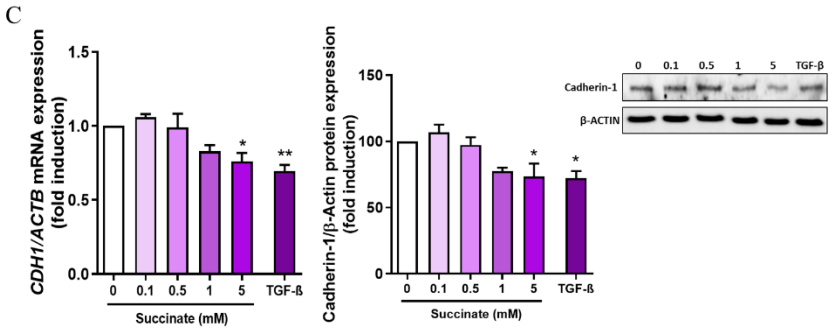


Figure IV. 10. Succinate induces gene expression of EMT markers in intestinal epithelial cells. *HT-29 cells were treated with different concentrations of succinate (0, 0.1, 0.5, 1 and 5 mM) or TGF-β 5 ng/ml for 48 h. (**A**) Graphs show the mRNA and protein expression of GPR91 receptor. Image of a representative Western Blot is shown (n=5). (**B**) Graphs show the mRNA expression of the EMT markers SNAI1, SNAI2, VIM and ITGB6 (n=5). (**C**) Graphs show the mRNA and protein expression of Cadherin-1. Image of a representative Western Blot is shown (n=5). Bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis test or unpaired t-test/Mann-Whitney test, *p<0.05, **p<0.01 and ***p<0.001 vs. vh HT-29 cells.*

These results were strongly reinforced with immunofluorescence studies which showed an increased intensity of Vimentin signal after succinate or TGF-β treatment (Figure IV.11), with a concomitant reduction of Cadherin-1 staining intensity. Of interest, in succinate-treated cells, a disturbed pattern of Cadherin-1 expression was observed in the cytoplasmic membrane.

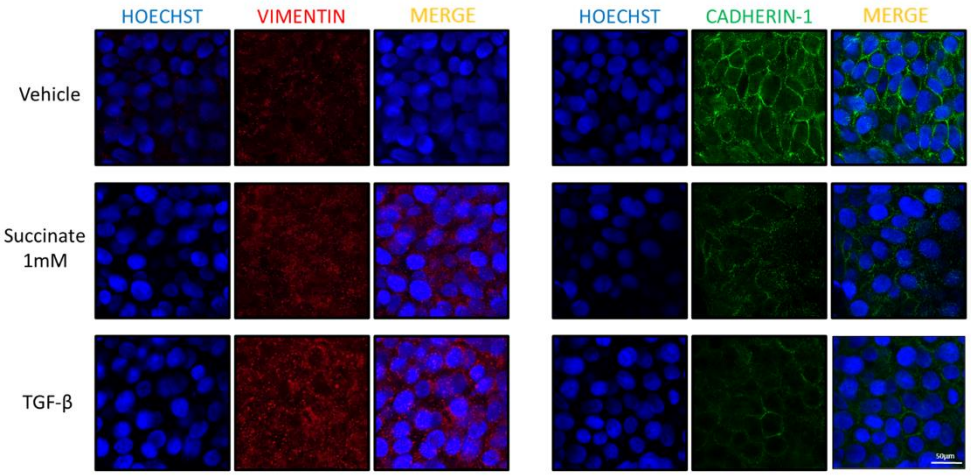


***Figure IV. 11. Immunofluorescence shows EMT induction by succinate**. Immunofluorescence of Vimentin and Cadherin-1 in HT-29 treated with succinate 1mM or TGF-β for 48 h. Representative pictures of a total of 3 independent experiments.*

Finally, to determine whether succinate activation of EMT was mediated by its receptor GPR91, HT-29 cells were transiently silenced with a specific GPR91 siRNA (Figure IV.12). To confirm the efficiency of transfection, gene and protein levels of GPR91 under transient silencing were analysed and a significant reduction was detected in both, as shown in Figure IV.12A. Results showed in succinate-treated cells, that siGPR91-HT-29 cells exhibited significantly lower levels of *SNAI1*, *SNAI2*, *ITGB6* and *VIM* than siCtrl-HT-29 cells, whereas the former showed significantly higher levels of *CDH1* (Figure IV.12B).

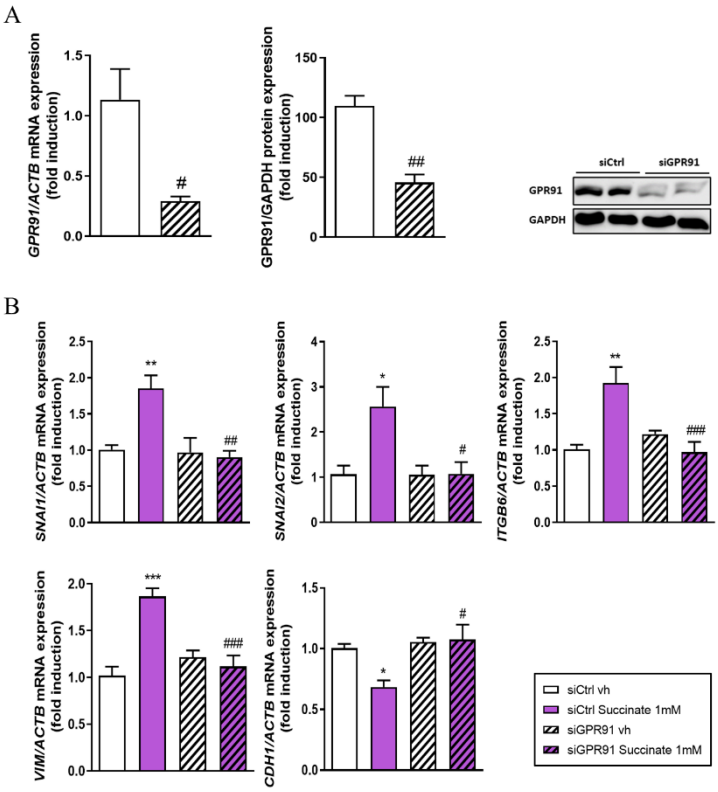
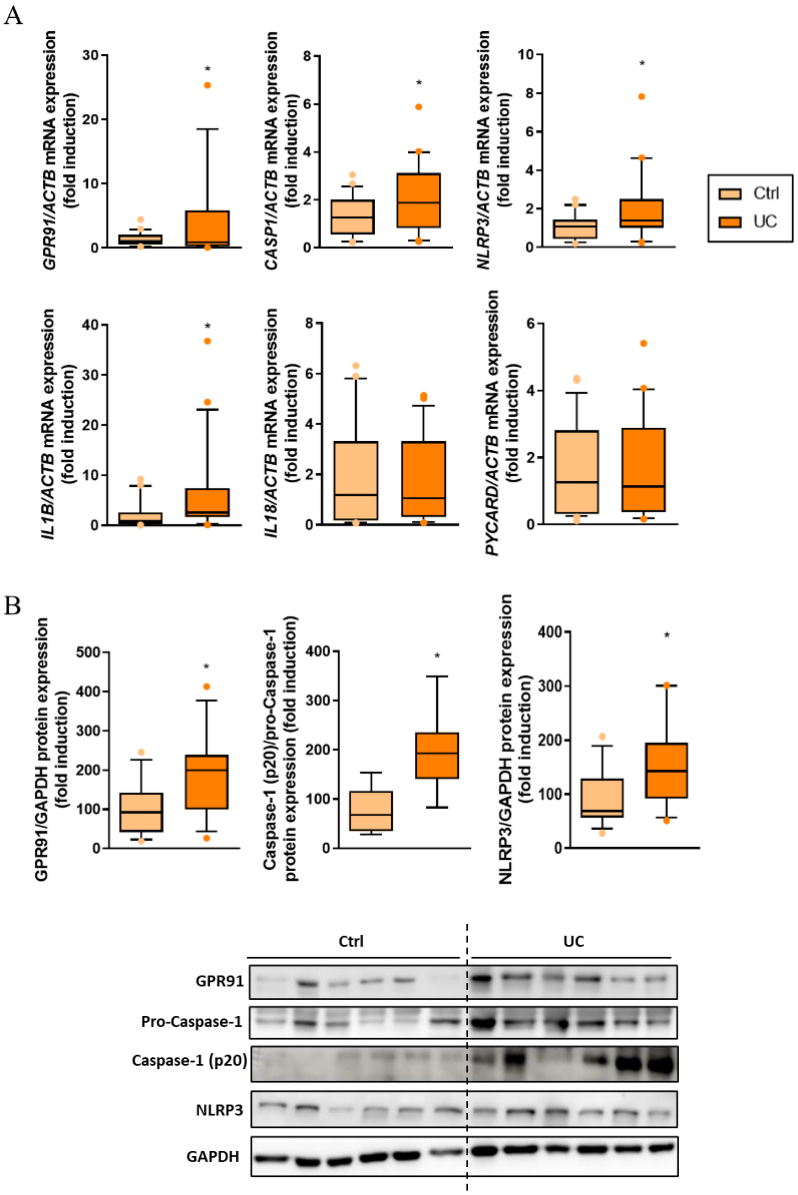


Figure IV. 12. Succinate induces EMT in intestinal epithelial cells through GPR91. *HT-29 cells were transiently silenced with a specific siRNA against GPR91, or control siRNA and treated with succinate 1 mM for 48 h post transfection. Graphs show (**A**) mRNA and protein expression of GPR91 after transfection and an image of a representative Western Blot is shown (n=5), (**B**) the expression of SNAI1, SNAI2, ITGB6, VIM and CDH1 (n=5). Bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis test. *p<0.05, **p<0.01 and ***p<0.001 vs. siCtrl vh HT-29 cells; #p<0.05, ##p<0.01 and ###p<0.005 vs. siCtrl-succinate treated HT-29 cells.*

IV.II.2 GPR91 in the priming step of inflammasome

IV.II.2.1 GPR91 and inflammasome components are increased and positively correlated in surgical resections from UC patients

Next, the role of GPR91 in inflammasome activation was also analysed in the present Doctoral Thesis. To achieve this, the expression of GPR91 and inflammasome components was analysed in surgical resections from UC patients. As shown in Figure IV.13, gene and protein expression of GPR91 was significantly higher in UC patients than in control colon. In a similar manner, gene expression of *CASP*1, *NLRP3* and *IL1B* was significantly higher in samples from UC patients than in control tissue, whereas no differences were detected in the expression of *IL18* nor *PYCARD* (Figure IV.13A). Of interest, protein expression of the inflammasome components such as NLRP3 and the ratio Caspase-1 (p20)/pro-Caspase-1 was also greater in UC patients (Figure IV.13B). To determine whether GPR91 was associated with inflammasome activation in human intestinal resections of UC patients, both pools of data were correlated. Results revealed a positive and significant correlation between the expressions of *GPR91* and all the inflammasome components analysed (*CASP1*, *NLRP3*, *IL1B*, *IL18* and *PYCARD*) (Figure IV.13C).


A
GPR91/ACTB mRNA expression (fold induction)
CASP1/ACTB mRNA expression (fold induction)
NLRP3/ACTB mRNA expression (fold induction)
IL1B/ACTB mRNA expression (fold induction)
IL18/ACTB mRNA expression (fold induction)
PYCARD/ACTB mRNA expression (fold induction)
Ctrl
UC
B
GPR91/GAPDH protein expression (fold induction)
Caspase-1 (p20)/pro-Caspase-1 protein expression (fold induction)
NLRP3/GAPDH protein expression (fold induction)
Ctrl
UC
GPR91
Pro-Caspase-1
Caspase-1 (p20)
NLRP3
GAPDH

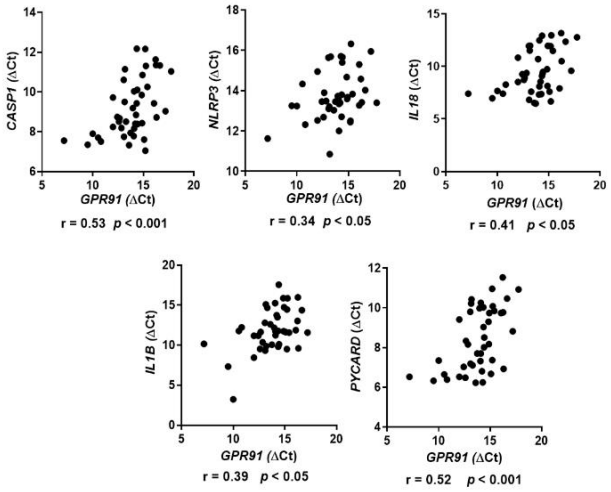


Figure IV. 13. GPR91 and inflammasome components are higher and positively correlated in surgical resections of UC patients*. (**A**) Graphs show mRNA expression of GPR91, CASP1, NLRP3, IL1B, IL18 and PYCARD in intestinal resections from UC patients (n=25) and non-IBD patients (n=30). (**B**) Graphs show protein expression of GPR91, Caspase-1/pro-Caspase-1 and NLRP3. Image of a representative Western Blot is shown (n=15). Data are represented in box and whisker diagrams. The line splitting the box in two represents the median value. The upper part of the box represents the upper quartile value, and the lower part the lower quartile value. Horizontal lines in edges represent the maximum and minimum values of data and single points indicate outliers. Data were analysed by unpaired t-test/Mann-Whitney test. *p<0.05 vs. controls. (**C**) Correlations between data relative to mRNA expression of GPR91 vs. mRNA expression of the inflammasome components CASP1, NLRP3, IL1B, IL18 and PYCARD (expressed as ΔCt). In each correlation, the value of the Spearman's correlation coefficient (r) and p value are shown.*

IV.II.2.2 Lack of GPR91 ameliorates DSS-chronic colitis

To analyse the role of GPR91 receptor in chronic colitis, WT and GPR91$^{-/-}$ mice were treated with four cycles of increasing percentage of DSS in drinking water over 7 days intercalated with 10 days of water. First, survival proportion at day 60 was significantly higher in GPR91$^{-/-}$ mice, in which none of the mice died, compared

with DSS-treated WT mice, in which a 66.67% rate of survival was observed (Figure IV.14A). In parallel, DSS-treated WT mice showed a significantly higher loss of body weight compared with DSS-treated GPR91$^{-/-}$ mice (Figure IV.14B). Next, DSS induced a significant reduction in the colon length in both WT and GPR91$^{-/-}$ mice (Figure IV.14C). However, DSS-treated GPR91$^{-/-}$ mice exhibited a significant reduction in the histological score compared to DSS-treated WT mice, as observed in Figure IV.14D.

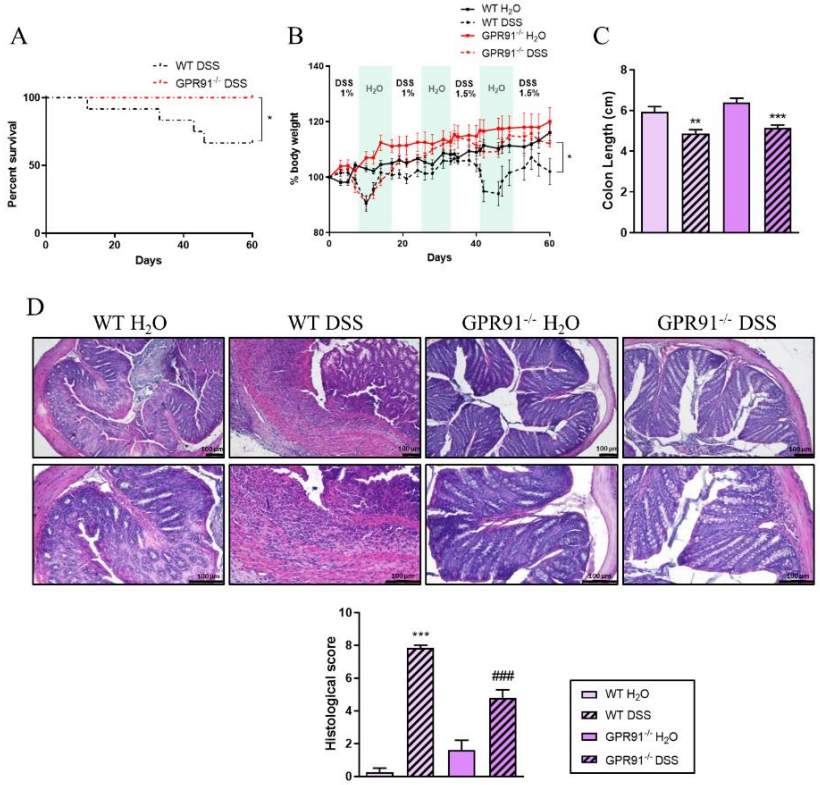


Figure IV. 14. Lack of GPR91 ameliorates DSS-chronic colitis. *Chronic intestinal colitis was induced in vivo in WT and GPR91$^{-/-}$ mice with four cycles of increasing percentage of DSS (1%, 1%, 1.5% and 1.5%) in drinking water over 7 days, intercalated with 10 days of water. At the end of the last cycle, on day 60, mice were euthanized, and colon tissue samples were collected. Graphs show (**A**) the survival percentage in mice after the four cycles of DSS,*

*(**B**) the evolution of body weight, and (**C**) the colon length of mice (n=10 per group). (**D**) Hematoxylin-Eosin staining performed on colons from WT and GPR91$^{-/-}$ mice (n=10). Representative pictures of each group are shown. Histological score to assess the integrity of the epithelium and the degree of infiltration was also performed following Obermeier et al. parameters. Bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis test. **p<0.01 and ***p< 0.001 vs. WT H_2O mice. ### p < 0.001 vs. DSS-treated WT mice.*

IV.II.2.3 GPR91 deficiency reduces intestinal inflammation and fibrosis in DSS-chronic colitis

First, DSS treatment in WT mice significantly increased the gene expression of the pro-inflammatory cytokines *Cox-2, Tnf, Nos2* and *Il6* in the colon. This effect was attenuated in DSS-treated GPR91$^{-/-}$ mice since gene expression of these cytokines was significantly reduced versus DSS-treated WT mice (Figure IV.15). Interestingly, the anti-inflammatory cytokine *Il10* was upregulated in DSS-treated GPR91$^{-/-}$ mice compared to DSS-treated WT mice.

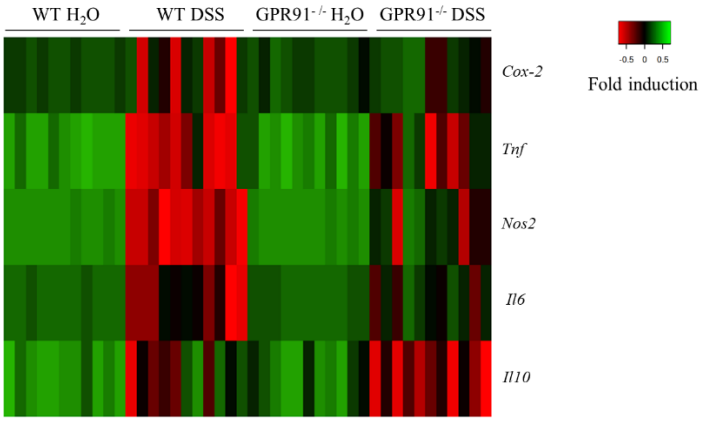


Figure IV. 15. GPR91 deficiency reduces intestinal inflammation in DSS-chronic colitis. *Heat map showing the mRNA expression of pro-inflammatory and anti-inflammatory cytokines including Cox-2, Tnf, Nos2, Il6 and Il10, in colons from WT and GPR91$^{-/-}$ mice after chronic administration with DSS (n=8).*

In addition, macrophage infiltration and macrophage phenotype were also analysed in these mice (Figure IV.16). First, DSS-treated WT mice exhibited an upregulation in the gene expression of *F4/80*, *Cd86*, *Ccr7*, *Arg1*, and *Fcgr3*. In contrast, DSS-treated GPR91$^{-/-}$ mice exhibited non-significant differences in *F4/80*, in parallel with a reduction in the *Cd86* expression and an increased expression of both *Mrc1* and *Arg1* compared to DSS-treated WT mice (Figure IV.16).

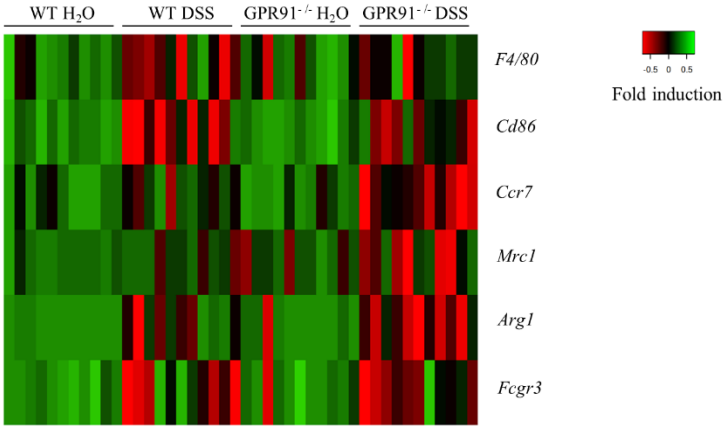


Figure IV. 16. GPR91 deficiency reduces intestinal macrophage infiltration in DSS-chronic colitis. *Heat map showing the mRNA expression of macrophage infiltration F4/80 and phenotype markers Cd86, Ccr7, Mrc1, Arg1 and Fcgr3 in colons from WT and GPR91$^{-/-}$ mice after chronic administration with DSS (n=8).*

Finally, the expression of pro-fibrotic markers was also analysed in these mice. Chronic DSS treatment induced a significant increase in *Col1a1*, *Col3a1*, *Col4a1*, *Tgfb1*, *Timp1* and *Mmp2* in WT mice, as shown in Figure IV.17. In the case of DSS-treated GPR91$^{-/-}$ mice, *Col1a1*, *Col3a1*, *Col4a1*, *Vim*, *Tgfb1* and *Mmp2* expression was significantly reduced versus DSS-treated WT mice.

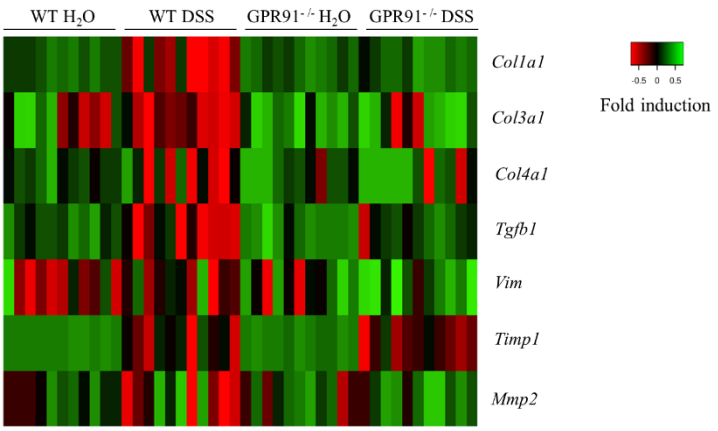


Figure IV. 17. GPR91 deficiency reduces pro-fibrotic markers in DSS-chronic colitis. *Heat map showing the mRNA expression of pro-fibrotic markers Col1a1, Col3a1, Col4a1, Tgfb1, Vim, Timp1 and Mmp2 in colons from WT and GPR91$^{-/-}$ mice after chronic administration with DSS (n=8).*

Next, the analysis of collagen layer was performed using Sirius Red staining. The percentage of red area and the analysis of the thickness of the collagen layer revealed that upon chronic DSS treatment, WT mice presented increased red percentage area and a significant thicker collagen layer versus DSS-treated GPR91$^{-/-}$ mice, as shown in Figure IV.18.

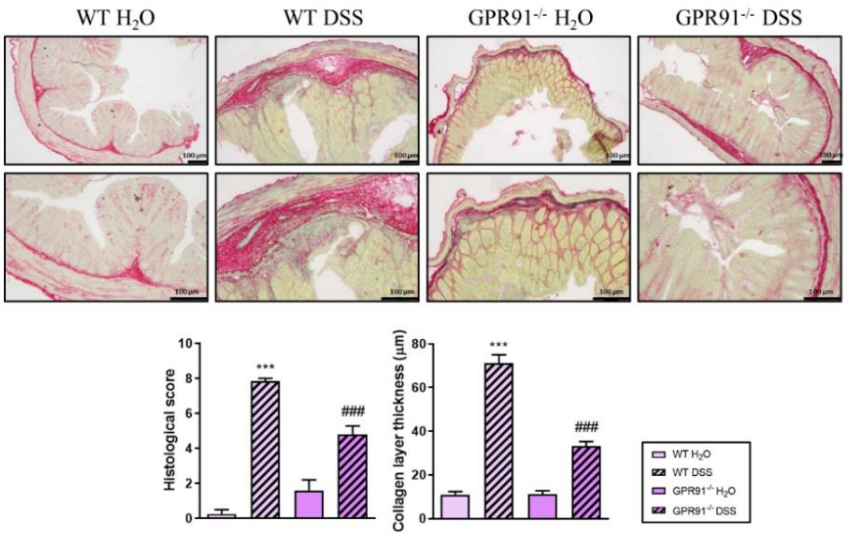


Figure IV. 18. GPR91 deficient mice show decreased collagen deposition in DSS-chronic colitis. *Representative images of Sirius-Red staining on colons from WT and GPR91$^{-/-}$ mice after chronic administration with DSS (n=8). Quantification of the collagen layer thickness and % of red area are also represented. Bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis test. ***p<0.001 vs. WT H_2O mice. ###p<0.001 vs. DSS-treated WT mice.*

IV.II.2.4 Lack of GPR91 impairs the expression of inflammasome components in DSS-chronic colitis

In addition, the role of GPR91 in the inflammasome activation *in vivo* was analysed in WT and GPR91$^{-/-}$ mice after the induction of DSS-chronic colitis (Figure IV.19). First, DSS-treated WT mice exhibited a significant increase in the mRNA expression of *Casp1*, *Nlrp3*, and *Il1b* (Figure IV.19A), while no changes were observed in *Pycard* compared with WT H_2O mice. In parallel, protein levels of pro-Caspase-1 were also significantly increased in DSS-treated WT mice versus WT H_2O mice (Figure IV.19B). Interestingly, DSS did not induce any of the previously observed effects in GPR91$^{-/-}$ mice, since a significant reduction in the gene

expression of *Casp1*, *Nlrp3*, and *Il1b*, as well as in the protein expression of pro-Caspase-1, was observed versus DSS-treated WT mice (Figure IV.19).

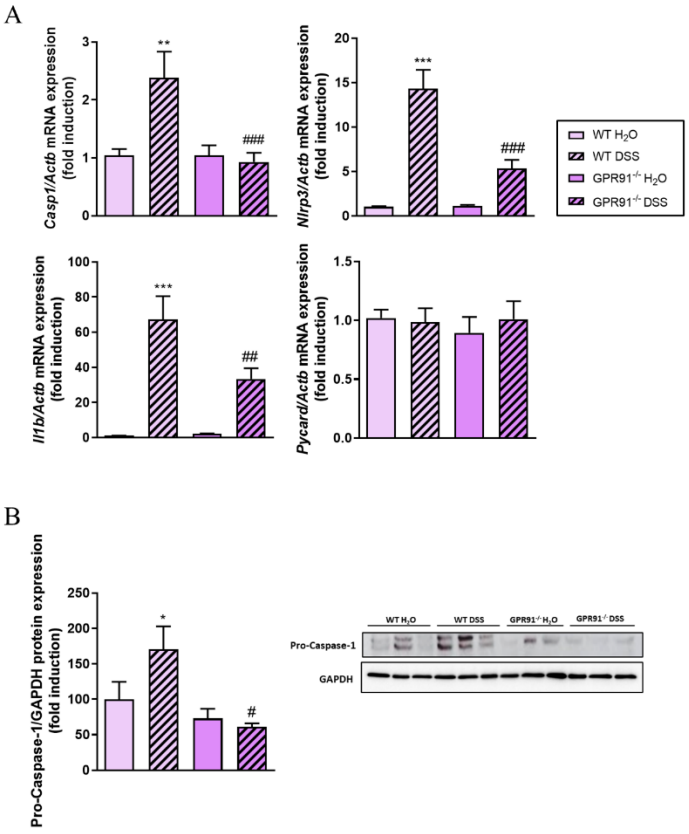


Figure IV. 19. Lack of GPR91 impairs the expression of inflammasome components in DSS-chronic colitis. ***(A)*** *Graphs show mRNA expression of Nlrp3, Casp1, Il1b and Pycard, in colons from WT and GPR91$^{-/-}$ mice after chronic administration with DSS (n=8).* ***(B)*** *Graph shows protein expression of pro-Caspase-1. Image of a representative Western Blot is shown (n=3). Bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis test. *p< 0.05, **p<0.01 and ***p<0.001 vs. WT H_2O mice; #p<0.05, ##p<0.01 and ###p< 0.0001 vs. WT DSS-treated mice.*

IV.II.2.5 GPR91 mediates the priming step of inflammasome in intestinal epithelial cells

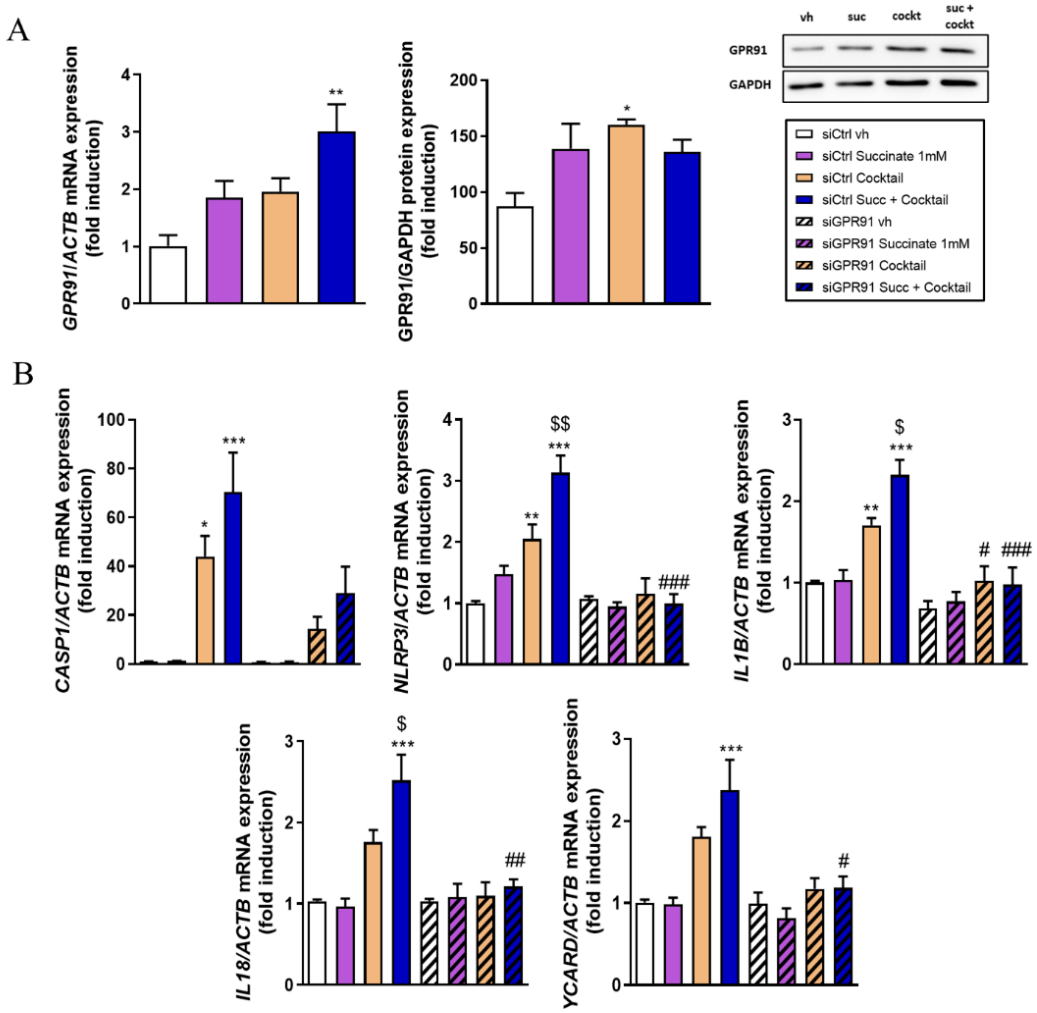
Once analysed the relevance of GPR91 in the priming step of the inflammasome *in vivo*, we sought to further study this role *in vitro*. To do that, HT-29 cells were treated with succinate, an inflammatory cocktail containing LPS (0.1 μg/ml), TNF-α (25 ng/ml) and IFN-γ (20 ng/ml) or a combination of both, for 24 h. First, gene and protein expression of GPR91 were increased under those treatments, although statistical significance was only obtained in the combined treatment for gene expression and in the cocktail for protein expression (Figure IV.20A).

Secondly, inflammasome components were not modified with the treatment of succinate (Figure IV.20B); however, inflammatory cocktail induced a significant increase in the expression of *CASP1, NLRP3* and *IL1B,* and the co-administration of succinate with the cocktail significantly potentiated the expression of *NLRP3, IL1B* and *IL18* (Figure IV.20B). To determine whether this effect was mediated by GPR91, HT-29 cells were transiently silenced with a specific GPR91 siRNA. In basal conditions, any inflammasome component was modified when GPR91 was transiently silenced, but inflammasome priming by the co-administration of succinate and the cocktail was impaired, since a significant reduction in the expression of *NLRP3, IL1B, PYCARD* and *IL18* was observed in siGPR91-treated cells versus siRNA control cells (Figure IV.20B).

In line with this, the cocktail *per se* and the combination induced a significant increase in the protein levels of pro-Caspase-1, an effect that was significantly reduced when cells were transiently GPR91 knocked-down (Figure IV.20C). In addition, in order to reinforce previous results, secreted IL-1β protein levels were quantified by ELISA. Results showed a significant increase in the supernatants of HT-29 cells treated with the inflammatory cocktail, which was potentiated with the

combination. Of interest, siGPR91-treated cells exhibited a significant decrease in secreted levels of IL-1β in both experimental conditions (Figure IV.20D).

Finally, as observed in Figure IV.20E, succinate levels were quantified in supernatants from HT-29 cells and no differences were detected in any of the experimental condition analysed detecting values around those detected in vehicle cells (130.4 ± 11.1 µM).



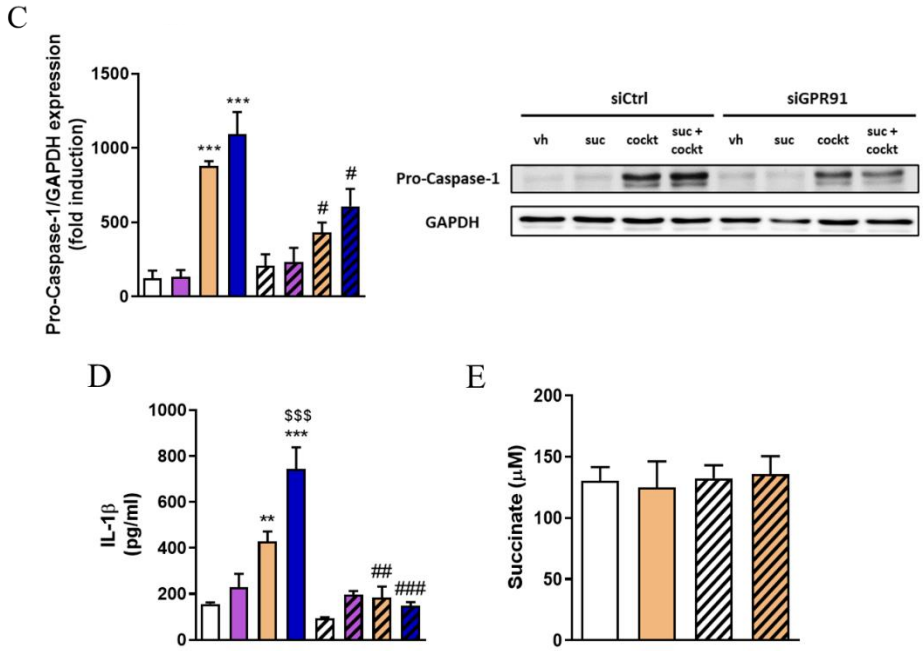


Figure IV. 20. GPR91 mediates inflammasome priming in intestinal epithelial cells. *(**A**) HT-29 cells treated with succinate 1 mM and/or an inflammatory cocktail for 24 h. Graphs show mRNA (n=7) and protein (n=3) expression of GPR91. Image of a representative Western Blot is shown. HT-29 cells were transiently silenced with a specific siRNA against GPR91, or ctrl and graphs show (**B**) mRNA expression of CASP1, NLRP3, IL1B, IL18 and PYCARD (n=5), (**C**) protein expression of pro-Caspase-1 and an image of a representative Western Blot(n=4), (**D**) secreted protein levels of IL-1β detected in supernatants of HT-29 cells (n=3) and (**E**) succinate levels in supernatants of HT-29 cells (n=6). In all cases, bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis test. *p<0.05, **p<0.01 and ***p<0.001 vs. siCtrl vh HT-29 cells; #p<0.05, ##p<0.01 and ###p<0.001 vs. respective siCtrl HT-29 cells. $p<0.05 and $$p<0.01, vs. siCtrl cocktail-treated HT-29 cells.*

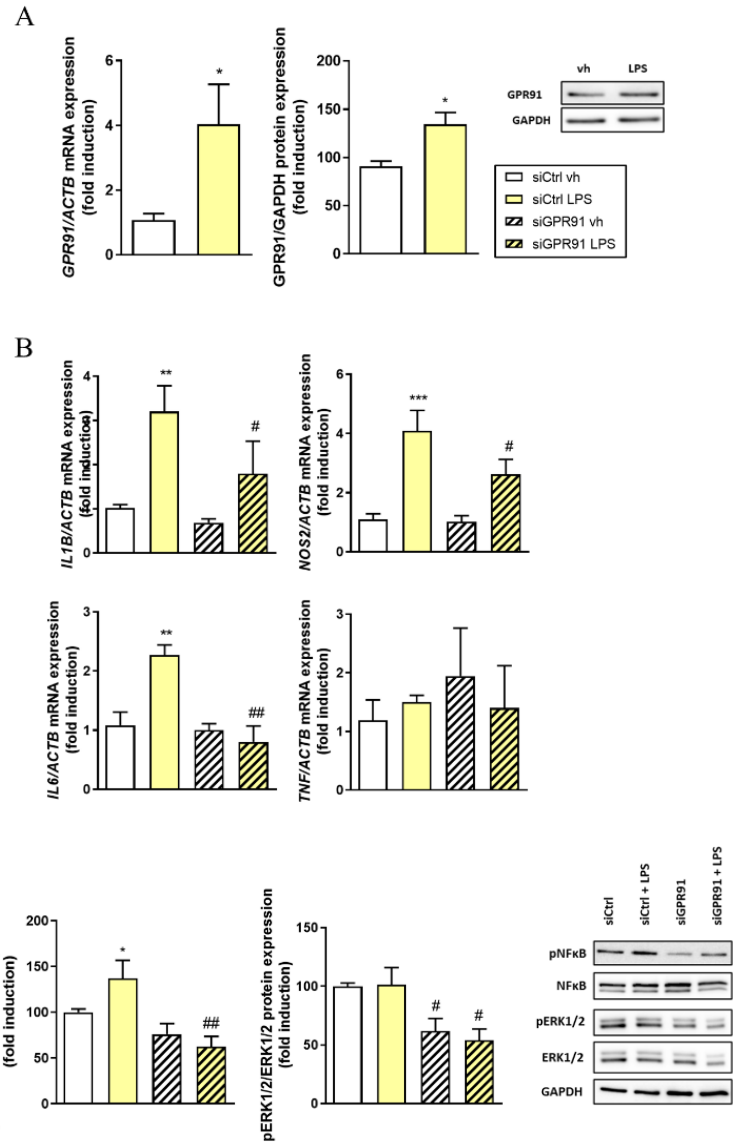
IV.II.2.6 GPR91 mediates basal and LPS-stimulated inflammatory pathways in intestinal epithelial cells

Once demonstrated the role of GPR91 in inflammasome priming step, we analysed the role of this receptor in the expression of inflammatory markers induced by LPS and characterized in depth the molecular pathways involved. Firstly, HT-29 cells were also treated with LPS (0.1 μg/ml) for 24 h and, in line with the increased expression of GPR91 after the treatment of the pro-inflammatory cocktail previously described, results showed a significant increase in both gene and protein GPR91 expression (Figure IV.21A).

Next, pro-inflammatory cytokines expression under LPS treatment was also analysed and data showed a significant increase in the gene expression of *IL1B*, *NOS2* and *IL6*, while *TNF* remained unchanged (Figure IV.21B). Of interest, siGPR91 cells treated with LPS showed a significant reduction in the expression of these genes in comparison with LPS-treated siCtrl cells, as observed in Figure IV.21B.

In addition, protein expression of inflammatory pathways was also analysed under these conditions. Hence, pNFκB/NFκB ratio was significantly increased when HT-29 cells were treated with LPS, an effect which was prevented in LPS-treated siGPR91 cells (Figure IV.21C). Regarding ERK1/2 pathway, LPS did not induce an increase in ERK1/2 phosphorylation, but when cells were GPR91 transiently silenced, pERK1/2/ERK1/2 ratio was significantly reduced in both vehicle and LPS-treated siGPR91 cells, as shown in Figure IV.21C. Next, the effects of pERK1/2 on NFκB activation were analysed and for that, HT-29 cells were treated with LPS and the MEK inhibitor U0126 10 μM during 2 h. LPS treatment did not change protein levels of pERK1/2, whereas significantly increased NFκB phosphorylation and slightly reduced the expression of IκB, as observed in Figure IV.21D. Interestingly, the addition of the MEK inhibitor U0126 had an effect on these cells since in this

case, levels of NFκB were significantly reduced, and the reduction of IκB was impaired compared with LPS treatment alone (Figure IV.21D). Finally, as in previous experiments, succinate levels remained unchanged in all the conditions analysed (Figure IV.21E).



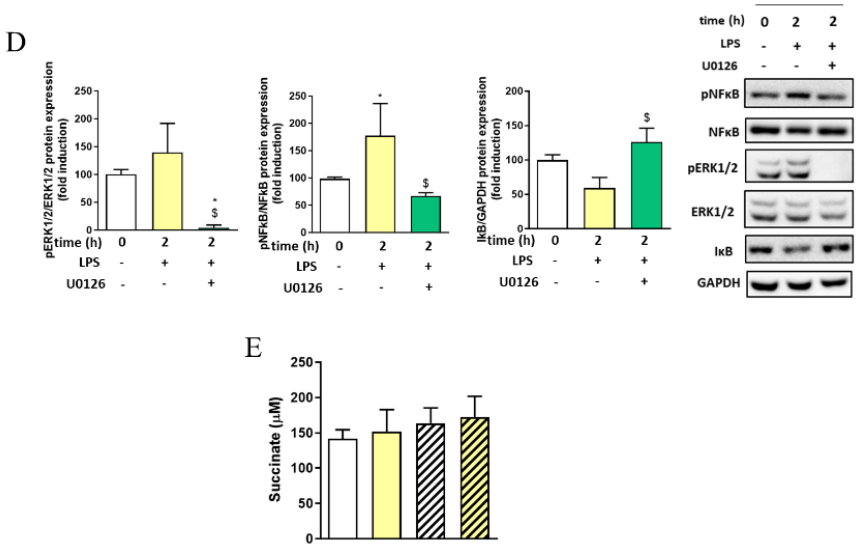


Figure IV. 21. GPR91 mediates basal and LPS-stimulated inflammatory pathways in intestinal epithelial cells. *HT-29 cells were treated with LPS 0.1 g/ml for 24 h. (**A**) Graphs show mRNA and protein expression of GPR91 (n=5). Image of a representative Western Blot of one independent experiment is shown. HT-29 cells were transiently silenced with a specific siRNA against GPR91 or ctrl and treated with LPS 0.1 μg/ml for 24 h. Graphs show (**B**) mRNA expression of IL1B, NOS2, TNF and IL6 (n=5) and (**C**) protein expression of pERK-1/2, ERK-1/2, pNFκB and NFκB. Image of a representative Western Blot is shown (n=6). (**D**) HT-29 cells were treated with vehicle or LPS (with or without the MEK inhibitor U0126 10 μM) over 2 h and graphs show protein expression of pERK-1/2, ERK-1/2, pNFκB, NFκB and IκB. Image of a representative Western Blot is shown (n=5). (**E**) Graph shows succinate levels in supernatant of HT-29 cells (n=4). In all cases, bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis test or unpaired t-test/Mann-Whitney test. *p<0.05, **p<0.01 and ***p<0.001 vs. siCtrl vh HT-29 cells; #p<0.05 and ## p<0.01 vs. respective siCtrl HT-29 cells; $p<0.05 vs. LPS treated without U0126 HT-29 cells.*

CHAPTER III: GPR109A IN IBD PATHOGENESIS

As previously described in the first chapter of this Doctoral Thesis, alterations in the expression of several metabolite-sensing GPCRs in IBD patients were found. Given the fact that some of those receptors have already been extensively studied, we decided to better study GPR109, which was significantly upregulated in both UC and CD compared with their respective controls, due to the scarce number of studies analysing this receptor in IBD pathogenesis. In addition, levels of its agonist, β-hydroxybutyrate, were also significantly increased in both pathologies. Besides, GPR109A expression positively correlated with most pro-inflammatory cytokines and pro-fibrotic markers. Hence, according to these preliminary data, we decided to deeper characterize the role of this receptor and determine its involvement in fibrotic and inflammatory processes associated to IBD pathology.

IV.III.1 GPR109A protein expression is increased in CD patients

Firstly, protein expression of GPR109A was analysed in human intestinal surgical resections from IBD patients. Western Blot analysis revealed no differences in the expression of this receptor between colon and ileum samples norbetween UC and colon samples. Of interest, a significantly higher protein expression of this receptor was detected in CD patients compared with ileum samples (Figure IV.22).

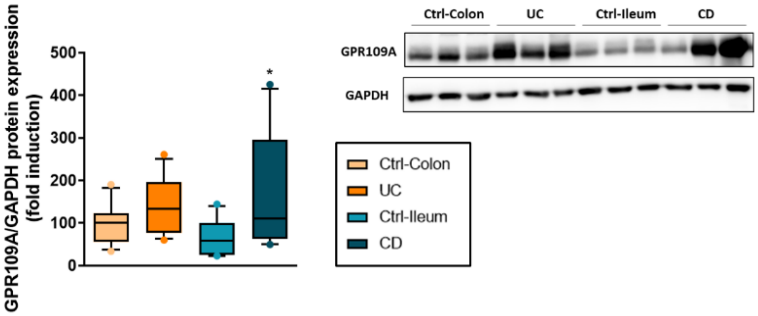


Figure IV. 22. GPR109A protein expression is increased in CD patients. *Graph shows protein expression of GPR109A in intestinal resections from colonic controls (n=12), UC*

*patients (n=12), ileal controls (n=12) and CD patients (n=12). Image of a representative Western Blot is shown. Data are represented in box and whisker diagrams. The line splitting the box in two represents the median value. The upper part of the box represents the upper quartile value, and the lower part the lower quartile value. Horizontal lines in edges represent the maximum and minimum values of data and single points indicate outliers. Data were analysed by one-way ANOVA test. *p<0.05 vs ctrl-ileum.*

To analyse the cellular distribution of this receptor, an immunohistochemistry of GPR109A was performed. As observed in Figure IV.23, GPR109A+ cells were mostly present in the intestinal epithelium of both colon and ileum control samples, while they were also detected in lamina propria of both UC and CD samples. The morphological analysis of these cells suggests that tissue-resident macrophages, among other type of cells such as leukocytes, neutrophils, or fibroblasts, expressed GPR109A in intestinal tissue of IBD patients.

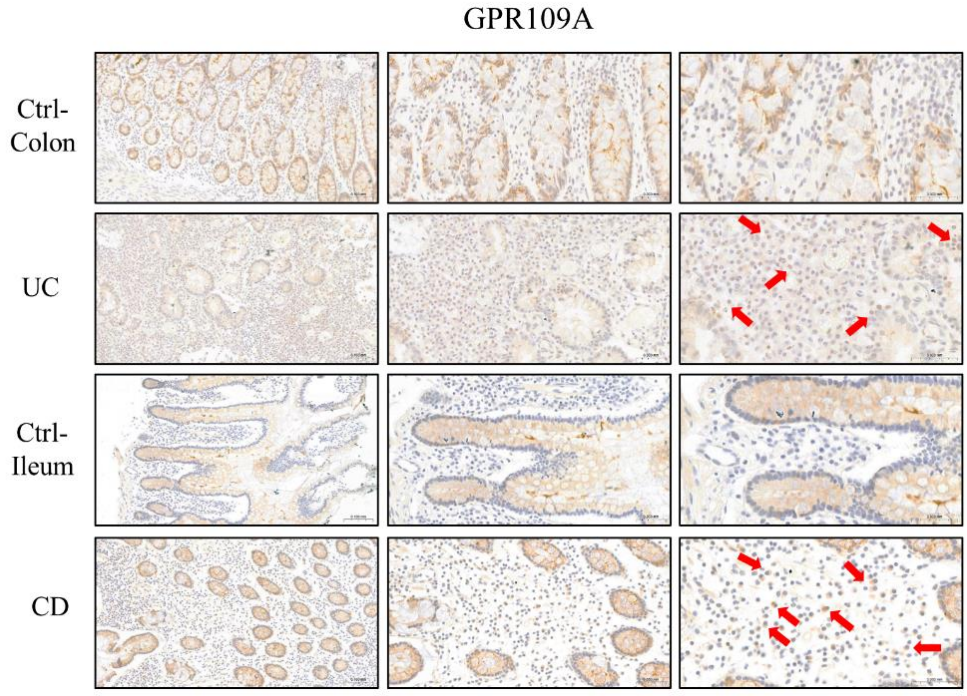


Figure IV. 23. GPR109A is expressed in intestinal epithelium and lamina propria of both UC and CD patients. *Immunostaining of GPR109A was performed in 5-µm sections of*

paraffin-embedded tissues, and representative pictures of colonic controls, UC patients, ileal controls, and CD patients are shown (n=3). Red arrows point to positive cells in lamina propria from both UC and CD patients.

IV.III.2 The GPR109A agonist, β-hydroxybutyrate, induced the expression of pro-inflammatory markers in macrophages

First, considering the positive immunostaining detected in cells of the lamina propria such as macrophages, we sought to determine the effect of β-hydroxybutyrate, a GPR109A agonist, on U937-derived macrophages. As observed in Figure IV.24A, treatment with β-hydroxybutyrate 10mM significantly increased the gene expression of *GPR109A* compared with vehicle macrophages. Next, the expression of the M1 macrophage marker *CD86* and the M2 marker *CD206* was analysed after treatment with β-hydroxybutyrate and increased expression of *CD86* with 10mM concentration was observed, whereas none of the concentrations of β-hydroxybutyrate modified the expression of the M2 marker *CD206* (Figure IV.24B). In addition, the expression of pro-inflammatory cytokines was also analysed and a significant upregulation of *IL1B* and *NOS2* gene expression was obtained after treatment with β-hydroxybutyrate. No differences in the expression of *IL6*, *IL8* nor *TNF* were observed after β-hydroxybutyrate treatment (Figure IV.24C).

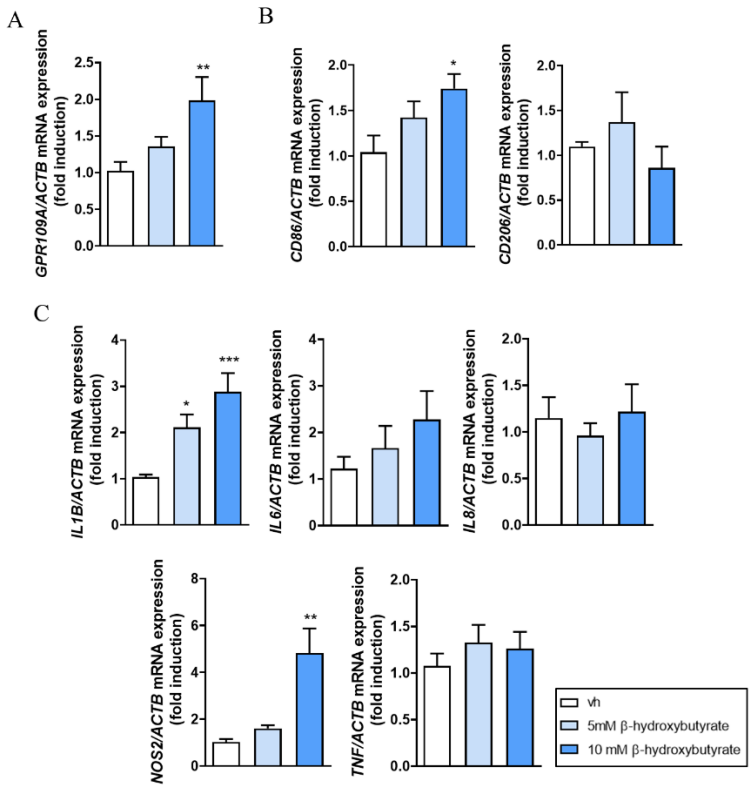


Figure IV. 24. Pro-inflammatory effects of β-hydroxybutyrate on macrophages. *U937 human monocytes were treated with PMA 0.01 μM for 48 h to differentiate them to macrophages. Then, U937-derived macrophages were treated with β-hydroxybutyrate 5 and 10 mM for 24 h. Graphs show mRNA expression of (**A**) GPR109A, (**B**) macrophage markers CD86 and CD206, and (**C**) pro-inflammatory markers IL1B, IL6, IL8, NOS2 and TNF (n=6). Bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis test. *p<0.05, **p<0.01 and ***p<0.001 vs. vh U937-derived macrophages.*

IV.III.3 GPR109A mediates the expression of pro-inflammatory markers in macrophages

Once demonstrated that β-hydroxybutyrate induces the expression of M1 markers, we sought to analyse the relevance of GPR109A in this macrophage phenotype. For that, MØ U937-derived macrophages were polarized towards M1

phenotype with LPS (0.1 μg/ml) and IFN-γ (20 ng/ml) for 24 h. As shown in Figure IV.25A, the gene expression of *GPR109A* was significantly increased in M1 macrophages versus MØ macrophages.

To determine the role of GPR109A on both MØ and M1 U937-derived macrophages, a GPR109A siRNA was used in order to transiently silence this receptor. The efficiency of the transfection was confirmed by analysing the mRNA expression of GPR109A, which was significantly reduced in both MØ-siGPR109A and M1-siGPR109A compared to the respective control groups (Figure IV.25A). The analysis of M1 markers showed that the mRNA expression of *CD86, IL1B, IL6, IL8, NOS2* and *TNF* was significantly upregulated in M1 macrophages compared to MØ macrophages (Figure IV.25B). The transient silencing of GPR109A provoked a significant reduction of the expression of *IL1B* and *TNF* compared with M1-siCtrl macrophages, whereas it did not significantly modify the expression of the rest of the genes analysed as observed in Figure IV.25B.

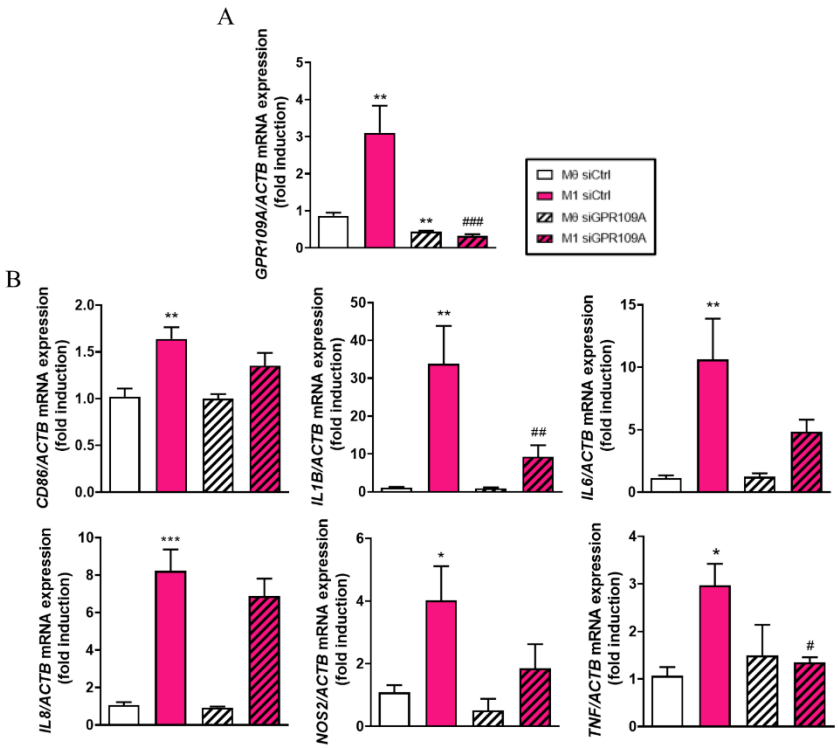


Figure IV. 25. Role of GPR109A on M1 macrophages activation. *U937 human monocytes were treated with PMA 0.01 μM for 48 h to differentiate them to macrophages. Then, U937-derived macrophages were transiently silenced with a specific siRNA against GPR109A and polarized towards M1 phenotype with LPS 0.1 μg/ml plus IFN-γ 20 ng/ml for 24 h post-transfection. Graphs show mRNA expression of (**A**) GPR109A and (**B**) the M1 marker CD86 and pro-inflammatory cytokines IL1B, IL6, IL8, NOS2 and TNF (n=6). Bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis test. *p<0.05, **p<0.01 and ***p<0.001 vs. siCtrl MØ-macrophages and #p<0.05, ##p<0.01 and ###p<0.001 vs. siCtrl M1-macrophages.*

To reinforce these results, secreted levels of several cytokines in supernatants (SN) from MØ siCtrl, M1 siCtrl and siGPR109A M1 macrophages were quantified by Luminex® Multiplex Assay in collaboration with the Department of Endocrinology and Nutrition from the Hospital Dr. Peset-FISABIO in València. As observed in Figure IV.26, secretion of IL-1β, IL-6, TNF-α and IL-8 was significantly

increased in SN from M1 macrophages versus SN from MØ macrophages, while no changes were observed in the levels of IL-17a, PDGF nor none of the TGF-β isoforms quantified. Of interest, when GPR109A receptor was transiently silenced in M1 macrophages, the secreted levels of IL-1β, IL-6 and TNF-α were significantly decreased when compared with SN from siCtrl M1 macrophages (Figure IV.26).

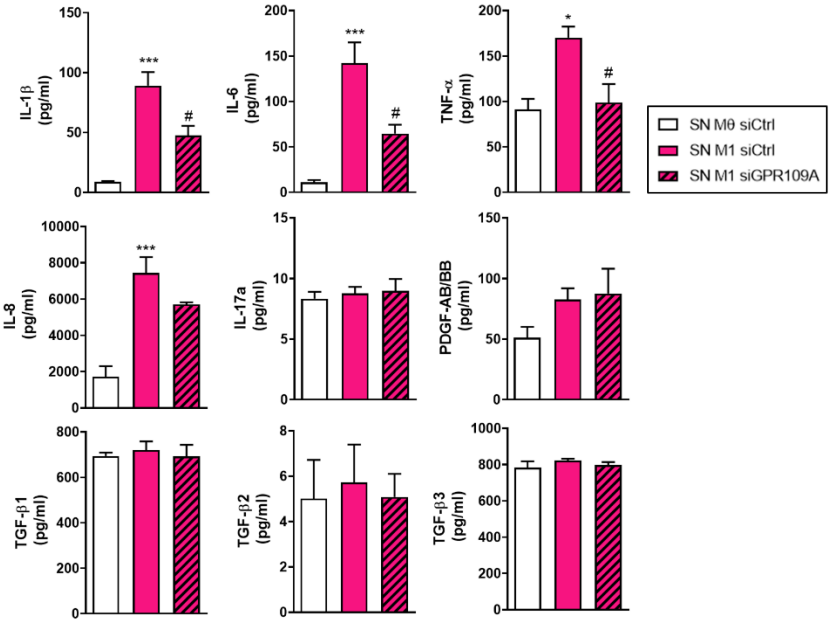


Figure IV. 26. Levels of several cytokines in supernatants from MØ, M1 and GPR109A-deficient M1 macrophages. *U937 human monocytes were treated with PMA 0.01 μM for 48 h to differentiate them to macrophages. Then, U937-derived macrophages were transiently silenced with a specific siRNA against GPR109A and polarized towards M1 phenotype with LPS 0.1 μg/ml plus IFN-γ 20 ng/ml for 24 h post-transfection and supernatants were collected. Graphs show secreted levels of a set of pro-inflammatory and pro-fibrotic cytokines in supernatants (SN) quantified by Luminex® Multiplex Assay (n=4). Bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis test. *p<0.05 and ***p<0.001 vs. SN from siCtrl MØ-macrophages and #p<0.05 vs. SN from siCtrl M1-macrophages.*

IV.III.4 GPR109A receptor modulates the ability of M1 macrophages to activate fibroblasts

Finally, we aimed to determine the effects induced by the secretome of M1 macrophages on intestinal fibroblasts activation and the role played by GPR109A in this effect. To achieve this aim, HSIFs were treated with SN of siCtrl MØ, siCtrl M1 and siGPR109A M1-macrophages for 24 h. Firstly, as observed in Figure IV.27A, *GPR109A* gene expression did not change after treatment with none of the SN used. In addition, treatment with SN from M1-siCtrl did not significantly modify the levels of any of the fibrotic markers analysed, although a tendency to increase gene expression of *ACTA2*, *COL1A1*, *COL3A1* and *COL4A1* was observed (Figure IV.27B). In contrast, HSIFs treated with the SN from siGPR109A-M1 macrophages exhibited a significant reduction of the mRNA expression of *COL1A1* and *COL3A1* compared with fibroblasts treated with the SN from siCtrl-M1 macrophages (Figure IV.27B).

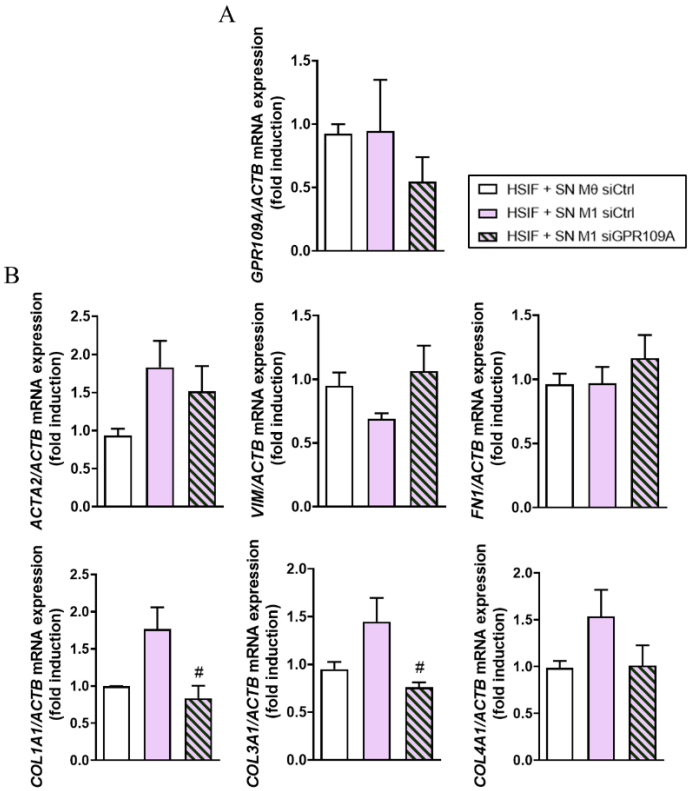


Figure IV. 27. HSIFs treated with supernatants from MØ, M1 and GPR109A-deficient M1 macrophages*. HSIFs were treated with SN of MØ siCtrl, M1 siCtrl and M1 siGPR10A macrophages for 24 h. Graphs show mRNA expression of (**A**) GPR109A, (**B**) pro-fibrotic markers ACTA2, VIM, FN1, COL1A1, COL3A1 and COL4A1 (n=3). Bars in graphs represent mean ± SEM and data were analysed by one-way ANOVA/Kruskal-Wallis. #p<0.05 vs HSIF + SN M1 siCtrl.*

V. DISCUSSION

During the last decades, biomedical research on IBD has contributed to improve diagnosis, patients' lifestyle, and pharmacological treatment; however, there is still a lack of an appropriate therapeutic approach which prevents or reverts clinical disease associated complications. It is widely known that IBD is a multifactorial disease, where several factors play crucial roles not only in the disease onset, but also on its development. For that, a better understanding of the molecular immune mechanisms involved in the inflammatory response and the development of complications is urgently needed. In the present Doctoral Thesis, we sought to analyse in depth in intestinal surgical resections from IBD patients, an important axis on the pathogenesis of the disease, which includes intestinal microbiota population, metabolites, and finally, metabolite-sensing GPCRs which are selectively activated and modulated by such metabolites. Given the different location of injuries in UC and CD, four different groups of samples were used including intestinal surgical resections from i) colonic controls, ii) UC patients, iii) ileal controls, and iv) CD patients. The aim of this study was to compare the potential differences among i) colonic and ileal controls, ii) UC and colonic controls, iii) CD and ileal controls, and iv) CD and UC.

To the best of our knowledge, most of the available studies characterizing the gut microbiome and gut microbiome-derived metabolites have been performed using biofluids such as plasma, serum or urine, feces, and few of them employed intestinal tissue samples, mostly mucosal biopsies. Although biofluids constitute a non-invasive way to measure the microbiome and metabolomic profile of patients, tissue samples provide consistent and representative data, to better characterize the cellular composition and structure, molecular pathways, and the functional alterations in the specific location of inflamed tissue. At this point, it is important to consider that differences in abundance have been reported between fecal microbiota and mucus-associated microbiota (28). Regarding the surgical intestinal resections employed in this Doctoral Thesis, they encompass all layers of the intestinal wall, and thus

constitute a significantly larger and even more representative tissue fraction compared to intestinal biopsies, which exclusively comprise the external mucosal epithelium. Moreover, mucosal biopsies can be obtained from patients in a state of remission, whereas surgical resections involve the removal of damaged and even non-functional tissue in complicated patients, constituting a pathologically more integrative tissue sample. In fact, we have also observed in certain cases that our findings diverged from prior published data derived from mucosal biopsies. Thus, disparities in results could be attributed to the varying tissue compositions of both sample types.

Once characterized the intestinal microbiota-metabolomic axis, since GPCRs have been described as possible modulators of inflammatory and fibrotic processes associated to IBD, in the present Doctoral Thesis we have better characterized the role of GPR91 and GPR109A on IBD pathogenesis.

V.1 Microbiota analysis

Firstly, it is broadly described that microbial dysbiosis is present in IBD patients (39, 40, 42-44). Thus, we sought to characterize the microbiota composition in surgical resections from both UC and CD patients. Interestingly, in line with previous results, we found significant differences in both bacterial richness and bacterial load in tissue samples from UC patients while a tendency, but not significant reduction in both parameters was observed in CD, in contrast with previous studies performed in different biological samples (40, 44).

Regarding differences at **genus level** of microbiota present in each group, it is important to highlight the huge increase in the abundance of *Cellulosimicrobium* detected in **UC patients**, which is 10-fold, indicating that inflammation of colon during UC is associated with an increase in *Cellulosimicrobium* proportions. The inclusion of controls for the DNA extraction and sequencing steps discarded the option for these bacteria being a contamination from external sources. In line with

this, unclassified *Cellulosimicrobium* species belongs to the group *Actinomycetes*, and previous studies reported increased abundance of this group in IBD patients (44, 247). To our knowledge, it is the first time that this genus of bacteria has been identified in intestinal surgical resections, and specifically in increased proportions in UC patients. *Cellulosimicrobium* genus is characterized by gram-positive, rod-shaped, aerobic or facultatively anaerobic, and non-motile bacteria which produce endospores. Five species have been identified including *C. cellulans*, *C. terreum*, *C. funkei*, *C. marinum* and *C. arenosum* (248, 249). They are commonly found in different environments such as soil, plant residues, water or decomposed organic matter, and they have a fermentative metabolism. Both *C. cellulans* and *C. funkei* are opportunistic bacteria known to infect immunocompromised patients producing neonatal bacterial sepsis (250), bacteraemia, peritonitis, meningitis, endocarditis (251), and infections caused by foreign bodies such as catheters, shunts, or prostheses (248, 252, 253).

Next, *Fusobacterium* genus was increased in **CD patients**, in line with previous studies reporting increased abundance of *Fusobacteriaceae* in affected tissue from IBD patients, especially in CD patients (48, 254-256). This increase has been associated with alterations in the autophagy-related *ATG16L1* gene, identified as a risk factor for IBD (257). Finally, contrary to previously reported data, in our analysis, *Escherichia/Shigella* and *Enterococcus* genera were found significantly reduced in both UC and CD patients respectively (258-261), differences that may be associated with variances in the type of biological samples used.

At **species taxonomic level**, unclassified *Pseudomonas* and *Stenotrophomonas maltophila*, were increased in UC patients, whereas unclassified *Sphingomonas* was slightly increased in CD patients. All these species belong to the phylum *Proteobacteria*, a phylum commonly increased in IBD patients and identified as possible causative agent (40, 71, 247, 262). We also found that abundance of *Bacteroides thetaiotaomicron* was slightly increased in UC individuals, a bacterium

with a protective role in DSS-colitis mice models (263, 264). Of interest, a previous study demonstrated that *B. thetaiotaomicron* communicates with immune cells through extracellular vesicles, which are disturbed due to IBD pathology (265). As a consequence, although abundance of *B. thetaiotaomicron* is increased in UC patients, these bacteria might not be able to communicate through extracellular vesicles and induce the ameliorative effects previously reported.

Regarding bacterial species whose abundance was found decreased, we highlight *Faecalibacterium prausnitzii* in CD patients, and *Coprococcus comes* in UC patients, both SCFA-producing bacteria. In addition, *Barnisella intestinihominis* was also reduced in UC patients and unclassified *Butyricimonas* in both UC and CD, species which belong to the anti-inflammatory phylum *Bacteroidetes*. Reduction of all these species has been extensively described in other biological samples from IBD and associated with disease progression (40, 49, 50, 266). In addition, several species from *Firmicutes* phylum, specifically from the class *Clostridia* and belonging to the family *Lachnospiraceae*, an anti-inflammatory group of bacteria, were significantly reduced in UC including unclassified *Lachnospiraceae UC-010*, unclassified *Lachnospiraceae UC-004, Dorea formicigenerans,* and the previously commented *Coprococcus comes*, whilst unclassified *Lachnospiraceae NK4A136* and *Dorea formicigenerans* were reduced in CD samples. In addition, several species also belonging to *Clostridia* inside *Oscillospiraceae* family, were also significantly reduced in both UC and CD patients. On the one hand, abundance of *Oscillibacter valericigenes, Butyricicoccus faecihominis* and unclassified *Ruminococcaceae UBA1819* was reduced in UC subjects, whereas *F. prausnitzii, Oscillospiraceae UCG-005 NA, Oscillospiraceae UCG-003 NA* and *Ruminococcus bromii* were decreased in CD subjects. Previous studies also reported a decrease in *Clostridia* in fecal and tissue samples from IBD patients (43, 51, 166, 247, 267), and associated a reduction in *Lachnospiraceae* with IBD progression (40, 44, 49). Moreover, these species have been described as modulators of the gastrointestinal immune response

and enhancers of intestinal epithelial barrier integrity (Frank DN, 2007). Interestingly, alterations in the cluster including *Faecalibacterium, Ruminococcus, Lachnospira, Blautia, Dorea, Coprococcus, Roseburia, Oscillospira* and *Bilophila* have been associated with an increased risk of recurrence in CD patients who underwent inflamed tissue resection (268). Regarding the class *Bacilli* inside the *Firmicutes* phylum, there is some controversy since unclassified *Gemella* in UC patients, and *Erysipelotrichaceae UCG-003 bacterium GAM147* in both UC and CD patients, were significantly reduced, whereas unclassified *Bacillus* and unclassified *Staphylococcus* were significantly increased in UC and CD respectively.

It is important to note that current techniques used for microbial characterization are very promising and provide useful information to identify the composition of the microbiota, but they fail to offer functional information, which is the next step needed to better understand the role of the gut microbiota in pathogenesis. We should also consider the so-called "microbial dark matter", which is the part of the microbiome that is unknown and has not yet been characterized. With the metagenomic techniques used nowadays, this part of the microbiome is underestimated when characterizing the gut microbiota composition of IBD patients, so there is a huge amount of data that is not accounted (39).

V.2 Metabolomic analysis

Next, we analysed the levels of metabolites, which can selectively activate GPCRs in all samples included in the study, and results are summarized in Table V.1.

Firstly, regarding **carboxylic acids** metabolites, no differences were found between the healthy colon and ileum. In line with previous data by our group (146), we detected increased levels of the TCA intermediate **succinate** in CD patients whereas other studies using intestinal biopsies reported decreased levels of this metabolite (144, 145). High levels of succinate in inflamed tissue have previously

been related to metabolic changes that perpetuate inflammation through HIF-1α, and sustained production of IL-1β (184); however, we did not detect enhanced levels of succinate in UC patients, which suggest that other factors, different to inflammation, may also be involved. In line with this, succinate accumulation has also been related to decreased abundance of *Phascolarctobacterium*, a succinate-utilizing bacteria, reported in IBD patients (148). Next, our data also showed significantly increased **β-hydroxybutyrate** levels in both UC and CD, in line with earlier studies in different biologic specimens of IBD patients (140, 154, 155, 269), except one in serum samples from CD patients (142). Of interest, Schicho R et al. showed in DSS-treated mice increased levels of ketone bodies, including β-hydroxybutyrate, in comparison to controls (270). Additionally, increased β-hydroxybutyrate levels have been identified in tumorigenesis since high energy demands result in enhanced β-oxidation of fatty acids, higher acetyl-CoA levels, and increased ketogenesis (271-274). Elevated ketone bodies can indicate changes in cellular metabolism linked with oxidative stress and cellular damage (140, 153). Thus, we hypothesize that inflammation may also increase levels of β-hydroxybutyrate, as the inflammatory process also implies a high energy demand and boosts fatty acids oxidation (275-278). Consequently, if the amount of acetyl-CoA exceeds the TCA cycle's capacity, ketone bodies like β-hydroxybutyrate are synthetized in the liver and excreted from the cell, as depicted in Figure V.1 (279). Finally, our findings revealed higher **lactic acid** levels in both UC and CD, in line with previous studies in different biological samples (140, 149-152). Indeed, increased levels of lactic acid are commonly found due to disturbances in metabolism of immune cells and inflammation (149-151). Conversely, other studies reported decreased levels of this metabolite in intestinal biopsies from IBD patients (144, 145), which could be related with the different cellular composition of samples analysed.

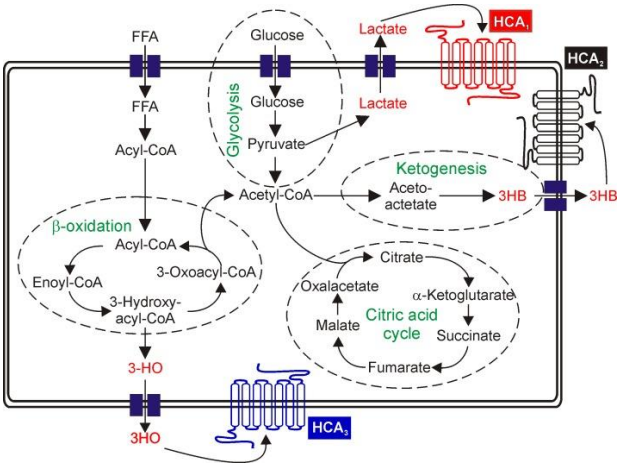


Figure V. 1. Hydroxycarboxylic acids metabolic pathways*. (279)*

Next, regarding **SCFAs,** we found higher levels of **butyrate** in the healthy ileum than in the colon. In addition, we detected higher amounts of **propionic acid** in both UC and CD patients, while **butyric acid** and **acetic acid** were increased in CD compared with UC. In contrast with these observations, SCFAs levels have been found decreased in different biological samples of IBD patients (51, 139, 140, 164, 165), which have been correlated with decreased abundance of fermentative gut microorganisms such as *Faecalibacterium prausnitzii* and *Roseburia hominis* (166). Our data also show decreased abundance of these bacteria, which seem to be in contradiction with both the increased levels of SCFAs detected and with the anti-inflammatory effects attributed to these compounds (158-161). However, other mechanisms may be involved in the regulation of SCFAs. In this line, a decreased expression of the MCT-1 transporter in colonocytes has been reported associated to intestinal inflammation (280), which could account for the accumulation of SCFAs in intestinal tissue. Reinforcing this observation, after long-term exposure to butyrate, stem cells in mouse intestinal crypts, can impair proliferation and wound healing as seen in IBD pathology (281).

Concerning **MCFAs**, our findings revealed an increase in **undecanoic acid** levels in healthy ileum in contrast with colon. Both metabolites of this group increased only in UC. In addition, decreased levels of **decanoic acid** and increased levels of undecanoic were found in CD compared to UC. To our knowledge, there is limited literature on the role of MCFAs in IBD pathogenesis, but a previous study has related decanoic acid with allergic inflammation (282).

Regarding **LCFAs,** we found lower levels of **eicosapentaenoic acid** and **oleic acid** in healthy ileum vs colon. Besides, increased levels of the omega-3 PUFAs **docosahexaenoic** and **α-linolenic acids** were found in UC patients. In line with this, different studies also reported significantly increased levels of omega-3 and omega-6 PUFAs in colonic biopsies of UC patients (283), and in plasma samples from both CD and UC patients (177), associated with higher energy demand under inflammatory conditions. Inflammation triggers increased expression of phospholipase A2, the responsible of hydrolysing phospholipids into PUFAs, such as arachidonic and docosahexaenoic acids, resulting in their accumulation in intestinal tissue (284). In contrast to our data, other studies have reported decreased levels of docosahexaenoic acid in serum samples of IBD patients compared to controls (135, 173), which has been associated with increased consumption of PUFAs due to oxidative stress and inflammation (285). On the other hand, in line with our findings, a previous study performed in mucosal biopsies showed no differences in the levels of **eicosapentaenoic acid** between IBD patients and respective controls, reporting lower levels of this metabolite in comparison with docosahexaenoic and α-linolenic acids (286). Next, although most studies reported decreased levels of **oleic acid** in different biological samples of IBD patients versus respective controls (170, 283), we did not find differences in the levels of this metabolite amongst the different groups analysed. In the case of **myristic acid**, we found higher levels in UC. A study performed by Barnes and colleagues reported that a high dietary intake of myristic acid which is found in palm oil or coconut oil,

positively correlates with increased risk of suffering flares in UC patients (287). Finally, all the LCFAs analysed except **N-oleylethanolamide**, were found decreased in CD versus UC samples.

Regarding **amino acids**, levels of **phenylalanine** and **aspartic acid** were higher in the healthy ileum than in colon, and the former was significantly increased in both UC and CD patients. Previous studies reported increased levels of phenylalanine in serum of active IBD when compared to remission IBD patients, associated with impaired phenylalanine-4-hydroxylase activity due to inflammation, the enzyme which transforms phenylalanine into tyrosine (140). Next, our data showed that aspartic acid and **glutamic acid** levels were significantly increased only in UC samples, in line with previous studies in mucosal colonic biopsies from UC patients (165). Indeed, glutamate, apart from constituting an energy source in metabolism, plays an important role in synthetizing the thiol antioxidant glutathione, an endogenous compound which protects the organism against oxidative stress, highly needed to face enhanced inflammation-associated oxidative stress, as occurs in UC (288). In contrast to our results, other studies reported decreased amino acids levels in intestinal biopsies (144, 145) and in serum samples from IBD patients compared to controls, while increased levels were detected in feces (135, 164, 165). Such differences could be attributed to gut malabsorption and microbiota dysbiosis that characterize this pathology (164, 165, 289).

Taking all together, although metabolomic profiling constitutes a promising tool to better understand IBD pathology, apply more individualized therapies, and unveil novel therapeutic targets, it still has some limitations. First, the type of biological sample used for the analysis is crucial, since changes in the levels of a metabolite may differ according to the sample analysed. In addition, levels of metabolites can also vary due to intraindividual alterations in intestinal absorption, microbial dysbiosis, and even pharmacological and surgical treatments received. Therefore, improvement in the experimental design and increasing the number of

studies using intestinal tissue will help to determine the exact role of metabolites when they are accumulated or diminished, and to confirm their potential involvement in molecular pathways, as well as using them as predictors for assessing treatment effectiveness.

Table V. 1. Summary of the alterations in the metabolomic profile of the different groups studied.

		Ileum vs Colon	UC vs Colon	CD vs Ileum	CD vs UC
Carboxylic acids	**Succinic acid**	=	=	↑	↑
	β-hydroxybutyric acid	=	↑	↑	↑
	Lactic acid	=	↑	↑	=
SCFAs	**Propionic acid**	=	↑	↑	=
	Butyric acid	↑	=	=	↑
	Acetic acid	=	=	=	↑
MCFAs	**Decanoic acid**	=	↑	=	↓
	Undecanoic acid	↑	↑	=	↑
LCFAs	**Docosahexaenoic acid**	=	↑	=	↓
	α-linolenic	=	↑	=	↓
	Eicosapentaenoic acid	↓	=	=	↓
	Oleic acid	↓	=	=	↓
	Myristic acid	=	↑	=	↓
	N-oleylethanolamide	=	=	=	=
Amino acids	**Phenylalanine**	↑	↑	↑	↑
	Aspartic acid	↑	↑	=	↑
	Glutamic acid	=	↑	=	=

V.3 Metabolite-sensing GPCRs analysis

In parallel with the expression of metabolites, we analysed the expression of metabolite-sensing GPCRs in the same biological samples and results are summarized in Table V.2.

Regarding **carboxylic acids-sensing GPCRs**, we found all of them significantly increased in both UC and CD, except for *GPR81*, which was significantly upregulated only in CD. Another study demonstrated *GPR109B* upregulation in IBD patients, which reverted upon effective infliximab treatment (290). We also detected high levels of both *GPR91* and *GPR109A* and its role in IBD etiopathogenesis will be analysed in depth in further lines of this discussion.

Regarding the **fatty acids-sensing receptors**, the expression of *GPR43, GPR41, GPR84, GPR40* and *GPR119* was significantly upregulated in CD, whereas in UC only *GPR43, GPR84* and *GPR40* were significantly increased. Several studies reported high levels of these receptors in inflammatory conditions and as a consequence, some of them also reported a higher anti-inflammatory effect of their agonists when they were administered. In this line, a study revealed that expression of **GPR40** was enhanced in periodontal tissues of patients suffering periodontitis and/or metabolic syndrome (291) and in an acute colitis murine model, agonist binding to GPR40 prevented inflammation (292). In addition, LPS-treated macrophages and monocytes increased **GPR41** expression and upon activation by butyrate, inflammation is decreased (160). In parallel, animal studies demonstrated that deletion of **GPR43** resulted in exacerbated immune response in acute colitis models (293-295). In line with our findings, **GPR84** expression was also increased in intestinal mucosa from UC patients and DSS-treated mice (296). In addition, it has been reported that its expression is upregulated by inflammation (297), and its activation by agonists enhances the pro-inflammatory effects in neutrophils and macrophages (298). Of interest, a pro-inflammatory role of this receptor has also been described in hepatic fibrosis or pulmonary inflammation (299, 300).

Finally, several receptors were down-regulated in pathological conditions such as, **GPR119** whose expression was significantly lower in UC patients while **GPR120** was downregulated in both UC and CD. Low levels of GPR120 have been reported in osteoarthritis patients and a role for this receptor has been shown in the decreased pro-inflammatory cytokines detected in omega-3 FA-treated macrophages (301, 302). Therefore, the loss of this receptor might contribute to the worsening of inflammation seen in patients with IBD.

Next**, GPCRs sensing to amino acids** and derivatives were analysed and decreased gene expression of *GPR35* was detected in UC patients. In line with this, studies have reported that activation of **GPR35** by its agonists ameliorates severity of DSS-induced colitis promoting wound repair mechanisms (303). Additionally, certain polymorphisms in this receptor have been found associated with an increased risk of developing IBD (157). Hence, the loss of expression of this receptor could also be involved in a perpetuated inflammatory response.

The last group of receptors analysed were **pH-sensing GPCRs**. Receptors sensitive to pH become activated under acidic conditions in inflammatory scenarios and trigger different responses. According to literature, intestinal tissue of IBD subjects shows increased expression of **GPR68** and **GPR4** (231, 304), similar results to that were obtained in the present study. Both receptors have been associated to inflammatory and fibrotic processes in intestinal cells of IBD patients (231, 304). Indeed, intestinal samples from fibrotic IBD patients exhibited in parallel higher levels of fibrotic markers and GPR68 expression (305). Our data also showed an increased gene expression of pro-fibrotic markers *COL1A1, COL3A1,* and *COL4A1* in intestinal tissue of CD patients. Last, we observed that **GPR65** expression, was upregulated in UC individuals. In line with this, a study demonstrated an upregulation of GPR65 in chronic DSS-colitis (232). However, most studies agree in its anti-inflammatory effect (306, 307) since its deletion increases the expression of pro-inflammatory mediators and immune cells infiltration in DSS-treated mice

(232). In addition, a genetic polymorphism in *GPR65* has also been associated with increased risk of IBD and severe course of the disease (233), pointing a role for this receptor in IBD pathogenesis.

Finally, we aimed to investigate the relationship between the metabolite-sensing GPCRs and the pro-inflammatory and/or pro-fibrotic markers in surgical resections from IBD patients, and the correlation analysis revealed for most receptors a positive and significant correlation with both pro-inflammatory and pro-fibrotic markers. In UC samples, we detected strong and positive correlations between receptors and pro-inflammatory cytokines, whereas these correlations were weaker with fibrosis markers whose expression was not significantly altered in samples from UC patients compared with colonic controls. Regarding CD, except for *IL1B*, strong positive correlations were observed between the receptors and pro-inflammatory cytokines as well as the profibrotic gene *COL4A1*. These findings led us to undertake a detailed analysis of the role played by two receptors, GPR91 and GPR109A in inflammatory and fibrotic processes associated to IBD.

Table V. 2. Summary of the alterations in GPCRs expression in the different groups analysed.

		Ileum vs Colon	UC vs Colon	CD vs Ileum	CD vs UC
Carboxylic acids	**GPR91**	=	↑	↑	=
	GPR109A	↑	↑	↑	↑
	GPR109B	=	↑	↑	↑
	GPR81	=	=	↑	↑
SCFAs	**GPR43**	=	↑	↑	=
	GPR41	↑	=	↑	↑
MCFAs	**GPR84**	=	↑	↑	=
LCFAs	**GPR40**	=	↑	↑	↑
	GPR120	=	↓	↓	=
	GPR119	=	↓	↑	↑
Amino Acids	**GPR35**	=	↓	=	↑
	TAAR1	=	=	↑	↑
	CaSR	=	=	↑	↑
	GPR142	=	=	=	=
pH	**GPR68**	=	↑	↑	↑
	GPR4	=	↑	=	↑
	GPR65	=	↑	=	=
	GPR132	=	=	↑	=

V.4 GPR91 in IBD pathogenesis

A previous study by our group demonstrated that GPR91 expression was increased in ileal resections of complicated CD patients compared with controls (146). The major complications of CD include the stenotic or B2 behaviour and the penetrating or B3 behaviour. In this Doctoral Thesis, we analysed whether GRP91

was differentially expressed among CD behaviours and results revealed a higher expression of both, GPR91 and its main ligand, succinate, in samples from B3-CD than in those from B2 CD.

The penetrating behaviour is characterized by the appearance of a fistula that connects the intestinal tract with other structure nearby and which is surrounded by squamous epithelial cells or myofibroblast-like cells, known as transitional cells. Our results demonstrated that both succinate levels and GPR91 are increased in the fistula tract. In parallel, we detected an increased gene expression of EMT markers *SNAI1* and *SNAI2*, a process which has been widely involved in fistula development (126, 127). Immunohistochemical analysis revealed the presence of transitional cells, fibroblasts and M2 macrophages expressing GPR91 in the fistula tract. These results, together with the positive and significant correlation between EMT markers and both succinate levels and GPR91, suggest a crosstalk between them. Hence, we analysed the EMT activation in the heterotopic transplant model using GPR91$^{-/-}$ mice and, interestingly, results showed that succinate accumulation and EMT activation were impaired in GPR91$^{-/-}$ mice. Furthermore, *in vitro* experiments were performed on intestinal epithelial cells and upon treatment with succinate, both GPR91 expression and EMT were induced. Indeed, a wide variety of studies have previously correlated succinate accumulation with other pathologies in which EMT is involved, such as cancer (308, 309). To better elucidate whether the effects of succinate were mediated by the receptor, a GPR91 siRNA was used to transiently silence the receptor in intestinal epithelial cells and results demonstrated a role for GPR91 in EMT activation.

Next, considering the enhanced expression of GPR91 in the ileum of CD patients, we aimed to analyse its expression in colonic resections from UC patients and its role in the inflammasome activation, a molecular mechanism that contributes to inflammation. We detected a higher expression of the receptor, as well as activation of the inflammasome in colonic UC samples, and a positive and significant

correlation between them was observed, which suggests that GPR91 might be associated with inflammasome activation in intestinal samples from UC patients.

In order to analyse *in vivo* the functional role of GPR91, a chronic murine model of colitis induced by DSS was performed in WT and GPR91$^{-/-}$ mice. Under DSS treatment, GPR91$^{-/-}$ mice exhibited higher survival and an ameliorated chronic colitis compared with WT mice. In line with this, DSS-treated GPR91$^{-/-}$ mice also reduced the expression of pro-inflammatory cytokines and M1 markers, and increased expression of M2 markers such as *Mrc1* and *Arg1*. In parallel, both pro-fibrotic markers, such as *Col1a1, Col3a1, Col4a1, Tgfb1* and *Vim,* and thickness of the collagen layer, were also significantly reduced in DSS-treated GPR91$^{-/-}$ mice. This data reinforces previously observed results in intestinal fibrosis in GPR91$^{-/-}$ mice (146). Moreover, in line with previous studies demonstrating the involvement of NLRP3 inflammasome in DSS-chronic colitis (108, 109), we also observed an enhanced mRNA expression of inflammasome components and increased protein levels of pro-Caspase-1 when chronic DSS-colitis was induced. Of interest, the absence of succinate receptor using GPR91$^{-/-}$ mice, impaired the upregulation of inflammasome components after DSS-treatment. Indeed, a pro-inflammatory role for GPR91 has been stablished in previous studies for acute colitis (146), arthritis (199, 310, 311), isoproterenol-induced myocardial ischemia (312), obesity, and diabetes (201). However, to our knowledge, this is the first time that GPR91 has been associated with the *in vivo* regulation of inflammasome priming, as well as intestinal fibrosis induced by chronic DSS treatment.

Moreover, in this study we have demonstrated *in vitro* that the first step of inflammasome activation in IECs is mediated by GPR91, since the increase in both gene and protein expression of inflammasome components induced by succinate and the inflammatory cocktail, was prevented when siGPR91 was transiently silenced in HT-29 cells. Inflammasome activation is an important line of defence in IECs since, as part of the mucosa layer, they are in direct contact with pathogens and intestinal

insults (313). Whether the inflammasome exerts a protective or a pro-inflammatory role is still an unaddressed question .There are several studies that have demonstrated a protective role of the inflammasome activation in epithelial cells in different tissues. For instance, in the eye, the activation of the inflammasome avoided *Staphylococcus aureus* infection (314), and in human airway epithelial cells inflammasome activation prevented influenza A viral infection (315). On the other hand, other studies have reported the contribution of NLRP3 inflammasome activity to inflammation-associated diseases such as chronic obstructive pulmonary disease and asthma (316), atherosclerosis (317), cancer, diabetes and obesity (318). Nonetheless, few studies have focused on the molecular pathways that participate in inflammasome priming. Data obtained in this study demonstrated that GPR91 is involved in this step of the inflammasome in IECs, suggesting that given the increased expression of GPR91, an overactivation and perpetuation of the inflammasome activity may contribute to chronic inflammation.

Next, we sought to better characterize the downstream molecular pathways GPR91-mediated. Hence, in line a previous study performed in endothelial cells (309), we observed that GPR91 was constitutively regulating the ERK1/2 pathway in IECs, since siGPR91 cells in basal conditions, exhibited a significant reduction of ERK1/2 phosphorylation. Our hypothesis is that the high levels of succinate quantified in the supernatant of epithelial cells are the responsible of the constitutive activation of GPR91. Indeed, we detected 141.6 ± 12.8 µM of succinate in the supernatant of HT-29 cells, levels that are enough to activate SUCNR1 in basal conditions, since those levels are higher than the reported EC50 of succinate which is 91 ± 14 µM (319). In addition, we also demonstrated that GPR91 regulates the basal phosphorylation of the pro-inflammatory transcription factor NFκB in IECs (Figure V.2), which may explain the reduced expression of pro-inflammatory cytokines in resting macrophages previously demonstrated in different studies (146, 199). In fact, it has been previously reported that NFκB mediates the transcription of

different inflammasome components such as Nlrp1, Nlrp3 or IL1B (320). Our data lead us to propose that GPR91 might regulate the inflammasome priming through NFκB Figure V.2.

Next, according to our results, HT-29 cells treated for 24 hours with LPS significantly increased *IL1B*, *NOS2* and *IL6*, as well as NFκB phosphorylation, an effect which was not observed when these cells were siGPR91 transiently transfected. However, ERK1/2 phosphorylation was not modified by LPS, despite it induced both an increase in NFκB phosphorylation and a reduction in IκB levels, suggesting that LPS induces inflammation via an ERK1/2-independent mechanism. However, when we used the ERK1/2 inhibitor U0126, we observed that NFκB phosphorylation was significantly reduced whilst IκB levels were increased. Thus, these results demonstrate that the activation of NFκB inflammatory pathways in both basal and LPS-stimulated conditions, is dependent of ERK1/2 phosphorylation constitutive cellular levels (Figure V.2).

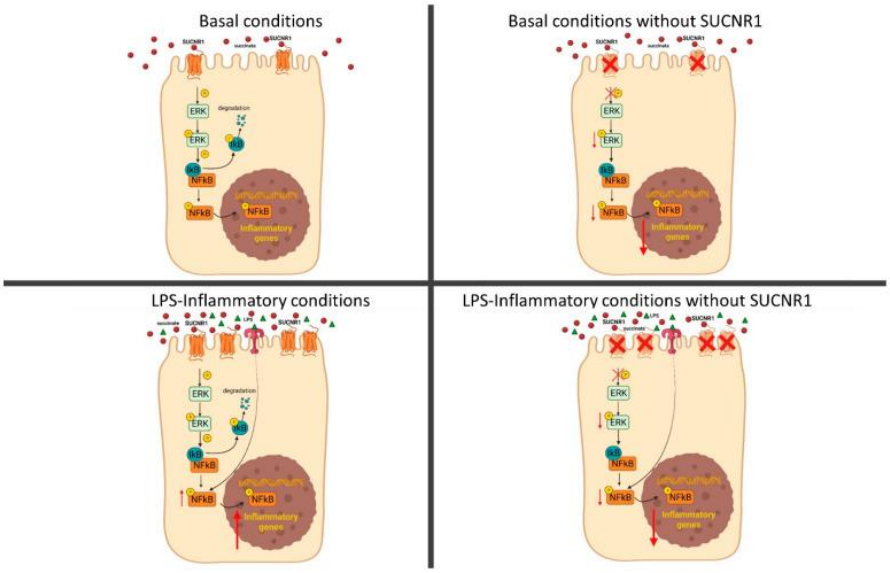


Figure V. 2. GPR91 activation. *The activation of GPR91 by constitutive levels of succinate regulates ERK and NFκB phosphorylation in non-stimulated epithelial cells. In LPS-treated*

cells, there is an upregulation of GPR91 which increases the phosphorylation of ERK and NFκB, with the subsequent expression of proinflammatory genes.

Finally, gene and protein expression of GPR91 in IECs was upregulated by both LPS and the inflammatory cocktail, as previously reported in bone marrow-derived macrophages (321). However, these stimuli failed to regulate extracellular succinate levels, opposite to what Littlewood-Evans et al. reported on macrophages (199). In this case, we should consider that Mu and colleagues reported that succinate levels vary among cell lines and primary cells, since succinate levels in human gastric cancer tissues and cancer cell lines (NCI-N87, BGC-823, AGS and SGC-7901) were higher compared with adjacent tissues and a normal gastric epithelial cell line (GES-1) (309). Indeed, we found 10-fold levels of succinate in HT-29 cells in comparison with levels detected in blood from carcinoma (322) or CD patients (146). Thus, we hypothesise that the limiting factor for the pro-inflammatory signalling pathway in HT-29 cells should be GPR91 instead of succinate levels. In fact, HT-29 cells treated with 1 mM of succinate did not increase inflammasome components, whereas the effect was potentiated when it was administered together with the inflammatory cocktail, possibly because GPR91 protein expression was increased due to the combination of both.

In summary, these findings demonstrate that GPR91 plays a crucial role in activating inflammatory pathways of IECs, and also participates in the priming step of inflammasome in human colitis, as well as a chronic murine colitis model. In addition, our research also demonstrates that fistulizing CD patients have higher expression of GPR91, and that deletion of this receptor impairs EMT activation in IECs. Considering that all these processes have been involved in the etiopathogenic mechanisms of UC, but also CD, we propose that the pharmacological blockade of GPR91 may be considered to prevent IBD complications.

V.5 GPR109A in IBD pathogenesis

In this study, we have demonstrated for the first time that the expression of GPR109A is significantly increased in intestinal tissue of both UC and CD patients. Immunohistochemical staining revealed that epithelial cells and cells of the lamina propria, which may include resident macrophages, dendritic cells, and lymphoid cells, were positive for GPR109A, suggesting an upregulated receptor expression in these cells during the pathology. In parallel, a prior study reported that GPR109A is expressed on intestinal epithelial cells in human colonic biopsies (220). To our knowledge, no studies of this receptor in IBD patients have been reported, but controversial results have been shown in cancer. In fact, a decreased expression of the receptor was described in patients with colon cancer (220), whereas in human skin cancer its expression was upregulated (323).

Next, based on the positive staining of GPR109A in cells of the lamina propria of IBD patients, we sought to analyse the effects of an agonist of this receptor, β-hydroxybutyrate, in U937-derived macrophages. Our results showed an increased gene expression of GPR109A after β-hydroxybutyrate treatment, in line with previous studies in alveolar macrophages (324). In parallel, β-hydroxybutyrate failed to modify the mRNA expression of *CD206*, a M2 macrophage marker (325, 326), while it significantly increased the expression of the M1 macrophage marker, *CD86*. In these cells, we also found an upregulation in the gene expression of pro-inflammatory cytokines *IL1B* and *NOS2* after β-hydroxybutyrate treatment, whilst *IL6*, *IL8* and *TNF* remained unchanged. In line with our results, previous studies demonstrated that β-hydroxybutyrate did not increased TNF-α nor IL-6 secretion in the same cell line, whilst acetoacetate, a different ketone body did (327, 328). However, in contrast to our data, other studies reported that treatment with 5 mM β-hydroxybutyrate failed to increase IL-1β secretion in Sw.71 cells, a cell line of human first-trimester trophoblast cells (329), a difference that may be attributed to variances in cell lines. Discrepancies among the final effects of β-hydroxybutyrate

on inflammation have been reported between humans and mice. Indeed, the exogenous administration of ketones to humans revealed, in blood supernatants, increased inflammation in monocytes after exogenous β-hydroxybutyrate intake (330). In contrast, β-hydroxybutyrate reduced inflammation in an inflammatory murine model of diabetes (331). Nevertheless, we have reported for the first time that MØ U937-derived macrophages treated with β-hydroxybutyrate increase mRNA expression of *CD86*, *IL1B* and *NOS2* in a dose-dependent manner.

Up to this point, we have demonstrated an increased expression of GPR109A in lamina propria cells of UC and CD patients, and also an increased gene expression of the M1 macrophage marker *CD86*, in both UC and CD patients. The relationship between the receptor and this macrophage phenotype was appreciated with the strong positive correlation we detected, especially in CD patients. Thus, to determine the role of GPR109A in M1 macrophage polarization, we transiently silenced the receptor and polarized macrophages. Our results showed an increased GPR109A mRNA expression in M1- macrophages and a significant reduction of the expression of pro-inflammatory cytokines *IL1B* and *TNF* together with a decreased protein secretion of IL-1β, IL-6, and TNF-α, in siGPR109A M1 macrophages. These observations report a role for GPR109A in the expression of pro-inflammatory cytokines in M1 macrophages. Previous studies demonstrated higher expression the GPR109A expression in murine macrophages by pro-inflammatory stimulus such as IFN-γ, LPS, TNF-α and IL-1β (214, 332). Furthermore, a study performed on RAW 264.7 macrophages transiently transfected with siGPR109A, showed an impaired NFκB translocation to the nucleus and decreased gene expression of *IL1B* in response to LPS (333). Nevertheless, our findings appear to contradict a study performed on murine BMDMs where LPS treatment and GPR109A deletion resulted in increased M1-macrophage polarization (334). In this case, it was 2 hours treatment of LPS, whilst polarization treatment of U937-derived macrophages towards the M1 phenotype requires 24 hours of treatment (92). As a result, these discrepancies in

timing as well as the possible differences between murine and human macrophages, potentially account for the conflicting outcomes observed.

Next, once elucidated the impact of GPR109A in the secretome of M1-macrophages, we wanted to determine its effects on intestinal fibroblasts. Hence, HSIFs were treated with secretome from both, M1 and siGPR109A-M1 macrophages for 24 hours and the expression of pro-fibrotic markers *COL1A1* and *COL3A1* tended to be higher in fibroblasts treated with the M1-macrophages secretome, an effect which was significantly reduced when fibroblasts were treated with M1-siGPR109A secretome. Therefore, detected disparities in the secretion of IL-1β, IL-6 and TNF-α could be accountable for the profibrotic outcomes observed in HSIFs, although other non-quantified cytokines cannot be ruled out. In line with this, previous data evidence that IL-1β, IL-6 and TNF-α promote fibrosis and increase their collagen deposition and pro-fibrotic markers expression (335, 336), not only in the intestinal scenario but also in skin (337), and the liver (338). Future studies are needed in order to deeply analyse the effect of each cytokine *per se* in the development of intestinal fibrosis.

Overall, we suggest that GPR109A plays a pro-inflammatory role in macrophages regulating the expression of pro-inflammatory cytokines, which has an impact on other key protagonists involved in IBD pathogenesis, notably fibroblasts. Currently, no studies have investigated the precise involvement of GPR109A in IBD patients, so additional research is required to better understand the relevance of its high expression in IBD samples, and to characterize how the molecular pathways that are activated by this receptor modulate inflammation and fibrogenesis. This information could be useful to identify the receptor as a possible target for improving the pharmacological approach of IBD.

VI. CONCLUSIONS

1. Intestinal surgical resections from IBD patients exhibit:
    a. Changes in gut microbiota composition, richness and load in UC patients together with an increased proportion of *Cellulosimicrobium*. In CD patients, there were no differences in gut microbiota composition, but a lower tendency in bacterial richness and load, together with decreased abundance of *Enterococcus*, was observed.
    b. Alterations in the GPCRs-agonist metabolomic profile.
    c. Higher gene expression of most of the metabolite-sensing GPCRs, being greater in CD versus UC patients.
2. Higher gene expression of most metabolite-sensing GPCRs is positively correlated with increased gene expression of pro-inflammatory cytokines, including *IL1B* and *IL8*, the M1 macrophage marker *CD86* and pro-fibrotic markers *COL1A1*, *COL3A1* and *COL4A1*.
3. Fistulizing CD patients exhibit high expression of GPR91 and EMT markers, including *SNAI1* and *SNAI2*, which are positively correlated.
4. Deletion of GPR91 impairs the EMT activation in both intestinal epithelial cells and a murine model of intestinal fibrosis.
5. UC patients exhibit increased expression of GPR91 and inflammasome components, including Caspase-1, NLRP3 and IL-1β which are positively correlated.
6. Deletion of GPR91 impairs inflammation and inflammasome priming in both intestinal epithelial cells and a murine model of chronic intestinal inflammation and fibrosis.

7. CD patients exhibit high levels of both β-hydroxybutyrate and its receptor, GPR109A, which is expressed in intestinal epithelial cells and cells of the lamina propria.

8. GPR109A mediates the expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in M1 macrophages and modulates their ability to activate intestinal fibroblasts.

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ANNEXES

Annex I. Ethics committee approval certificate of the clinical study

La Fe
Departament de Salut

CEIm-F-PE-01-11 v01

DICTAMEN DEL COMITÉ DE ÉTICA DE LA INVESTIGACIÓN CON MEDICAMENTOS

MARÍA TORDERA BAVIERA, titular de la Secretaría Técnica del Comité de Ética de la Investigación con medicamentos del **CEIM - HOSPITAL UNIVERSITARIO Y POLITÉCNICO LA FE,**

CERTIFICA

Que este Comité ha evaluado en su sesión de fecha 08/09/2021 el Proyecto de Investigación:

Título: **"IDENTIFICACIÓN DE METABOLITOS CLAVE EN LA ENFERMEDAD INFLAMATORIA INTESTINAL: APUNTANDO A LOS GPCRS COMO DIANAS FARMACOLÓGICAS."**
Nº de registro: **2021-545-1**
Investigador Coordinador: **JESÚS COSÍN ROGER**

Documento	Fecha - Versión
Protocolo	sin versión ni fecha
Hoja de Información al Paciente y Consentimiento Informado	Versión 2. Fecha de la versión 02-08-2021

Que dicho proyecto se ajusta a las normativas éticas sobre investigación biomédica con sujetos humanos y es viable en cuanto al planteamiento científico, objetivos, material y métodos, etc, descritos en la solicitud, así como la Hoja de Información al Paciente y el Consentimiento Informado.

En consecuencia, este Comité acuerda emitir **INFORME FAVORABLE** de dicho Proyecto de Investigación que será realizado en los siguientes centros:

CENTRO	INVESTIGADOR PRINCIPAL
HOSPITAL UNIVERSITARI I POLITÈCNIC LA FE	MÓNICA MILLÁN SCHEIDING
HOSPITAL DE MANISES	FRANCISCO NAVARRO VICENTE

Que el CEIM - HOSPITAL UNIVERSITARIO Y POLITÉCNICO LA FE, tanto en su composición como en sus procedimientos, cumple con las normas de BPC (CPMP/ICH/135/95) y con la legislación vigente que regula su funcionamiento, y que la composición del CEIM - HOSPITAL UNIVERSITARIO Y POLITÉCNICO LA FE es la indicada en el anexo I, teniendo en cuenta que, en el caso de que algún miembro participe en el estudio o declare algún conflicto de interés, no habrá participado en la evaluación ni en el dictamen de la solicitud de autorización del estudio clínico.

Lo que firmo en Valencia, a 08/09/2021

Firmat per Maria Tordera Baviera el 14/09/2021 14:47:33

GENERALITAT VALENCIANA

Fdo.: MARÍA TORDERA BAVIERA
Secretario/a Técnica del Comité de Ética de la Investigación con medicamentos

ANEXO I
COMPOSICIÓN CEIm

Presidente:
ADELA CAÑETE NIETO - Facultativo Especialista en Pediatría. Jefe Sección de Unidad de Hematología y Oncología Pediátrica

Vicepresidente:
SALVADOR F ALIÑO PELLICER - Catedrático de Farmacología. Facultativo Especialista en Farmacología Clínica

Secretario:
MARÍA TORDERA BAVIERA - Farmacéutica Especialista en Farmacia Hospitalaria. Farmacéutica adjunta del Servicio de Farmacia

Vocales:
MARÍA VICTORIA PARICIO GÓMEZ - Diplomada Enfermería. Supervisora del Servicio de Hematología y Trasplante de Progenitores Hemáticos
JAVIER LLUNA GONZÁLEZ - Facultativo Especialista en Cirugía Pediátrica. Médico adjunto del Servicio de Cirugía Pediátrica
PAULA RAMÍREZ GALLEYMORE - Facultativo Especialista en Medicina Intensiva. Médico adjunto del Servicio de Medicina Intensiva
JOSÉ MARÍA CANELLES GAMIR - Farmacéutico de Atención Primaria del Departamento de Salud Valencia La Fe
SERAFÍN RODRÍGUEZ CAPELLÁN - Licenciado en Derecho. Técnico de Función Administrativa adscrito a la Dirección de Investigación
VICENTE INGLADA ALCAIDE - Miembro Lego, representante de los intereses de los pacientes
MIGUEL ÁNGEL CANO TORRES - Licenciado en Derecho. Técnico de Función Administrativa adscrito a la Dirección de Investigación
LUIS VICENTE MARTÍNEZ DOLZ - Facultativo Especialista en Cardiología. Jefe de Servicio de Cardiología
BONAVENTURA CASANOVA ESTRUCH - Facultativo Especialista en Neurología. Médico adjunto del Servicio de Neurología
SARA BRUGGER FRIGOLS - Facultativo Especialista en Radiodiagnóstico. Médico adjunto del Área de Imagen Médica
Mª ISABEL IZQUIERDO MACIÁN - Facultativo Especialista en Pediatría (Neonatología). Jefa de Servicio de Neonatología
MATTEO FRASSON - Facultativo Especialista en Cirugía. Médico adjunto del Servicio de Cirugía General y Digestiva
JOSÉ VICENTE SOLANAS PRATS - Facultativo Especialista en Medicina de Familia y Comunitaria. Médico del Centro de Salud Trinitat
ANTONIO ORDUÑA GALÁN - Ing. de aplicaciones y sistemas. Responsable Área de Seguridad y Calidad de Sistemas de inforamcion
OSCAR DÍAZ CAMBRONERO - Facultativo especialista en Anestesiología y Reanimación. Jefe Sección Anestesiología y Reanimación

Annex II. Informed consents

Instituto de Investigación Sanitaria La Fe

HOJA DE INFORMACIÓN AL PACIENTE ADULTO

Versión: 1	Fecha de la versión: 01/07/2021
Investigador Principal: MÓNICA MILLÁN SCHEIDING. SERVICIO: CIRUGÍA GENERAL Y DEL APARATO DIGESTIVO monicamillan72@gmail.com	
CENTRO: HOSPITAL LA FE	
Título del proyecto de investigación: Identificación de Metabolitos Clave en la Enfermedad Inflamatoria Intestinal: apuntando a los GPCRs como dianas farmacológicas	

1. INTRODUCCIÓN:

Nos dirigimos a usted para informarle sobre un estudio en el que se le invita a participar. El estudio ha sido aprobado por el Comité de Ética de la Investigación con medicamentos del Hospital Universitario y Politécnico La Fe (CEIm La Fe).

Nuestra intención es tan sólo que usted reciba la información correcta y suficiente para que pueda evaluar y juzgar si quiere o no participar en este estudio. Para ello lea esta hoja informativa con atención y nosotros le aclararemos las dudas que le puedan surgir después de la explicación. Además, puede consultar con las personas que considere oportunas.

2. PARTICIPACIÓN VOLUNTARIA:

Debe saber que su participación en este estudio es voluntaria y que puede decidir no participar y retirar el consentimiento en cualquier momento, sin que por ello se altere la relación con su médico ni se produzca perjuicio alguno en su tratamiento.

3. DESCRIPCIÓN GENERAL DEL ESTUDIO:

El presente proyecto de investigación plantea: 1) la identificación de algún receptor GPCR como posible diana farmacológica para el tratamiento de la Enfermedad Inflamatoria Intestinal y de la Fibrosis Intestinal y 2) la identificación de algún metabolito o molécula indicadora de la severidad de la Enfermedad Inflamatoria Intestinal presente en la sangre circulante. Ambos objetivos centrales se plantean con el fin de poder desarrollar un posible tratamiento farmacológico que mejore cualitativamente la calidad de vida de los pacientes con EC.

Con el fin de desarrollar por completo el presente proyecto de investigación y con el fin de analizar exactamente qué ocurre en la patología humana, necesitamos recoger un total de 40 resecciones intestinales. En dichos pacientes se recogerá, siempre y cuando el cirujano lo considere oportuno y posible, un trozo de mucosa inflamada y otro trozo de mucosa no inflamada. Además, también se necesitan 20 muestras de tejido sano de pacientes con cáncer de colon con el fin de disponer de

unas muestras que consideraremos como grupo control. En todos los casos, se tratará de pacientes que se necesiten someter a una intervención quirúrgica para eliminar un trozo de intestino en el momento en el que el médico lo considere más adecuado para cada paciente.

En dichas muestras, se analizará: 1) los perfiles metabólicos mediante metabolómica y 2) por diferentes técnicas de biología molecular (PCR cuantitativa, WesternBlot, inmunohistoquímica e inmunofluorescencia) la expresión de receptores GPCR, así como la expresión de diferentes marcadores proinflamatorios y profibróticos con el fin de poder estudiar si existe algún tipo de correlación entre la expresión de estos receptores con marcadores proinflamatorios o profibróticos. Además, también se aislarán macrófagos y fibroblastos intestinales primarios de dichas muestras para poder analizar mediante experimentos de cultivos celulares la relevancia de los receptores GPCR en dichas células primarias.

Por lo tanto, la participación en este proyecto de investigación NO supone ningún inconveniente al paciente porque en todo momento NO se va a alterar ni el tratamiento ni la forma de actuar de los médicos con cada paciente, ni tampoco se necesita que se extraiga una muestra de mayor tamaño que la necesaria. Simplemente, de aquella resección intestinal que el cirujano considere oportuno extirpar de cada paciente, nos proveerá de un trozo de zona no inflamada y otro trozo de zona inflamada para su posterior análisis en el proyecto de investigación. Tampoco se va a alterar el número de visitas ni el número de pruebas complementarias a las que el paciente se someterá. Únicamente, del trozo que necesite extraerse de cada paciente, el cirujano nos proporcionará un trozo de la zona no inflamada y un trozo de la zona inflamada para su análisis en el estudio.

4. BENEFICIOS Y RIESGOS DERIVADOS DE SU PARTICIPACIÓN EN EL ESTUDIO:

Debe saber que su participación en este estudio puede no aportarle beneficios directos.

En cambio, debe saber que los beneficios a corto plazo del presente proyecto de investigación pueden ser la identificación de una nueva diana farmacológica, lo que permitiría poder desarrollar algún compuesto farmacológico específico contra la misma permitiendo reducir tanto la inflamación como la aparición de fibrosis intestinal. Además, cabe destacar que, pese a todos los avances que se han hecho hasta la fecha, todavía se desconoce los mecanismos moleculares implicados en la fibrosis intestinal, complicación muy frecuente en pacientes con EC y contra la que no se dispone de ningún tratamiento farmacológico que permita su desarrollo o reversión. De hecho, en el momento en el que un paciente desarrolla fibrosis intestinal, la única opción de tratamiento del que se dispone en la actualidad es la cirugía, lo que limita y reduce considerablemente la calidad de vida del paciente. Por todo ello, consideremos que dicho proyecto de investigación pueda suponer un gran beneficio para toda la sociedad, concretamente para todos los pacientes afectados con Enfermedad Inflamatoria Intestinal.

Además, la participación en este proyecto no supone ningún riesgo derivado puesto que los pacientes no se van a someter a ningún tratamiento/prueba diagnóstica

adicional ni específica. De modo que ni el seguimiento ni el tratamiento va a verse modificado por la participación en dicho estudio.

5. TRATAMIENTOS ALTERNATIVOS:

En este caso, no existe ningún tratamiento o alternativa terapéutica más eficaz ya que, como se ha comentado anteriormente, la participación del paciente en el presente estudio no supone una alteración de su tratamiento. De modo que únicamente podrán participar aquellos pacientes que necesiten someterse a una cirugía puesto que su médico lo ha considerado más oportuno en función de su situación. Si usted lo desea, el médico del estudio le dará más información y le resolverá cualquier duda que pueda tener durante su participación en el proyecto.

6. Nº DE URGENCIA PARA PROBLEMAS DEL ESTUDIO:

En caso de que desee formular preguntas acerca del estudio o daños relacionados con el mismo, puede contactar con el médico del estudio, la Dra. Mónica Millán Scheiding, en el número de teléfono 638107948.

7. CONFIDENCIALIDAD:

El tratamiento, la comunicación y la cesión de los datos de carácter personal de todos los sujetos participantes se ajustará a lo dispuesto en la Ley Orgánica 3/2018, de 5 de diciembre de protección de datos y garantía de los derechos digitales y en el Reglamento (UE) 2016/679 del Parlamento europeo y del Consejo de 27 de abril de 2016 de Protección de Datos (RGPD) . De acuerdo a lo que establece la legislación mencionada, usted puede ejercer los derechos de acceso, modificación, oposición, cancelación de datos, limitar el tratamiento de datos que sean incorrectos y solicitar una copia o que se trasladen a un tercero (portabilidad) para lo cual deberá dirigirse al investigador principal del estudio o al/a la Delegado/a de Protección de Datos de la Institución, con quién podrá comunicarse a través del teléfono y/o dirección de correo electrónico: Mónica Millán Scheiding al email monicamillan72@gmail.com o al número de teléfono 638107948.

Le recordamos que los datos no se pueden eliminar, aunque deje de participar en el estudio, para garantizar la validez de la investigación y cumplir con los deberes legales. Así mismo tiene derecho a dirigirse a la Agencia de Protección de Datos si no quedara satisfecho.

Tanto el Centro como el Promotor son responsables respectivamente del tratamiento de sus datos y se comprometen a cumplir con la normativa de protección de datos en vigor. Los datos recogidos para el estudio estarán identificados mediante un código, de manera que no se incluya información que pueda identificarle, y sólo su médico del estudio/colaboradores podrá relacionar dichos datos con usted y con su historia clínica. Por lo tanto, su identidad no será revelada a ninguna otra persona salvo a las autoridades sanitarias, cuando así lo requieran o en casos de urgencia médica. Los Comités de Ética de la Investigación con medicamentos, los representantes de la Autoridad Sanitaria en materia de inspección y el personal autorizado por el Promotor, únicamente podrán acceder para comprobar los datos personales, los procedimientos

del estudio clínico y el cumplimiento de las normas de buena práctica clínica (siempre manteniendo la confidencialidad de la información).

El Investigador y el Promotor están obligados a conservar los datos recogidos para el estudio al menos hasta 25 años tras su finalización. Posteriormente, su información personal solo se conservará por el centro para el cuidado de su salud y por el promotor para otros fines de investigación científica si usted hubiera otorgado su consentimiento para ello, y si así lo permite la ley y requisitos éticos aplicables.

Sólo se tramitarán a terceros y a otros países los datos recogidos para el estudio, que en ningún caso contendrán información que le pueda identificar directamente, como nombre y apellidos, iniciales, dirección, nº de la seguridad social, etc... En el caso de que se produzca esta cesión, será para los mismos fines del estudio descrito y garantizando la confidencialidad como mínimo con el nivel de protección de la legislación vigente en nuestro país.

Si realizáramos transferencia de sus datos codificados fuera de la UE a las entidades de nuestro grupo, a prestadores de servicios o a investigadores científicos que colaboren con nosotros, los datos del participante quedarán protegidos con salvaguardas tales como contratos u otros mecanismos por las autoridades de protección de datos. Si el participante quiere saber más al respecto, puede contactar con el Delegado de Protección de Datos del promotor al email dpd@gva.es.

8. COMPENSACIÓN ECONÓMICA:

El promotor del estudio es el responsable de gestionar la financiación del mismo, por lo que su participación en éste no le supondrá ningún gasto.

9. OTRA INFORMACIÓN RELEVANTE:

Cualquier nueva información que se descubra durante su participación y que pueda afectar a su disposición para participar en el estudio, le será comunicada por su médico lo antes posible.

Si usted decide retirar el consentimiento para participar en este estudio, no se añadirá ningún dato nuevo a la base de datos y, en caso de que se hubieran recogido muestras, puede exigir la destrucción de todas las muestras identificables, previamente obtenidas, para evitar la realización de nuevos análisis.

También debe saber que puede ser excluido del estudio si el promotor o los investigadores del mismo lo consideran oportuno, ya sea por motivos de seguridad o porque consideren que usted no está cumpliendo con los procedimientos establecidos. En cualquiera de los casos, usted recibirá una explicación adecuada del motivo por el que se ha decidido su retirada del estudio.

El promotor podrá suspender el estudio siempre y cuando sea por alguno de los supuestos contemplados en la legislación vigente.

Al firmar la hoja de consentimiento adjunta, se compromete a cumplir con los procedimientos del estudio que se le han expuesto. Cuando acabe su participación

recibirá el mejor tratamiento disponible y que su médico considere el más adecuado para su enfermedad.

10. OBTENCIÓN Y UTILIZACIÓN DE MUESTRAS BIOLÓGICAS:

Su participación en este estudio conlleva la obtención y utilización de muestras biológicas con fines de investigación, para lo que se observará la Ley 14/2007 de investigación biomédica y el Real Decreto 1716/2011, normativas que garantizan el respeto a los derechos que le asisten.

Al firmar este documento, revisado y evaluado favorablemente por el Comité de Ética de la Investigación con medicamentos que ha aprobado este estudio, usted acepta que se utilicen sus muestras para las finalidades del presente estudio.

Las muestras biológicas se custodiarán en el laboratorio de Farmacología de la Facultad de Medicina (Universidad de Valencia), durante la realización del presente estudio.

Instituto de Investigación Sanitaria La Fe

CONSENTIMIENTO POR ESCRITO

Título del estudio: Identificación de Metabolitos Clave en la Enfermedad Inflamatoria Intestinal: apuntando a los GPCRs como dianas farmacológicas

Yo, *(nombre y apellidos).*

- He leído la hoja de información que se me ha entregado.
- He podido hacer preguntas sobre el estudio.
- He recibido suficiente información sobre el estudio.
- He hablado con: *(nombre del Investigador)*
- Comprendo que mi participación es voluntaria.
- Recibiré una copia firmada y fechada de este documento de consentimiento informado.

Comprendo que puedo retirarme del estudio:

1º Cuando quiera
2º Sin tener que dar explicaciones.
3º Sin que esto repercuta en mis cuidados médicos.

Presto libremente mi conformidad para participar en el estudio.

Firma del Participante	***Firma del Investigador***
Nombre: _____	***Nombre:*** _____
Fecha: ____/____/____	***Fecha:*** ____/____/____

Deseo que me comuniquen la información derivada de la investigación que pueda ser relevante para mí salud: ☐ **Sí** ☐ **No**

Firma del representante _____ Fecha: ____/____/____ *(Nombre, firma y fecha de puño y letra del representante legal)*

Firma del investigador: _____ Fecha: ____/____/____

Deseo que me comuniquen la información derivada de las pruebas genéticas realizadas (<u>únicamente</u> para aquellos estudios que incluyan este tipo de pruebas, siempre que estén validadas y que puedan tener relevancia para la salud del paciente): ☐ **Sí** ☐ **No**

Firma del representante _____ Fecha: ____/____/____ *(Nombre, firma y fecha de puño y letra del representante legal)*

Firma del investigador: _____ Fecha: ____/____/____

Instituto de Investigación Sanitaria La Fe

CONSENTIMIENTO ORAL ANTE TESTIGOS

Título del estudio: Identificación de Metabolitos Clave en la Enfermedad Inflamatoria Intestinal: apuntando a los GPCRs como dianas farmacológicas

Yo, _____ *(nombre y apellidos),*

- He recibido la hoja de información sobre el estudio.
- He podido hacer preguntas sobre el estudio.
- He recibido suficiente información sobre el estudio.
- He sido informado por: _____ *(nombre del investigador)*
- Recibiré una copia firmada y fechada de este documento de consentimiento informado.

Declaro bajo mi responsabilidad que: _____ *(nombre del participante del estudio)*

- Comprende que su participación es voluntaria.
- Comprende que puede retirarse del estudio:
  1º Cuando quiera
  2º Sin tener que dar explicaciones.
  3º Sin que esto repercuta en sus cuidados médicos.
- Y ha expresado libremente su conformidad para participar en el estudio.

Firma del testigo (Nombre, firma y fecha de puño y letra)

Nombre: _____

Fecha: ____/____/____

Firma del Investigador:

Nombre: _____

Fecha: ____/____/____

El paciente desea que le comuniquen la información derivada de la investigación que pueda ser relevante para su salud (si procede): ☐ Sí ☐ No

Firma del testigo (Nombre, firma y fecha de puño y letra)

Nombre: _____

Fecha: ____/____/____

Firma del Investigador:

Nombre: _____

Fecha: ____/____/____

El paciente desea que le comuniquen la información derivada de las pruebas genéticas realizadas (<u>únicamente</u> para aquellos estudios que incluyan este tipo de pruebas, siempre que estén validadas y que puedan tener relevancia para la salud del paciente): ☐ Sí ☐ No

Firma del testigo (Nombre, firma y fecha de puño y letra)

Nombre: _____

Fecha: ____/____/____

Firma del Investigador:

Nombre: _____

Fecha: ____/____/____

Instituto de Investigación Sanitaria La Fe

CONSENTIMIENTO DEL REPRESENTANTE LEGAL

Título del estudio: Identificación de Metabolitos Clave en la Enfermedad Inflamatoria Intestinal: apuntando a los GPCRs como dianas farmacológicas

Yo, _____ *(nombre y apellidos)*

- He leído la hoja de información que se me ha entregado.
- He podido hacer preguntas sobre el estudio.
- He recibido suficiente información sobre el estudio.

He hablado con: _____ *(nombre del Investigador)*

Comprendo que la participación de _____ *(nombre del participante)* es voluntaria.

Comprendo que _____ *(nombre del participante)* puede retirarse del estudio:

1º Cuando quiera
2º Sin tener que dar explicaciones.
3º Sin que esto repercuta en sus cuidados médicos.

En mi presencia se ha dado a _____ *(nombre del participante)*, toda la información pertinente adaptada a su nivel de entendimiento y está de acuerdo en participar.

Recibiré una copia firmada y fechada de este documento de consentimiento informado.

Y presto mi conformidad con que _____ *(nombre del participante)* participe en el estudio.

Firma del Representante	***Firma del Investigador***
Nombre: _____	***Nombre:*** _____
Fecha: ____/____/____	***Fecha:*** ____/____/____

Deseo que me comuniquen la información derivada de la investigación que pueda ser relevante para mí salud: ☐ **Sí** ☐ **No**

Firma del representante _____ Fecha: ____/____/____ *(Nombre, firma y fecha de puño y letra del representante legal)*

Firma del investigador: _____ Fecha: ____/____/____

Deseo que me comuniquen la información derivada de las pruebas genéticas realizadas (<u>únicamente</u> para aquellos estudios que incluyan este tipo de pruebas, siempre que estén validadas y que puedan tener relevancia para la salud del paciente): ☐ **Sí** ☐ **No**

Firma del representante _____ Fecha: ____/____/____ *(Nombre, firma y fecha de puño y letra del representante legal)*

Firma del investigador: _____ Fecha: ____/____/____

Instituto de Investigación Sanitaria La Fe

HOJA DE INFORMACIÓN AL PACIENTE ADULTO

Versión: 1	Fecha de la versión: 01/07/2021
Investigador Principal: FRANCISCO NAVARRO VICENTE. SERVICIO: CIRUGÍA GENERAL Y DEL APARATO DIGESTIVO fran.navarro.vicente@gmail.com	
CENTRO: HOSPITAL DE MANISES	
Título del proyecto de investigación: Identificación de Metabolitos Clave en la Enfermedad Inflamatoria Intestinal: apuntando a los GPCRs como dianas farmacológicas	

1. INTRODUCCIÓN:

Nos dirigimos a usted para informarle sobre un estudio en el que se le invita a participar. El estudio ha sido aprobado por el Comité de Ética de la Investigación con medicamentos del Hospital Universitario y Politécnico La Fe (CEIm La Fe).

Nuestra intención es tan sólo que usted reciba la información correcta y suficiente para que pueda evaluar y juzgar si quiere o no participar en este estudio. Para ello lea esta hoja informativa con atención y nosotros le aclararemos las dudas que le puedan surgir después de la explicación. Además, puede consultar con las personas que considere oportunas.

2. PARTICIPACIÓN VOLUNTARIA:

Debe saber que su participación en este estudio es voluntaria y que puede decidir no participar y retirar el consentimiento en cualquier momento, sin que por ello se altere la relación con su médico ni se produzca perjuicio alguno en su tratamiento.

3. DESCRIPCIÓN GENERAL DEL ESTUDIO:

El presente proyecto de investigación plantea: 1) la identificación de algún receptor GPCR como posible diana farmacológica para el tratamiento de la Enfermedad Inflamatoria Intestinal y de la Fibrosis Intestinal y 2) la identificación de algún metabolito o molécula indicadora de la severidad de la Enfermedad Inflamatoria Intestinal presente en la sangre circulante. Ambos objetivos centrales se plantean con el fin de poder desarrollar un posible tratamiento farmacológico que mejore cualitativamente la calidad de vida de los pacientes con EC.

Con el fin de desarrollar por completo el presente proyecto de investigación y con el fin de analizar exactamente qué ocurre en la patología humana, necesitamos recoger un total de 40 resecciones intestinales. En dichos pacientes se recogerá, siempre y cuando el cirujano lo considere oportuno y posible, un trozo de mucosa inflamada y otro trozo de mucosa no inflamada. Además, también se necesitan 20 muestras de tejido sano de pacientes con cáncer de colon con el fin de disponer de

unas muestras que consideraremos como grupo control. En todos los casos, se tratará de pacientes que se necesiten someter a una intervención quirúrgica para eliminar un trozo de intestino en el momento en el que el médico lo considere más adecuado para cada paciente.

En dichas muestras, se analizará: 1) los perfiles metabólicos mediante metabolómica y 2) por diferentes técnicas de biología molecular (PCR cuantitativa, WesternBlot, inmunohistoquímica e inmunofluorescencia) la expresión de receptores GPCR, así como la expresión de diferentes marcadores proinflamatorios y profibróticos con el fin de poder estudiar si existe algún tipo de correlación entre la expresión de estos receptores con marcadores proinflamatorios o profibróticos. Además, también se aislarán macrófagos y fibroblastos intestinales primarios de dichas muestras para poder analizar mediante experimentos de cultivos celulares la relevancia de los receptores GPCR en dichas células primarias.

Por lo tanto, la participación en este proyecto de investigación NO supone ningún inconveniente al paciente porque en todo momento NO se va a alterar ni el tratamiento ni la forma de actuar de los médicos con cada paciente, ni tampoco se necesita que se extraiga una muestra de mayor tamaño que la necesaria. Simplemente, de aquella resección intestinal que el cirujano considere oportuno extirpar de cada paciente, nos proveerá de un trozo de zona no inflamada y otro trozo de zona inflamada para su posterior análisis en el proyecto de investigación. Tampoco se va a alterar el número de visitas ni el número de pruebas complementarias a las que el paciente se someterá. Únicamente, del trozo que necesite extraerse de cada paciente, el cirujano nos proporcionará un trozo de la zona no inflamada y un trozo de la zona inflamada para su análisis en el estudio.

4. BENEFICIOS Y RIESGOS DERIVADOS DE SU PARTICIPACIÓN EN EL ESTUDIO:

Debe saber que su participación en este estudio puede no aportarle beneficios directos.

En cambio, debe saber que los beneficios a corto plazo del presente proyecto de investigación pueden ser la identificación de una nueva diana farmacológica, lo que permitiría poder desarrollar algún compuesto farmacológico específico contra la misma permitiendo reducir tanto la inflamación como la aparición de fibrosis intestinal. Además, cabe destacar que, pese a todos los avances que se han hecho hasta la fecha, todavía se desconoce los mecanismos moleculares implicados en la fibrosis intestinal, complicación muy frecuente en pacientes con EC y contra la que no se dispone de ningún tratamiento farmacológico que permita su desarrollo o reversión. De hecho, en el momento en el que un paciente desarrolla fibrosis intestinal, la única opción de tratamiento del que se dispone en la actualidad es la cirugía, lo que limita y reduce considerablemente la calidad de vida del paciente. Por todo ello, consideremos que dicho proyecto de investigación pueda suponer un gran beneficio para toda la sociedad, concretamente para todos los pacientes afectados con Enfermedad Inflamatoria Intestinal.

Además, la participación en este proyecto no supone ningún riesgo derivado puesto que los pacientes no se van a someter a ningún tratamiento/prueba diagnóstica

adicional ni específica. De modo que ni el seguimiento ni el tratamiento va a verse modificado por la participación en dicho estudio.

5. TRATAMIENTOS ALTERNATIVOS:

En este caso, no existe ningún tratamiento o alternativa terapéutica más eficaz ya que, como se ha comentado anteriormente, la participación del paciente en el presente estudio no supone una alteración de su tratamiento. De modo que únicamente podrán participar aquellos pacientes que necesiten someterse a una cirugía puesto que su médico lo ha considerado más oportuno en función de su situación. Si usted lo desea, el médico del estudio le dará más información y le resolverá cualquier duda que pueda tener durante su participación en el proyecto.

6. Nº DE URGENCIA PARA PROBLEMAS DEL ESTUDIO:

En caso de que desee formular preguntas acerca del estudio o daños relacionados con el mismo, puede contactar con el médico del estudio, el Dr. Francisco Navarro Vicente, en el número de teléfono 696789968.

7. CONFIDENCIALIDAD:

El tratamiento, la comunicación y la cesión de los datos de carácter personal de todos los sujetos participantes se ajustará a lo dispuesto en la Ley Orgánica 3/2018, de 5 de diciembre de protección de datos y garantía de los derechos digitales y en el Reglamento (UE) 2016/679 del Parlamento europeo y del Consejo de 27 de abril de 2016 de Protección de Datos (RGPD) . De acuerdo a lo que establece la legislación mencionada, usted puede ejercer los derechos de acceso, modificación, oposición, cancelación de datos, limitar el tratamiento de datos que sean incorrectos y solicitar una copia o que se trasladen a un tercero (portabilidad) para lo cual deberá dirigirse al investigador principal del estudio o al/a la Delegado/a de Protección de Datos de la Institución, con quién podrá comunicarse a través del teléfono y/o dirección de correo electrónico: Francisco Navarro Vicente al email fran.navarro.vicente@gmail.com o al número de teléfono 696789968.

Le recordamos que los datos no se pueden eliminar, aunque deje de participar en el estudio, para garantizar la validez de la investigación y cumplir con los deberes legales. Así mismo tiene derecho a dirigirse a la Agencia de Protección de Datos si no quedara satisfecho.

Tanto el Centro como el Promotor son responsables respectivamente del tratamiento de sus datos y se comprometen a cumplir con la normativa de protección de datos en vigor. Los datos recogidos para el estudio estarán identificados mediante un código, de manera que no se incluya información que pueda identificarle, y sólo su médico del estudio/colaboradores podrá relacionar dichos datos con usted y con su historia clínica. Por lo tanto, su identidad no será revelada a ninguna otra persona salvo a las autoridades sanitarias, cuando así lo requieran o en casos de urgencia médica. Los Comités de Ética de la Investigación con medicamentos, los representantes de la Autoridad Sanitaria en materia de inspección y el personal autorizado por el Promotor, únicamente podrán acceder para comprobar los datos personales, los procedimientos

del estudio clínico y el cumplimiento de las normas de buena práctica clínica (siempre manteniendo la confidencialidad de la información).

El Investigador y el Promotor están obligados a conservar los datos recogidos para el estudio al menos hasta 25 años tras su finalización. Posteriormente, su información personal solo se conservará por el centro para el cuidado de su salud y por el promotor para otros fines de investigación científica si usted hubiera otorgado su consentimiento para ello, y si así lo permite la ley y requisitos éticos aplicables.

Sólo se tramitarán a terceros y a otros países los datos recogidos para el estudio, que en ningún caso contendrán información que le pueda identificar directamente, como nombre y apellidos, iniciales, dirección, nº de la seguridad social, etc... En el caso de que se produzca esta cesión, será para los mismos fines del estudio descrito y garantizando la confidencialidad como mínimo con el nivel de protección de la legislación vigente en nuestro país.

Si realizáramos transferencia de sus datos codificados fuera de la UE a las entidades de nuestro grupo, a prestadores de servicios o a investigadores científicos que colaboren con nosotros, los datos del participante quedarán protegidos con salvaguardas tales como contratos u otros mecanismos por las autoridades de protección de datos. Si el participante quiere saber más al respecto, puede contactar con el Delegado de Protección de Datos del promotor al email dpd@gva.es.

8. COMPENSACIÓN ECONÓMICA:

El promotor del estudio es el responsable de gestionar la financiación del mismo, por lo que su participación en éste no le supondrá ningún gasto.

9. OTRA INFORMACIÓN RELEVANTE:

Cualquier nueva información que se descubra durante su participación y que pueda afectar a su disposición para participar en el estudio, le será comunicada por su médico lo antes posible.

Si usted decide retirar el consentimiento para participar en este estudio, no se añadirá ningún dato nuevo a la base de datos y, en caso de que se hubieran recogido muestras, puede exigir la destrucción de todas las muestras identificables, previamente obtenidas, para evitar la realización de nuevos análisis.

También debe saber que puede ser excluido del estudio si el promotor o los investigadores del mismo lo consideran oportuno, ya sea por motivos de seguridad o porque consideren que usted no está cumpliendo con los procedimientos establecidos. En cualquiera de los casos, usted recibirá una explicación adecuada del motivo por el que se ha decidido su retirada del estudio.

El promotor podrá suspender el estudio siempre y cuando sea por alguno de los supuestos contemplados en la legislación vigente.

Al firmar la hoja de consentimiento adjunta, se compromete a cumplir con los procedimientos del estudio que se le han expuesto. Cuando acabe su participación

recibirá el mejor tratamiento disponible y que su médico considere el más adecuado para su enfermedad.

10. OBTENCIÓN Y UTILIZACIÓN DE MUESTRAS BIOLÓGICAS:

Su participación en este estudio conlleva la obtención y utilización de muestras biológicas con fines de investigación, para lo que se observará la Ley 14/2007 de investigación biomédica y el Real Decreto 1716/2011, normativas que garantizan el respeto a los derechos que le asisten.

Al firmar este documento, revisado y evaluado favorablemente por el Comité de Ética de la Investigación con medicamentos que ha aprobado este estudio, usted acepta que se utilicen sus muestras para las finalidades del presente estudio.

Las muestras biológicas se custodiarán en el laboratorio de Farmacología de la Facultad de Medicina (Universidad de Valencia), durante la realización del presente estudio.

Instituto de Investigación Sanitaria La Fe

CONSENTIMIENTO POR ESCRITO

Título del estudio: Identificación de Metabolitos Clave en la Enfermedad Inflamatoria Intestinal: apuntando a los GPCRs como dianas farmacológicas

Yo, *(nombre y apellidos).*

- He leído la hoja de información que se me ha entregado.
- He podido hacer preguntas sobre el estudio.
- He recibido suficiente información sobre el estudio.
- He hablado con: *(nombre del Investigador)*
- Comprendo que mi participación es voluntaria.
- Recibiré una copia firmada y fechada de este documento de consentimiento informado.

Comprendo que puedo retirarme del estudio:

1º Cuando quiera
2º Sin tener que dar explicaciones.
3º Sin que esto repercuta en mis cuidados médicos.

Presto libremente mi conformidad para participar en el estudio.

Firma del Participante	***Firma del Investigador***
Nombre: _____	***Nombre:*** _____
Fecha: ____/____/____	***Fecha:*** ____/____/____

Deseo que me comuniquen la información derivada de la investigación que pueda ser relevante para mí salud: ☐ **Sí** ☐ **No**

Firma del representante _____ Fecha: ____/____/____ *(Nombre, firma y fecha de puño y letra del representante legal)*

Firma del investigador: _____ Fecha: ____/____/____

Deseo que me comuniquen la información derivada de las pruebas genéticas realizadas (únicamente para aquellos estudios que incluyan este tipo de pruebas, siempre que estén validadas y que puedan tener relevancia para la salud del paciente): ☐ **Sí** ☐ **No**

Firma del representante _____ Fecha: ____/____/____ *(Nombre, firma y fecha de puño y letra del representante legal)*

Firma del investigador: _____ Fecha: ____/____/____

Instituto de Investigación Sanitaria La Fe

CONSENTIMIENTO ORAL ANTE TESTIGOS

Título del estudio: Identificación de Metabolitos Clave en la Enfermedad Inflamatoria Intestinal: apuntando a los GPCRs como dianas farmacológicas

Yo, _____ *(nombre y apellidos)*,

- He recibido la hoja de información sobre el estudio.
- He podido hacer preguntas sobre el estudio.
- He recibido suficiente información sobre el estudio.
- He sido informado por: _____ *(nombre del investigador)*
- Recibiré una copia firmada y fechada de este documento de consentimiento informado.

Declaro bajo mi responsabilidad que: _____ *(nombre del participante del estudio)*

- Comprende que su participación es voluntaria.
- Comprende que puede retirarse del estudio:
  1º Cuando quiera
  2º Sin tener que dar explicaciones.
  3º Sin que esto repercuta en sus cuidados médicos.
- Y ha expresado libremente su conformidad para participar en el estudio.

Firma del testigo (Nombre, firma y fecha de puño y letra)

Nombre: _____

Fecha: ____/____/____

Firma del Investigador:

Nombre: _____

Fecha: ____/____/____

El paciente desea que le comuniquen la información derivada de la investigación que pueda ser relevante para su salud (si procede): ☐ Sí ☐ No

Firma del testigo (Nombre, firma y fecha de puño y letra)

Nombre: _____

Fecha: ____/____/____

Firma del Investigador:

Nombre: _____

Fecha: ____/____/____

El paciente desea que le comuniquen la información derivada de las pruebas genéticas realizadas (<u>únicamente</u> para aquellos estudios que incluyan este tipo de pruebas, siempre que estén validadas y que puedan tener relevancia para la salud del paciente): ☐ Sí ☐ No

Firma del testigo (Nombre, firma y fecha de puño y letra)

Nombre: _____

Fecha: ____/____/____

Firma del Investigador:

Nombre: _____

Fecha: ____/____/____

Instituto de Investigación Sanitaria La Fe

CONSENTIMIENTO DEL REPRESENTANTE LEGAL

Título del estudio: Identificación de Metabolitos Clave en la Enfermedad Inflamatoria Intestinal: apuntando a los GPCRs como dianas farmacológicas

Yo, _____ *(nombre y apellidos)*

- He leído la hoja de información que se me ha entregado.
- He podido hacer preguntas sobre el estudio.
- He recibido suficiente información sobre el estudio.

He hablado con: _____ *(nombre del investigador)*

Comprendo que la participación de _____ *(nombre del participante)* es voluntaria.

Comprendo que _____ *(nombre del participante)* puede retirarse del estudio:

1º Cuando quiera
2º Sin tener que dar explicaciones.
3º Sin que esto repercuta en sus cuidados médicos.

En mi presencia se ha dado a _____ *(nombre del participante)*, toda la información pertinente adaptada a su nivel de entendimiento y está de acuerdo en participar.

Recibiré una copia firmada y fechada de este documento de consentimiento informado.

Y presto mi conformidad con que _____ *(nombre del participante)* participe en el estudio.

Firma del Representante	***Firma del Investigador***
Nombre: _____	***Nombre:*** _____
Fecha: ____/____/____	***Fecha:*** ____/____/____

Deseo que me comuniquen la información derivada de la investigación que pueda ser relevante para mí salud: ☐ **Sí** ☐ **No**

Firma del representante _____ Fecha: ____/____/____ *(Nombre, firma y fecha de puño y letra del representante legal)*

Firma del investigador: _____ Fecha: ____/____/____

Deseo que me comuniquen la información derivada de las pruebas genéticas realizadas (únicamente para aquellos estudios que incluyan este tipo de pruebas, siempre que estén validadas y que puedan tener relevancia para la salud del paciente): ☐ **Sí** ☐ **No**

Firma del representante _____ Fecha: ____/____/____ *(Nombre, firma y fecha de puño y letra del representante legal)*

Firma del investigador: _____ Fecha: ____/____/____

Annex III. Ethics committe approval certificate for the murine models

GENERALITAT VALENCIANA
Conselleria de Agricultura, Desarrollo Rural, Emergencia Climática y Transición Ecológica

Dirección General de Agricultura, Ganadería y Pesca
Ciudad Administrativa 9 de Octubre
Calle de La Democracia, 77 · 46018 Valencia
www.gva.es

UNIÓN EUROPEA

AUTORIZACION PROCEDIMIENTO 2019/VSC/PEA/0290

Vista la solicitud realizada en fecha **17/12/19** con nº reg. entrada **836319** por D/Dª. **Carlos Hermenegildo Caudevilla**, **Vicerrector de Investigación y Política Científica**, centro usuario **ES462500001003**, para realizar el procedimiento:

"Relevancia del receptor de succinato y la autofagia en la fibrosis intestinal. "

Teniendo en cuenta la documentación aportada, según se indica en el artículo 33, punto 5 y 6, y puesto que dicho procedimiento se halla sujeto a autorización en virtud de lo dispuesto en el artículo 31 del Real Decreto 53/2013, de 1 de febrero,

Vista la propuesta del jefe del servicio de Producción y Sanidad Animal.

AUTORIZO:

la realización de dicho procedimiento al que se le asigna el código: **2019/VSC/PEA/0290** tipo **2,** de acuerdo con las características descritas en la propia documentación para el número de animales, especie y periodo de tiempo solicitado. Todo ello sin menoscabo de las autorizaciones pertinentes, por otras Administraciones y entidades, y llevándose a cabo en las siguientes condiciones:

Usuario: **Universidad de Valencia-Estudio General**

Responsable del proyecto: **M.ª Dolores Barrachina Sancho**

Establecimiento: **Animalario Unidad Central de Investigación (Fac. Medicina y Odontología) Valencia**

Necesidad de evaluación restrospectiva:

Condiciones específicas:

Observaciones:

Valencia a, fecha de la firma electrónica
El director general de Agricultura, Ganadería y Pesca

Firmat per Rogelio Llanes Ribas el
09/02/2020 10:50:09
Càrrec: Dirección General

Annex IV. Bibliographic production of the PhD candidate

1. Seco-Cervera M, Ortiz-Masiá D, Macias-Ceja DC, Coll S, Gisbert-Ferrándiz L, Cosín-Roger J, **Bauset C**, Ortega M, Heras-Moran B, Navarro-Vicente F, Millan M, Esplugues JV, Calatayud S, Barrachina MD. Resistance to Apoptosis in Complicated Crohn's Disease: Relevance in Ileal Fibrosis. *Under major revision in Molecular Basis of Disease*.

2. Lis-López L, **Bauset C**, Seco-Cervera M, Macias-Ceja D, Navarro F, Álvarez Á, Esplugues JV, Calatayud S, Barrachina MD, Ortiz-Masià D, Cosín-Roger J. P2X7 Receptor Regulates Collagen Expression in Human Intestinal Fibroblasts: Relevance in Intestinal Fibrosis. Int J Mol Sci. 2023 Aug 18;24(16):12936. doi: 10.3390/ijms241612936.

3. Macias-Ceja DC, Coll S, **Bauset C**, Seco-Cervera M, Gisbert-Ferrándiz L, Navarro F, Cosin-Roger J, Calatayud S, Barrachina MD, Ortiz-Masia D. IFNγ-Treated Macrophages Induce EMT through the WNT Pathway: Relevance in Crohn's Disease. Biomedicines. 2022 May 8;10(5):1093. doi: 10.3390/biomedicines10051093.

4. **Bauset C**, Lis-Lopez L, Coll S, Gisbert-Ferrándiz L, Macias-Ceja DC, Seco-Cervera M, Navarro F, Esplugues JV, Calatayud S, Ortiz-Masia D, Barrachina MD, Cosín-Roger J. SUCNR1 Mediates the Priming Step of the Inflammasome in Intestinal Epithelial Cells: Relevance in Ulcerative Colitis. Biomedicines. 2022 Feb 24;10(3):532. doi: 10.3390/biomedicines10030532.

5. Lis-López L, **Bauset C**, Seco-Cervera M, Cosín-Roger J. Is the Macrophage Phenotype Determinant for Fibrosis Development? Biomedicines. 2021 Nov 23;9(12):1747. doi: 10.3390/biomedicines9121747.

6. **Bauset C**, Gisbert-Ferrándiz L, Cosín-Roger J. Metabolomics as a Promising Resource Identifying Potential Biomarkers for Inflammatory Bowel Disease. J Clin Med. 2021 Feb 6;10(4):622. doi: 10.3390/jcm10040622.

7. Ortiz-Masiá D, Gisbert-Ferrándiz L, **Bauset C**, Coll S, Mamie C, Scharl M, Esplugues JV, Alós R, Navarro F, Cosín-Roger J, Barrachina MD, Calatayud S.

Succinate Activates EMT in Intestinal Epithelial Cells through SUCNR1: A Novel Protagonist in Fistula Development. Cells. 2020 Apr 29;9(5):1104. doi: 10.3390/cells9051104.