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RELEVANCE OF THE VITAMIN D RECEPTOR IN COMPLICATIONS ASSOCIATED WITH CROHN'S DISEASE

Doctoral Thesis

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Que el trabajo titulado "Relevance of the vitamin D receptor in complications associated with Crohn's disease", presentado por la Licenciada en Farmacia **Laura Gisbert Ferrándiz**, ha sido realizado bajo nuestra dirección en el Departamento de Farmacología de la Facultad de Medicina y Odontología de la Universidad de Valencia.

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Abbreviations & acronyms

5-ASA	5-Aminosalicylic Acid
A	Adenine
ABC	Avidin-Biotinylated peroxidase Complexes
a-SMA (ACTA2)	Smooth muscle actin
ACTB	Beta Actin
AJ	Adherent Junction
AMP	Antimicrobial Peptide
APS	Ammonium Persulfate
ATG16L1	Autophagy Related 16 Like 1
bFGF	Basic Fibroblast Growth Factor
BP	Base Pairs
BSA	Bovine Serum Albumin
C	Cytosine
CARD15	Caspase Recruitment Domain-containing protein 15
CCL	Chemokine Ligand
CCR	Chemokine Receptor
CD	Crohn's Disease
CD40	Cluster of Differentiation 40
CDC42	Cell Division Control protein 42 homolog
CDH1	Cadherin-1
CTGF	Connective Tissue Growth Factor
CXC	Chemokine (C-X-C motif)
CYP	Cytochrome p450
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAMP	Damage-Associated Molecular Pattern
DBP	Vitamin D Binding Protein
DC	Dendritic Cell
DEFB1	Beta-defensin 1

DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphosphate
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EMT	Epithelial Mesenchymal Transition
EndoMT	Endothelial Mesenchymal Transition
ER	Endoplasmic Reticulum
ERK	Extracellular signal-Regulated Kinase
FAP	Fibroblast Activation Protein
FGF	Fibroblast Growth Factor
FITC	Fluorescein isothiocyanate
FOX3P	Forkhead box P3
G	Guanine
GALT	Gut-Associated Lymphoid Tissue
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GWAS	Genome-Wide Association Study
H	Hour
HBSS	Hanks' Balanced Salt solution
HIER	Heat-induced epitope retrieval
HLA	Human leukocyte Antigen
IBD	Inflammatory Bowel Disease
IC	Indeterminate Colitis
IEC	Intestinal Epithelial Cell
iFBS	Inactivated Fetal Bovine Serum
IFNG	Interferon gamma
IGF	Insulin-like Growth Factor

IHC	Immunohistochemistry
IL-	Interleukin
IL23R	Interleukin 23 Receptor
Ip	Intraperitoneal
IPAA	Ileal Pouch-Anal Anastomosis
IRGM	Immunity-Related GTPase family M protein
IκBα	Nuclear Factor kappa-light-chain-enhancer of activated B cells Inhibitor, alpha
JAK	Janus Kinase
KO	Knock Out
LAMB1	Laminin subunit Beta-1
LGR5	Leucine-rich repeat-containing G-protein coupled Receptor 5
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
MAGI1	Membrane-Associated Guanylate kinase 1
MALT	Mucosa-Associated Lymphoid Tissue
MAP	Mitogen-Activated Protein
MC	Microscopic Colitis
MHC	Major Histocompatibility Complex
Min	Minute
miRNA	MicroRNA
MMP	Metalloproteinase
mRNA	Messenger RNA
MUC2	Mucin 2
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NFAT	Activated T cell Nuclear Factor
NLR	NOD-Like Receptors

NOD2	Nucleotide Binding Oligomerization Domain 2
NOS	Nitric Oxide Synthase
NPT	Sodium-Phosphate cotransporter
OPG	Osteoprotegerin
OR	Odds Ratio
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PDGF	Platelet-Derived Growth Factor
PDIA3	Protein-Disulfide Isomerase-Associated 3
PE	Phycoerythrin
PMA	Phorbol Myristate Acetate
PRDM1	PR Domain zinc finger protein 1
PRR	Pattern Recognition Receptor
PTPN22	Protein Tyrosine Phosphatase Non-Receptor Type 22
RANKL	Receptor Activator of Nuclear factor kappa-B Ligand
RHOB	Ras Homolog gene family, member B
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Specie
ROS	Reactive Oxygen Specie
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
RT-qPCR	Real-time quantitative PCR
RXR	Retinoid-X Receptor-alpha

SDS	Sodium Dodecyl Sulfate
Sec	Second
SEM	Standard Error of the Mean
siRNA	Small interfering RNA
SMC	Smooth Muscle Cell
SNP	Single Nucleotide Polymorphism
STAT3	Signal Transducers and Activators of Transcription
T	Thymine
TAE	Tris Acetate-EDTA
TBS-T	Tris-buffered saline-Tween
TEMED	N,N,N',N'-tetramethylethylenediamine
TER	Transepithelial mucosal Resistance
TGF-β	Transforming Growth Factor beta
TH	T-helper
TIMP	Tissue Inhibitor of Metalloproteinases
TJ	Tight Junction
TLR	Toll-Like Receptor
TNBS	Trinitrobenzenesulfonic Acid
TNF	Tumour Necrosis Factor
TRPV	Transient Receptor Potential cation channel
UC	Ulcerative Colitis
UVB	Ultraviolet B
VD	Vitamin D
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
VEGF	Vascular Endothelial Growth Factor
VEO IBD	Very Early Onset Inflammatory Bowel Disease
WT	Wild Type

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Abstract

Crohn's disease (CD) is a chronic gastrointestinal inflammatory disorder with an unknown aetiology with alternating periods of relapses and remissions. The transmural involvement of CD leads to complications, such as strictures or fistulas, that often require surgery. Fibrosis is usually detected in CD and is characterized by a dysregulation of the equilibrium between the production and the degradation of the extracellular matrix. There is no effective treatment for intestinal fibrosis in CD, which makes a better understanding of the etiopathogenesis of CD essential.

GWAS studies have revealed single nucleotide polymorphisms (SNP) in different genes associated with CD. Among them, TaqI SNP in the vitamin D receptor (VDR) gene has been reported as a risk factor for CD. VDR is a member of the nuclear receptor family of transcription factors, and vitamin D constitutes its main ligand. VD deficiency and defective signalling have been reported in CD patients. In recent years, PDIA3 has emerged as a vitamin D receptor.

The aim of this work was to analyse the relevance of genetic polymorphisms and the VD/VDR/PDIA3 pathway in CD complications. First, we detected that homozygosity for the C allele in CD patients was associated with reduced VDR protein levels and higher expression of IL-1β in PBMCs, as well as an elevated lymphocyte activation and a higher probability of deriving towards a penetrating phenotype.

We also observed a lower expression of VDR and a higher expression of PDIA3 in intestinal resections and in isolated intestinal fibroblasts from CD patients *vs* controls. PDIA3 levels correlated with COL1A1 levels in intestinal tissue, and down-regulation of the PDIA3 receptor in control fibroblasts was associated with decreased COL1A1 expression and increased VDR protein levels. In addition, a reduced expression of VDR and an increase in PDIA3

were detected in fibroblasts from control subjects who were homozygous for the C allele in the TaqI SNP of VDR, providing strong evidence of a crosstalk between the two VD receptors.

VD treatment increased VDR protein levels in control fibroblasts. VD also induced a slight increase in VDR expression and a decrease in PDIA3 in fibroblasts isolated from the damaged mucosa of CD patients.

Finally, the systemic treatment of mice with VD prevented intestinal fibrosis induced by the heterotopic transplant model. The effects of VD were mediated by modulation of the immune system and a direct action of VD on intestinal fibroblasts.

In summary, this study demonstrates reduced VDR protein levels in PBMCs and fibroblasts from CD patients carrying the TaqI SNP of the VDR gene, and shows that these patients are more prone to develop a penetrating phenotype. VD increased VDR and decreased PDIA3 expression in intestinal fibroblasts from CD patients, which correlates with a reduced expression of markers of fibrosis. These effects may be involved in the anti-fibrotic effect of VD demonstrated in the in vivo murine model.

Resumen

La Enfermedad Inflamatoria Intestinal (EII) comprende un grupo de enfermedades crónicas que cursan con inflamación y afectan al tracto gastrointestinal. Están caracterizadas por periodos de brotes y de remisiones clínicas. Aunque su causa es desconocida, se sabe que las complejas interacciones entre factores inmunológicos, microbianos, ambientales, nutricionales y genéticos juegan un papel importante en su etiología. Las dos principales manifestaciones son la Enfermedad de Crohn (EC) y la Colitis Ulcerosa (CU). Aunque comparten características, son patologías claramente diferenciadas. La CU se asocia con inflamación y ulceraciones en las capas mucosa y submucosa principalmente del colon. La EC puede aparecer en cualquier parte del tracto gastrointestinal de forma discontinua y la inflamación es asimétrica y transmural, formando granulomas no necróticos y fístulas. La mayoría de los pacientes con EC debutan con un fenotipo considerado inflamatorio (B1 en la clasificación de Montreal). Sin embargo, la inflamación subclínica presente en los periodos de remisión de la EC lleva en muchas ocasiones a complicaciones de la enfermedad como pueden ser estenosis (patrón estenosante o B2 en la clasificación de Montreal) y fístulas o abscesos (patrón penetrante o B3 en la clasificación de Montreal), que frecuentemente requieren de cirugía. Ambos patrones se caracterizan por la presencia de fibrosis intestinal. Este proceso es esencial en la cicatrización de la herida, un mecanismo fisiopatológico de reparación que conduce al depósito de diferentes proteínas en la matriz extracelular (MEC, red de macromoléculas extracelulares formadas por colágeno, glicoproteínas y enzimas, cuyo objetivo es proporcionar soporte a las células circundantes). Sin embargo, el desequilibrio entre la producción y la degradación de estas proteínas puede convertirse en una fibrogénesis incontrolada y persistente, causando un depósito excesivo de la MEC. Las citocinas, las quimiocinas y diversos tipos celulares como los fibroblastos están implicados en el proceso

de fibrogénesis intestinal, que incluye la activación de miofibroblastos y otras células productoras de colágeno, la producción de TGF-β1, el daño celular, el reclutamiento de células inflamatorias y la producción de especies reactivas de oxígeno (ERO).

La EII puede ser considerada una enfermedad global con diferente prevalencia e incidencia en el mundo. Inicialmente, su incidencia se encontraba en los países más desarrollados, sin embargo, en los últimos años cada vez es más frecuente en países de Asia o el este de Europa.

Los factores principalmente implicados en el desarrollo de la EII son elementos ambientales como el tabaco, la dieta o el estrés sumado a una alteración en la microbiota intestinal y en la respuesta inmune que lleva a una disrupción en la barrera intestinal y a un fallo en la defensa contra los microbios intestinales. La penetración de las bacterias en el contenido luminal, estimula respuestas del sistema inmunitario adaptativo promoviendo procesos inflamatorios crónicos, causando los daños característicos de la EII.

El componente genético también ha sido ampliamente estudiado en la EII como un factor etiológico de la enfermedad. Un pequeño porcentaje de los pacientes con EII poseen historia familiar de la enfermedad y se ha demostrado que el riesgo de padecerla depende del grado de parentesco. Se han descrito más de 50 genes que predisponen a padecer EII. Gracias a estudios de asociación del genoma completo (GWAS) se han descubierto numerosas variaciones genéticas asociadas a la EII. La mayoría de estas variaciones implican el cambio en una sola base del ADN, son llamadas polimorfismos de nucleótido simple (SNP). El primer y más estudiado gen con susceptibilidad a la EC es un receptor de reconocimiento de patrones, llamado dominio de oligomerización por unión de nucleótidos que contiene

la proteína 2 (NOD2). Además de incrementar el riesgo de sufrir la enfermedad, mutaciones en NOD2 están asociadas a pacientes con una EC ileal, estenótica y con enfermedad perianal.

La mayoría de los genes implicados pertenecen a procesos relacionados con la EII, como la regulación de la respuesta inmune, la barrera intestinal o la autofagia. Entre ellos se ha descrito un polimorfismo genético en el receptor de la vitamina D, VDR como factor de riesgo para sufrir la enfermedad de Crohn, el *rs731236* o TaqI. Este polimorfismo consiste en el cambio de una citosina por una timina en la secuencia de ADN en el exón 9 del gen VDR, lo que no produce ninguna alteración en la secuencia de aminoácidos de la proteína, es sinónima. La vitamina D (VD) o colecalciferol, es una hormona secoesteroide con funciones paracrinas y autocrinas esenciales en el metabolismo del calcio y la mineralización ósea. Esta forma es una pro-hormona no activa que se puede sintetizar bajo la acción de los rayos ultravioleta u obtenerse de la dieta. El colecalciferol sufre dos hidroxilaciones, una en el hígado y otra en los riñones, antes de convertirse en su metabolito activo, el calcitriol. Esta molécula se une al VDR que es un factor de transcripción perteneciente a la familia de receptores nucleares. Una vez en el citosol, VDR forma un heterodímero con el receptor X retinoide y se une al elemento de respuesta a hormonas (HRE) en el ADN, dando lugar a la expresión o trans-represión de determinados genes. La proteína disulfuro-isomerasa A3 (PDIA3), una isomerasa que actúa como chaperona en el plegamiento de proteínas, también se ha descrito como receptor de la Vitamina D.

Existe una clara relación entre la Vitamina D y la EII. Diversos estudios describen niveles más bajos de esta vitamina en los pacientes con EII, especialmente con EC. Además, los pacientes deficitarios en Vitamina D

poseen un mayor riesgo de incremento en la actividad clínica y de cirugías. En modelos murinos de colitis en ratones deficitarios de VDR se ha descrito una colitis más severa y mayor mortalidad. Con todo esto, la asociación entre esta vitamina y la enfermedad es clara, sin embargo, los mecanismos celulares y moleculares detrás de estos efectos todavía se desconocen.

OBJETIVOS

El objetivo general de este trabajo es analizar la relevancia de los polimorfismos genéticos y los efectos de la vía VD/VDR en las complicaciones de la EC.

Los objetivos específicos son:

- Analizar la relación que poseen diferentes SNP en genes relacionados con la EC, con las características clínicas de los pacientes con EC.
- Evaluar la influencia de SNP TaqI en el gen *VDR* en las características clínicas de los pacientes con EC así como, en el curso clínico de la enfermedad.
- Determinar los efectos que tiene en células mononucleares de sangre periférica (PBMC) de pacientes con EC el SNP TaqI de *VDR* en: la expresión de VDR, en las citocinas inflamatorias y en la activación de linfocitos.
- Evaluar la influencia de la EC en la expresión de VDR, PDIA3 y COL1A1 en resecciones intestinales humanas.
- Analizar los efectos del SNP TaqI de *VDR* en el gen VDR en la expresión de VDR, PDIA3 y COL1A1 en fibroblastos intestinales humanos.
- Estudiar la influencia de la EC y los efectos de la vitamina D en la expresión de VDR y PDIA3 en fibroblastos intestinales humanos.

- Caracterizar los efectos de la vitamina D en la fibrosis intestinal murina.

METODOLOGÍA Y RESULTADOS

Relación entre las características clínicas de los pacientes de EC y los diferentes SNP en genes relacionados con la enfermedad.

Los pacientes con EC y los controles fueron genotipados y las frecuencias genotípicas se analizaron para diferentes SNPs en genes relacionados con procesos relevantes en la patogénesis de la EII. En los SNP de los genes de NOD2, LAMB1 y ECM1 el genotipo homocigoto para el alelo de riesgo fue significativamente más frecuente en pacientes con EC que en los controles. El análisis de asociaciones entre las características clínicas de la enfermedad y el genotipo, a excepción de *rs731236* en el gen VDR, no mostró ninguna relación significativa y relevante con la localización o el comportamiento en la EC. Sin embargo, algunas tendencias no significativas como, por ejemplo, en el SNP del gen CDH1 o del gen LAMB1, el genotipo considerado como de riesgo, podría asociarse con pacientes con EC que poseen un mejor pronóstico.

El SNP *rs731236* en el gen *VDR* está asociado con niveles disminuidos de proteína en PBMCs y con un patrón en la EC penetrante (B3).

Los pacientes con EC homocigotos para el alelo C de riesgo en el SNP *rs731236* o TaqI fueron diagnosticados a una edad más temprana, lo que es indicativo de un peor pronóstico de la enfermedad. Además, los pacientes con este genotipo exhibieron un mayor riesgo de tener un fenotipo de la enfermedad penetrante (B3) así como de sufrir cirugía. Esta asociación no se observó cuando los pacientes tenían una fístula perianal.

Cuando se analizaron las frecuencias genotípicas del TaqI SNP entre pacientes con EC y donantes sanos se demostró que los pacientes con EC que presentan un fenotipo de comportamiento B3 eran significativamente más propensos a ser homocigotos para el alelo C que los controles, lo que reforzó la observación obtenida en la población de pacientes.

El SNP TaqI no altera la secuencia de aminoácidos de la proteína VDR, es una mutación sinónima. Por ello, el primer análisis fue observar si el polimorfismo cambiaba la expresión de VDR. Para este propósito, el análisis se realizó en dos muestras diferentes.

Primero, se genotiparon para el TaqI SNP dos líneas celulares de monocitos diferentes, y se detectó que la línea celular THP1 poseía un genotipo TT, por el contrario, los monocitos U937 eran genotipo CC. Aprovechando los genotipos de estas células, se analizó el ARNm de VDR y los resultados mostraron una reducción significativa en las células THP1. Sin embargo, las células U937 expresaron niveles más altos de citocinas proinflamatorias que los monocitos THP1.

Posteriormente, se analizaron los niveles de ARNm y proteína VDR en PBMCs de pacientes con genotipo TT y CC. Los resultados no mostraron cambios en los niveles de ARNm de ninguno de los grupos de pacientes. Como el SNP se encuentra en el extremo 5' del exón 9 en el ARNm de VDR, estudiamos si podría estar afectado el proceso de *splicing* y que el cambio de base generara una proteína truncada o no funcional. Sin embargo, el análisis por PCR usando cebadores para amplificar una región entre los exones 7 y 9 reveló el mismo producto de PCR en muestras de pacientes con genotipos TT y CC. Al realizar el análisis de los niveles de proteína VDR, sí se observaron diferencias, los pacientes homocigotos C mostraron niveles más bajos de esta proteína en comparación con el otro grupo.

La disminución de la expresión de la proteína VDR en pacientes con EC homocigotos para el alelo C llevó a investigar los niveles de citocinas pro-inflamatorias en las PBMC de estos pacientes. Cuando los datos se expresaron en relación a los de donantes sanos, los pacientes con genotipo CC exhibieron un aumento en la expresión de ARNm de IL1-β, IL-8, IL-18, IFN-γ y TNF-α. Con esto, se decidió analizar el factor de transcripción NFκB. En los extractos citosólicos de PBMC de pacientes con EC, los niveles de proteína IκBα fueron más bajos en pacientes homocigotos para el alelo C que en aquellos homocigotos para el alelo T. Además, los pacientes con 2 alelos C presentaron niveles más altos de la proteína NFκB p65 en el núcleo, lo que indica un aumento de la translocación nuclear. Tomados en conjunto, los datos sugieren que la disminución de los niveles de proteína VDR en pacientes con EC con 2 copias del alelo C de riesgo en el SNP *rs731236* lleva a un aumento tanto de la activación de la vía de señalización de NFκB como de la expresión de citocinas pro-inflamatorias en las PBMC.

Por último se analizó, por citometría de flujo, sangre entera de pacientes de ambos genotipos. Los resultados mostraron un incremento en los niveles de las integrinas CD11a, CD11b y CD49d en linfocitos de los sujetos CC. Además, la expresión de CD62L (L-selectina) en este tipo de células fue significativamente menor en pacientes CC que en aquellos con el genotipo TT, lo que es indicativo de una mayor interacción de leucocitos con células endoteliales.

En conclusión, estos resultados indican que los pacientes con EC homocigotos para el alelo C en el SNP TaqI llevan asociados niveles reducidos de proteína VDR y una mayor expresión de IL-1β en PBMC, mayor activación de linfocitos y un mayor riesgo de derivar hacia un fenotipo penetrante.

Niveles inferiores de VDR en resecciones intestinales de pacientes con EC

Después del análisis de VDR en PBMC, la investigación se centró en el tejido intestinal, la estructura afectada en la EC. El análisis de los niveles de proteína VDR mostró una disminución significativa tanto en el tejido intestinal como en las criptas epiteliales de pacientes con EC en comparación con tejido control. Paralelamente, también se observó una disminución en la expresión de ARNm de *VDR* y un aumento en la expresión de ARNm de *PDIA3* y *COL1A1* en el tejido de pacientes con EC en comparación con el tejido de control. Finalmente, los resultados mostraron una correlación significativa entre el ARNm de *COL1A1* y la expresión del ARNm de *PDIA3* en el intestino de pacientes con EC.

Esta disminución de VDR en pacientes también se observó en el análisis inmunohistoquímico de los cortes histológicos. No se detectaron diferencias significativas en la expresión de la proteína VDR entre los diferentes fenotipos de la enfermedad.

Existe una acción cruzada entre VDR y PDIA3 en fibroblastos intestinales humanos

Se obtuvieron fibroblastos intestinales de resecciones intestinales de pacientes control y se transfectaron con un ARN pequeño de interferencia (ARNip) de PDIA3. Se consiguió reducir en un 80% la expresión del ARNm de PDIA3, así como una ligera reducción en los niveles de proteína PDIA3. En estas condiciones, los resultados mostraron un aumento significativo tanto en los niveles de ARNm como de proteína de VDR en paralelo con una disminución significativa en la expresión de ARNm y proteína de COL1A1.

Los fibroblastos aislados fueron cultivados 24 horas y 7 días. Los fibroblastos cultivados durante 7 días expresaron niveles de ARNm

significativamente más altos de *VDR*, *PDIA3* y *COL1A1* en comparación con 24 h. Estos fibroblastos cultivados 7 días fueron genotipados y clasificados según el genotipo para TaqI y aquellos homocigotos para el alelo C presentaban una expresión reducida de la proteína VDR, así como niveles aumentados de PDIA3 y COL1A1 en comparación con las células que portaban el alelo T.

Esta clasificación por genotipo también se llevó a cabo en monocitos obtenidos de PBMC de donantes sanos. Estas células se polarizaron con IFN-γ, lo que indujo un aumento en la expresión de la proteína VDR en los macrófagos que portaban el alelo T, que no se detectó en los macrófagos que portaban el alelo C. La menor expresión de VDR detectada en las células CC, iba en paralelo con una mayor expresión de IL-1β e IL-6.

Bajos niveles de proteína VDR, tanto basales como estimulados por vitamina D, se detectan en fibroblastos de pacientes con EC

Se obtuvieron fibroblastos de resecciones intestinales de pacientes con EC tanto de la zona dañada como de la zona no dañada del paciente. Al analizar la expresión proteica de VDR, los niveles fueron significativamente más bajos en las células obtenidas de la mucosa dañada por la EC que en los fibroblastos tanto del tejido no dañado del mismo paciente con EC como de los fibroblastos control. También se detectó una reducción significativa en la expresión de ARNm de un gen diana de VDR, el *CYP24A1*, en fibroblastos de pacientes en comparación con los controles. No se detectaron cambios significativos en los niveles de proteína de PDIA3 y COL1A1 entre los diferentes fibroblastos analizados.

El tratamiento con vitamina D, aumentó significativamente los niveles de proteína VDR en los fibroblastos control y también en fibroblastos obtenidos

del tejido no dañado de pacientes con EC, pero solo causó una ligera acumulación de VDR en fibroblastos del tejido dañado de los pacientes con EC. Por el contrario, la vitamina D disminuyó significativamente los niveles de proteína PDIA3 en fibroblastos de la mucosa dañada mientras que no lo hizo en fibroblastos control.

La expresión de ARNm de *VDR*, *PDIA3*, *COL1A1*, *COL3A1* y α-*SMA* no fue significativamente diferente entre los fibroblastos control y los fibroblastos obtenidos de pacientes con EC, pero los niveles de *MMP2* fueron significativamente más altos en los fibroblastos del tejido no dañado de pacientes. El tratamiento con vitamina D aumentó ligeramente los niveles de ARNm de *VDR* en todas las células analizadas y también indujo un aumento significativo en la expresión de ARNm de *PDIA3* y *COL1A1* en fibroblastos obtenidos de la parte dañada del paciente con EC mientras que no modificó la expresión de *COL3A1*, α-SMA o *MMP2*. Sin embargo, se detectó una correlación positiva y significativa entre la expresión de ARNm de *PDIA3* y *COL1A1*, así como entre la expresión de ARNm de *PDIA3* y *COL3A1*, tanto en fibroblastos tratados con vehículo como con vitamina D. Del mismo modo, se detectaron correlaciones significativas entre la expresión génica de VDR y marcadores de fibrosis; *MMP2* se correlacionó positivamente y α-*SMA* negativamente, con los niveles de ARNm de *VDR*.

Se realizaron experimentos para evalúan la capacidad de migración y proliferación de los fibroblastos. Los resultados demostraron que, tras 48 horas, los fibroblastos procedentes de la zona dañada de pacientes con EC migran más rápido que las células control. Por otro lado, el tratamiento de estas células procedentes de pacientes con EC con vitamina D, disminuyó su tasa de migración.

La vitamina D es capaz de reducir la fibrosis intestinal en un modelo murino

Finalmente, analizamos los efectos del tratamiento con vitamina D sobre la fibrosis intestinal en un modelo *in vivo*, mediante el uso de un trasplante heterotópico de un trozo de colon de ratón.

La evaluación de la expresión de VDR en el modelo de fibrosis reveló que los injertos intestinales obtenidos 7 días después del trasplante mostraron una disminución significativa en los niveles de proteína VDR en comparación con los injertos intestinales en el día 0. Además, estos injertos de 7 días mostraron un aumento en la expresión de proteínas de COL1A1 y la relación pSTAT3 / STAT3.

Se administraron diferentes dosis diarias de vitamina D (1 µg / kg ó 2 µg / kg, i.p.) o su vehículo a ratones receptores y se obtuvieron sus injertos 7 días después del trasplante. El análisis histológico del colon mostró una disminución en la deposición de colágeno y una arquitectura histológica preservada en los injertos de colon obtenidos de ratones tratados con vitamina D en comparación con los ratones tratados con vehículo. El tratamiento con vitamina D a la dosis de 1 µg / kg no alteró significativamente la expresión de ARNm de ninguno de los marcadores analizados, pero a la dosis de 2 µg / kg, la vitamina D redujo significativamente la expresión de ARNm de *Col1a1* y *Pdia3* mientras que no modificó significativamente la expresión de *Vdr*. Se detectó un aumento leve pero no significativo en la expresión proteica de VDR en injertos intestinales de ratones tratados con vitamina D, pero encontramos una ligera disminución en los niveles de proteína PDIA3 en paralelo con niveles de proteína significativamente más bajos de COL1A1 en injertos de ratones tratados con 2 µg / kg de VD. Estos injertos también exhibieron una reducción significativa en los niveles de proteína vimentina en comparación con los injertos de ratones tratados con

vehículo. Además, se detectó una correlación significativa y positiva entre la expresión de ARNm de *Pdia3* y *Col1a1*.

El tratamiento con vitamina D no alteró significativamente la infiltración de macrófagos según lo analizado por la expresión de ARNm de F4/80. Sin embargo, ambas dosis de vitamina D alteraron el fenotipo de macrófagos y el perfil inflamatorio en comparación con el vehículo. Se detectó una disminución significativa en la expresión de ARNm de *Cd86* en paralelo con una disminución significativa en la expresión de ARNm de *Il-6*, mientras que se observaron diferencias no significativas en la expresión de ARNm de *Tgf-*β.

En resumen, nuestro estudio demuestra niveles reducidos de proteína VDR en PBMC y fibroblastos aislados de pacientes con EC que son homocigotos para el alelo C en el gen VDR, lo cual va asociado con una mayor activación de estas células y una predisposición de estos pacientes hacia un comportamiento penetrante en la EC. Además, los datos también muestran que la vitamina D aumenta VDR y disminuye PDIA3 en fibroblastos intestinales de pacientes con EC, lo que se correlaciona con una expresión reducida de marcadores de fibrosis. Estos resultados apoyan firmemente que los pacientes con EC se beneficiarían del valor terapéutico anti-fibrótico de la vitamina D, claramente demostrado en modelos experimentales. Son necesarios estudios adicionales destinados a conocer los efectos de la vitamina D en pacientes con EC con el polimorfismo TaqI en el gen VDR para así, comprender mejor la relevancia que tendría el genotipar para este SNP de VDR en la decisión del médico a la hora de recetar suplementos de vitamina D a pacientes con EC.

CONCLUSIONES

1. Los pacientes con EC homocigotos para el alelo C en el SNP TaqI del gen *VDR* mostraron:
   - **a)** Niveles reducidos de proteína VDR y mayor expresión de citocinas proinflamatorias en PBMC.
   - **b)** Mayor activación de linfocitos.
   - **c)** Disminución en los niveles de proteína VDR en fibroblastos intestinales.
   - **d)** Mayor riesgo de desarrollar un comportamiento penetrante en la EC.
2. En fibroblastos humanos intestinales existe una acción cruzada independiente de VD entre VDR y PDIA3 que regula la expresión de COL1A1.
3. La EC está asociada con niveles disminuidos de proteína VDR en resecciones intestinales, células epiteliales y fibroblastos intestinales los cuales exhibieron una alta tasa de migración.
4. El tratamiento con VD aumenta los niveles de proteína VDR y disminuye la expresión de la proteína PDIA3 en fibroblastos de pacientes de EC. Ambas situaciones se correlacionan con marcadores de fibrosis reducidos.
5. El tratamiento con VD previno la fibrosis intestinal en un modelo murino.
6. Teniendo en cuenta los resultados en las muestras humanas y los experimentos *in vivo*, nuestros datos respaldan que los pacientes con EC se podrían beneficiar del valor terapéutico antifibrótico de la VD.

Index

I. Introduction

1. INFLAMMATORY BOWEL DISEASE (IBD)

Inflammatory Bowel Disease (IBD) includes a group of chronic gastrointestinal disorders characterised by alternating periods of relapses and remissions with and unknown aetiology. The two major forms are Crohn's Disease (CD) and Ulcerative Colitis (UC). It has been a global health-care problem with a continually increasing incidence. The complex interaction between immunological, genetic and environmental factors play a role in the pathogenesis of both diseases. However, they present several differences and they can be handled as different entities[1].

UC is characterised by inflammation and ulceration only in the mucosal and submucosal layers. It usually starts in a continuous manner from the rectum to the more proximal colon with variable extents. The majority of the patients (50-60%) present at diagnosis rectum and sigmoid colon affectation (distal colitis), 20-30% presented with left-sided colitis and 20% with pancolitis (entire colon)[2].

CD can affect any part of the gastrointestinal tract but the ileum constitutes the most common location. In contrast to UC, the inflammation is segmental, asymmetrical and transmural and lesions can co-localize with healthy areas. CD can be distinguished into three main phenotypes: inflammatory, fistulizing, and stricturing[3,4].

1.1. Epidemiology of IBD

IBD can be considered as a global disease. It occurs around the world but with differences between prevalence and incidence. This can be explained by the variability in the distribution of susceptibility genes, environmental

exposures, advances in the health-care system and the differences in the surveillance of IBD among the countries.

Since the middle of the twentieth century, the incidence and prevalence of UC and CD have increased in North America, Europe, Australia and New Zealand[5]. Now, 3.1 million Americans and 2.2 million Europeans suffer from IBD, rates that are increasing annually[6]. Similarly, the incidence of IBD is highest in the Western world, ranging from 10 to 30 per 100,000. However, in the last decades, the incidence is increased, not only in the more common industrialized countries but also, even more in countries such as Asia and Eastern Europe suggesting the influence of the environment in the occurrence of the disease[7].

Overall, the prevalence in UC and CD is different among countries. Europe has a higher prevalence of UC and Australia of CD; in North America, both diseases are equally prevalent[5].

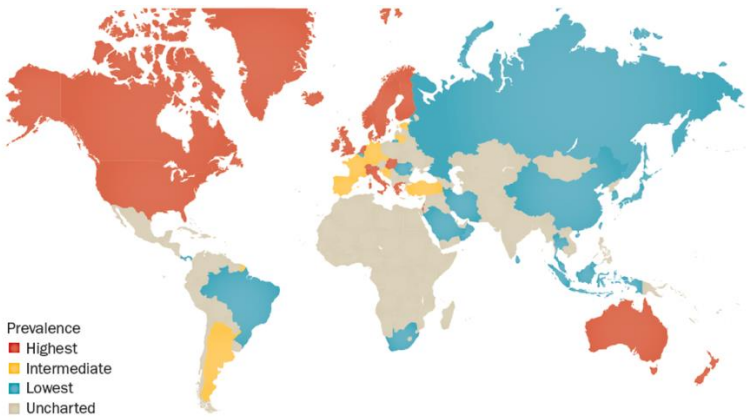


Figure I. Worldwide prevalence of IBD[5].

According to the age of on-set, UC is diagnosed around 30-40 years and CD between 20-30 years of age[7]. There is not an evident conclusion about

differences in the incidence of IBD related to gender. The same happens with ethnicity; the risk of IBD is three-fold higher in Jewish, especially in Ashkenazi population than in non-Jewish populations[8]. In spite of that, IBD affects individuals of all ethnic groups whose families have lived in the Western world for many generations.

There is no curative treatment for these diseases and treatment only persecutes to extend the time of clinical remission, which increases the prevalence and the health-care cost. Before the introduction of biological therapy, the costs were driven by surgery and hospitalizations. In the last years, surgery has decreased and the quality of life of patients has increased due to the biologic agents. However, the use of these therapies has led to a considerable rise in health-care costs. In Europe, the direct health-care cost from IBD patients is estimated to be €4.6-5.6 billion annually[5].

1.2. Aetiology of IBD

Several factors have been associated to IBD aetiology, but it remains largely unclear. The development of the disease results from the interplay between genetic susceptibility, alterations in the composition of the intestinal microbiota, a dysregulation of the immune system, and environmental factors such as diet, smoke or stress, resulting in an abnormal mucosal immune response and compromised epithelial barrier function.

1.2.1. Environmental factors

The gastrointestinal tract is under constant environmental exposure, and a large number of environmental factors play an essential role in the pathogenesis of IBD. Among others, smoking, diet, drugs, stress, air pollution, geography and psychological elements have been reported[9]. Smoking is one

of the risk factors most studied. In 1982, it was described for the first time the protective effect of smoking in the development of UC[10,11]. The percentage of smokers (more than seven cigarettes per week) between UC patients is about 10-15%, lower if it is compared with the control population (25%-40%). Currently, smoking decreased the risk for UC (Odds ratio [OR]: 0.58). In contrast, smoking increases both the risk of developing CD (OR: 1.76)[12] and the recurrence after surgery. There are several hypotheses to explain the protection and the risk in IBD smokers. Probably it can be explained by the differences in aetiology, location and immune response between these diseases. However, none have demonstrated the reason behind the divergent effect on CD and UC convincingly[8,13].

Appendectomy seems to have the same controversial effect in both diseases, protecting from the development of UC. This protection can be caused by the inflammation of the appendix rather than the mere removal of the organ.

Life-style and stress can influence gut inflammation and causes IBD. There were described various mechanisms via the hypothalamus-pituitary-adrenal axis, resulting in the activation of inflammatory cells and alteration of the permeability in the gut[14].

Some pathogens have been studied as an indirect trigger of IBD but there is not a clear mechanism. Furthermore, studies demonstrated that the infection of some pathogens such as *Clostridium difficile* are frequently associated with the relapse in those with established IBD[8]. There is not diet responsible for IBD, but it is assumed that some nutrients like meat and fats, especially polyunsaturated fatty acids and omega-6 fatty acids in higher amounts increase the risk of developing IBD while others such as fibre, fruits,

and vegetables would reduce it[15,16]. Of note, the consumption of higher levels of fibre, mainly fruits, had 40% less risk of developing CD whereas no relationship was observed for UC[6,17]. Further research is required to clarify whether diet alone is a cause of IBD or if it is the interplay between diet and the microbiota.

Malnutrition is one of the complications associated with IBD, and it is responsible for weight loss. This condition affects the 65-75% of patients with CD and in 18-62% of patients with ulcerative colitis[18]. This complication is worse in the paediatric population and it is mainly caused by the reduced food intake, medications and malabsorption, among others. The malabsorption leads to the deficiency of micronutrients and vitamins in IBD patients. Several studies suggest that vitamin D (VD) deficiency might have a role in the pathogenesis and development of IBD. This deficiency is common in patients at the diagnosis. Different studies have examined in different populations that low levels of this vitamin are associated with IBD, especially with CD[19,20]. VD deficiency has been demonstrated to be a risk for the development of colorectal cancer in people with IBD and the increased of each 1 ng/ml of VD in plasma reduce the cancer risk in an 8%[21].

1.2.2. Epigenetic and genetic factors

The genetic component in the aetiology of IBD has been widely studied. It is reported that between the 10-20% of IBD patients have a family story of IBD and this risk is directly dependent on degree relative. Indeed, studies in monozygotic and dizygotic twins have provided the best evidence for genetic predisposition to IBD, especially in CD, showing a concordance of 37% in monozygotic and 4% in dizygotic twins[22]. Nevertheless, these differences in monozygotic twins put in relevance the role not only of genetics in IBD but

also the epigenetics and the environment. Indeed, genetic variants only explain 20-25% of all IBD cases[23].

From the genetic point, IBD is complex to study due to the polygenic condition (except for rare cases)[24] of the disease. Since 2005, more than 50 candidate genes and more than 240 risk loci predisposing to IBD have been discovered thanks to the genome-wide association studies (GWAS). The aim of these studies is the evaluation of the most common genetic variants in different individuals to see whether any variant is associated with a specific trait. The majority of variants analysed are the changes in one DNA's nucleotide, called single nucleotide polymorphism (SNP).

CD and UC share some susceptibility loci, but they differ in most of them. The first gene associated to IBD was an intracellular pattern recognition receptor of bacteria, the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) in which the presence of an insertion mutation at position 3020, increased the susceptibility to developing CD (OR: 3.01)[25]. The activation of the protein NOD2 leads to the nuclear factor-κB (NFκB) activation and induces autophagy in dendritic cells (DCs); in cells with susceptibility variants in NOD2 gene, deficient autophagy induction and reduced localization of bacteria in autophagolysosomes was detected. Strong genetic associations were also seen in other genes such as interleukin 23 receptor (IL-23R) in IBD (OR: 2.01) and human leukocyte antigen (HLA) in UC (OR: 1.44)[26]. The majority of susceptibility genes are involved in the regulation of the innate immune response (NOD2, CARD15), autophagy (ATG16L1, IRGM), cytokines (IL-23R, IL-10) or intestinal barrier disruption (ECM1, LAMB1)[27].

Although there is much to discover in IBD susceptibility genes, the influence of SNPs in patient characteristics, the clinical course and the functional relevance of them, has been seldom studied. Few studies have related these genes with a specific clinical phenotype[28-30], for instance, NOD2 is associated with a fibrostenotic ileal CD and with perianal fistula complication while Janus kinase 2 (JAK2), an intracellular tyrosine kinase that transduces cytokine-mediated signals via the JAK-STAT pathway, has an increased risk of ileal and stenosing CD behaviour[31].

Epigenetics is the study of the mechanisms of gene expression regulation without alterations in the DNA sequence. It can establish the relationship between genetics and environment that determines a specific phenotype. As interactions with the environment can caused IBD, epigenetics may play an important role in this pathology and its study could provide new insight into the pathogenesis, the diagnoses and new therapies of IBD.

The most studied epigenetic mechanism is DNA methylation and an early studied showed a global hypomethylation in UC patients compared with healthy controls[32]. This situation and hypermethylation of certain tumour suppressor genes can increase the risk of colon cancer. Another study that evaluated changes in DNA methylation in peripheral blood and tissue from IBD patients showed five genes in UC patients (CXCL14, CXCL5, GATA3, IL-17C and IL-4R) that were hypermethylated compared to healthy controls. The methylation pattern was different in CD patients. The detection of changes in methylation of the DNA in some specific genes in peripheral blood can be used as potential diagnose biomarker of the disease[33].

MicroRNAs (miRNAs) are small non-coding RNAs, which act as post-transcriptional regulators of gene expression. It is estimated that miRNAs

regulate more than 60% of protein-coding mRNAs and that more than one-third of human genes are targets for miRNA regulation. These molecules play an important role in the development, regulation and differentiation of the immune system so are differentially expressed in chronic inflammatory diseases like IBD. A previous study that compared colonic tissue from CD patients and healthy controls found that 23 miRNAs were remarkably upregulated in the former[34]. One of the most studied miRNA associated with IBD was miR-21. There is also murine studies with a miR-21 knock-out (KO), where the induction of colitis increased the survivor rate and generated less inflammation compared to wild type group (WT), due to the modulation of the RhoB and CDC42 expression, that regulates the intestinal barrier function[35]. Finally, some miRNAs such as miR-192, miR-122, miR-29 y miR-146a, that modulate the expression of genes that regulate autophagy like NOD2, ATG16L1 or IRGM, have been associated with a major risk of IBD development[36].

1.3. Diagnosis and classification

IBD is diagnosed according to symptoms, biochemical analysis and histological, endoscopic and radiological investigations. The biochemical studies included the determination of erythrocyte sedimentation, C-reactive protein or complete blood count. The confirmed diagnoses in most cases is a sigmoidoscopy or a colonoscopy that allows the visualisation of typical damage from IBD. A biopsy is also taken in the endoscopic technique for histological analysis.

In addition to the most frequent entities, CD and UC, other pathologies are also included in IBD such as pouchitis, microscopic colitis (MC) and indeterminant colitis (IC).

The main symptoms of UC are rectal bleeding, diarrhoea and abdominal pain and some patients develop extraintestinal manifestations. Disease distribution is stratified by the extent of inflammation involvement and severity, according to Montreal's classification[37] (**Table 1**).

CD is characterised by a segmental inflammation that can even go through the mucosa and causing a transmural affection. Some patients present at diagnosis an inflammatory phenotype; however, stenosis and penetrating complications are frequent in the disease and require surgery in many cases. Extraintestinal manifestations are also present in CD. Patients are classified by age at diagnosis and those with very early onset (VEO-IBD) usually exhibit a complicated course. Location of the damaged areas is also important, being the ileum the most frequent, but it is also detected in the colon, ileocolonic and upper regions of the gastrointestinal tract. All these characteristics are present in Montreal's classification[37] (**Table 2**).

Although Montreal's classification has been established as a helpful tool for clinicians, the recent discovery of serological markers, specific genetic and epigenetic markers may modify this classification, to adapt it better to the reality of the disease and to improve the treatment and the management of the patients.

In some cases, a potentially curative surgery, especially for UC patients, is the total proctocolectomy followed by ileal pouch-anal anastomosis (IPAA). Pouchitis is the non-specific inflammation of this faecal reservoir and it is a common complication that affects the 30% of UC patients[38]. The symptoms of pouchitis are many and can include increased bowel frequency, urgency, tenesmus, incontinence, rectal bleeding, and abdominal pain[39].

In the MC the bowel mucosa usually appears normal during colonoscopy. This term includes the Lymphocytic Colitis and Collagenous Colitis. These conditions are very similar and tend to cause the same symptoms, causing chronic diarrhoea[40].

It is called IC when there is an inflammatory entity but it results difficult to diagnose the patient into UC or CD. It affects 10-15% of cases of IBD[41].

Table 1. Montreal's classification of UC.

EXTENT		SEVERITY	
E1	Proctitis (rectum)	**S0**	Clinical remission
E2	Left sided UC (distal UC)	**S1**	Mild
E3	Extensive UC (pancolitis)	**S2**	Moderate
		S3	Severe

Table 2. Montreal's classification of CD.

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)

1.4. Treatment

As previously commented, IBD is characterised by the alternation of inflammatory episodes with phases of clinical remission. Therefore, the current treatment has two objectives: control the symptoms in a short-term and prolong the periods of remission and restrain the course of the disease. First, therapy is adjusted to the type and severity of the pathology and it is changed according to the clinical evolution of the patients. Therapy is based on nutrition, anti-inflammatory and immunomodulatory drugs and biological therapy alone or in combination following a "step-up" principle.

For mild IBD, patients are usually treated at induction and maintenance with 5-aminosalicylic acid (5-ASA) and/or budesonide. For moderate diseases, immunosuppressants such as corticosteroids or azathioprine and, in some cases, biological therapies are typically used. In severe or refractory IBD, biologic therapy such as anti-tumour necrosis factor (TNF) drugs (infliximab, adalimumab), anti-IL-12 and IL-23 cytokines (ustekimumab) or anti-integrin $\alpha_4\beta_7$ (vedolizumab) are the most used alone or in combination with azathioprine. In some cases, patients do not respond to the different therapies and surgery is their option. In spite of that, especially in CD patients, after surgery the disease appears again, becoming in a pathology with a complicated managed.

2. IBD AETIOPATHOGENESIS

IBD can be the result of an impaired intestinal mucosal barrier, an altered gut microbiota and dysregulated host immune response, that can lead to a failure to defend against intestinal microbes. As a consequence, bacterial translocation and the penetration of the luminal content is produced,

stimulating adaptative immune responses and the promotion of chronic inflammatory processes, causing the characteristics injuries of IBD.

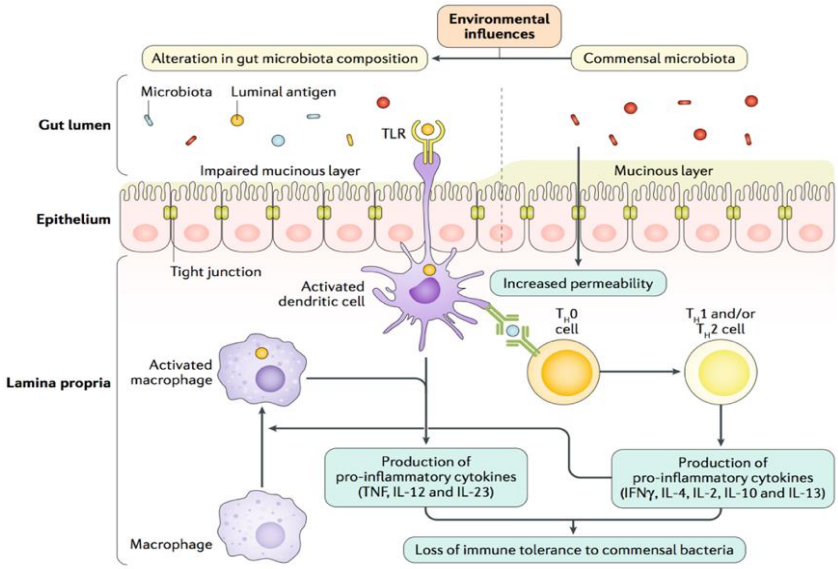


Figure II. Mechanisms in IBD pathogenesis[17]

2.1. Microbiota

The microbiota in the intestinal tract is formed by up to 1,000 different species including 10^{14} bacteria but also some eukaryote, viruses and archaea making a weight of 1-2 kg. At the birth, the digestive tract is sterile but it starts promptly to be colonized. The gastric and bile acid complicated the life of the bacteria in the stomach, duodenum and jejunum, so they are mainly in the ileum and colon. The colon has the majority of the bacteria, more than 10^{12} per gram predominated by anaerobic groups such as *Bacteroides* or *Bifidobacterium* that can be influenced by the diet, lifestyle or host genetics, among others.

In a healthy person, a host-microbiota mutualism exists, in which both receive benefits. Bacteria are benefited with a positive environment and nutrients and the host receives the decomposition of nondigestible nutritional components or the synthesis of certain vitamins. In the eubiosis, where there is a balanced commensal microbiota, intestinal epithelial cells secrete mucins and antimicrobial peptides (AMPs), and pathogen-associated molecular patterns (PAMPs) induce the secretion of epithelial cytokines (IL-33, IL-25 and transforming growth factor β, TGF-β) that promote the development of tolerogenic DCs and macrophages. A physiological state of intestinal inflammation (tolerance) is induced and maintained[42].

In CD, abnormalities in the gut microbiota causing a dysbiosis have been demonstrated. An increased in the presence of Bacteroidetes and Proteobacteria and a decreased of Firmicutes and bacterial diversity are detected[43]. In addition, a decrease in some specific and beneficial bacteria such as *Faecalibacterium prausnitzii*, which have anti-inflammatory properties, was found in patients with CD associated with an increased risk of postoperative recurrence after resection for ileal disease[44]. A question that is not even clear is if IBD-associated dysbiosis is a cause or a consequence of the pathology and if dysbiosis alone is sufficient to induce IBD, or it needs environmental and immune dysregulations. Overall, it is essential a non-problematic host-bacteria coexistence, for the integrity of the epithelial intestinal barrier[42].

2.2. Disruption of the intestinal barrier mucosa

The intestinal barrier mucosa regulates the exchange of nutrients, water and ions between the lumen and the underlying tissues. At the same time, their function also avoids the penetration of exogenous antigens and

microbes, acting as a physical and immunological defence barrier. The elements that form this barrier are: a mucus layer with commensal gut microbiota, the AMPs and secretory immunoglobulin A; the intestinal epithelium formed by different intestinal epithelial cells (IECs); and immune cells in the lamina propria such as T and B cells, macrophages and DCs.

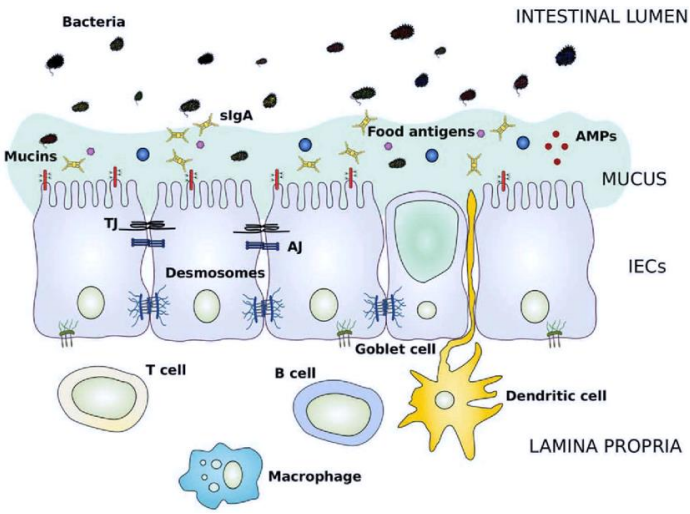


Figure III. Main components of the intestinal barrier[45].

The intestinal epithelium is organized as a single layer of cells forming crypts and villi. It has a high capacity of renewed thanks to the stem cells Lgr5+ present in the base of the crypts from which, all cells types are derived. Two signalling pathways, Notch and Wnt, are crucial for maintaining the proliferative zone in the crypt. The majority of epithelial cells are enterocytes with the main function of nutrient absorption. Other cells are responsible for the secretion to the lumen of different molecules; Goblet cells secrete mucin, Paneth cells secrete AMPs (α-defensins, β-defensins and cathelicidins) and the enteroendocrine cells secrete various gastrointestinal hormones. Microfold cells (M cells) are found in the gut-associated lymphoid tissue

(GALT) of the Peyer's patches in the small intestine, and in the mucosa-associated lymphoid tissue (MALT) of other parts of the gastrointestinal tract. M cells are responsible for the antigen presentation to the underlying immune cells[45].

To maintain the physical intestinal barrier, junctional complexes exists which allow the connection between epithelial cells. The more important are the tight junctions (TJs), the adherents junctions (AJs) and desmosomes. Alterations in the expression or the conformation, for instance, in TJs have been associated with the development of IBD[46]. Moreover, an increased TJ permeability caused by pro-inflammatory cytokines, such as TNF-α and interferon (IFN)-γ, induce apoptosis of IECs[47]. Studies in patients responding to anti-TNF therapy demonstrated a restored intestinal permeability[48]. All these changes provoke the disruption of the epithelial barrier and creates the damage presented in the disease.

IECs also expressed recognition molecules, called pattern recognition receptors (PRR). PRR detected components of the bacteria and PAMPs, such as lipopolysaccharide (LPS), and induced defence mechanisms. The most common PRR are the Toll-like receptors (TLRs) situated in the cell surface and the intracellular NOD-like receptors (NLRs) where is located the NOD2, a gene widely associated with CD[49].

The mucus layer is composed of highly glycosylated mucin proteins that form a gel-like net over the intestinal epithelium. The colon has two mucus layer, whereas the small intestine has only one. Mucin 2 (MUC2), secreted by Goblet cells is the most abundant mucin in both intestines. This protein is critical in the IBD protection, and studies show an exacerbated experimental colitis in Muc2 KO[50]. Within this mucus, there are the AMPs secreted to

protect bacteria from reaching the epithelial surface. In the small intestine, α-defensins predominate. In contrast, in the colon, the most important AMP is the β-defensin. These molecules are relevant in the mucosal homeostasis and a defective expression has been shown in CD patients[51].

2.3. Inflammatory response

The maintenance of the intestinal homeostasis depends on a balance between the intestinal microbiota and the immune response, including innate and adaptative immunity. A dysregulation between them is associated with the development of IBD[52]. As the mechanisms of defence weaken, the intestinal permeability is increased, and also the interaction between the immune system and the microbiota. The persistence of the interaction, allows the loss of the tolerance to the microbiota, and the development of a chronic intestinal inflammation[53].

2.3.1. Innate immunity

The innate immune system is the first line of defence, producing an immediate response against any infection and helping to trigger the adaptative immune response. It is mediated by non-immune cells, such as epithelial, endothelial and mesenchymal cells, and immune cells such as neutrophils, monocytes, macrophages and DCs. The innate immune system is non-specific and does not confer lasting immunity (memory). The infiltration in the mucosa by neutrophils is one of the earliest signs of inflammation, a situation that stays over time. Neutrophils are implicated in the IBD pathogenesis through the impairment of epithelial barrier function, tissue destruction through oxidative and proteolytic damage, and the persistent release of inflammatory mediators[54].

DCs and macrophages derived from different precursors and have different functions, though both present PRR, like TLR and NLR, to respond to pathogens. DCs present in the lamina propria, have a limited response in basal status with less expression of PPR and higher expression of IL-10. Once activated, DCs migrate from their resident tissue to the peripheral lymphoid tissue, where they initiate the adaptative immune response collaborating in the generation of T helper, the differentiation of regulatory T cells and supporting the production of IgA, among others. Nevertheless, tissue macrophages generally do not migrate and they activate adaptative responses locally, presenting antigens to the T cells[55].

In CD and UC patients, increased levels of the TLR2 and TLR4 in human mucosal DCs are shown. In addition, DC from CD patients exhibit higher levels of CD40 and the chemokine receptor CCR7, and produce more IL-12 and IL-6 than control DCs. All these findings are compatible with an activated state of DCs joined to the elevated expression of chemokines that could contribute to the attraction and retention of the cells in the inflamed mucosa and promote the inflammation in CD patients. Interestingly, anti-TNF treatment of CD patients decreased the elevated levels of CD40 on DCs in the intestinal mucosa[42,56,57].

2.3.1.1. Macrophages

Macrophages are a major component of the innate immune system and, with dendritic cells, present foreign antigens to the cells of the adaptative immune system. They are very plastic cells and play distinct functions depending on the microenvironment.

In the intestine, macrophages are known because of their high phagocytic role and for clearing apoptotic cells. They express CD64, CD11c, major

histocompatibility complex (MHC) class II and F4/80. Intestinal macrophages are localized in the lamina propria working to maintain the protection of the intestinal mucosa barrier and to eliminate microorganisms due to their close localization to the epithelial layer and the lumen[58]. Macrophages that reside in the lamina propria express different markers from the peripheral monocytes, they maintain the microbicide effects but they do not produce inflammatory mediators under the microbial stimulus. They have low expression of CD80, CD86 and CD40 and PRRs with a high expression of IL-10. In mice, it has been demonstrated that intestinal macrophages are continuously renewed by peripheral-blood monocytes $LY6C^{Hi}$ and, once differentiated into macrophages upregulate pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF) and produce reactive oxygen species in response to TLR agonist stimulation. These monocytes are probably recruited in response to the low inflammation caused by commensal gut microbiota[59].

There is a traditional macrophage classification, the inflammatory macrophages (M1) and resolving-repair macrophages (M2) which in turn, includes different sub-phenotypes that are involved in wound healing and tissue repair. The process of differentiation to different phenotypes is known as macrophage polarization. This classification nowadays is fairly used however, it will become outdate due to the fact that macrophages represent a highly heterogeneous group of immune cells and the complexity of macrophage subsets far exceeds such simple classification.

IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and/or LPS polarize macrophages towards the pro-inflammatory phenotype which is characterized by the production of cytokines such as IL-1β, TNF-α, IL-6, IL-12, IL-18 and IL-23, chemokines (CXCL9, CXCL10), reactive oxygen-

species (ROS) and nitrogen-species (RNS)[55]. The production of these molecules triggers T-helper (Th)1- and Th17- mediated immune responses and improve the macrophage capacity to eliminate pathogens. Although pro-inflammatory macrophages are essential to fight in an infection, they also produce pro-inflammatory cytokines implicated in the IBD pathogenesis[60].

The other important subgroup of macrophages is induced by IL-4 or IL-13, regulating the activation of Th2 cells, secreting IL-10 and chemokines (CCL17, CCL22, CCL24) and playing a key role in immune regulation. Studies in mice demonstrated that the injection of this type of macrophages in a model of colitis reduced the severity of the disease and increased the production of IL-10[61], which allowed mice to resist inflammation and preserve the intestinal homeostasis.

In the last years, studies in IBD patients have demonstrated how abnormal innate response and altered macrophagic function leads to defective bacterial clearance and granuloma formation. It has been shown that circulating monocytes from CD patients secrete higher levels of pro-inflammatory cytokines, like IL-23 or TNF-α[62] while CD patients with an anti-TNF therapy accumulated macrophages that produced anti-inflammatory cytokines and express the regulatory macrophage marker, CD206[63]. A recent study reports an increased number of CD16+ macrophages that seems to contribute to intestinal fibrosis in the damaged mucosa of CD patients with stenotic or penetrating complications[64].

2.3.2. Adaptative immunity

In addition to the innate response, the organism has a much-sophisticated defence, the adaptative immunity, which is highly specific and confers long-lasting immunity, called memory. The principal cells implicated

are the T and B lymphocytes. In normal intestinal conditions, the macrophages and DCs present the antigen to the cells Th $CD4^+$, which activate the cell T-regulatory (Treg) that migrate to the intestine to induce a tolerogenic and immune response. Once they arrived and mature, these cells can regulate the maintenance and function of IECs.

During wound healing and inflammation, the stimulation of different cytokines differentiates the naïve T-cells $CD4^+$ (Th0) into T helper cell types (Th1, Th2, Th17). IL-12 can induce Th1 and Th2 cells. Th1 cells produced IFN-γ and TNF-α, which causes apoptosis of IEC and differentiation of stromal cells into myofibroblasts which secretes the metalloproteinases (MMPs) and causes extracellular matrix and tissue degradation. Th2 release IL-13, increasing the permeability of the intestinal barrier and IEC apoptosis. Induction of the Th17, in the presence of IL-6, IL-23 and TGF-β, mediated by the activation of the signal transducer and activator of transcription 3 (STAT3), produce IL-17, IL-21 and IL-22 which recruits neutrophils and induces MMPs[65].

Traditionally, it was accepted that CD was mediated by Th1 cells, while Th2 cells were more relevant in UC. In the last years, strong evidence support that Th17 cells also play an important role in the CD pathogenesis. The production, by mucosal lymphocytes, of IFN-γ and IL-17A has redefined this form of IBD as a mix Th1 and Th1/Th17 condition[66]; the higher expression of IL-17 and IL-21 in mucosa and serum of CD compared to UC patients strongly reinforces this observation[67,68]. Furthermore, GWAS have identified genes involved in the differentiation and development of Th17 cells with a CD susceptibility[69]. Different family members of IL-17 cytokines have been described: IL-17A might protect from the inflammation[70] and the IL-17F can

drive to it[71]. Different murine models of colitis were associated with a high production of IL-21 and IL-21 KO mice were protected from colitis [72].

Another important lymphocyte that collaborates to maintain the intestinal homeostasis is the Treg that produces IL-10 and TGF-β and promotes the tolerance and controls the inflammation through the downregulation of the expression of MHC molecules. The transcription factor Fox3p is critical for the development and function of Treg cells and high levels of TGF-β induce it. An impaired frequency and function of these cells has been detected in IBD, which contributes to the development of the immune response against the commensal bacteria. The loss of tolerance generates a chronic inflammation, which is a main characteristic of IBD[73].

Lymphocytes B, generally known for their capacity to produce antibodies, can express pro-inflammatory or anti-inflammatory cytokines, depending on the signals that are present in the mucosa. The lymphocyte B regulatory (Breg), is a subset of cell in charge of the IL-10 production and perform their anti-inflammatory function mainly depending on their secretion of IL-10. They have been implicated in the IBD pathogenesis, where a reduced frequency of $CD19^{high}$ $CD1d^{high}$ Breg cells has been observed in CD patients. These cells can reduce intestinal inflammation in colitis in a Treg-independent manner[52,74].

2.4. Defects in wound healing

Under normal conditions, an injury in the intestinal barrier triggers an intrinsic repair system that can regenerate and heal the wound once the inflammation is controlled. In IBD, this tissue regeneration can be altered, which impairs the healing of the intestinal ulcers caused by the disease.

The epithelial repair response starts with an acute phase called "epithelial restitution" where the existing IECs surrounding the wounded area lose their polarity and quickly migrate to the lesion to initiate wound healing covering the ulcer and restoring the integrity of the epithelial layer. The most important factor in this phase is the TGF-β and other molecules like TGF-α, epidermal growth factor (EGF), IL-1β, and IFN-γ augmented epithelial restitution by induction of TGF-β[75].

Then, there is a later phase of cell proliferation and crypt division promoted by various growth factors such as, EGF or fibroblast growth factor (FGF); cytokines such as IL-1β, IL-6 or IL-22, may modulate this process by inducing the activation of transcription factors such as NFκB or STAT3, which promote the proliferation of IECs[76]; in contrast, IFN-γ and TNF block the proliferation and even the apoptosis of these cells impairing wound healing[77].

As previously commented, the proliferation of the new IECs come from the stem cells LGR5+ located at the bottom of the crypts and the process of differentiation to the different types of IECs is strictly regulated by Wnt and Notch signalling pathways. Studies have discovered that the activation of these pathways is indispensable during tissue repair. Expression of Notch1 receptor and their ligands are increased in the IECs of the inflamed mucosa of the colon[78] and the blockade of Notch activation in mice induces severe exacerbation of the DSS-induce colitis attributable to the loss of the regenerative response within the epithelial layer[79]. The activation of the Wnt signalling pathway by the STAT6-dependent macrophage phenotype promotes the repair of the mucosa in a murine colitis model[80]. Besides,

Wnt5a secreted by the mesenchymal cells in the injury colonic tissue plays a critical role in the regeneration of the crypt structure[81].

3. IBD COMPLICATIONS

IBD are characterized by alternated periods of clinical remission and recurrence. The persistent subclinical inflammation that exists in the remission periods is thought to lead to complications (stricture, fistula, abscesses) that are more frequent in CD and often require surgery. In CD, the location tends to be stable over time, although the behaviour can change. Stricture or fistula is present in 20% of CD patients at diagnosis. The prevalence of a stricturing phenotype is 14.3% and 19.6% of a penetrating behaviour[82]. Unfortunately, surgery is not curative; clinical recurrence is reported in 50% of patients, endoscopic recurrence in 80%, and surgical recurrence in 30%[83]. Other complications include perianal affectation, present in a third of CD patients and skin, joint or eye extraintestinal manifestations present in 50% of patients[4].

In IBD, chronic inflammation runs into fibrosis, causing the narrowing of the intestinal tract, strictures, affecting the 30% of CD and 5% of UC patients[84]. The presentation of fistulas in the course of the disease affects 17-50% of CD patients. Usually, it seems that the presence of fibrosis and strictures leads to the formation of a fistula due to a chronic inflammatory infiltrate and fibrosis is commonly detected surrounding the fistula tract. Together, fistula and stenosis affect 70% of CD patients during their life.

3.1. Intestinal fibrosis

Fibrosis is an essential step of the wound healing, a pathophysiological mechanism of repair that leads to the deposition of different proteins in the

extracellular matrix (ECM). The ECM is a network of extracellular macromolecules formed by collagen, glycoproteins and enzymes among others, which aim is to provide support of surrounding cells. An altered equilibrium between the production and the degradation of these proteins can become in uncontrolled and persistent fibrogenesis, causing an excessive deposition of the ECM. Cytokines, chemokines and diverse types of cells are implicated in the intestinal fibrogenic process, which includes the activation of myofibroblast and other collagen-producing cells, production of TGF-β1, cellular damage, recruitment of inflammatory cells and the production of reactive oxygen species (ROS)[85]. In parallel, MMPs are in charge of the ECM degradation, and they and its inhibitor, the tissue inhibitor of metalloproteinases (TIMP), seems to be altered in the intestinal fibrosis creating a disequilibrium[86]. The inhibition of some MMPs, such as MMP-9 can prevent experimental fibrosis in mice[87]. Nevertheless, the mechanism of regulation of these factors is not clear.

Also, the composition and stiffness of the fibrotic tissue seem to have a role in the evolution of fibrosis. The stiffness can be increased by the release of growth factors like TGF-β with the activation of myofibroblasts. *In vitro* studies suggest this stiffness can trigger the activation of more myofibroblast, generating more fibrosis and demonstrated the stiff ECM as a result but also a source of fibrosis[88].

Despite the therapeutic advances in the disease, there is not a specific anti-fibrotic therapy. Evidence suggests that inflammation is necessary for establishing the fibrosis but it seems to be less important in its progression; therefore, the anti-inflammatory treatments should be ineffective once the fibrosis has started[89]. The presence of inflammation that precedes fibrosis is

also demonstrated in a mouse model of intestinal fibrosis with *Salmonella* infection, in which the delayed elimination of the pathogen represses inflammation, but not fibrosis. Of interest, the early elimination does not prevent fibrosis but ameliorate it[90].

Overall, the intestinal damage in IBD is the result of a dysregulation in mucosa and submucosa of the whole wound healing process, causing chronic inflammation and ulcers. The excess of ECM deposition occurs deeper in the intestinal wall, which may have a role in confining inflammation and prevent tissue destruction and may be responsible of the formation of not only strictures, but also fistulas.

3.1.1. Cellular players in fibrosis

3.1.1.1. Mesenchymal cells

Intestinal mesenchymal cells (fibroblast, myofibroblast, smooth muscle cell) play a role in the intestinal fibrosis and when they are stimulated by inflammation, promote the deposition of collagen in the ECM.

Fibroblast is the most common cell of the connective tissue in animals. Their function in the intestine is the maintaining of the structural integrity of the tissue and they have an important role in wound healing, in regeneration and also in fibrosis. Fibroblast express vimentin, but not α-smooth muscle actin (α-SMA) and desmin[91]. They mainly synthesize collagen and other proteins, being the type of cells not contractile that secrete ECM components. Fibroblasts also produce cytokines and chemokines that modify the inflammatory process. Under the combination of different triggers, IL-1β, TGF-β1 and mechanical tension, fibroblasts are activated into myofibroblasts[92].

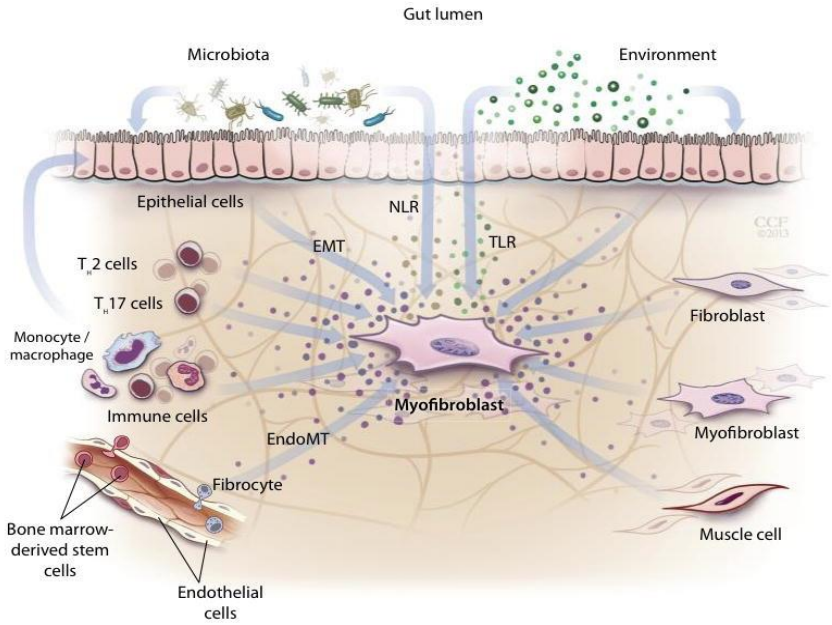


Figure IV. Sources of myofibroblasts[93].

Myofibroblast is the most relevant cell in fibrogenesis. They are highly contractile and they also express both vimentin and α-SMA. When they are activated, synthesize high levels of ECM, which are modulated by pro-fibrotic or anti-fibrotic factors. In contrast with other tissues, in intestinal fibrosis, ECM-produced myofibroblast are derived from various types of cells, becoming more complicated the fibrosis situation. These cells include resident mesenchymal cells (fibroblasts, subepithelial myofibroblasts and smooth muscle cells) also stellate cells, pericytes, bone marrow stem cells and epithelial or endothelial cells through the process called epithelial-mesenchymal transition (EMT)/endothelial-mesenchymal transition (EndoMT)[94]. During EMT and EndoMT, epithelial/endothelial cells get a more spindle shape, loss their classic markers and get the myofibroblasts ones. This transition is induced by cytokines, ECM components, fibronectin and fibrin,

among others[95]. A recent study reveals an increased EMT associated with a WNT2b/FZD4 interaction in CD patients with a penetrating behaviour[96].

Myofibroblast can be activated through multiple mechanisms including paracrine and autocrine signals, PAMP, damage-associated molecular patterns (DAMP) which include DNA, RNA, ATP, high-mobility group box proteins, microvesicles and fragments of ECM molecules. The intestinal microbiota has also been described as a modulator of fibrosis[97,98].

Another important source of myofibroblasts derives from migration and proliferation within the inflamed gut. It has been shown that myofibroblasts from IBD patients proliferate faster than those from controls[99]. In CD strictures there is an overexpression of fibronectin[100] and N-cadherin[101] which enhance myofibroblast migration.

Finally, smooth muscle cells (SMCs) have also a role in fibrosis. They express α-SMA and desmin; but, in pathological conditions, they can differentiate into a phenotype of myofibroblast close to SMCs expressing desmin and vimentin. SMCs in response to TGF-β and IL-1β release IL-6[102] and induce collagen and MMP production, contributing to the fibrogenesis process.

3.1.1.2. Immune cells

Immune cells play an essential role in fibrosis because they activate and differentiate different cells into myofibroblasts through the expression of different pro-inflammatory and pro-fibrotic factors.

Macrophages and DCs act directly over the ECM to regulate the activation of myofibroblasts and their progenitors[103]. After the inflammatory response, the subset of macrophages recruit and their concentrations in the damaged

tissue determine if there is tissue repair or fibrosis. A phenotype of pro-inflammatory macrophages activated myofibroblasts and induce fibrosis; in addition, they may cause more tissue damage and provoke a resistance of myofibroblast to apoptosis. TNF-α is an essential factor in the myofibroblast activation in the wound healing but it is also associated with the chronic inflammation in fibrosis. Macrophages with a repair phenotype are in charge of the production of anti-inflammatory and pro-fibrotic molecules such as IL-4, IL-13, IL-10, TGF-β1, FGF, connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), and Insulin-like growth factor (IGF). Another subtype of macrophages has an anti-fibrotic effect, via STAT1 and NFκB inhibition. These macrophages release IL-10 and Arginase-1 which inactivated other pro-inflammatory macrophages and myofibroblasts. As previously explained, macrophages are very plastic cells and they change the phenotype just as the fibrosis or the tissue repair is moved forward. Nevertheless, the specific factors that regulate these changes *in vivo* are not well-defined yet[104,105].

After damage, neutrophils are the first cells to reach the tissue and their function is to remove tissue debris and eliminate invading microorganisms. If inflammatory neutrophils are not eliminated, they can exacerbate tissue-damage and promote the activation of myofibroblasts through the release of pro-fibrotic cytokines, chemokines, ROS and RNS. Mast cells and eosinophils promote fibrosis by recruiting inflammatory leukocytes and producing pro-fibrotic mediators such as TGF-β1 and IL-13. The role of basophils in fibrosis is less clear, although they may be a source of cytokine[104].

Adaptive cells include subsets of Th cells (Th1, Th2, Th17), Tregs and B cells. The Th17 cells related to CD have been reported with a pro-

inflammatory and a pro-fibrotic immune response. In fibroblasts obtained from strictures from CD patients, elevated levels of IL-17A, MMP3, MMP12 and TIMP1 are found[106]. IL-7 may be regulating fibrosis through the activation of TGF-β and CTGF. Th2 cells are also considered fibrogenic since they secrete IL-13, a pro-fibrotic mediator that stimulates the production of collagen in myofibroblasts. Instead, the Th1 type immune response characterised by IFN-γ expression may have anti-fibrotic activity. The role of Treg in fibrogenesis is unclear, although it could suppress fibrosis induced by Th17 and Th2 cells[104].

3.1.2. Molecular mediators in fibrosis

3.1.2.1. Growth factors

Among the growth factors implicated in fibrosis, TGF-β is the most relevant in almost all tissues and organs. It is produced mainly by macrophages and fibroblasts and its function is to increase the production of ECM proteins especially collagen I and III, to express TIMP1 and α-SMA, and to promote EMT and EndoMT. In physiological conditions, TGF-β regulates the immune homeostasis.

TGF-β is synthesized as a precursor that mature in the Golgi apparatus. During its synthesis and maturation, it is attached to a protective complex in which it remains inactive and allows its association with the ECM. The release and activation of TGF-β are mediated by proteases, such as plasmin, MMP2, MMP9, integrins or thrombospondin. SMAD proteins mediate the canonical TGF-β intracellular signal transduction pathway. In IBD, this pathway may be defective and CD patients overexpress SMAD7, a TGF-β signalling inhibitor and exhibit a reduced levels of phosphorylated SMAD3[107]. In addition to SMAD, TGF-β also modulates other signal transduction pathways, including

mitogen-activated kinases ERK, cJUN and p38. There are three isoforms of TGF-β and two of them, TGF-β1 and 2, stimulate the synthesis of collagen and up-regulate TIMPs, while TGF-β3 has the opposite effect. TGF-β1 also promote fibroblasts contraction[108]. TGF-β and its receptors have been reported to be up-regulated in different intestinal cells of CD patients [109]. This situation might indicate a repair of CD damage by enhancing the process of reepithelization, but might also be detrimental and contribute to the excess of ECM generation and the subsequent fibrosis.

Another pro-fibrotic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), have been shown up-regulated in the serum of CD and UC patients[110]. In vitro studies have demonstrated that bFGF up-regulates collagen production in fibroblasts from CD patients[111]. bFGF modulates fibroblasts proliferation and promotes wound healing, whereas VEGF increases vascular permeability and capillary angiogenesis.

3.1.2.2. Fibroblast activation protein (FAP)

Fibroblast activation protein (FAP) is not detected in normal adult tissues but it is expressed in fibroblasts when they get activated in wound healing. In addition, FAP has also been shown up-regulated in the strictures of CD patients compared to non-stricture CD tissue. This protein is mainly located in the submucosa in α-SMA+ cells and its inhibition can reduce collagen I production[112,113].

3.1.2.3. Cytokines

There are a number of cytokines implicated, directly or indirectly in the process of fibrosis. They are summarised in **Table 3**[104].

Table 3. Cytokines in fibrosis.

CYTOKINE	ACTIONS RELATED FIBROSIS
IL-1	Induces myofibroblast activation. ECM and chemokines and MMPs secretion. Increases TGF-β-induced EMT.
IL-4	Collagen production and fibroblast differentiation.
IL-5	Induces IL-13 and TGF-β production.
IL-6	Stimulates TGF-β production and induces the activation and proliferation of fibroblasts. It is necessary for Th17 cells development.
IL-7	Increases Smad7, inhibits TGF-β production.
IL-10	Inhibits collagen deposition.
IL-12	Induces IFN-γ production.
IL-13	Induces monocyte and macrophage production of TGF-β, activates fibroblast differentiation, collagen production and induces chemokines.
IL-17	Induces collagen, chemokine production and EMT TGFβ1-dependent.
IL-21	Promotes Th2 cells survival and stimulates IL-4 e IL-13 receptor in macrophages. It is necessary for Th17 cells development.
IL-23	Induces IL-17 and TNF-α production.
IL-33	Induces angiogenesis and fibrosis.
TNF-α	Inhibits MMPs and induces TIMP1 expression. In combination with IGF-1, stimulates myofibroblast proliferation and collagen production.
IFN-γ	Inhibits TGF-β activity, fibroblast proliferation and collagen production.

3.1.2.4. Chemokines

Chemokines are chemotactic cytokines that cooperate with cytokines in fibrogenesis recruiting myofibroblasts, macrophages and other relevant cells. The most critical chemokine pathway in fibrosis is the CC- and CXC-

chemokine receptor families; CCL2, CCL3, CCL4 and CCL20 are profibrotic mediators which are elevated in IBD[104].

3.1.2.5. ROS

ROS are involved in any acute or chronic inflammatory process. Oxidative stress is common in IBD patients. ROS regulate several transcription factors by activating protein kinases, inactivating phosphatases or direct redox reactions with transcription factors. They seem to be mediators in the complex process of collagen gene regulation[114].

3.2. Fistula

Fistula is another frequent complication in IBD. It is defined as an abnormal communication between the intestinal tract and another epithelized surface such as other intestinal loops, urologic and genital structures, the gallbladder, the abdominal wall, and any other anatomical structure nearby. The most common fistula in CD is the perianal fistula, which is frequently detected in patients with rectal involvement. Inflammation is associated with fistula development. Notwithstanding, animal models that cause inflammation sometimes cause fibrosis but rarely develop fistula or strictures. Pharmacological treatment in fistula has a limited effect; only anti-TNF seems to have good results, even though in some of the cases surgery is needed.

The particular mechanisms behind the fistula formation are still unknown. The most important process studied in fistula formation is EMT which seems to be activated as a consequence of an impaired wound healing. When the CD-associated fistulas start their formation, epithelial cells migrate to the fistula tract and differentiate into transitional cells. TNF-α, IL-13 and TGF-β

are mediators with a role in this process and in fistula formation[115,116]. In tissue around the fistula, a strong expression of MMP3 and MMP9 and a lower expression of TIMP1, TIMP2 and TIMP3 have been reported which suggest an imbalance in the ECM[117].

4. VITAMIN D

Vitamin D (VD) or cholecalciferol, is a secosteroid hormone with paracrine and autocrine functions essential in calcium metabolism and bone mineralization. This form is a pro-hormone non-active that can be synthesized in the skin from the 7-dehidrocholesterol under the action of ultraviolet rays; 10% of VD is obtained directly from the diet as it is present in some food such as milk, egg or some fish.

4.1. Synthesis and metabolism

Cholecalciferol is bound to the vitamin D-binding protein (DBP) and transported to the liver where, cytochrome p450 (Cyp) catalyses the hydroxylation of vitamin D to convert cholecalciferol into $25(OH)D_3$ or calcifediol. Then, this form is transported by the DBP to the kidneys and it suffers another hydroxylation by the 1α-hydroxylase Cyp27B1 and becomes $1,25(OH)_2D_3$ or calcitriol, the active metabolite of the VD. To control the concentrations of calcitriol, Cyp24A1 hydroxylates the hormone and it becomes it in a less effective metabolite[118].

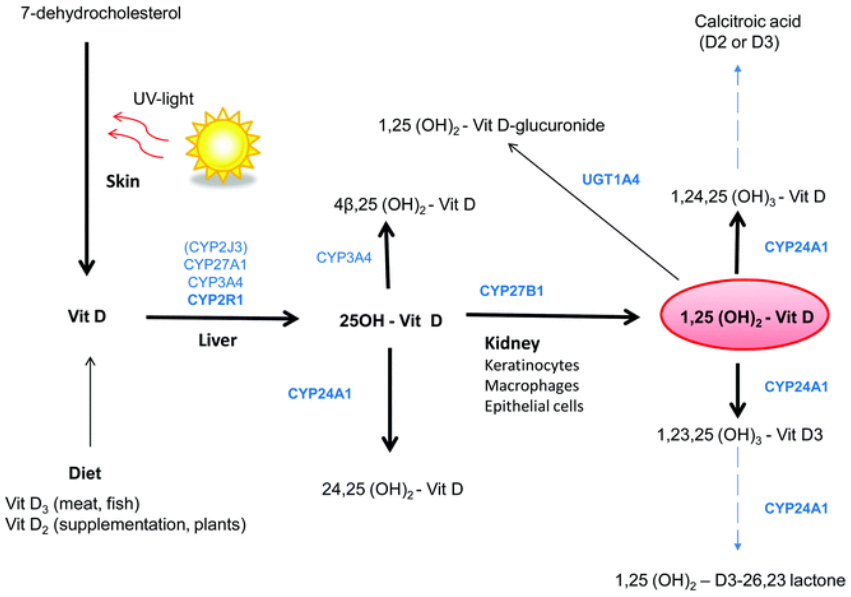


Figure V. Vitamin D synthesis [119]***.***

4.2. Functions

VD has numerous and varied functions. The most important is the maintaining of calcium homeostasis by facilitating the absorption of calcium in the intestine. In addition, calcitriol is essential for the development of bone growth plate, the maintenance of the mineralized skeleton and the control of bone homeostasis, by regulating the balance between osteoblastic formation and osteoclastic resorption. In calcium deficient circumstances, calcitriol induces the RANKL cytokine that causes differentiation to osteoclasts, producing bone resorption and release of calcium that is transferred from the bone matrix to the blood. Calcitriol also acts by inhibiting the expression of osteoprotegerin (OPG), a protein that prevents the binding of RANKL to its receptor[120]. VD also has a role in the parathyroid gland where inhibits the synthesis and secretion of the parathyroid hormone.

Besides to calcium, VD also contributes in regulating phosphate homeostasis in bone, intestine and kidneys[121].

VD has different functions in cellular proliferation and differentiation. Some studies have demonstrated that VD slows the rate of tumour growth through differentiation or apoptosis. Besides, its role in angiogenesis, adhesion and cell migration has also been demonstrated[122].

Anti-infectious and anti-inflammatory properties of VD have been also reported through the upregulation of the expression of antimicrobial peptides and the decrease in the expression of inflammatory cytokines by immune cells[123]. Finally, VD also modulates the innate and adaptative immune system through the regulation of several genes implicated in the function of these cells[124]. The ability of VD to act in numerous tissues has been related to the regulation of the 3% of known genes[125].

The optimal levels of this vitamin are controversial. The majority of experts consider vitamin D insufficiency when levels are below 20-30 ng/ml[126]. Determinants of vitamin D status such as sunlight exposure or diet can modify these levels, but some diseases like calcium-related disorders, diabetes, cardiovascular diseases, cancer and autoimmune diseases which includes rheumatoid arthritis or IBD, have been associated with VD insufficiency[120].

4.3. VD receptors

4.3.1. Vitamin D receptor (VDR)

Several rapid and non-genomic actions of VD have been described[127] but, the majority of the effects induced by this hormone are mediated through VDR[119], which is a member of the nuclear receptor family of transcription

factors. Calcitriol enters into the cell and binds to VDR in the cytosol. Then, the complex VD/VDR is translocated into the nucleus where forms a heterodimer VDR-RXR with the retinoid-X receptor-alpha. This complex binds to specific DNA-sequences called vitamin D responsive elements (VDRE) modifying gene expression. Several VDRE have been shown in the human genome, although the relevance in vivo for most of them remains unknown[128].

VDR is expressed in many cells types and tissues, including skin, immune cells, intestine, kidneys, and reproductive tissue which suggest the different and complex functions that VD and VDR have in the human organism.

VDR is abundantly expressed in epithelial cells of the intestine where mediates the calcium (30%) and phosphate (80%) absorption induced by VD. These processes are mediated by the active transport through canals such as transient receptor potential cation channel (TRPV6 and TRPV5) and the phosphate transport by the sodium-phosphate cotransporter type II (NPT2)[129]. In addition, VDR also plays an immunoregulatory role in the gut[121].

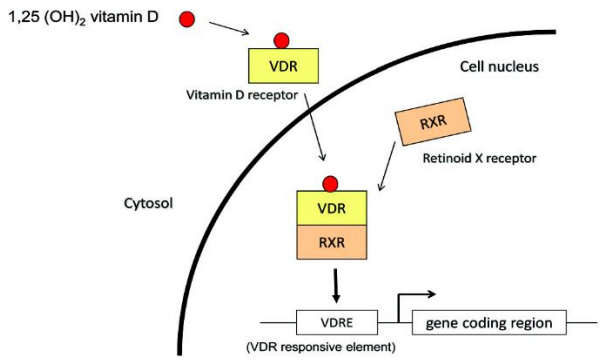


Figure VI. Classic Vitamin D signalling pathway[119].

4.3.2. Protein-disulfide isomerase-associated 3 (PDIA3)

Protein-disulfide isomerase-associated 3 (PDIA3, also known as ERp57) has been revealed in last years as a VD receptor. The first association was discovered in Pdia3 purified from chick intestinal epithelium that showed a binding to VD [130]. PDIA3 is an isomerase enzyme localized in the endoplasmic reticulum (ER), nucleus, extracellular matrix and the caveolae structure of the plasma membrane in many different cells types. It acts as a chaperone in charge of the modulation of folding new glycoproteins and is also part of the MHC I. In addition, PDIA3 is associated with cellular division, cytoskeleton proteins and monocyte/macrophage differentiation[131]. It has been demonstrated that VD binds to PDIA3 and initiates a rapid non-genomic response, a membrane-related cascade reaction, including calcium and phosphate uptake[132]. This binding is also involved in genomic responses that modulate gene transcription and it has been reported that VD initiates the nuclear translocation of PDIA3-STAT3, with a subsequent transcriptional change of STAT3 transcription factor[133]. VD may act as an autocrine regulator and the complex VD-PDIA3 is involved in the reorganization of extracellular matrix in osteoblasts[134]. PDIA3 has also been described as a mediator in the collagen I synthesis in epithelial cells[135].

4.4. VD-VDR and IBD

The relationship between IBD and VD comes from epidemiological studies in which it was shown that the decrease in UVB exposure was associated with a higher incidence of CD. Along these lines, the incidence of IBD is higher in the population living in Nordic countries[136,137]. Several studies have associated low levels of VD in IBD patients and this association may be stronger in CD patients. In addition, low levels of VD influence the disease

course with an increase in clinical activity and surgeries and therefore, a higher rate of hospital admission and prolongation of hospitalization time[19,138]. In a similar manner, low levels of VDR were also shown in IBD patients, in which an inverse correlation between the receptor VDR and tissue inflammation was detected[139]. It is not clear if VD deficiency in IBD patients is a cause or a consequence, due to reduced intake, absorption or solar exposition. On the other hand, VD supplementation in IBD patients with deficiency has been associated with a lower risk of CD surgery and complications[138]. However, the cellular and molecular mechanisms involved in the reported effects of VD in IBD patients are largely unknown.

The relevance of VD in IBD has also been demonstrated in murine models of colitis. Colitis-induced by DSS in VDR knockout mice exhibited increased mucosal damage and pro-inflammatory markers that led to increased mortality[140]. This severe colitis is also observed when VDR is selectively deleted only in epithelial cells[141]. The double KO IL-10/VDR develops accelerated and severe colitis with mortality at 8 weeks[142]. The administration of VD in IBD mouse models has been shown to reduce TNF-α and suppress colitis which strongly reinforces the role of VD/VDR in the intestinal homeostasis and the inflammatory process[143].

4.5. VD-VDR and intestinal barrier

VD/VDR has a protective effect in maintaining the mucosal barrier in the intestine. Vitamin D increases the expression of intercellular junction proteins such as occludins and claudins, as well as E-cadherin, thereby increasing transepithelial mucosal resistance (TER) an indicator of barrier integrity[121]. Besides, it has been demonstrated that VDR signalling has a role in maintaining mucosal barrier in the intestine suppressing epithelial cell

apoptosis[144]. Finally, this hormone through VDR activation modulates NOD2 expression and antimicrobial peptide synthesis[145].

4.6. VD-VDR and microbiota

Alterations in the intestinal barrier allow the translocation into the mucosa of microorganisms and the activation of the immune system. VD can regulates the microbiota, and a deficiency in this vitamin or its receptor, VDR, results in a dysbiosis in the gut[146]. VD deficiency in mice shows an increase of 50 times in the number of intestinal bacteria compared to the group of mice with adequate levels of VD[147]. Moreover, mice without VDR have reduced amounts of bacteria from the *Firmicutes* phylum and increased amounts from the *Bacteroides* and the *Proteobacteria* phyla in the faeces[146]. In addition, it has been demonstrated that a probiotic treatment increases VDR protein[148].

4.7. VD-VDR and inflammation

The majority of cells from the immune system express VDR, whose activation has been related to the activation of innate immunity and attenuation of adaptive response[123]. It is assumed that the VD-VDR pathway exerts an anti-inflammatory action characterized by the decrease of both Th1/Th17 T cells and pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, IFN, and TNF; in addition it promotes Th2 response, increasing Tregs, downregulating T cell-driven IgG production, inhibiting DC differentiation, and helping maintain self-tolerance, while enhancing protective innate immune responses[149]. These actions have been related to the interaction of VDR with other transcriptional pathways, such as p38 MAP kinase, activated T cell nuclear factor (NFAT), NFκB or glucocorticoid receptor. Although the interaction between VDR and NFκB signalling pathways seems to be very

complex, different mechanisms have been described: VDR interference at NFκB DNA binding sites[150], decreased expression of the NFκB components[151] and increased expression of their inhibitors[152], the kidnapping of NFκB in the cytosol by physical interaction with VDR and improved protein stabilization of the NFκB inhibitor, IκBα[153]. On the other hand, the DNA binding of VDR to the NFAT sites inhibits NFAT-dependent expression of IL-2 and IL-17A in lymphocytes[154].

4.8. VD-VDR and fibrosis

In human fibroblasts from control patients, vitamin D regulates the expression of genes involved in cellular functions, such as migration, adhesion, wound healing and expression of inflammatory molecules[155]. A recent study shows that a diet rich in vitamin D prevents intestinal fibrosis associated with a chronic murine model of TNBS-induced colitis. In this model, vitamin D increases the expression of VDR, which modulates the synthesis of collagen, induced by TGF-β1[156].

4.9. VD-VDR and genetics

The VDR gene is located on chromosome 12, which transcribes a protein with protein interaction surfaces formed after VD binding that facilitates interaction with the heterodimer required for specific DNA binding. Genetic studies have described several SNPs in the VDR gene. The most important, called by the restriction enzyme that identifies them, are TaqI (*rs731236*), ApaI (*rs7975232*), BsmI (*rs1544410*) and FokI (*rs2228570*). Some of these polymorphisms are reported to be associated with different diseases such as diabetes[157], psoriasis[158], some types of cancer[159,160] and CD. Although their relation with CD depends on ethnic group, sex or SNP, there is strong evidence that TaqI has a susceptibility to CD in European countries[161,162].

These SNPs have also been shown to contribute to the variation of VD serum levels in IBD patients[163,164].

Few studies report the functional relevance of these VDR SNPs. For instance, FokI polymorphism changes the VDR translation initiation site and it alters functional properties of the VDR altering the VD-mediated gene activation and the enzymes involved in the VD pathway, which seems to affect the bone mineral density and calcium absorption[165]. Furthermore, initial studies reported low mRNA expression of VDR associated with TaqI [166,167] and a higher expression of IL-12 in monocytes of CD patients with TaqI SNP[168]. TaqI is the presence of T or C nucleotides in the exon 9 of the VDR gene. This mutation does not involve changes in the amino acid sequence of the protein, so it is not clear how TaqI affects VD signalling through VDR or if may modify the disease course in CD patients.

Although there is extensive research related to IBD and susceptibility genes, the functional effects that these mutations can produce in the pathway where the gene, in this case, VDR, is implicated and if this finally has an implication in the disease course, is seldom investigated.

II. Aims

The general aim of this work is to analyse the relevance of genetic polymorphisms and the effects of the VD/VDR pathway in CD complications.

The specific aims are:

- To analyse the influence of different SNPs in genes related to CD on the clinical characteristics of CD patients.
- To evaluate the influence of TaqI *VDR* SNP on clinical characteristics of CD patients and the clinical course of the disease.
- To determine the effects of TaqI *VDR* SNP on the expression of both VDR and inflammatory cytokines in PBMCs from CD patients and in lymphocyte activation.
- To evaluate the influence of CD in VDR, PDIA3 and COL1A1 expression in human intestinal resections.
- To analyze the effects of TaqI *VDR* SNP on the expression of VDR, PDIA3 and COL1A1 in human intestinal isolated fibroblasts.
- To explore the influence of CD and the effects of VD in VDR and PDIA3 expression in human intestinal fibroblasts.
- To characterize the effects of VD on murine intestinal fibrosis.

III. Materials & methods

1. REAGENTS

All general analytical grade chemical reagents were purchased from Sigma Chemicals (Steinheim, Germany), PanReac Química S.L.U. (Barcelona, Spain), Merck (Darmstadt, Germany) and Roche Life Science (Penzberg, Germany).

Media and supplements for cell culture were obtained from Gibco™ (Thermo Fisher Scientific, Waltham, MA, USA), Sigma Chemicals and Lonza (Basel, Switzerland).

2. EXPERIMENTS WITH HUMAN SAMPLES

2.1. Blood samples

Blood from CD patients recruited from the Gastroenterology Department of the Hospital of Manises (Valencia, Spain) was collected in BD Vacutainer® tubes (BD™ Biosciences, Franklin Lakes, NJ, USA) with EDTA or citrate. In the study were included European Caucasian patients who had been followed-up and treated in the hospital. Individuals with a disease follow-up less than two years were excluded from the study. This study followed the Helsinki declaration's recommendations and was approved by the hospital's Institutional Review Board. Also, written informed consent was obtained from all patients. A group of 103 CD patients was analysed, and demographic and clinical data from them were collected, including age, sex, age at diagnosis, family history, smoking status, disease location, disease behaviour, presence of perianal fistula, surgery related to CD, appendicectomy, development of extraintestinal manifestations and pharmacological treatment. In addition, 72 European Caucasian healthy controls were recruited. All clinical and demographic data are summarised in **Table 4** and **Table 5.**

Table 4. CD patients characteristics.

CD PATIENTS (n=103)			
GENDER		**BEHAVIOUR**	
Female	57	B1 (Inflammatory)	42
Male	46	B2 (Stricturing)	31
AGE	39.27±12.14 (18-68)	B3 (Penetrating)	30
AGE AT DIAGNOSIS		**PERIANAL FISTULA**	
< 16 years	15	Yes	38
17-40 years	70	No	64
> 40 years	16	**SURGERY**	
FAMILY HISTORY		Yes	44
Yes	11	No	57
No	74	**APPENDICECTOMY**	
SMOKING STATUS		Yes	23
Yes	31	No	76
Ex	7	**EXTRAINTESTINAL MANIFESTATIONS**	
No	54	Yes	26
LOCATION		No	75
Ileal (L1)	55	**TREATMENT**	
Colorectal (L2)	15	Salicylates	4
Ileocolonic (L3)	33	Azathioprine	45
Upper GI (L4)	9	6-mercaptopurine	3
		Biological therapy	81
		No treatment	7

Table 5. Healthy donors characteristics.

HEALTHY DONORS (n=72)			
AGE		**GENDER**	
	39.6 ± 13.00 (18-65)	Female	38
		Male	34

2.1.1. DNA extraction

Genomic DNA isolation and purification from EDTA or citrated blood was performed using the QIAamp® DNA Mini supplied by Qiagen (Hilden, Germany), according to manufacturer's instructions. Briefly, 200 µl of whole blood, 20 µl of proteinase K and 200 µl of Lysis buffer were mix and incubated 10 min at 56°C. Then, 200 µl of 100% ethanol were added and after the mix was transferred into a column with a silica membrane and centrifuged 6000 *x g* for 1 min. After two column washes, DNA was eluted in 200 µl of distilled water. The purity and concentration of the DNA were determined by spectrophotometry, using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific).

2.1.2. Genotyping

The genotyping was done in SNP of genes related to autophagy and the barrier function, two essential processes in the pathogenesis of IBD. The genes analysed were: *ATG16L1, CDH1, ECM1, IRGM, LAMB1, LRRK2, NOD2, PTPN2* and *VDR*.

Genotyping was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). First, DNA fragments containing the different SNPs were amplified by Polymerase Chain Reaction (PCR). In a final volume of 10 µl per sample, were used: 1 µl PCR Buffer (Mg^{2+} plus), 0.8 µl dNTP Mixture (2.5 mM each) and 0.05 µl Taq™ DNA polymerase (0.25

Units), which were all included in the kit TaKaRa Taq™ (TaKaRa Bio Inc., Shiga, Japan); 0.5 µl of the sense primer and 0.5 µl of the antisense primer 10 µM both, 1 µl of genomic DNA and water. The reaction was carried out in the thermal cycler GeneAmp® PCR System 2400 (PerkinElmer Inc. Waltham, MA, USA), following: initial denaturalisation 98°C for 2 min, followed by 35 cycles of 98°C 10 sec, 60°C 30 sec, 72°C 1 min, and a final phase of 72°C for 5 min. The primer pairs for human genes used were synthesized by IDT® (Integrated DNA Technologies, Coralville, IA, USA). Their sequences and the size of the amplified products are shown in **Table 6**.

PCR products were restricted with their respective restriction enzyme mixed with CutSmart™ Buffer 10 X and incubated 2 h. All the enzymes and the CutSmart™ Buffer were from New England BioLabs Inc. (Ipswich, MA, USA). Enzyme incubation temperature and the cuts they perform in the PCR product are summarised in **Table 7.**

Then, restriction fragments were load in 2% agarose gels made with Tris Acetate-EDTA 1X buffer (TAE; Tris 20 mM, pH 7.8; EDTA 0.5 mM and sodium acetate 10 mM) containing GELRED® DNA stain (Biotium, Fremont, CA, USA) to visualise DNA bands. The loading buffer used was 10X Loading Buffer (TaKara Bio Inc.). Also, a 100 bp DNA ladder (TaKara Bio Inc.) was load beside the samples to check the size of the bands. Samples were run in electrophoresis at 100 volts for 40 mi. To visualise them in the gel was used a luminescent image analyser Fujifilm LAS-3000 Imager (Fujifilm, Tokyo, Japan).

Table 6. Primers for SNP genotyping.

GENE	SNP	PRIMERS (SENSE-ANTISENSE)	SIZE (BP)*
ATG16L1	*rs2241880*	5'-TTTCCTTTGCCCCATCCCTC-3' 5'-TCCACAGGTTAGTGTGCAGG-3'	595
CDH1	*rs10431923*	5'-TGTTGCGCAGGCTCAAAATC-3' 5'-TTGCTGACACCAGGACTGTG-3'	438
ECM1	*rs3737240*	5'-CTGCCTTAGTGCTCAGGACC-3' 5'-GCTGGACTGTGGTAGGTTCC-3'	492
ECM1	*rs13294*	5'-GAGAGAAGGGGCCAAGTGTC-3' 5'-ATGTTGTGGATCAGCCCAGG-3'	455
IRGM	*rs13361189*	5'-ATGAACAAGGCCTGGGGATG-3' 5'-TCTGCATGGATAGCTGGCAG-3'	666
LAMB1	*rs886774*	5'-GCCCAAAATCAGCCTGTGTG-3' 5'-ACGTGAAGCAGGGAGGAAAG-3'	489
LRRK2	*rs11175593*	5'-GATGGCCAGAGGGTGTGATG-3' 5'-TTCCTGAGTGGCACAAAGCT-3'	507
NOD2	*rs2066844*	5'-GCTTCTTTGCCGCGTTCTAC-3' 5'-GCTCCTCCTGCATCTCGTAC-3'	425
NOD2	*rs2066845*	5'-ACTTGTGGGTGGCAGTTACC-3' 5'-CCAATGGATTGGAATTGGTC-3'	731
NOD2	*rs2066847*	5'-CTAAGGGACAGGTGGGCTTC-3' 5'-TGCAACCTGCTCCTAACCTG-3'	602
PTPN2	*rs2542151*	5'-AGTTTCCAGGATGAGCAGGC-3' 5'-AAGTGTGGAGCTACACGGTG-3'	478
VDR	*rs731236*	5'-ATGCACGGAGAAGTCACTG-3' 5'-GGTCGGCTAGCTTCTGGATC-3'	328

**Base pairs of the PCR product.*

Table 7. Restriction enzymes.

GENE	RESTRICTION ENZYME	TEMP	RESTRICTION FRAGMENTS
ATG16L1	Bci VI (10.000 U/ml)	37°C	T: 326 + 269 **C**: 595
CDH1	Bts$^{\alpha}$ I (10,000 units/ml)	55°C	G: 323 + 115 **T**: 438
ECM1 (rs3737240)	Bsm I (10,000 units/ml)	65°C	C: 332 + 156 **T**: 287 + 156 + 49
ECM1 (rs13294)	Bsi EI (10,000 units/ml)	60°C	G: 214 + 241 **A**: 455
IRGM	Mlu CI (10,000 units/ml)	37°C	**C**: 355 + 311 T: 355 + 241 + 70
LAMB1	Bsa I (10,000 units/ml)	37°C	**G**: 273 + 216 A: 489
LRRK2	Msl I (10,000 units/ml)	37°C	C: 507 **T**: 193 + 314
NOD2 (rs2066844)	Msp I (20,000 units/ml)	37°C	C: 220 + 65 + 65 + 30 + 25 + 20 **T**: 220 + 130 + 30 + 25 + 20
NOD2 (rs2066845)	Hha I (20,000 units/ml)	37°C	G: 731 **C**: 372 + 359
NOD2 (rs2066847)	Nla IV (20,000 units/ml)	37°C	Ø: 306 + 205 + 91 **C**: 306 + 165 + 91+ 40
PTPN2	Ban II (10,000 U/ml)	37°C	T: 202 + 122 + 154 **G**: 202 + 276
VDR	Taq$^{\alpha}$ I (20,000 units/ml)	65°C	T: 328 **C**: 215 + 113

Restriction fragments: Size in base pairs of the amplified DNA sequence after the restriction. In red: risk allele. T: thymine, C: cytosine, G: guanine, A: adenine, Ø: no base insertion. TEMP: Temperature of incubation.

2.1.3. Isolation of peripheral blood mononuclear cells (PBMCs) and monocytes

Human PBMCs from CD patients and healthy donors were isolated from citrated blood samples. Whole blood was diluted in the same volume of PBS (without Ca^{2+} or Mg^{2+}). Then, the blood was placed carefully over Ficoll®-Paque Plus (GE Healthcare Life Science, Chicago, IL, USA) and centrifuged 400 *x g*, 40 min without acceleration or brake. After centrifugation, PBMCs were separated by gradient density and were contained in the white layer. This layer was collected, washed with PBS and centrifuged 10 min 400 *x g*. The pellet was resuspended in RPMI 1640 medium with L-glutamine and HEPES supplemented with 10% inactivated foetal bovine serum (iFBS), 1% penicillin/streptomycin and 1% sodium pyruvate.

Monocytes were isolated from healthy donor buffy coats obtained in the Dutch National Blood Bank. After PBMCs isolation, Percoll® (Sigma) gradient centrifugation was used to isolate monocytes from the cell suspension. These cells were cultured for 6 days with RPMI culture medium in the presence of IFN-γ (20 ng/ml) or vehicle. The macrophages were treated with VD 100 nM dissolved in ethanol (D1530; Sigma-Aldrich) or vehicle 24 h before collecting cells. These experiments were performed in a research stay in the Tytgat Institute (Amsterdam).

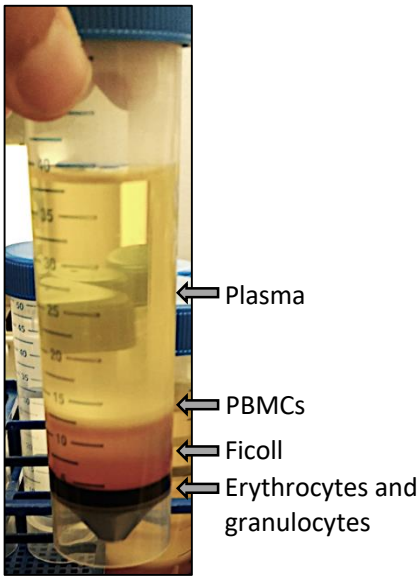


Figure VII. PBMCs isolation after centrifugation with Ficoll.

2.2. Intestinal resection

Intestinal resections from CD patients and controls were obtained from the Surgical Service of the Hospital of Manises (Valencia, Spain), following the Helsinki declaration recommendations. The Institutional Review Board of the hospital approved the study and the written informed consent was obtained from all patients. Demographic and clinical data from these patients, including age, gender and disease behaviour was collected and it is shown in **Table 8.**

Intestinal resections from the stricture in CD patients presenting a stenotic behaviour (B2) or from the damaged area of CD patients with a penetrating behaviour (B3) were obtained after surgery. In general, the last pharmacological treatment dose was administered at least 3 weeks before surgery. Non-damaged resections of colon carcinoma patients were used as control samples.

Table 8. Characteristics of control and CD patients from which fibroblasts were obtained.

	CONTROL	CD
Number of patients	10	12
AGE		
17-40 years	3	5
>40 years	7	7
GENDER		
Female	4	5
Male	6	7
BEHAVIOUR		
B1 (Inflammatory)	-	-
B2 (Stricturing)	-	6
B3 (Penetrating)	-	6

2.2.1. Epithelial cell and fibroblast isolation

Surgery samples from CD patients and controls were obtained in cold PBS. After several washes with PBS, muscular and adipose tissues were removed, and the sample was cut in pieces of approximately 1 mm. Epithelial cells were isolated after incubation in HBSS without Ca^{2+} and Mg^{2+} supplemented with EDTA 2mM and 0.5% of iFBS during 30 min at 37°C in agitation at 150 rpm. After this, the supernatant was collected and centrifuged to obtain the epithelial cells. Then, a digestion of the pieces in 1 ml of PBS with collagenase I 1 mg/ml (Sigma), hyaluronidase 2 mg/ml (Sigma), and DNase 1 µl/ml (GE Healthcare Life Science) was performed during 30 min at 37°C in agitation at 150 rpm. Finally, in a Petri dish with lines done with a needle to allow the pieces stack in, the explants were seeded. After the pieces were dried, the culture medium was added and cells were cultured in a cell culture incubator (MCO-19AICUV-PE, Panasonic Healthcare Co. Ltd., Gunma, Japan) at 37°C, with a humidified atmosphere of 5% CO_2 and 95% air (AirLiquide Medical,

Valencia, Spain). The medium (DMEM high glucose, Sigma) was supplemented with 20% iFBS, Penicillin 100 IE/ml (Gibco), Streptomycin 100 µg/ml (Gibco), Gentamycin 100 µg/ml (Sigma), Amphotericin B 2 µg/ml (Gibco) and Ciprofloxacin 16 µg/ml (Sigma). The medium was changed every 3 days. When fibroblasts migrated from the tissue and proliferated in the Petri dish reaching an 80-90% confluency they were passed into a flask, using 0.25% Trypsin-EDTA. Intestinal fibroblasts from passages 6 to 8 were used in all experiments. Fibroblasts, from controls and patients, were treated with calcitriol (D1530, Sigma) 10 nM and 100 nM for 24h or 7 days. A control with the vehicle ethanol was used in all cases.

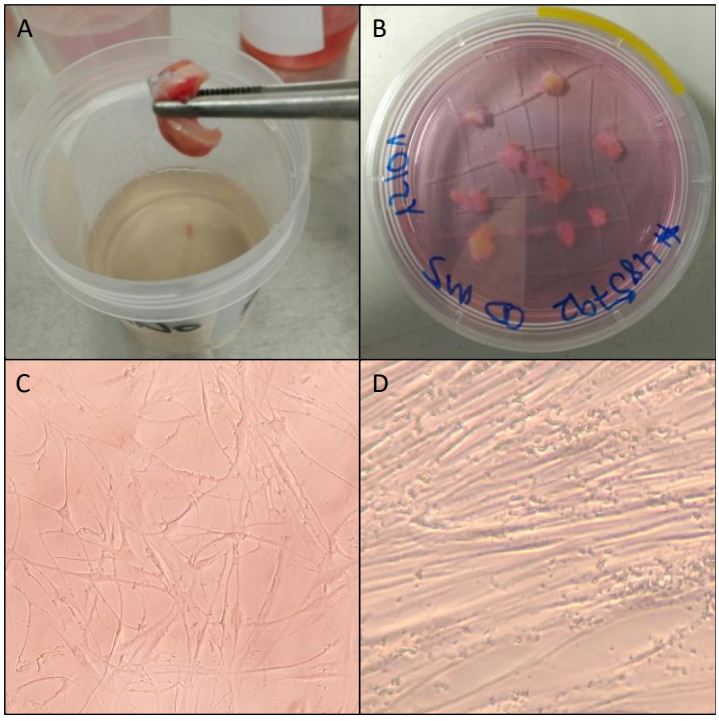


Figure VIII. Intestinal fibroblast isolation and culture.
A*. Intestinal resection,* ***B.*** *Explants seeded in the Petri dish,* ***C*** *and* ***D*** *Fibroblasts at different confluency.*

2.2.2. Fibroblasts wound healing assay

The capacity of wound healing of fibroblasts from CD patients and controls was analysed in an *in-vitro* wound healing assay in the presence of vitamin D or its vehicle. These cells, from control and CD patients, were seeded in 6-well plate to obtain a cell monolayer. Prior to the experiment, fibroblasts were treated with calcitriol 100 nM (D1530, Sigma) or vehicle. After 24 h, in the centre of the fibroblasts monolayer, a single scraping with a disposable pipette tip of 10 µl was done. After washing with fibroblasts with PBS to eliminate cell debris, fibroblasts medium without iFBS was added. Then, photos were taken at different time points. In all cases, the wounded area was determined (ImageJ; National Institutes of Health, Bethesda, MD, USA) from 3 representative photographs taken of each well at 0, 24 h and 48 h. Results were expressed as the percentage of the wound at each time point for the maximal wounded area (time 0, 100%).

These experiments were performed using an Olympus IX81 (Hamburg, Germany) fluorescence inverted microscope and the Cell^R software v.2.8 was employed to take images manually.

2.2.3. Small interfering (siRNA) transfection

Primary human intestinal fibroblasts were transfected with 25 pmol of specific PDIA3 siRNA (#107677, Ambion, Thermo) in order to down-regulated PDIA3 expression. Fibroblasts were seeded in 6-well plate and 15 min before the transfection; the medium was removed and, Opti-MEM® medium was added. According to the manufacturer's instructions, 9 µl of Lipofectamine RNAiMAX reagent (Invitrogen, Thermo) were added to 150 µl of Opti-MEM® per well. In another tube, 150 µl of Opti-MEM® were mixed with 3 µl of the siRNA PDIA3 10 µM per well. The two mixes were combined and incubated

at room temperature (RT). After 5 min, the mix with lipofectamine and siRNA was added to the cells and incubated at 37°C with 5% CO2 during 16 h. Then, the mix was removed from the cells and fibroblast medium was added. A mock transfection without the siRNA was also done as a control. The efficiency of transfection was determined by analysing the *PDIA3* mRNA expression.

3. HUMAN CELL LINES

Two human cell lines were used in different experiments. Both were human monocytes (U937 and THP1) from the European Collection of Cell Culture, Salisbury, UK. They were cultured in RPMI medium with 10% iFBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Monocytes were differentiated into macrophages by culturing them in the presence of phorbol myristate acetate (PMA, Sigma) for 48h.

Cell cultures were maintained in a cell culture incubator at 37°C, with a humidified atmosphere of 5% CO_2 and 95% air. All cell lines were sub-cultured when they reached 90-95% confluence, using 0.25% Trypsin-EDTA (Sigma) when they are attached. Sub-confluent cell cultures of passage number lower than 30 were used for all the experiments.

4. ANIMAL STUDIES

4.1. Mice

C57BL/6 WT mice supplied by Charles-River were used in all the experiments, and they were maintained under specific pathogen-free conditions and were co-housed to reduce potential differences in the microbiota. Animals were given *ad libitum* access to water and chow diet, they were kept at 21°C ± 1°C under a standard 12 h light/dark regimen. The

experiments were performed with mice of 10-12 weeks old with 20-25 g of body weight. Mice were always randomly divided into different experimental groups. All experiments were performed in compliance with the European Animal Research Law, and the protocols were approved by the institutional care and use laboratory animals committees of the University of Valencia (authorization code 2018/VSC/PEA/0179).

4.2. Induction of intestinal fibrosis

The *in vivo* model of intestinal fibrosis was induced in C57BL/6 mice using a heterotopic intestinal transplant as previously described[169,170]. In this protocol, small colon resections from donor mice were extracted and subcutaneously transplanted into the dorsal neck region of recipient mice. All procedures were performed using a sterile technique. Donor mice were sacrificed by neck dislocation. Then, after an abdominal incision, the colon was extracted. The colon was clean with 5 mL of 0.9% NaCl to remove stool and divided into 6 equal 10-mm parts (**Figure IX.A**). Resections were kept moist with 0.9% NaCl solution, whereas the recipient animals were prepared for transplantation by shaving a small area of their back to avoid contamination with hair. Two subcutaneous pouches were prepared through a small incision perpendicular to the body axis on either side of the neck and a colon resection was implanted into each of the subcutaneous pocket (**Figure IX.B**). These colon resections from the same donor mouse were introduced in the two experimental groups. Two adjacent segments of the colon from each donor were kept to be used as autologous control tissue (named as day 0). After 7 days, recipient mice were sacrificed by neck dislocation and the intestinal grafts were obtained (**Figure IX.C**).

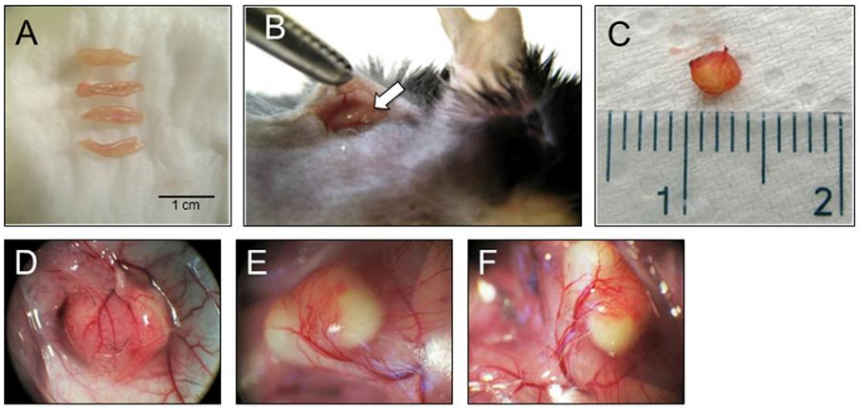


Figure IX. Protocol for the heterotopic transplant model to induce intestinal fibrosis. (Adapted from [171]).

4.3. Treatment of recipient mice

Recipient mice were separated into two experimental groups, one received calcitriol and the other received its vehicle, ethanol. A daily intraperitoneally (i.p.) dose of calcitriol (D1530, Sigma), dissolved in ethanol at a concentration of 20 µM, was administered into mice (1 µg/kg or 2 µg/kg in a 0.9% NaCl solution) from the day of surgery (day 0) until sacrifice (day 7). The solutions were prepared freshly every day.

5. HISTOLOGICAL ANALYSIS

Samples were washed in saline solution and fixed using 10% neutral-buffered formalin solution (Histofix® Preservative, PanReac) for 48-72 h. Samples were then dehydrated, embedded in paraffin and subsequently cut in a microtome at 3-5 µm of thickness.

5.1. Sirius red/fast green staining

Sirius red is a stain used to identify fibrillar collagen networks in tissue sections. This staining is useful to characterise collagen network alterations in tissues and allows the identification of different collagen subtypes

according to their colours under polarised light[172]. The sulphonic acid groups of Sirius red molecule react with basic amino groups such as lysine and hydroxylysine present in the collagen molecule. Slides with colon grafts from recipient mice were used in this stain to assess fibrosis among the different experimental groups. We did not distinguish between different collagen subtypes and all the sections were analysed under light microscopy, where collagen was stained in red and the background of the tissue in green.

The slides were incubated at 55°C for 30 min, after which they were deparaffinized and rehydrated. This process was done by 3 immersions in xylene for 3 min each, following by immersions in decreasing ethanol grades finishing in distilled water for 3 min each. After hydration, slides were incubated with 0.01% Fast Green (Fast Green FCF F7258, Sigma) in a saturated aqueous solution of picric acid (1.3% in water, Sigma) for 15 min at room temperature and in the darkness. Then, samples were washed 3 times in distilled water and incubated 30 min at RT in a fast green 0.04% solution + 0.1% Sirius red (Direct red 80 365548, Sigma) in picric acid. After that, samples were washed in distilled water 3 times and dehydrated by immersions in increasing ethanol grades during 3 min each finishing in 3 xylene immersion 3 min each. Mounting of the slides was done placing a coverslip over the slice with mounting medium (DPX® Mountant for histology, Sigma). Images were acquired with a digital light microscope (Leica DMD108; Leica Microsystems, Barcelona, Spain) at different magnifications.

5.2. VDR immunohistochemistry (IHC)

Immunohistochemical staining is useful to identify specific markers in a tissue section using antibodies. This technic allows assessing the localization and distribution of a protein in biological tissue. Slides from intestinal human

resections of CD patients and controls were used in the immunohistochemistry to analyse the VDR protein.

Deparaffinization and hydration

The slides were incubated at 55°C for 30 min and then they were deparaffinized and rehydrated, as previously described.

Heat-induced epitope retrieval (HIER)

Formaldehyde covalently binds to tissue protein and creates cross-link of proteins and peptides that can mask the antigens and hind the binding of the antibody. Therefore, in formalin-fixed tissues is necessary an antigen retrieval step before immunohistochemical staining. The antigen retrieval step breaks the aforementioned bonds, exposing the antigenic sites, allowing antibodies to bind. For this purpose, slides were immersed in a pH 6 buffer citrate unmasking solution 1x, Dako Target Retrieval Solution 10x (Agilent, Santa Clara, CA, USA) and heated in a water bath at 95°C during 20 min. Then, slides were allowed to cool down for 10 min and washed in distilled water twice for 3 min and twice in Tris-buffered saline-Tween (TBS-T, 20 mM Tris-HCl pH 7.2, 150 mM NaCl and 0.1% Tween-20 v/v) for 5 min.

Endogenous peroxidase blocking

Endogenous peroxidases are present in some tissues. These enzymes can react with a chromogenic substrate solution used in IHC, leading to non-specific staining. In order to avoid false positives, slides were incubated in peroxidase blocking solution (3% H_2O_2, in distilled water, Sigma) for 15 min at RT and washed twice in TBS-T (5 min each).

Blocking and primary antibody incubation

All the incubations were carried out in a humidified chamber. In order to block non-specific binding sites, samples were incubated for 1 h with the blocking buffer. This buffer contains 1% of bovine serum albumin (BSA, Fisher Scientific, Hampton, NH, USA) and 5% of the serum of the species in which the secondary antibody was generated in this case normal horse serum (Vector Laboratories, Peterborough, UK) diluted in PBS with 0.5% Triton X. Afterwards, slides were incubated overnight with the primary antibody VDR (12550; Cell Signaling Technology, Danvers, MA, USA) diluted 1:200 in blocking solution at 4°C.

The following day, samples were rinsed twice for 5 min in TBS-T and incubated 45 min at RT with the secondary antibody: Horse Anti-Mouse/Rabbit IgG Biotinylated (Vectastain® Universal Elite ABC Kit; Vector Laboratories) diluted 1:100 in blocking buffer. Then, a washing step was performed twice in TBS-T for 5 min.

Signal amplification

Vectastain® Universal Elite ABC Kit (Vector Laboratories) was used to amplify the signal of the VDR antibody. This method is based on the formation of avidin-biotinylated peroxidase complexes (ABC), which bind to the biotinylated secondary antibody through the unoccupied binding sites on avidin, which is a tetravalent protein[173]. Following the manufacturer's instructions, slides were incubated in ABC solution for 30 min and washed twice with PBS for 5 min.

Chromogenic detection

For the detection of the target antigen, we employed the chromogenic substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB Enhanced Liquid

Substrate System, Sigma). DAB is a precipitating substrate, which produces an intense brown stain by peroxidase action (enzyme present in secondary antibody and avidin-biotin complexes). DAB was prepared following the manufacturer's protocol and then was added to the sample for 2 min checking the appearance of the brown stain. Once the tissue was properly stained, the reaction was stopped immersing slides in water.

All tissue sections were counterstained with hematoxylin (Hematoxylin solution, Gill No. 3; Sigma) for 30 sec. Next, tissues were rinsed with water for 10 sec and, sequentially, slides were immersed in 0.5% Ethanol-HCl for 10 sec and in distilled water for 5 min.

Mounting and image acquisition

Tissue sections were dehydrated (in graded alcohols and xylene) and then allowed to dry. Finally, coverslips were placed onto the slides just after applying the mounting medium on them (DPX® Mountant for histology, Sigma).

IHC images were acquired using a digital light microscope (Leica DMD 108, Leica Microsystems) at different magnifications.

6. FLOW CYTOMETRY ANALYSIS

The expression of leukocyte adhesion molecules in whole blood samples was determinate by flow cytometry using a flow cytometer (FACSCalibur, BD™). Leukocyte populations (neutrophils, monocytes and lymphocytes) were identified based on their characteristics of size and granularity (Forward Scatter -FS- and Side Scatter -SS-, respectively). Median fluorescence intensity was used as an expression marker of the different adhesion molecules analysed and data were relativized to the value from the group

that was homozygous for the T allele. Per sample, 10,000 events were analysed.

Whole blood samples with TT or CC genotype for the VDR *rs731236* were incubated 20 min (4ºC, dark) in the presence of specific fluorescence-labelled FITC or PE antibodies (BD™) against CD11a, CD11b, CD11c, CD18, CD49d and CD62L. After incubation, red blood cells were lysed and leukocytes were fixed with BD FACS™ Lysing Solution (BD™), and samples were then run in the cytometer.

7. CYTOMETRIC BEAD ARRAY (CBA)

A human Inflammatory Cytokine Kit (BD™) was used to measure the release of cytokines (IL-1β, IL-6) by different IFN-γ-treated macrophages. The Cytometric Bead Array was performed according to the manufacturer's instruction on supernatant collected after 6 days of culture. The cytokine levels were analysed by flow cytometry (FACS BD Fortessa™).

8. GENE EXPRESSION ANALYSIS

8.1. RNA isolation from cell cultures

Cells were detached from plates mechanically, washed with PBS and centrifuged 3 min at 500 *x g* to obtain cell pellets. RNA was isolated and purified from cultured cells with the Illustra RNAspin Mini Kit (GE Healthcare), according to manufacturer's protocol. Briefly, the cell pellet was resuspended in 350 µl of lysis buffer with β-mercaptoethanol and homogenised by passage through a 25-gauge needle. Then, 350 µl of 70% ethanol were added and transfer into a column which retains RNA. After incubation with DNase to eliminate genomic DNA, columns were washed 3 times and RNA was eluted in 30 µl RNase-free water. The purity and

concentration of the RNA were determined by spectrophotometry, using a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific).

8.2. RNA isolation from murine tissue

RNA from mice colon was isolated using Tripure Isolation Reagent (Roche Life Science). Colon samples were homogenised with MACS™ Dissociator (MACS Miltenyi Biotec; Bergisch Gladbach, Germany) in 750 µL of TriPure and then were centrifuged at 12000 *x g* for 15 min at 4°C. After, 150 µl of chloroform were added into the supernatant mixed and incubated 15 min on ice. Another centrifugation at 12000 *x g* for 15 min at 4°C was done and the colourless aqueous upper phase, which contains RNA, was transferred into a new tube. In this tube, 375 µl of isopropanol were added to precipitate the RNA with incubation of 1 h at -20°C. Then, the samples were centrifuged at 12000 *x g* for 15 min at 4°C and the precipitated was washed with ethanol 70% and resuspended in 30 µl RNase-free water. The purity and concentration of the RNA were determined by spectrophotometry, using a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific).

8.3. Complementary DNA (cDNA) synthesis by reverse transcription

cDNA was synthesized by reverse transcription, employing the PrimeScript™ RT Reagent Kit (Perfect Real Time) (TaKaRa Bio Inc.). Under the manufacturer's protocol, the reaction was performed in a final volume of 20 µl using 1 µg RNA and 4 µl of 5X PrimeScript Buffer, 1 µL PrimeScript RT Enzyme Mix I, 1 µl (50 pmol) Random hexamers and 1µl (25 pmol) Oligo dT Primer. The reaction was carried out in the thermal cycler GeneAmp® PCR System 2400 (PerkinElmer Inc.) according to the following conditions: 15 min at 37°C, 5 sec at 85°C and 4°C to infinity.

8.4. Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed with SYBR® *Premix Ex Taq™* (TaKaRa Bio Inc.) which contains TaKaRa Ex Taq HS DNA polymerase, dNTP mixture, Mg^{2+}, Tli RNase H and TB Green I (DNA intercalator that emits fluorescence only when bound to dsDNA, and thus, detection of its fluorescent signal allows quantification of the amplification products). The reaction was carried out mixing 1 µl cDNA, 5 µl SYBR premix Ex Taq 2X, 2 µM of sense and antisense primers and RNase-free water in a final volume of 10 µl. The RT-qPCR was carried out in a Lightcycler® 96 Real-Time PCR System (Roche Life Science), following the protocol: 95°C for 30 sec; 95°C for 5 sec; 60°C for 20 sec (50 cycles); 95°C for 1 sec; 65°C for 15 sec; 95°C for 1 sec and 40°C for 30 sec. All experiments were performed in duplicate with a negative control with RNase-free water instead of cDNA. The specific mice and human primer pairs were designed (Sigma) and show in **Table 9** and **Table 10**, respectively. Before their use in qPCR, primers were tested performing a melting curve analysis and standard electrophoresis in a 2% agarose gel in TAE 1X buffer containing GELRED® DNA stain. The bands were detected in a LAS-3000 camera (Fujifilm).

The relative gene expression was analysed using the cycle threshold (C_t) for the calculation of the fold change. Fold change = $2^{-\Delta(\Delta Ct)}$, where ΔCt = Ct (target gene) - Ct (housekeeping gene), and Δ(ΔCt) = ΔCt (experimental group) - ΔCt (control). As a housekeeping gene, ACTB/Actb (β-actin) was used.

8.4.1. Splicing variation

In order to investigate splicing variation, a fragment of the VDR mRNA spanning exons 7 to 9 was amplified by real-time PCR using cDNAs from

patients with TT and CC genotypes and the primers 5'-TGCGCTCCAATGAGTCCTTC (sense) and 5'-GGTCGGCTAGCTTCTGGATC (antisense). PCR products were visualised on 2% agarose gels.

Table 9. List of human primer pairs used in qRT-PCR.

GENE	SENSE (5'-3')	ANTISENSE (5'-3')
ACTA2 (α -SMA)	GACCTTTGGCTTGGCTTGTC	AGCTGCTTCACAGGATTCCC
ACTB	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
CD14	AAGCACTTCCAGAGCCTGTC	TCGTCCAGCTCACAAGGTTC
CCL2	CATAGCAGCCACCTTCATTCC	CACAGCTTCTTTGGGACACTTG
COL1A1	GGAGCAGACGGGAGTTTCTC	CCGTTCTGTACGCAGGTGAT
COL3A1	CGCCCTCCTAATGGTCAAGG	TTCTGAGGACCAGTAGGGCA
CYP24A1	ACCAGGGGAAGTGATGAAGC	TCATCCTCCCAAACGTGCTC
IFNG	CGTTTTGGGTTCTCTTGGCGT	TCCGCTACATCTGAATGACCTG
IL1B	TTCGACACATGGGATAACGAGG	TTTTTGCTGTGAGTCCCGGAG
IL6	ATGAGGAGACTTGCCTGGTG	CTGGCATTTGTGGTTGGGTC
IL8	CAGAGACAGCAGAGCACACA	TGGGGTGGAAAGGTTTGGAG
IL18	ATCGCTTCCTCTCGCAAC	CCAGGTTTTCATCATCTTCAGC
MMP2	CATTCCCTGCAAAGAACACA	GTATTTGATGGCATCGCTCA
PDIA3	TGCTAGAACTCACGGACGAC	CACCTGCTTCTTCACCATCTC
TNF	AGCCGCATCGCCGTCTCCTA	CAGCGCTGAGTCGGTCACCC
VDR	TGGAGACTTTGACCGGAACG	AAGGGGCAGGTGAATAGTGC

Table 10. List of murine primer pairs used in qRT-PCR.

GENE	SENSE (5'-3')	ANTISENSE (5'-3')
Actb	GCCAACCGTGAAAAGATGACC	GAGGCATACAGGGACAGCAC
Adgre1 (F4/80)	CTTCCCAGAATCCAGTCTTTCC	TGACTCACCTTGTGGTCCTAA

Cd86	GCACGGACTTGAACAACCAG	CCTTTGTAAATGGGCACGGC
Col1a1	CAGGCTGGTGTGATGGGATT	AAACCTCTCTCGCCTCTTGC
Il6	GAGTCCTTCAGAGAGATACAGAAAC	TGGTCTTGGTCCTTAGCCAC
Pdia3	TGGAACTGACGGACGAAAAC	TAGCCACTGACCCCATACTT
Tgfb	GCGGACTACTATGCTAAAGAGG	TCAAAAGACAGCCACTCAGG
Vdr	ACAAGACCTACGACCCCACCT	AGCCGATGACCTTTTGGATGCT

9. PROTEIN EXPRESSION ANALYSIS

9.1. Total protein extraction from cells

First, attached cells were detached mechanically prior extraction. Then, washed with PBS and centrifuged 3 min at 500 *x g* to obtain cell pellets which were re-suspended in 80-120 µL of complete lysis buffer (50 mM Tris-HCl pH 7.8, 137 mM NaCl, 1 mM EDTA, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1% Triton X-100, 0.2% N-Lauroylsarcosine and 10% Glycerol). Protease inhibitors were added immediately before their use (cOmplete™, Mini Protease Inhibitor Cocktail; Roche, Basilea, Sweden). Samples were then vortexed twice and incubated on ice for 15 min, vortexed again and centrifuged at 16000 *x g* for 15 min at 4°C. Pellets were discarded and supernatants (whole-cell protein extracts) were collected.

9.2. Nuclear and cytosolic protein extraction from cells

Cells were washed with PBS and centrifuged 3 min at 500 *x g* to obtain cell pellets. To obtain nuclear and cytosolic extracts, pellets were re-suspended in lysis buffer (10 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 10 mM NaCl, 10 mM EDTA, 1 mM EGTA, 10 mM NaF, and 0.1 mM Na_3VO_4). After centrifugation (16,000 *x g* 10 min, 4°C), supernatants (cytosolic fraction) were collected. Pellets were then re-suspended in nuclear extraction buffer (25 mM HEPES pH 7.5, 500 mM NaCl, 1mM DTT, 10 mM NaF and 10%

glycerol). Finally, the supernatants of nuclear fraction were collected after centrifugation. Both buffers (nuclear and cytosolic) were supplemented with 0.2% NP-40 Surfacts-Amps and 5 mM of protease inhibitors (cOmplete™ Mini and Pefabloc®, both from Roche).

9.3. Total protein extraction from tissue

A small piece of mouse colon was homogenised in 800 µl of lysis buffer containing 10 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM NaCl, 0.1 mM Na_3VO_4. A cocktail of 1 mM DTT, 5 mM broad-spectrum serine and cysteine protease inhibitors (cOmplete™ Mini and Pefabloc®) and 0.1% of detergent solution (NP-40 Surfact-Amps™, Thermo Fisher) were added immediately prior their use. The homogenization was done in gentleMACS™ Dissociator (MACS Miltenyi Biotec). Then, the samples were centrifuged (16000 *x g* 20 min at 4°C) and supernatants (whole protein extracts) were collected.

9.4. Protein quantification (Bradford assay)

Bradford protein assay was used to quantify the protein content of the extracts. This method is a colorimetric assay based on the formation of a complex between Coomassie Brilliant Blue G-250 dye and the proteins in solution. This dye exists in an anionic (blue), neutral (green) and cationic (red) form. Under acidic conditions, the red form of the dye is converted into its blue form, after binding to the protein being assayed. If there is no protein to bind, then the solution remains brown. The protein concentration can be evaluated by determining the amount of blue dye and measuring the absorbance of the solution at 595 nm using a spectrophotometer.

This test was performed following the manufacturer's instructions. A standard protein curve was prepared by serial dilutions of BSA (31.25-1000 mg/mL). In a 96-well plate, 10 µl of samples diluted 1:10 or standard dilutions were added per well. Afterwards, 200 µl of Bradford reagent (Bio-Rad Protein Assay Hercules, CA, USA) diluted 1:5 in distilled water was added per well. Both samples and standard curve points, were analysed in duplicate. After 5 min of incubation at RT, the absorbance was measured at 595 nm using a Multiskan™ Ascent 354 microplate spectrophotometer (Thermo Labsystems, Thermo Fisher).

9.5. Western blotting

Sample preparation

Before loading in the polyacrylamide gel, the proteins need to be denaturalised. Thus, a 6X sample buffer (0.5 mM Tris-HCl pH 6.8, 25% glycerol v/v, 10% SDS, 0.5% β-mercaptoethanol and 0.5% bromophenol blue) was added to samples and then the mix was boiled at 100°C during 5 min to achieve the protein denaturation.

SDS-polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels were made using a mixture of acrylamide/bis-acrylamide solution (37.5:1) (Norgen Biotek; Schmon Pkwy, ON, Canada). Resolving gels were prepared with different percentage of polyacrylamide (8-12%) in 0.375 M Tris-HCl pH 8.8 and 0.1% SDS, whereas stacking gels were always prepared with 3.75% polyacrylamide in 0.125 M Tris-HCl pH 6.8 and 0.1% SDS. The gel polymerisation reaction was catalysed by 0.1% ammonium persulfate (APS) (SERVA, Heidelberg, Germany) and N,N,N',N'-tetramethylethylenediamine (TEMED, Fisher). The SDS-PAGE was performed in the Mini-PROTEAN Tetra Cell System (Bio-Rad). Extracts with equal protein

amounts were loaded and molecular weight marker EZ-Run™ Pre-Stained Rec Protein Ladder (Fisher) was also loaded in the gel to determine the molecular weight of the proteins. Electrophoresis was performed in a buffer tank with running buffer (25 mM Tris pH 8.3, 0.1% SDS and 192 mM glycine) at a constant voltage of 120 V.

Protein transfer to nitrocellulose membrane

After electrophoresis, proteins were transferred from the polyacrylamide gel to a 0.45 µm nitrocellulose blotting membrane (GE Healthcare Life Science) using a Mini Trans-Blot® Cell (Bio-Rad). The transference was performed at 4°C in a transfer buffer (25 mM Tris pH 8.3, 192 mM glycine and 20% methanol) for 70 min at a constant amperage of 0.35 A.

Ponceau

To verify the transfer efficiency, membranes were immersed for 1 min in a Ponceau solution which stains proteins in red. This solution contains 0.1% Ponceau S (Sigma) and 5% acetic acid solution (Sigma).

Antibodies incubation

First, we incubated samples with a blocking solution that prevents antibodies from binding to the membrane non-specifically. Blocking is made with 5% non-fat dried milk or 5% BSA (Fisher) diluted in TBS-T with continuous gentle shaking, at least for 1 h, at RT.

Once the membrane was blocked, it was incubated with the primary antibody, prepared in blocking solution supplemented with 0.02% NaN_3 (Sigma) overnight at 4°C with continuous gentle shaking. The day after, the membrane was washed four times in TBS-T for 5 min at RT and with vigorous shaking, incubated with a secondary antibody in fresh blocking solution at RT

for 1 h, and washed again. All primary and secondary antibodies used are listed in **Table 11.**

Table 11. List of antibodies employed in WB.

PRIMARY ANTIBODY	SUPPLIER	DILUTION
COL1A1	84336S, Cell Signaling	1:1000
GAPDH	G9545, Sigma-Aldrich	1:10000
IκBα	sc-371, Santa Cruz Biotechnology inc. (Dallas, TX, USA)	1:1000
NFκB p65	8242, Cell Signaling	1:1000
PDIA3	ab13506, Abcam	1:2000
Phospho STAT3	ab76315, Abcam	1:1000
STAT3	ab68153, Abcam	1:1000
VDR	12550, Cell Signaling	1:1000
Vimentin	ab92547, Abcam	1:1000
SECONDARY ANTIBODY	**SUPPLIER**	**DILUTION**
Goat Anti-Mouse IgG	Thermo Fisher (31430)	1:2000
Goat Anti-Rabbit IgG	Vector (PI-1000)	1:5000

Chemiluminescence detection

Immunolabeling was detected by enhanced chemiluminescence, employing Luminata™ Crescendo Western HRP substrate (Merck Millipore) or SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo), following manufacturer's instructions. This detection method is based in an oxidation reaction of luminol catalysed by the enzyme horseradish peroxidase (HRP), conjugated to the secondary antibody, in the presence of hydrogen peroxide, and giving rise to 3-aminophthalate that emits light at 425 nm.

Immunolabeling was visualised with a digital luminescent image analyser, Fujifilm LAS-3000 Imager (Fujifilm). Densitometric analyses were performed using Multi Gauge V3.0 software (Fujifilm). The protein expression was normalised versus that of the β-actin or GAPDH (employed as loading controls).

Stripping method

We next proceed to remove antibodies from the membrane in order to reuse the membrane for incubating other antibodies, by the stripping method. This method consists in the incubation of the membrane with 0.5 M glycine pH 2.5 for 1 h at RT with vigorous shaking. Then, the membrane was washed with TBS-T twice and then blocked and incubated with primary and secondary antibodies, according to the protocol described above.

10. STATISTICAL ANALYSIS

Data were expressed as mean ± SEM and compared by a t-test for comparisons between two groups, one-way analysis of variance (ANOVA) with Newman-Keuls *post hoc* correction for multiple comparisons. A P value <0.05 was considered to be statistically significant. The correlation between different data obtained in human samples was analysed using Spearman's correlation coefficient. For clinical data analysis, contingency tables of the 3 genotypes were analysed by $\chi 2$ test (3 × 2 tables). When significant differences were obtained, the comparison was made between the homozygous groups (CC *vs* TT) or the group that was homozygous for the risk allele with the rest of the patients (CC vs TT+TC) using the Fisher exact test (2 × 2 tables). Data were analysed using GraphPad Prism® 7.0a (GraphPad Prism® Software Inc., La Jolla, CA, USA).

IV. Results

1. RELATIONSHIP BETWEEN THE GENOTYPE OF SNP IN GENES RELATED TO IBD PATHOGENESIS AND CLINICAL CHARACTERISTICS IN CD PATIENTS

1.1. Frequencies of risk allele

CD patients and controls were genotyped and frequencies were analysed in SNPs of different genes related to relevant processes in the pathogenesis of IBD. In **Table 12,** the genotypic frequencies of the homozygous risk allele in each SNP are displayed. This risk allele is the allele described that conferred susceptibility to CD. All the genotyped SNPs were in Hardy-Weinberg equilibrium in patients and controls except for *rs11175593, rs13361189* and *rs2066847* where any subject with the risk allele was found. In the SNPs *rs2066845, rs886774* and *rs13294* the genotype homozygous for the risk allele was significantly more frequent in CD patients than in controls (**Table 12**Table 12).

Table 12. Genotypic frequencies.

GENE	SNP	Genotypic frequency[a]		
		Control	CD	P value[b]
ATG16L1	*rs2241880*	0.64	0.65	0.9335
PTPN2	*rs2542151*	0.042	0.051	0.4429
LRRK2	*rs11175593*	0	0	-
IRGM	*rs13361189*	0	0	-
NOD2	*rs2066844*	0	0.02	0.3166
NOD2	*rs2066845*	0	0.01	**0.0221**
NOD2	*rs2066847*	0	0	0.9278
CDH1	*rs10431923*	0.35	0.26	0.2660
LAMB1	*rs886774*	0.083	0.21	**0.0235**
ECM1	*rs13294*	0.43	0.305	**0.0289**

ECM1	*rs3737240*	0.19	0.17	0.4997
VDR	*rs731236*	0.125	0.175	0.5989

[a] The genotypic frequency is for the homozygous risk allele. [b] P value corresponding to statistical analysis by χ2 test of a contingency table for the 3 genotypes in controls and CD patients. In bold, analysis with P < 0.05.

1.2. Association between genotypes and clinical characteristics in CD patients

The analysis of potential associations between clinical characteristics and genotype, with the only exception of *rs731236* in the *VDR* gene, didn't show any significant and relevant relationship with location or behaviour. However, some non-significant tendencies could suggest associations in studies with a higher number of patients. For example, in the SNP of the *CDH1* gene, TT genotype considered as a risk genotype, could be associated with CD patients who have a better prognostic: they have more risk to have an inflammatory phenotype (B1) and less risk to need surgery or suffer from perianal fistula (**Figure X.A**). Besides, *rs886774* in the *LAMB1* gene, the presence of the risk allele G, could be associated with CD patients suffering from a colonic (L2) and inflammatory (B1) disease (**Figure X.B**).

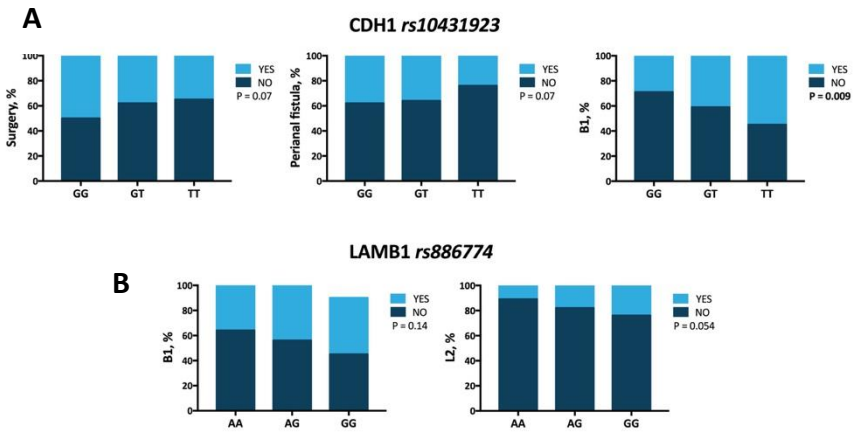


Figure X. CD patients with risk genotype for SNPs in CDH1 and LAMB1 genes have a better disease course.

A*. Graphs represent percentages of CD patients with each genotype for the rs10431923 (CDH1) who needed surgery, with perianal fistula or with B1 phenotype.* ***B****. Graphs represent percentages of CD patients with an inflammatory phenotype (B1) or colonic location (L2) by genotype.* ***A-B****. The χ2 test was used for statistical analysis.*

2. A SNP IN THE *VDR* GENE IS ASSOCIATED WITH DECREASED LEVELS OF THE PROTEIN AND A PENETRATING PATTERN IN CD

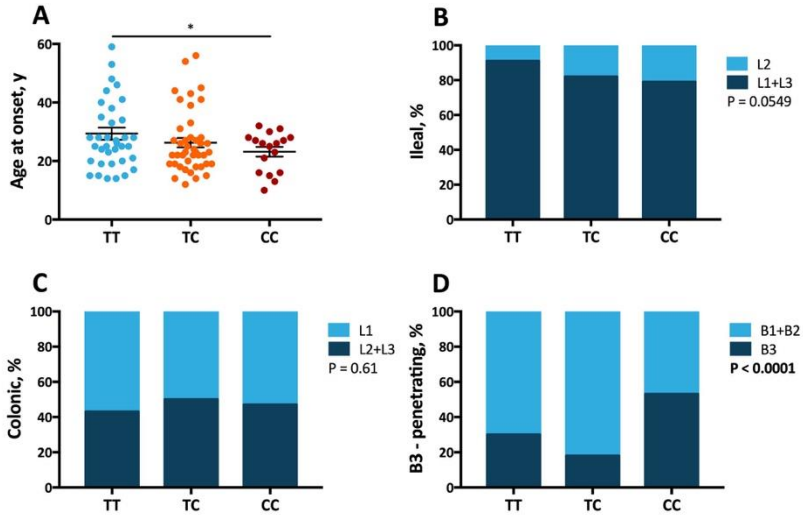
2.1. CD patients carrying the allele C in the SNP rs731236 of the VDR gene run a higher risk of developing a penetrating phenotype

The study of relationships between clinical characteristics in CD patients and alleles in the SNP *rs731236* or TaqI in the *VDR* gene showed interesting points. The data indicated that CD patients homozygous for the risk allele C were diagnosed at an earlier age (**Figure XI.A**), which is an indication of a worse disease course. According to clinical location, no associations with the location of the disease and the genotype were found (**Figure XI.B-C**). Nevertheless, patients homozygous C exhibited a significantly higher risk of having a B3-penetrating phenotype (CC *vs* TT+TC, OR: 3.6; P = 0.0229, Fisher test) (**Figure XI.D, Table 13)** and also of needing to undergo CD-related

surgery (CC *vs* TT+TC: OR = 3.3; P = 0.0390, Fisher test) (**Figure XI.F, Table 13**). This association was not observed when patients had a perianal fistula (**Figure XI.E**). When only considered patients with a complicated behaviour (B2 or B3), those with CC genotype also had a higher risk of developing a B3 phenotype (CC *vs* TT+TC, OR: 4.7; P = 0.0311, Fisher test).

The analysis of genotypic frequencies of the TaqI SNP between CD patients and healthy donors showed that CD patients presenting a B3 behaviour phenotype were significantly more likely to be homozygous for the C allele than controls (**Table 13**), which reinforced our observation in the patient population.

VDR *rs731236*



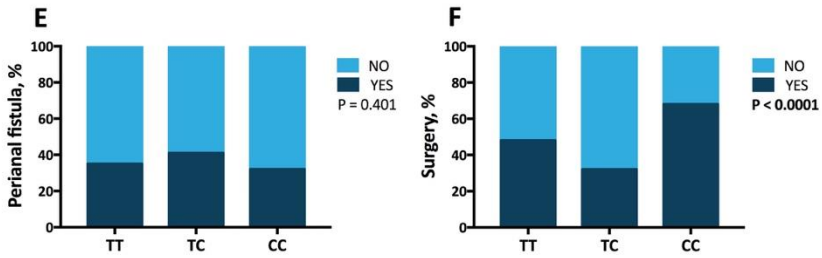


Figure XI. CD patients with CC genotype for the TaqI SNP have a worse disease course. ***A****. Graph shows the age at diagnosis of CD patients with different genotypes and mean ± SEM for each group. The Student t-test was used for statistical analysis of the results of each group with respect to those of all the other groups (P = 0.0229, TT vs CC).* ***B-C****. Graph represents percentages of CD patients with ileal (L1+L3)* ***B*** *or colonic (L2+L3)* ***C*** *involvement vs other locations according to genotype.* ***D****. Graph represents percentages of CD patients with a penetrating behaviour (B3) or not (B1 and B2) by genotype.* ***E****. Graph represents percentages of CD patients with a perianal fistula or not by genotype.* ***F****. Graph represents the percentages of CD patients with each genotype who needed surgery or not.* ***B-F*** *The χ^2 test was used for statistical analysis.*

Table 13. TaqI genotypic frequencies in donors and CD patients.

Group	n	TT	TC	CC	P-value[a]
Healthy donors	72	**32** (44.4%)	**31** (43.1%)	**9** (12.5%)	
CD patients	103	**40** (38.8%)	**44** (42.7%)	**19** (18.5%)	0.5321
B1 inflammatory	42	**15** (35.7%)	**21** (50.0%)	**6** (14.3%)	0.6587
B2 stricturing	31	**13** (41.9%)	**15** (48.4%)	**3** (9.7%)	0.8533
B3 penetrating	30	**12** (40.0%)	**8** (26.7%)	**10** (33.3%)	**0.0384**

[a] P-value corresponding to statistical analysis by χ2 test of a contingency table for the 3 genotypes between healthy donors and the rest of groups. In bold, analysis with P<0.05.

2.2. Low VDR protein levels are detected in PBMCs from CD patients homozygous for the C allele

The TaqI SNP doesn't alter the amino acid sequence of the VDR protein. It is a synonymous mutation that involves a nucleotide change in the VDR

gene (T *vs* C). The first analysis was to observe if the polymorphism changed VDR expression. For this purpose, the analysis was done in two different samples.

First, we genotyped for the TaqI SNP two different monocytes cell lines, and detected that the THP1 cell line had a TT genotype, by contrast, monocytes U937 were CC genotype. Taken advantage of these cell lines genotypes, both were cultured and treated with PMA to differentiate them into macrophages. VDR mRNA was analysed and results showed a significant reduction in THP1 cells compared with U937. However, U937 cells expressed higher levels of pro-inflammatory cytokines and less CD14 than THP1 monocytes. Treatment with PMA increases the levels of pro-inflammatory cytokines and in CD14 this increase is significantly higher in THP1 cells (**Figure XII**).

Then, mRNA and protein levels in PBMCs of TT (n=14) and CC (n=13) patients were analysed. Results showed no changes in the mRNA levels of either group of patients (**Figure XIII.A**). As the SNP is located in the 5' terminus of the exon 9 in the VDR mRNA, we studied whether it could affect the splicing process and generate a truncated or non-functional protein. PCR analysis using primers to amplify a region between exons 7 and 9 revealed the same PCR product in samples from patients with TT and CC genotypes (**Figure XIII.B**). However, the analysis of VDR protein levels showed lower levels in patients carrying 2 C alleles compared with patients with 2 T alleles (100.0 ± 8.9% vs 28.5 ± 8.9%, P = 0.0003 by Student t-test) (**Figure XIII.C**).

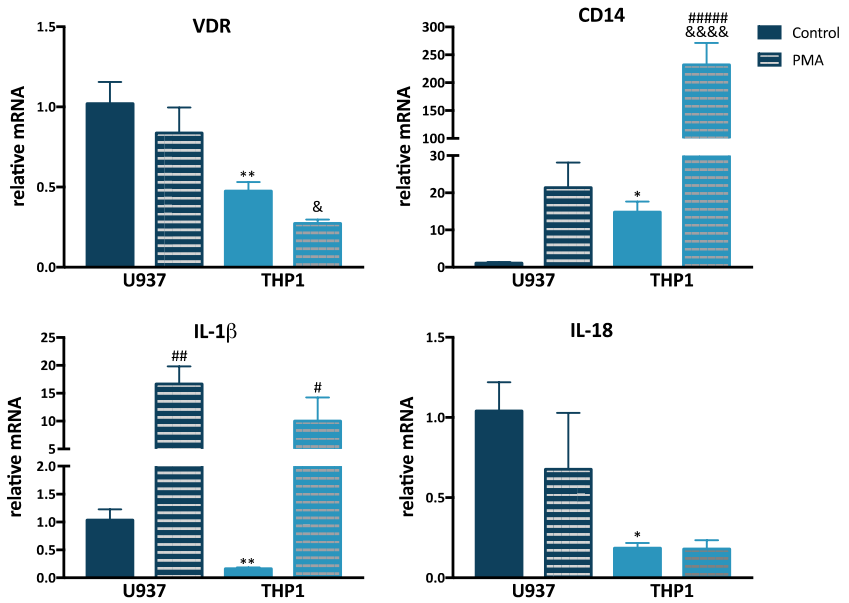


Figure XII. Increased pro-inflammatory cytokines in U937 cells with a CC genotype. *Graphs showed the relative mRNA expression of different gene vs β-ACTIN in U937 (n=3) and THP1 (n=4) cells treated or not with PMA. Data are represented as fold induction vs β-ACTIN and vs control U937 monocytes. Bars in graphs represent mean ± SEM and significant differences vs the control U937 group are shown by *P < 0.05 or **P < 0.01; vs the respective control cell group by #P < 0.05, ##P < 0.01 or ####P < 0.0001 and vs PMA U937 group by &P < 0.05 or &&&&P < 0.0001.*

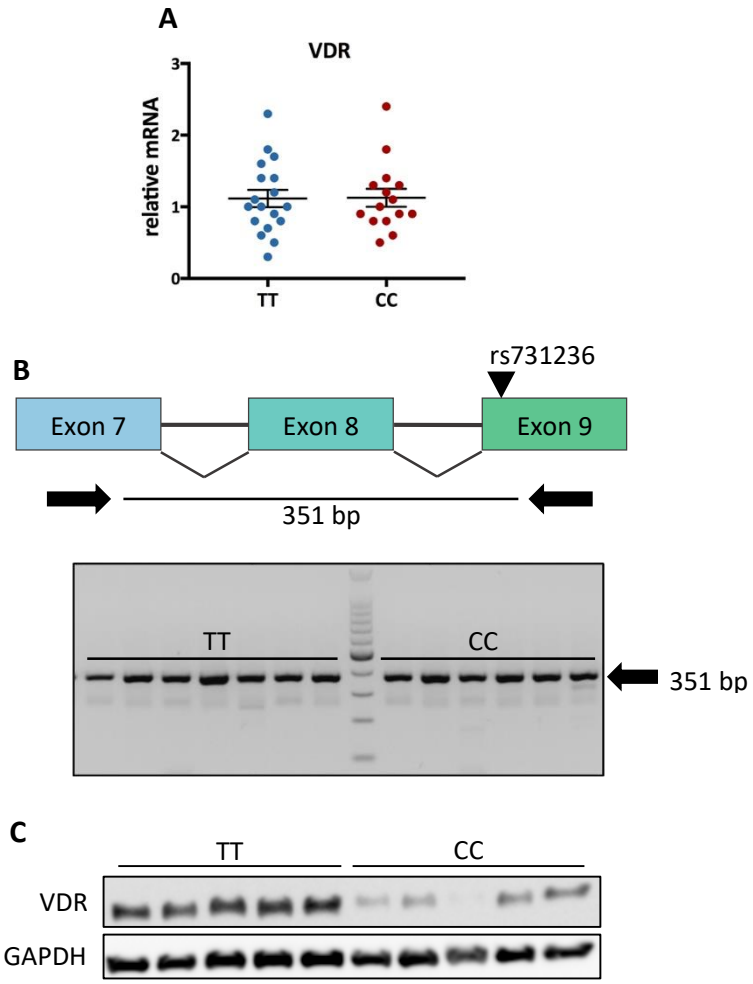


Figure XIII. Decreased VDR protein levels in PBMCs from CC CD patients without changes in mRNA or splicing.

A*. Graph shows the relative mRNA expression of VDR/β-actin (mean ± SEM) by fold induction method.* ***B****. Fragment amplified of the region surrounding the SNP and including exons 7, 8, and 9. Diagram showing the position of primers and the expected amplicon length. Image shows a 2% agarose gel with the same PCR products for the 2 genotypes.* ***C.*** *Representative images of VDR and GAPDH protein expression in lysates from PBMCs of CD patients.*

2.3. Pro-inflammatory cytokines are increased in PBMCs from CD patients homozygous for the C allele

The decreased expression of VDR protein in CD patients with homozygous C allele led to investigate the levels of pro-inflammatory cytokines in PBMCs of these patients. When data were expressed relative to that of healthy donors, results show that patients with the CC genotype exhibited a significant increase in *IL1-β* mRNA expression compared with that of patients with the TT genotype, while a non-significant increase was detected in mRNA levels of *IL-8*, *IL-18*, *IFN-γ* and *TNF-α* (**Figure XIV.A**). To study the influence of the NFκB signalling pathway on this regulation, protein levels of IκBα and NFκB p65 were analysed. In cytosolic extracts from PBMCs of CD patients, IκBα protein levels were lower in patients homozygous for the C allele than in those homozygous for the T allele (**Figure XIV.B**). In addition, patients with 2 C alleles presented higher levels of the NFκB p65 protein in the nucleus, indicating increased nuclear translocation (**Figure XIV.B**). Taken together, data suggest that decreased VDR protein levels in CD patients with 2 copies of the risk allele C in the SNP *rs731236* lead to enhancement of both activation of the NFκB signalling pathway and expression of pro-inflammatory cytokines in PBMCs.

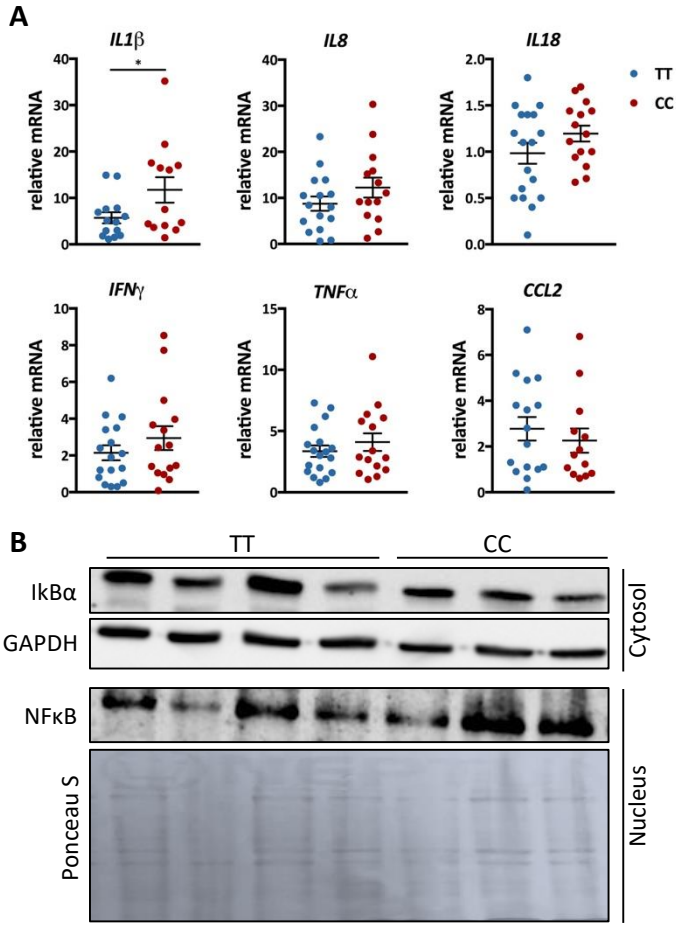


Figure XIV. Increased expression of pro-inflammatory cytokines in PBMCs from CD patients homozygous for the C allele in the TaqI SNP.

A*. Graph shows the relative mRNA expression of several cytokines (gene/ β-actin) in CD patients using a group of 6 healthy donors as a reference by fold induction method (mean ± SEM). *P < 0.05 by Student t-test.* ***B****. Representative immunoblots of IκBα and GAPDH proteins in cytosolic extracts, and NFκB p65 protein in nuclear extracts. Ponceau S staining was used as the loading control for nuclear extracts.*

2.4. Lymphocytes from CD patients with CC genotype are more activated than those with TT genotype

Flow cytometry analysis of whole blood of CD patients (n=4 for TT and n=3 for CC) showed that levels of the integrins CD11a, CD11b, and CD49d in lymphocytes were higher in CC- than in TT-homozygous subjects (**Table 14, Figure XV**). Besides, the expression of CD62L (L-selectin) in this cell type was significantly lower in CD patients with the CC than in those with the TT genotype (**Table 14**, **Figure XV**) which is indicative of a higher interaction of leukocytes with endothelial cells. Nevertheless, there was no change in neutrophilic and monocytic populations of these adhesion molecules between both genotypes (**Table 14**).

Table 14. Levels of adhesion molecules in neutrophils, monocytes and lymphocytes from CD patients.

	NEUTROPHILS		**MONOCYTES**		**LYMPHOCYTES**	
	% of CC[a]	**P-value**	**% of CC[a]**	**P-value**	**% of CC[a]**	**P-value**
CD11a	102.6 ± 5.241	0.7551	84.30 ± 4.300	0.4580	139.0 ± 17.53	0.0565
CD11b	92.23 ± 16.41	0.8092	67.27 ± 8.225	0.3295	151.2 ± 20.55	0.0525
CD11c	76.33 ± 11.45	0.4906	73.47 ± 7.332	0.4476	79.63 ± 2.976	0.4185
CD18	85.37 ± 21.39	0.5463	73.27 ± 10.36	0.2450	116.7 ± 16.44	0.3923
CD49d	78.23 ± 2.232	0.0569	55.63 ± 3.656	**<0.0001**	180.7 ± 24.24	**0.0472**
CD62L	110.5 ± 18.91	0.5927	107.4 ± 34.35	0.8203	26.87 ± 1.093	**0.0083**

[a]Columns show the percentage of expression relative to the mean ± SEM of TT genotype group. P-value was calculated by Student's t-test. In bold, analysis with P<0.05.

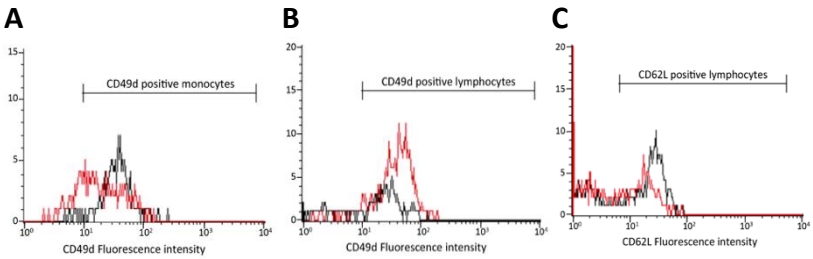


Figure XV. Representative histograms from flow cytometry experiments.
In black, TT genotype; in red, CC genotype. ***A****. CD49d expression levels in monocytes.* ***B****. CD49d expression levels in lymphocytes.* ***C****. CD62L expression levels in lymphocytes.*

3. INTESTINAL RESECTIONS FROM CD PATIENTS EXPRESS LOWER LEVELS OF VDR

3.1. The expression of VDR is diminished and that of PDIA3 is increased in intestinal resections of CD patients

After the analysis of VDR in PBMCs, the investigation was focused on the intestinal tissue, the structure affected in CD. First, the analysis of VDR protein levels showed a significant decrease in both intestinal tissue and epithelial crypts from CD patients compared to control tissue (**Figure XVI.A-B**). In parallel, a decrease in *VDR* mRNA expression and an increase in both *PDIA3* and *COL1A1* mRNA expression were also observed in tissue from CD patients compared with control tissue (**Figure XVI.C**). Finally, results reveal a significant correlation between *COL1A1* mRNA and *PDIA3* mRNA expression in CD intestine (**Figure XVI.D**).

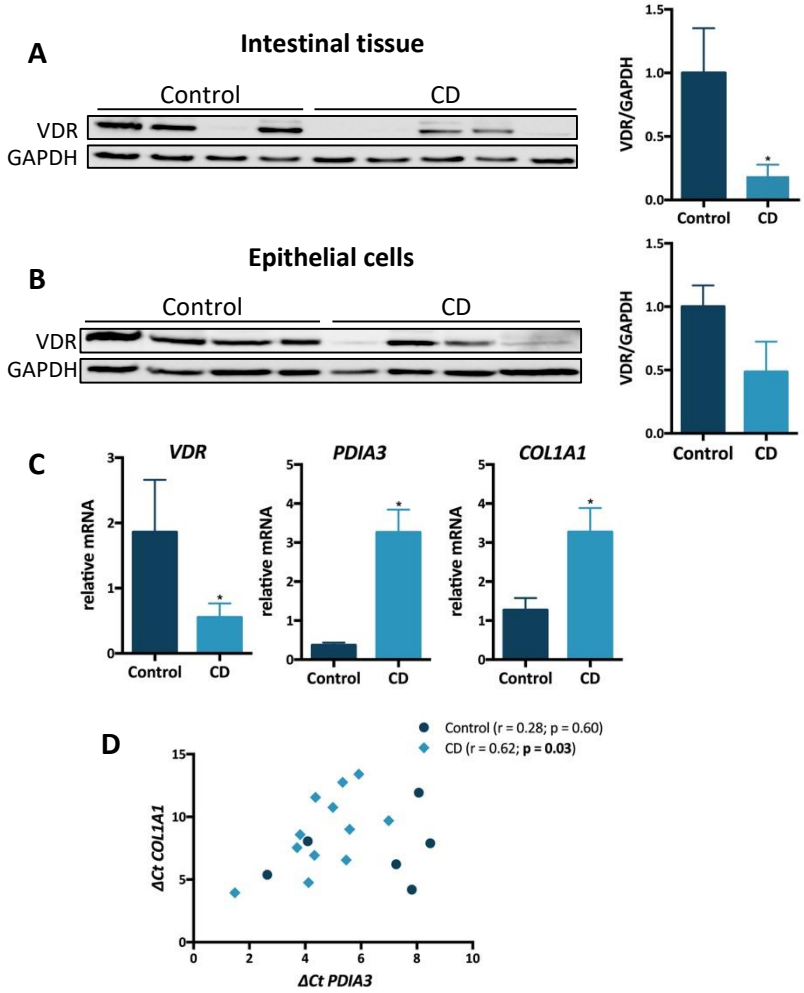


Figure XVI. Diminished VDR expression in intestinal resections from CD patients.
A*. Representative western blot image of VDR protein in lysates from total mucosa from control (n=8) and CD patients (n=10). Graph shows VDR protein expression vs GAPDH represented as a fold induction vs control mucosa.* ***B****. Immunoblot of VDR protein levels of intestinal epithelial cells isolated from controls and CD patients. Graph shows VDR protein expression vs GAPDH represented as a fold induction vs control cells.* ***C****. mRNA expression (expressed as fold induction vs β-actin) of different genes in total mucosa from control (n=5) and CD patients (n=10). In (**A**), (**B**) and (**C**), bars in graph represent mean ± SEM and significant differences vs the control group are shown by *P < 0.05.* ***D****. A positive and significant correlation between PDIA3 and COL1A1 expression (showed as Ct gene-Ct β-ACTIN) in total mucosa from CD patients.*

Immunohistochemical analysis of VDR showed that control tissue exhibited cytosolic and nuclear VDR staining in epithelial cells as well as in cells of the lamina propria. In contrast, in intestinal tissue from CD patients, a slight nuclear VDR staining was detected, which was lost in many epithelial cells and cells of the lamina propria (**Figure XVII.A**). No significant differences were detected in VDR protein expression among CD behaviours (**Figure XVII.A, B**).

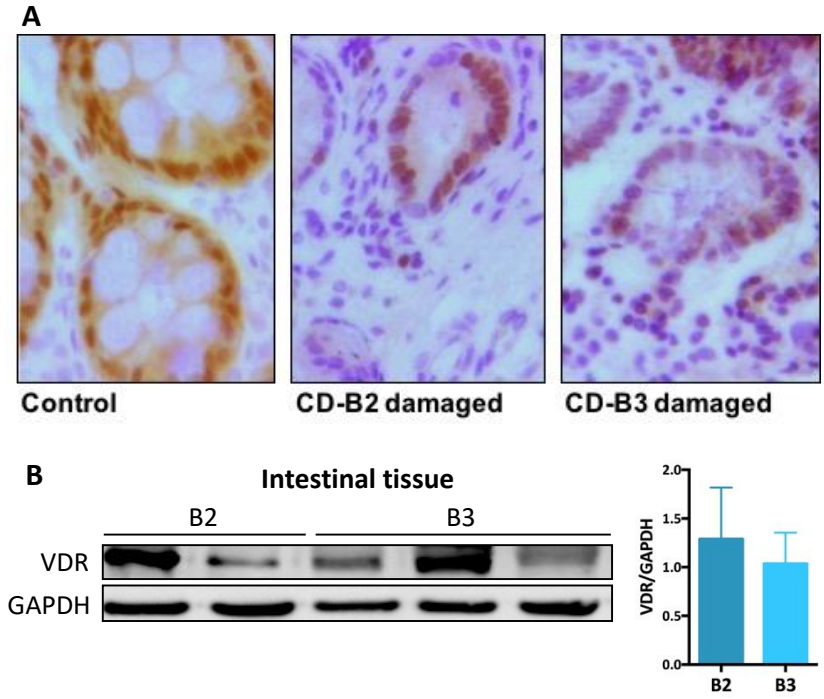


Figure XVII. VDR expression is lower in damaged intestinal resections from CD patients than in control tissue and no differences were detected between phenotypes.
A*. Representative images showing VDR immunostaining in the mucosa of control and CD patients with B2 and B3 phenotypes.* ***B****. Representative immunoblot of VDR protein levels of intestinal resections from B2 and B3 CD patients. Graph shows VDR vs GAPDH protein expression.*

4. A CROSS-TALK BETWEEN VDR AND PDIA3 IS DETECTED IN HUMAN INTESTINAL FIBROBLASTS

4.1. Silencing of PDIA3 increases VDR expression in human intestinal fibroblasts

Intestinal fibroblasts were obtained from intestinal resections of control patients and transfected with PDIA3 siRNA. As shown in **Figure XVIII**, the efficiency of the transfection reached an 80% reduction in the expression of *PDIA3* mRNA and a slight reduction in PDIA3 protein levels. Under these conditions, results show a significant increase in both VDR mRNA and protein levels in parallel with a significant decrease in COL1A1 mRNA and protein expression.

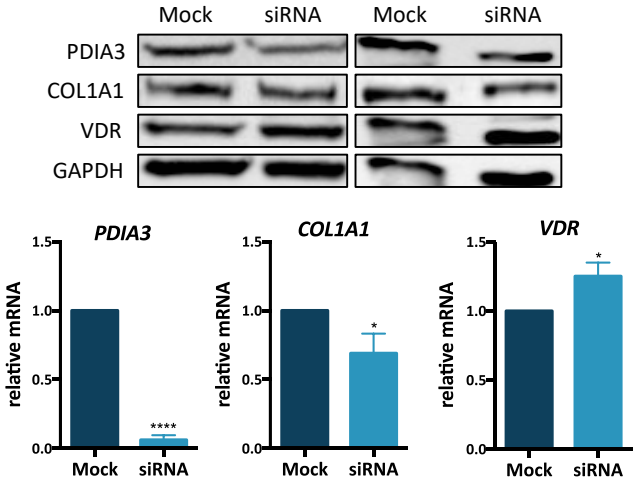


Figure XVIII. PDIA3 gene silencing in control human intestinal fibroblasts increases VDR and decreases COL1A1 expression.

*Control fibroblasts were transfected with specific PDIA3 siRNA (n=4). A pair of representative western blot images showing protein expression. Graphs show the relative mRNA expression of different genes vs β-ACTIN represented as a fold induction vs non-transfected cells (mock). Bars in graphs represent mean ± SEM *P < 0.05 or ****P < 0.0001 vs mock transfected cells.*

4.2. Reduced VDR expression and increased PDIA3 expression is detected in human intestinal fibroblasts carrying the C allele in the VDR gene

Fibroblasts were cultured 24 h and 7 days. As shown in **Figure XIX**, seven days-cultured fibroblasts expressed significantly higher mRNA expression levels of *VDR*, *PDIA3* and *COL1A1* compared to 24h-cultured cells. Protein levels were slightly, but not significantly increased by 7 days of culture. Also, mRNA expression of pro-inflammatory cytokines such as *IL-1β* and *IL-6* were higher in cells cultured for 7 days than in those culture during 24h.

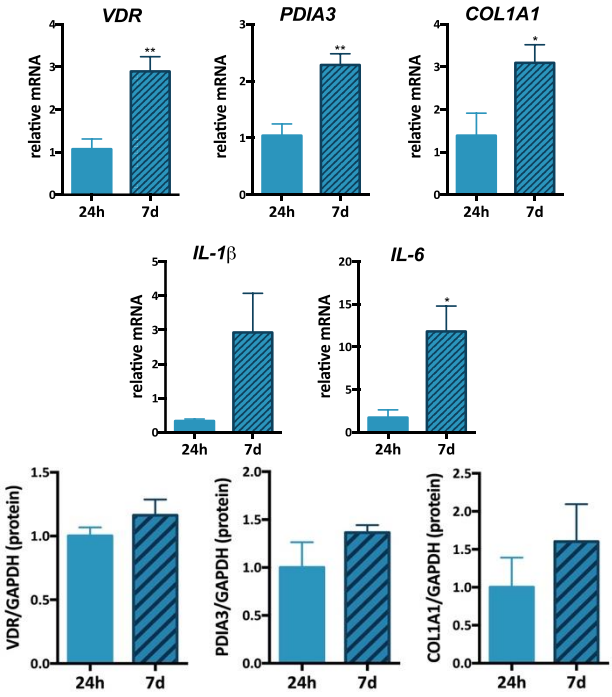


Figure XIX. Seven days-culture of fibroblasts results in the activation of the cells.
*Graphs show the relative mRNA expression of genes (expressed as fold induction vs β-ACTIN) and protein expression vs GAPDH in fibroblasts cultured during 24 h (n=4) or seven days (n=4), represented as a fold induction vs 24h control cells. Bars in graph represent mean ± SEM and significant differences vs the 24 h group are shown by *P < 0.05 or **P < 0.01.*

Control fibroblasts were classified according to the presence of the T or C allele in the SNP *rs731236* of the *VDR* gene and they were activated culturing them for seven days. As shown in **Figure XX.A**, seven days-cultured fibroblasts carrying the C allele presented a reduced VDR protein expression in parallel with increased levels of both PDIA3 and COL1A1 protein levels compared with cells carrying the T allele.

In addition, we also classified according to their genotype, monocytes obtained from fresh PBMCs from healthy donors; treatment with IFN-γ induced an increase in VDR protein expression in macrophages carrying the T allele, but it was not detected in macrophages carrying the C allele (**Figure XX.B**). The lower VDR expression detected in CC cells, paralleled with a higher significant expression of IL-1β and increased levels of IL-6 (**Figure XX.C**).

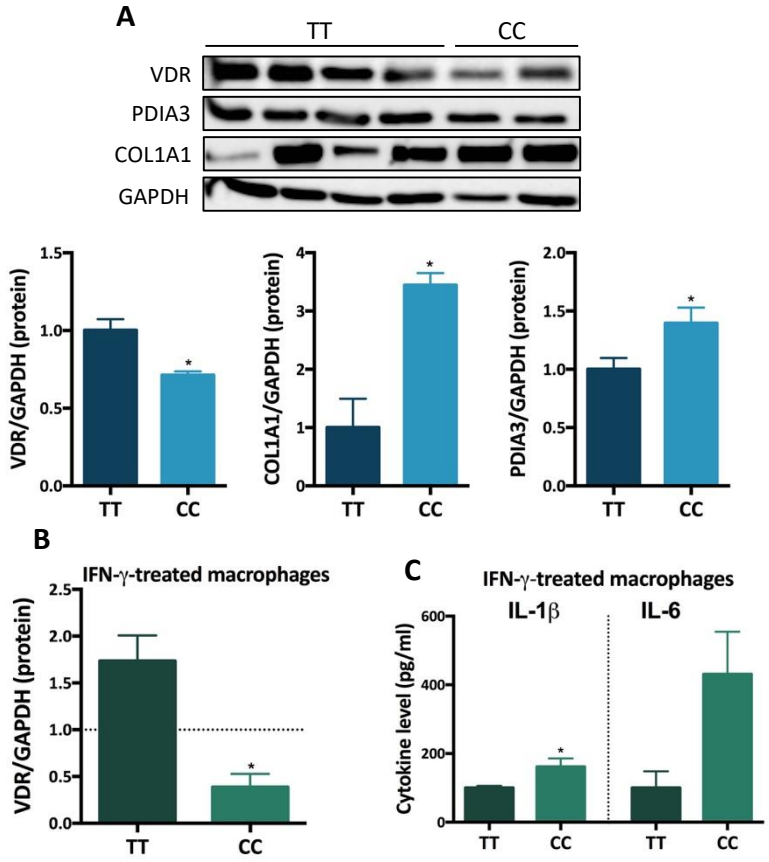


Figure XX. The CC genotype in the TaqI SNP of VDR exhibited a reduced VDR expression in human intestinal fibroblasts from controls.

A*. Western blot images showing protein expression in seven-days cultured fibroblast homozygous for the T (n=4) or C (n=2) allele in the SNP rs731236. Graphs show protein levels vs GAPDH represented as fold induction vs TT group.* ***B****. Monocytes classified according to the VDR genotype (TT or CC) were cultured for six days with IFN-γ (n=3). Graph show protein expression vs GAPDH in IFN-γ-treated cells represented as fold induction vs non-treated macrophages (represented as a horizontal line).* ***C****. IL-1β and IL-6 levels in six-days cultured IFN-γ-treated monocytes homozygous for the T (n=3) or C (n=4) allele. Graph show cytokine levels in pg/ml expressed as a fold induction vs TT group. In all cases, bars in graph represent mean ± SEM and significant differences vs the respective TT group are shown by *P < 0.05.*

5. CROHN´S DISEASE AFFECT BOTH BASAL AND VD-STIMULATED VDR PROTEIN LEVELS IN INTESTINAL FIBROBLASTS

5.1. Reduced VDR protein levels in fibroblasts from CD patients

In addition to control subjects, fibroblasts were also obtained from intestinal resections of damaged and non-damaged intestinal tissue of CD patients. The analysis of VDR protein expression reveals that levels of VDR were significantly lower in cells obtained from CD affected mucosa than in fibroblasts from both, the non-damaged tissue of the same CD patient or control fibroblasts (**Figure XXI.A**). A significant reduction was also detected in the mRNA expression of a *VDR* target gene, *CYP24A1* in CD fibroblasts compared with controls (**Figure XXI.B**). No significant changes in protein levels of PDIA3 and COL1A1 were detected among different fibroblasts analysed (**Figure XXI.A**).

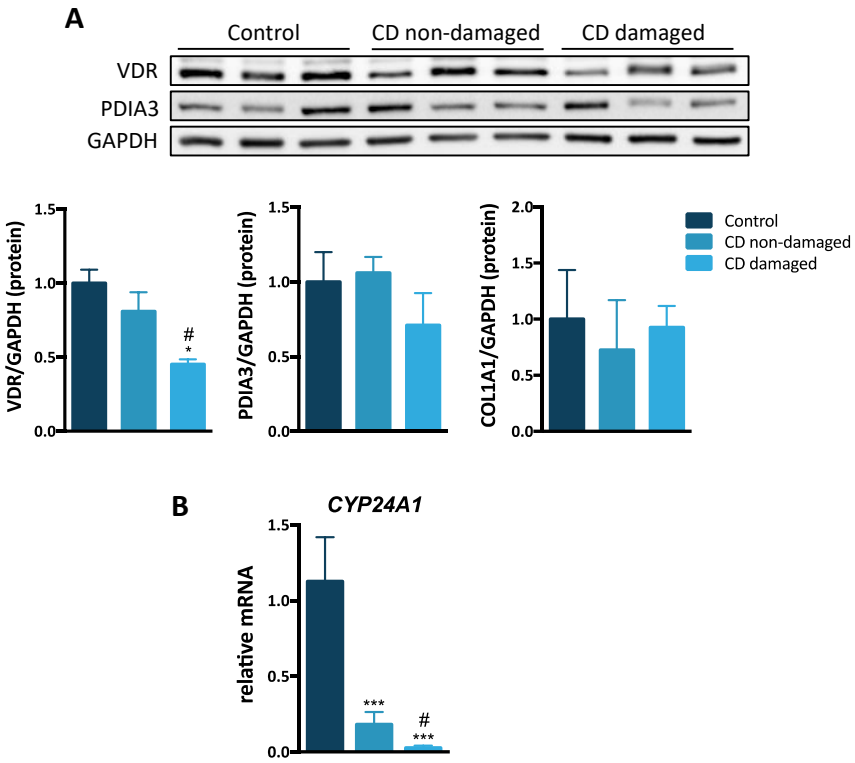


Figure XXI. Reduced VDR expression and VDR signalling in intestinal fibroblasts of CD patients.

A*. Immunoblot of protein levels in fibroblasts isolated from non-damaged tissue of control patients (n=3), non-damaged and damaged tissue of CD patients (n=3). Graphs show protein expression vs GAPDH represented as fold induction vs control cells.* ***B****. Graph shows the relative mRNA expression of CYP24A1 gene vs β-ACTIN in control (n=4) and CD (n=7) fibroblasts. Data are represented as fold induction vs control fibroblasts. Bars in graphs* ***A*** *and* ***B*** *represent mean ± SEM and significant differences vs the control group are shown by *P < 0.05 or ***P < 0.001 and vs the non-damaged group by #P < 0.05.*

5.2. VD increased VDR and decreased PDIA3 protein levels in fibroblasts from CD patients

Treatment of control fibroblasts with VD significantly increased VDR protein levels (**Figure XXII.A**). VD also enhanced VDR protein levels in fibroblasts obtained from the non-damaged tissue of CD patients, but only

caused a slight accumulation of VDR in fibroblasts from the damaged tissue of the same patients (**Figure XXII.A**). In contrast, VD significantly decreased PDIA3 protein levels in fibroblasts from CD-damaged mucosa while not in control fibroblasts (**Figure XXII.B**).

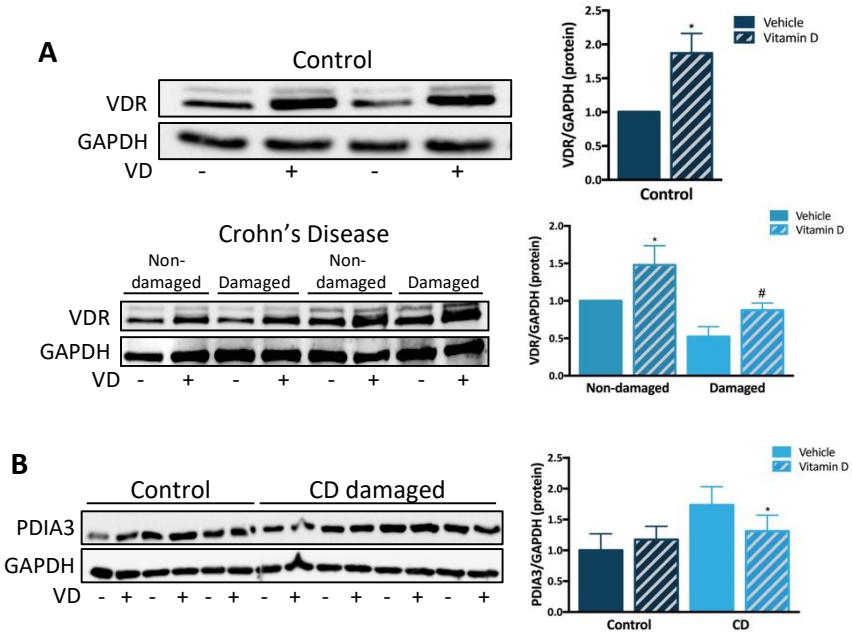


Figure XXII. VD increased VDR and decreased PDIA3 protein levels in fibroblasts from CD patients.

Fibroblasts were treated for 24 h with VD 100 nM or vehicle. ***A****. Immunoblot showing protein levels in fibroblasts isolated from control mucosa (n=4) or the non-damaged and damaged tissue of CD patients (n=4) treated with vehicle or VD. Graphs show VDR protein expression vs GAPDH represented as fold induction vs vehicle in control cells and vs non-damaged vehicle in CD cells. Bars represent mean ± SEM and significant differences vs the respective vehicle group are shown by *P < 0.05 and vs non-damaged fibroblasts treated with VD by #P < 0.05.* ***B****. Western blot showing protein levels in control fibroblasts (n=3) or fibroblasts from damaged mucosa of CD patients (n=4) treated with vehicle or VD. Bars in graphs represent protein level vs GAPDH in control or CD cells. Significant difference vs the respective vehicle group is shown by *P < 0.05.*

The mRNA expression of *VDR, PDIA3, COL1A1*, *COL3A1 and α-SMA* was non-significantly different between control and CD fibroblasts, but *MMP2* levels were significantly higher in fibroblasts from non-damaged tissue of CD patients. Treatment with VD slightly increased *VDR* mRNA levels in all cells analysed and it also induced a significant increase in the mRNA expression of *PDIA3* and *COL1A1* in fibroblasts obtained from CD-damaged tissues while it failed to modify the expression of *COL3A1, α-SMA* or *MMP2* significantly in any cell examined (**Figure XXIII.A**).

A positive and significant correlation was detected between *PDIA3* and *COL1A1* mRNA expression as well as between *PDIA3* and *COL3A1* mRNA expression, in both, vehicle and VD-treated fibroblasts. Similarly, significant correlations were detected between gene expression of VDR and markers of fibrosis; *MMP2* mRNA correlated positively and *α-SMA* mRNA correlated negatively with *VDR* mRNA levels (**Figure XXIII.B**).

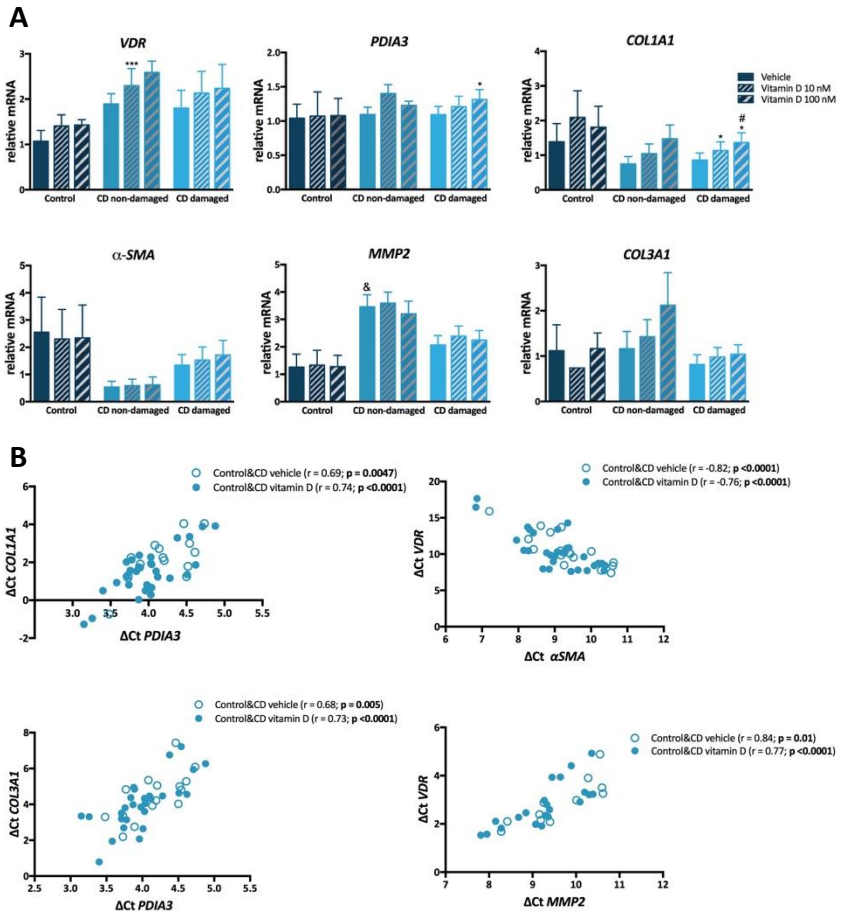
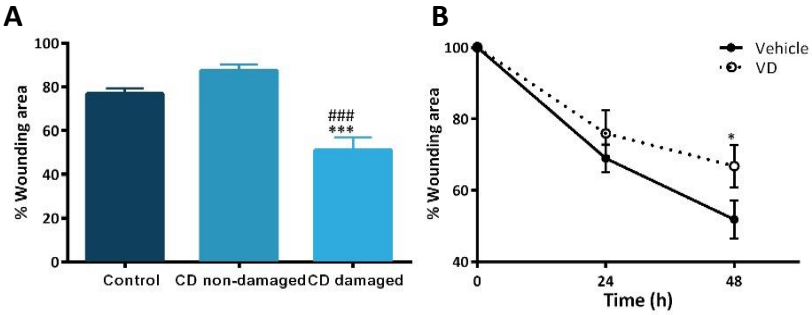


Figure XXIII. VDR and PDIA3 correlate with fibrosis markers.
Fibroblasts were treated 24 h with VD 10 nM, 100 nM or vehicle. ***A****. Graphs show the relative mRNA expression (expressed as fold induction vs β-ACTIN and vs vehicle control group) of different genes in fibroblasts from control mucosa (n=4), CD non-damaged (n=6) and damaged (n=7) tissue. Bars in graph represent mean ± SEM and significant differences from vehicle-treated group are shown by *P < 0.05, ***P < 0.001; from VD 10 nM by #P < 0.05 and from vehicle control by &P < 0.05.* ***B****. Significant correlations (showed by Ct gene-Ct β-ACTIN) detected between PDIA3 or VDR and markers of fibrosis in intestinal fibroblasts.*

5.3. The increased migration of CD fibroblasts is impaired by treatment with VD

The wound healing assay allows to determine the ability of fibroblasts to migrate/proliferate and close the wound. Results demonstrated that CD fibroblasts from damaged tissue migrate faster than control cells, resulting in a reduced wounding area, 48 h later (**Figure XXIV.A**). Treatment of these CD cells with VD decreased their migration rate, and 48h later cells exhibited a higher wounding area compared to vehicle (**Figure XXIV.B-C**).



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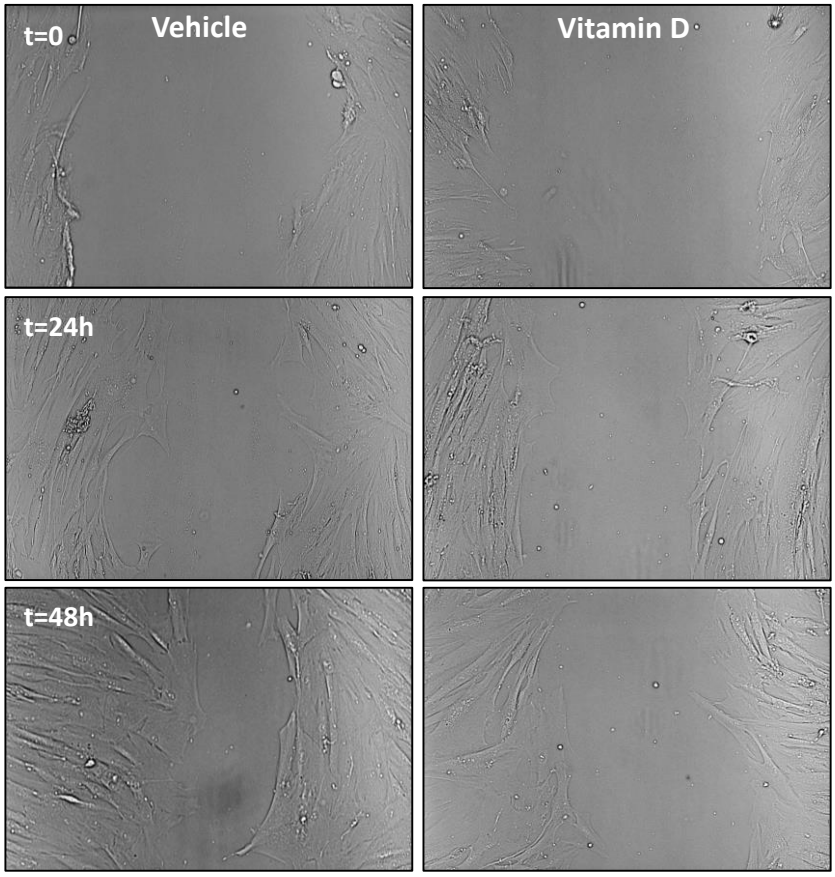


Figure XXIV. VD impaired the increased migration of CD fibroblasts.
Wound healing experiments were performed in fibroblasts from controls, CD non-damaged mucosa and CD damaged mucosa. ***A****. Graph represents the percentage of the wounding area after 48h of control (n=4), non-damaged CD (n=3) and damaged CD (n=4) fibroblasts. Bars in graph represent mean ± SEM and significant difference from control group is shown by ***P < 0.001 and from non-damaged CD by ###P < 0.001.* ***B****. Graph represents the different wounding areas in CD damaged fibroblasts at 24h and 48h treated with vehicle or VD. Points represent mean ± SEM and significant difference from 48h vehicle group is shown by **P < 0.01.* ***C****. Representative photos of the wounding area in CD fibroblasts treated with VD or vehicle at different time points.*

6. VD REDUCED INTESTINAL FIBROSIS IN A MURINE MODEL

Finally, we analysed the effects of VD treatment *in vivo*, by using a heterotopic transplant mouse model of intestinal fibrosis.

6.1. Reduced VDR expression in murine intestinal fibrosis

The evaluation of VDR expression in the fibrosis model revealed that intestinal grafts obtained seven days after transplantation exhibited a significant decrease in VDR protein levels compared with intestinal grafts on day 0. In addition, these seven-days graft showed an increase in protein expression of COL1A1 and the ratio pSTAT3/STAT3 (**Figure XXV**).

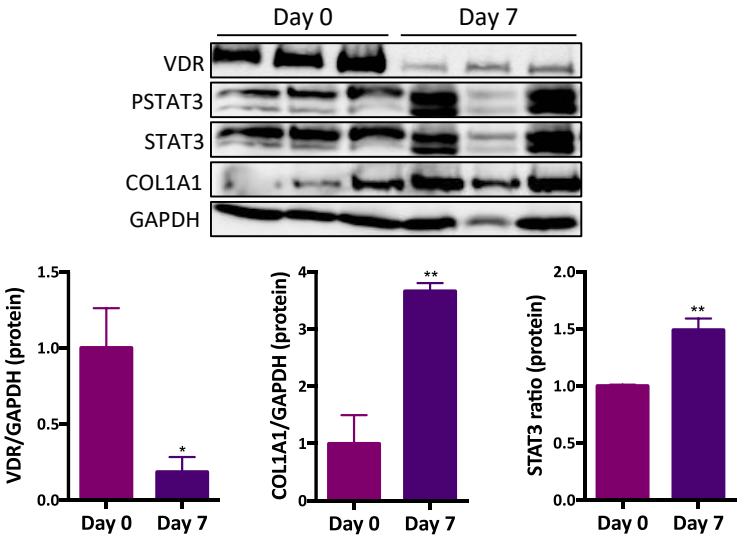
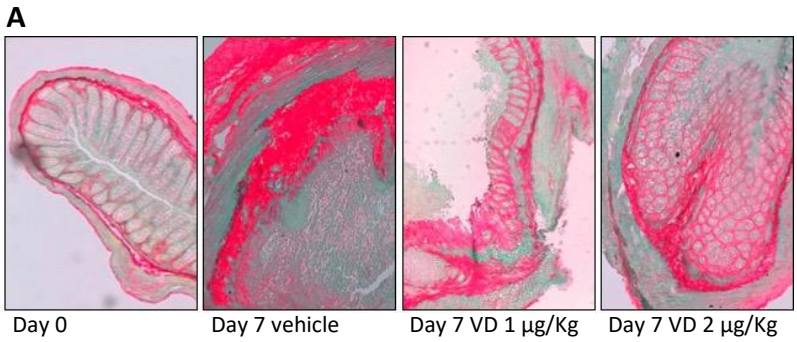


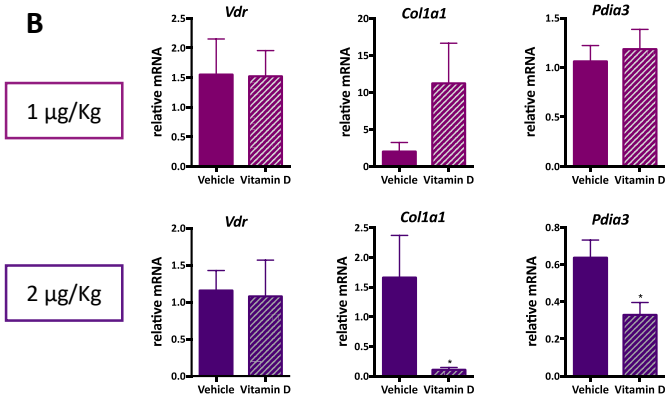
Figure XXV. VDR is reduced in a murine model of intestinal fibrosis.
*Western blot images showed protein levels in total lysates from intestinal grafts at day 0 (control) (n=3) or seven days after transplantation (n=3). Graphs show protein expression vs GAPDH represented as fold induction vs day 0. Bars in graph represent mean ± SEM and significant differences vs day 0 are shown by *P < 0.05 or **P < 0.01.*

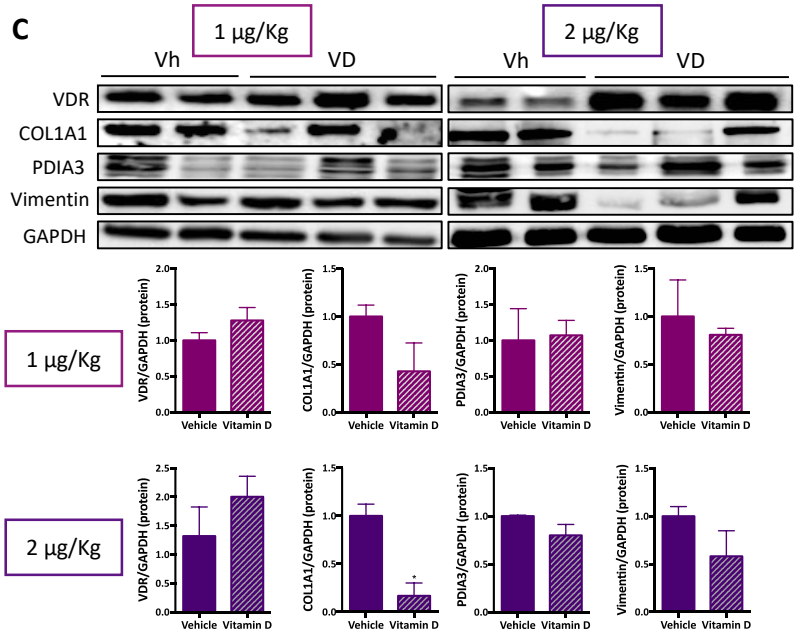
6.2. VD reduces murine intestinal fibrosis

Daily different doses of VD (1 µg/kg or 2 µg/kg, i.p.) or its vehicle were administered to receptor mice and grafts were obtained seven days after transplantation. The histological analysis of the colon shows a decrease in collagen deposition and a preserved histological architecture in colon grafts obtained from VD-treated mice compared with vehicle-treated mice (**Figure XXVI.A**). Treatment with VD at the dose of 1 µg/kg did not significantly alter the mRNA expression of any of the markers analysed but at the dose of 2 µg/kg, VD significantly reduced the mRNA expression of *Col1a1* and *Pdia3* while it did not significantly modify *Vdr* mRNA expression (**Figure XXVI.B**). A slight but not significant increase was detected in protein expression of VDR in intestinal grafts from VD-treated mice but we found a minor decrease in PDIA3 protein levels in parallel with significant lower protein levels of COL1A1 in grafts from mice treated with 2 µg/kg of VD (**Figure XXVI.C**). These grafts also exhibited a significant reduction in vimentin protein levels compared with grafts from vehicle-treated mice. Of interest, a significant and positive correlation was detected between the mRNA expression of *Pdia3* and *Col1a1* (**Figure XXVI.D**).

VD treatment did not significantly alter macrophage infiltration as analysed by the mRNA expression of *F4/80* at any dose analysed. However, both doses of VD altered the macrophage phenotype and the inflammatory profile compared with vehicle. A significant decrease in the mRNA expression of *Cd86* was detected in parallel with a significant diminution in the mRNA expression of *Il-6* while non-significant differences in *Tgf-β* mRNA expression were observed (**Figure XXVI.E**).


A
Day 0
Day 7 vehicle
Day 7 VD 1 µg/Kg
Day 7 VD 2 µg/Kg


B
1 µg/Kg
2 µg/Kg
Vdr
Col1a1
Pdia3
relative mRNA
Vehicle
Vitamin D


C
1 µg/Kg
2 µg/Kg
Vh
VD
VDR
COL1A1
PDIA3
Vimentin
GAPDH
VDR/GAPDH (protein)
COL1A1/GAPDH (protein)
PDIA3/GAPDH (protein)
Vimentin/GAPDH (protein)
Vehicle
Vitamin D

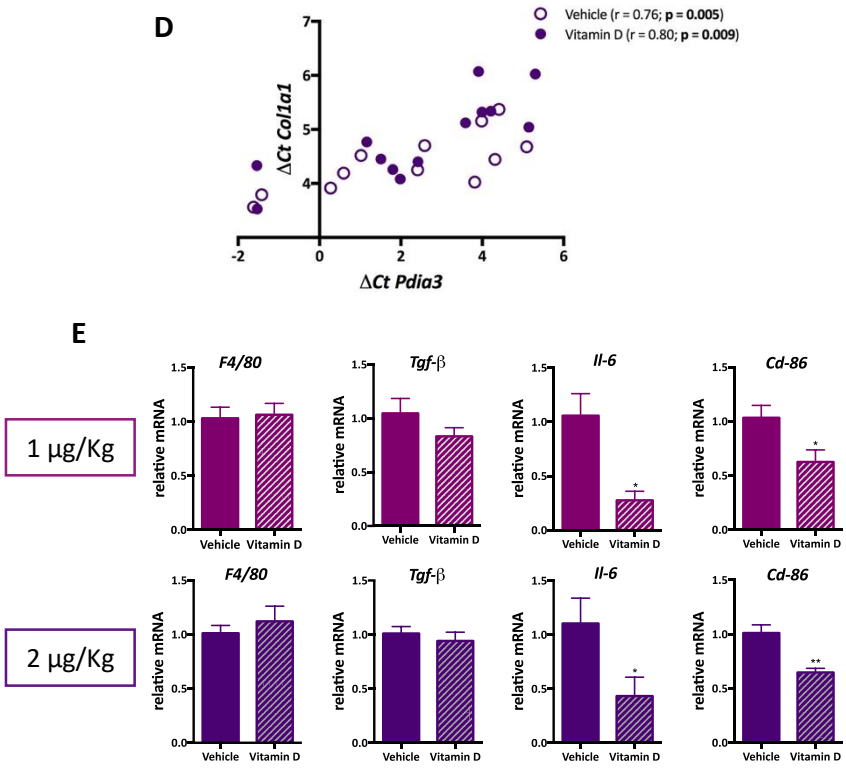


Figure XXVI. VD reduces murine intestinal fibrosis.

A*. Sirius Red staining was performed in paraffin-embedded tissues. Representative pictures under transmission and polarized light.* ***B****. Graphs show the relative mRNA expression (expressed as fold induction vs ß-actin) of different genes in intestinal explants from mice seven-days treated with VD at doses of 1 µg/kg (n=7), 2 µg/kg (n=6) or vehicle (n=6).* ***C****. Western blot images of protein expression from seven-days grafts from mice treated with VD at doses of 1µg/kg (n=2), 2 µg/kg (n=2) or vehicle (n=3). Graphs represent protein vs GAPDH quantification expressed as fold induction. In* ***B*** *and* ***C****, bars in graph represent mean ± SEM and significant differences vs the vehicle group are shown by *P<0.05.* ***D****. A positive and significant correlation between Pdia3 and Col1a1 expression (showed as Ct gene-Ct ß-actin).* ***E****. Graphs show the mRNA expression (expressed as fold induction vs ß-actin) of different genes in seven-days grafts from mice treated with VD at doses of 1 µg/kg (n=7), 2 µg/kg (n=6) or vehicle (n=6). Bars in graph represent mean ± SEM and significant differences vs the vehicle group are shown by *P < 0.05 or **P < 0.01.*

V. Discussion

Innovative technics and advances in biomedical research have pointed to the relevance of genetics in multifactorial diseases such as IBD. GWAS studies have revealed a high number of mutations that generate susceptibility to CD and UC. Nevertheless, these studies usually finish at that point, and they do not deepen in the functional relevance of the SNPs and the role that they may have in the clinical course of the disease.

In the present study, by using a cohort of 103 patients from Hospital of Manises, we aimed first to determine the expression of different SNPs of genes reported to increase susceptibility to CD[69] and its relationship with the phenotype or the clinical complications of the disease. The results of the present study are in line with previous studies[29,69] and demonstrate an increased genotypic frequency in SNPs of *NOD2*, *LAMB1* and *ECM1* in CD patients. In addition, we found a relationship between different genotypes of *LAMB1* and *CDH1* and clinical characteristics of the disease. In particular, the risk genotype in *rs10431923* of *CDH1* gene did not show CD susceptibility in our patients; however, this genotype was associated with CD patients with an inflammatory behaviour. In contrast to a previous study showing that the risk genotype in *rs886774* in *LAMB1* was associated with an early onset in UC[174], our data show a tendency to colonic and inflammatory CD phenotype associated with this alteration which suggests a less complicated course of the disease. Further studies with a higher population will help to understand better whether these mutations only predispose to the clinical onset of the disease or they are associated with specific clinical phenotypes.

Different is the situation of *rs731236* SNP (TaqI) in the *VDR* gene, and because of that the rest of the study was focused in this SNP and the relevance of VD/VDR pathway in CD. Our study did not reveal an increased expression of the *rs731236* SNP (TaqI) in the *VDR* gene in CD patients

compared with controls. However, we found that CD patients carrying two copies of the C allele in the *rs731236* SNP presented a more than three-fold increased risk of developing a B3-penetrating phenotype. As previously described, the majority of CD patients start with an inflammatory phenotype (B1 in Montreal classification) at diagnosis. However, approximately half of them are progressively acquiring a more complicated phenotype, including the development of fibrotic areas that lead to stenosis (B2 or stricturing phenotype) and the appearance of fistulas and abscesses (B3 phenotype). Of interest, our data show that the higher risk for developing a fistula in patients carrying the CC genotype should be only considered for non-perianal fistulas because there was not an association between genotypes and the development of perianal disease.

These complications lead to surgery in most cases and we found that patients with a CC genotype have a more than a three-fold increased risk to undergo surgery related to the disease. Taking together, our data show that the SNP in the *VDR* gene increases the risk of CD patients to progress towards a B3 phenotype and to require surgery, although our data did not reveal a higher frequency of the CC genotype in CD patients than in healthy donors, in contrast to that previously reported[161,162]. A possible explanation for this discrepancy would be the small cohort of patients used in the present study compared with the previous ones. Nevertheless, the fact that we did obtain a significant increase in the risk of developing a B3 CD-phenotype with the small cohort of patients, strongly reinforces the significance of this observation.

Next, we proceed to determine the functional relevance of the TaqI polymorphism in the expression of both VDR and inflammatory cytokines in PBMCs from CD patients. The TaqI SNP is a synonymous mutation and the

exchange of a T nucleotide by a C nucleotide in the coding region does not involve a change in the amino acid sequence in the protein. A preliminary analysis in cell lines demonstrated a higher inflammatory state in monocytes with CC genotype than in those with TT genotype, which lead us to determine the specific role of the genotype in PBMCs from CD patients and controls.

Our data reveal in PBMCs from CD patients homozygous for the C allele, similar levels of *VDR* mRNA than those detected in cells from patients homozygous for the T allele, suggesting that mRNA stability is not compromised with the change of a T for a C. In addition, we also discarded that the change of nucleotide altered the splicing pattern near the SNP *rs731236.* In contrast, the analysis of VDR protein levels, showed a decrease in CD patients with the CC genotype compared to TT, indicating that this mutation mainly affects VDR protein. Several mechanisms have been suggested to explain whether a synonymous mutation can decrease protein levels[175-177]. For instance, a decrease in translation speed could affect protein stability and their folding, which can lead to misfolded proteins that are recognized by the quality control machinery of cells. Also, the translation rate could be diminished by the change to a rare codon or by the alterations of mRNA secondary structure.

The decreased levels of VDR protein are in parallel with increased mRNA expression of pro-inflammatory cytokines, such as *IL-1β* in PBMCs from CD patients, homozygous for the C allele. Previous studies have reported that the activation of VDR by its ligand, exerts anti-inflammatory properties and decreases the expression of pro-inflammatory cytokines in different cells[178,179]. The NFκB pathway is a major transcriptional regulator of cytokine expression, which has been modulated by VDR[152]. We detected in PBMCs from CC CD-patients an increased amount of the NFκB p65 protein in the

nucleus, which is in concordance with lower levels of IκBα, an inhibitor of the nucleus translocation of NFκB. This data suggests an increased nuclear translocation of this transcription factor in CC-patients. Although the interaction of VDR and NFκB at their DNA binding sites in the IL-1β promoter cannot be ruled out, it seems likely that the low VDR levels detected in the CC homozygous result in lower levels of IκBα and subsequent NFκB release.

In addition to decreased VDR protein levels and increased cytokines expression, patients with CC genotype in the *rs731236* SNP presented a different expression of adhesion molecules in lymphocytes. Of interest, the pattern of expression of these molecules would be indicative of a higher interaction of lymphocytes with the vascular endothelium and increased migration to the inflamed tissue, a main characteristic of chronic inflammatory diseases like CD. In fact, biological therapy targeting several adhesion molecules has been postulated as an alternative to anti-TNF drugs for the treatment of CD[180,181].

Another important aspect is that VDR signalling influences gut microbiota, which is a relevant factor in inflammatory bowel disease[182]. Although our study focused on the impact of allelic variants in the VDR SNP on immune blood cells, we cannot rule out that low VDR protein levels in patients homozygous for the allele C were reproduced in the intestinal epithelium. This circumstance may provoke microbiome alterations and host response in the gut, which could lead to the indirect immune activation of blood cells.

In summary, these results show that homozygosity for the C allele in CD patients is associated with reduced VDR protein levels and higher expression of IL-1β in PBMCs, greater lymphocyte activation and a higher risk to derive towards a penetrating phenotype. Previous studies have related the presence of SNPs in genes such as *NOD2, MAGI1, JAK2, PRDM1, IL23R*,

ATG16L1 and *DEFB1*[28-31,183] with a complicated CD behaviour. Interestingly, some of these genes have been described as VDR targets, such as NOD2[184] and ATG16L1[148], indicating that defects in a specific genetic network could define the development of a complicated disease course. Our results strongly suggest that *rs731236* SNP in the *VDR* gene should also be considered when searching an association between the most significant loci and different CD clinical phenotypes.

Intestinal fibrosis constitutes one of the most important complications associated with CD and it is detected in both, the stenotic and penetrating CD behaviour. After the characterization of the VDR expression and the analysis of TaqI SNP in the *VDR* gene in immune cells from CD patients, we proceed to determine the VDR expression and its modulation by VD in intestinal fibrosis.

We found a reduced VDR protein expression in both intestinal resections and isolated epithelial cells from CD patients with stenotic or penetrating complications with regard to control tissue. In addition, we also detected lower VDR protein levels in control isolated fibroblasts carrying the C allele in the VDR gene, after activation by seven-days culture, than in those carrying the T allele which would be in line with that detected in PBMCs from CD patients and suggest the epigenetic regulation of VDR, specially associated to the TaqI polymorphism. The reduced VDR expression paralleled with higher levels of the other known VD-receptor, PDIA3, and also of COL1A1 expression in both intestinal tissue and isolated fibroblasts. Of interest, a positive and significant correlation was detected between the expression of PDIA3 and COL1A1. PDIA3 has been involved in COL1A1 transcription[135] and we found that a transient transfection of PDIA3 siRNA in primary human fibroblasts from controls provoked a significant reduction in COL1A1 protein

levels suggesting a role for PDIA3 in the regulation of COL1A1. However, it is important to note that reduced PDIA3 expression was associated with a significant increase in the VDR expression, and this VDR has also been shown to down-regulate markers of fibrosis in lung fibroblasts and epithelial cells[185,186]. Our results are not conclusive about the specific role played by each VD receptor in COL1A1 transcription but strongly support a VD-independent crosstalk between VDR and PDIA3 regulating COL1A1 expression in human intestinal fibroblasts.

Next, the influence of CD in the expression of VD receptors in intestinal fibroblasts was analysed in cells isolated from patients who do not carry the C allele in the VDR gene to avoid the genetic influence reported above. Our data demonstrate, for the first time, that fibroblasts obtained from the damaged intestine of CD patients present lower VDR protein levels than those obtained from the non-damaged tissue of the same patient or from control subjects. The difference detected between samples of the same patient excludes genetics or changes in serum VD levels as responsible for the VDR down-regulation observed in cells coming from damaged tissues. Of interest, this reduced VDR protein expression occurred without changes in VDR mRNA expression and remained stable over several passages. Again, these results lead us to speculate that epigenetic mechanisms concerning protein degradation or post-transcriptional regulation by miRNA may be involved[187]. In this line, the overexpression of both miR-125b[188] and miR27b[189], differentially regulated by CD[190] have been associated with reduced VDR protein levels. Further studies are needed to better understand this regulation.

VD is at present widely used for the treatment of CD patients because of its immunomodulatory effects in the gut, but little is known about its role in

intestinal fibrosis. The subsequent study was focused on the analysis of the effects of VD in the expression of VDR and PDIA3 in intestinal fibroblasts. VD increased VDR protein levels in both control and CD fibroblasts, although the increase detected in cells obtained from the affected mucosa of CD patients was lower than that detected in control fibroblasts. All these changes occurred without significant alterations in VDR mRNA expression, which reinforces that VD accumulates VDR because it inhibits the proteasomal degradation of VDR[191], a process that may be overactive in fibroblasts from the CD-damaged tissue. In addition, VD significantly reduced PDIA3 protein levels, while increased its mRNA expression, but only in CD fibroblasts. We speculate that the low levels of VDR detected in these cells allow VD to bind to PDIA3 and favour its degradation. In this line, it has been shown that binding of VD to PDIA3, located in caveolae, allows the internalization of the complex[192] which may result in lysosomal degradation[193]. Previous studies have shown a diminution of PDIA3 expression by VD in several cell types[192,194,195] but the mechanisms involved in this complex interaction require more investigation. Of note, VD failed to significantly modify the expression of markers of fibrosis in CD fibroblasts but, both the increase in VDR and the decrease in PDIA3 expression induced by VD in these cells strongly correlates with reduced markers of fibrosis which suggest that the reported anti-fibrotic effects of VD[156] may also be mediated by a direct effect of this hormone on intestinal fibroblasts.

Finally, changes induced by CD in intestinal fibroblasts have also been detected in the wound healing assay where fibroblasts from CD-damaged mucosa migrates faster than control cells, in line with previous studies [99,196], and treatment with VD prevented this process. Despite the use of a medium lacking growth factors, we cannot rule out the possibility that cell

proliferation is involved in this assay and that the prevention induced by VD is due to an anti-proliferative effect of this hormone, previously reported in cancer cells[198] which would also be of interest in the treatment of fibrosis.

As a final point, we aimed to analyse the relevance of the VD/VDR/PDIA3 signalling pathway in the development of intestinal fibrosis, and we tested the effects of VD in a murine model. The heterotopic transplant of colonic tissue provokes submucosal and subserosal fibrosis, significant ECM deposition and important cellular infiltration, and thus resembles some of the CD characteristics[170]. In line with the results obtained in human intestinal samples from CD patients, we found reduced VDR expression in murine fibrotic tissue. Treatment of mice with daily doses of VD preserved the histological architecture of the colon, reduced collagen deposition and prevented intestinal fibrosis which reinforces a previous study in a different experimental model[156]. The modulation of the immune response by VD may be involved in its protective effects since intestinal grafts from VD-treated mice showed an altered pattern of macrophage expression characterised by a reduced expression of the M1 macrophage marker, Cd86, in parallel with a diminished expression of Il-6, a known pro-fibrotic cytokine[199]. However, the fact that this immune modulation was also detected at VD doses that did not prevent fibrosis joined to a recent study reporting that fibrosis obtained by the heterotopic transplant model is not dependent on the severity of inflammation[200], strongly support additional mechanisms activated by VD. Considering the direct effects of VD on fibroblasts reported above and the positive and significant correlation also detected in murine tissue between Pdia3 and Col1a1, our results strongly suggest a role for VD, acting on fibroblasts, in its anti-fibrotic effects.

In summary, our study demonstrates reduced VDR protein levels in both PBMCs and fibroblasts isolated from CD patients carrying the homozygosity for the C allele in the VDR gene which is associated with higher activation of these cells and the final predisposition of these patients towards a penetrating CD behaviour. In addition, data also show that VD increases VDR and decreases PDIA3 in intestinal fibroblasts from CD patients which correlates with reduced expression of markers of fibrosis. These results strongly support that CD patients would benefit of the VD anti-fibrotic therapeutic value clearly demonstrated in experimental models. Further studies aimed at knowing the effects of VD in CD patients carrying the TaqI polymorphism in the VDR gene help to better understand the relevance of VDR genotyping in the clinician's decision to prescribe vitamin D supplements to CD patients.

VI. Conclusions

1. CD patients homozygous for the C allele in the SNP TaqI in the *VDR* gene exhibited:
   - **a)** Reduced VDR protein levels and increased pro-inflammatory cytokines expression in PBMCs.
   - **b)** Higher lymphocyte activation.
   - **c)** Diminished VDR protein levels in isolated intestinal fibroblasts.
   - **d)** Increased risk of developing a penetrating CD behaviour.

2. A VD-independent cross talk between VDR and PDIA3 regulating COL1A1 expression is detected in human intestinal fibroblasts.

3. CD is associated with decreased VDR protein levels in intestinal resections, epithelial cells and intestinal fibroblasts which exhibited a high rate of migration.

4. Treatment with VD increases VDR protein levels and decreases PDIA3 protein expression in CD fibroblasts and both situations correlate with reduced markers of fibrosis.

5. Treatment with VD prevented intestinal fibrosis in a murine model.

6. Taking into account results from both, human samples and in vivo experiments, our data strongly support that CD patients would benefit of the VD anti-fibrotic therapeutic value.

VII. Bibliography

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GENERALITAT VALENCIANA
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AUTORIZACION PROCEDIMIENTO 2018/VSC/PEA/0179

Vista la solicitud realizada en fecha **20/07/18** con nº reg. entrada **172112** 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: **2018/VSC/PEA/0179** 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: **Universitat de Valencia**

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

Establecimiento: **Animalario Facultad de Medicina – Campus Blasco Ibañez**

Necesidad de evaluación restrospectiva:

Condiciones específicas:

Observaciones:

Valencia a, fecha de la firma electrónica

El director general de Agricultura, Ganadería y Pesca

INFORME DE LA COMISIÓN DE INVESTIGACIÓN DEL HOSPITAL DE MANISES

D. Carlos Rodrigo Benito, Presidente de la Comisión de Investigación del Departamento de Salud de Manises,

CERTIFICA:

Que esta comisión ha analizado los aspectos éticos y científicos relacionados con el proyecto de investigación titulado:

"MODULACIÓN DE LA FIBROSIS INTESTINAL POR EL SISTEMA INMUNE INNATO EN LA ENFERMEDAD DE CROHN: RELEVANCIA DE LA AUTOFAGIA."

Que será llevado a cabo en el Servicio de Medicina Digestiva del Hospital de Manises, cuyo investigador principal es el Dr. Joaquín Hinojosa del Val. Que reúne las características adecuadas referentes a información a los pacientes y cumplimiento de los criterios éticos para la investigación médica y biomédica.

Manises a 20 de abril 2016

Dr. Carlos Rodrigo Benito
Director Médico de Atención Primaria
Presidente de la Comisión de Investigación
Hospital de Manises

Academisch Medisch Centrum
Universiteit van Amsterdam

CERTIFICADO DE REALIZACIÓN DE ESTANCIAS BREVES PREDOCTORALES / *MOBILITY PREDOCTORAL CERTIFICATE*

1. DATOS PERSONALES / PERSONAL INFORMATION

Apellidos y nombre / *Last and First Name*: Gisbert Ferrándiz, Laura	**NIF/*ID*:** 48666658-S
Centro de realización del doctorado / *Predoctoral institution*: Departamento de Farmacología, Universidad de Valencia.	

2. CENTRO DE REALIZACIÓN DE LA ESTANCIA BREVE O TRASLADO TEMPORAL / *RECEIVING CENTRE DURING THE MOBILITY*

Nombre del Organismo receptor / *Name of the host institution*: Academic Medical Centre (AMC)
Nombre del centro / *Name of the centre*: Tytgat Institute for Liver and Intestinal Research
Ciudad y País / *City and Country*: Amsterdam (Netherlands)
Investigador/a responsable de la Estancia Breve / *Researcher in charge during the mobility*: Dr. A. A. te Velde
Cargo o Categoría del o de la responsable / *Position of the researcher in charge*: Principal Investigator (PI)

3. DURACIÓN DE LA ESTANCIA BREVE O TRASLADO TEMPORAL / *DURATION OF THE MOBILITY*

Fecha inicio / *Start date*: 01/05/2018
Fecha fin / *End date*: 31/07/18

El abajo firmante certifica que el/la estudiante predoctoral al que se refiere el presente documento ha permanecido en el centro de trabajo desde el día 1 de mayo de 2018 al 31 de julio de 2018 / *The undersigned certifies that the Ph.D student has remained in this centre from the 1st May 2018 until the 31st July 2018.*

Fecha / *Date:* 31st July 2018

Firma / *Sign*

MINISTERIO
DE EDUCACION,
CULTURA Y DEPORTE

SECRETARÍA GENERAL DE
UNIVERSIDADES

SUBDIRECCIÓN GENERAL DE FORMACIÓN
DEL PROFESORADO UNIVERSITARIO Y
ATENCIÓN A LOS ESTUDIANTES

SERAFIN LARRIBA CABEZUDO, JEFE DE SERVICIO DE LA SUBDIRECCIÓN GENERAL DE FORMACIÓN DEL PROFESORADO UNIVERSITARIO Y ATENCIÓN A LOS ESTUDIANTES.

CERTIFICA:

Que de acuerdo con la información existente en nuestras bases de datos, Dª. Laura GISBERT FERRÁNDIZ, con DNI 48666658-S es beneficiaria de una ayuda predoctoral con Ref: FPU2014-05678, concedida por Resolución de 20 de agosto de 2015, (BOE-28-8-2015) de la Secretaría de Estado de Educación, Formación Profesional y Universidades, por la que se conceden ayudas para contratos predoctorales para la formación de profesorado universitario, de los subprogramas de Formación y Movilidad dentro del Programa Estatal de Promoción del Talento y su Empleabilidad.

Dicha ayuda está adscrita a la Universidad de Valencia y tiene efectos económicos y administrativos con fecha de inicio el 16 de octubre de 2015 y fecha fin prevista el 15 de octubre de 2019.

Además la dotación mensual correspondiente a su ayuda es de 1.173,00 € con 14 pagas anuales.

También dispondrá de una ayuda para la estancia en PAISES BAJOS entre el 01/05/2018 y el 31/07/2018, por la cuantía de 4.290 €.

Para que conste a petición de la interesada, y para que cumpla los efectos oportunos, expido el presente certificado en Madrid, a 29 de mayo de 2018.