EFECTO DE LA ADMINISTRACIÓN ORAL DEL AGENTE ESTABILIZADOR DEL MASTOCITO, CROMOGLICATO DISÓDICO, SOBRE LA EVOLUCIÓN CLÍNICA Y BIOLÓGICA EN PACIENTES CON SÍNDROME DEL INTESTINO IRRITABLE CON PREDOMINIO DE DIARREA

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HACEN CONSTAR

Que la memoria titulada "Efecto de la administración oral del agente estabilizador del mastocito, cromoglicato disódico, sobre la evolución clínica y biológica en pacientes con síndrome del intestino irritable del subtipo diarrea" presentada bajo su dirección y, al considerarla concluida, autorizan su presentación para ser juzgada por el tribunal correspondiente.

Y para que conste a los defectos, firman la presente en Barcelona, Julio de 2013

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ÍNDICE

Ag, Antígeno

BMI, Body mass index

CP, Células de Paneth

CPAs Células presentadoras de antígenos

DCs, Células dendríticas

EII, Enfermedad inflamatoria intestinal

HPA, Hipotálamo hipofisario adrenal

IBSSS, Irritable Bowel Syndrome Severity Score

Ig, Inmunoglobulina

IL, Interleuquina

LIE, linfocito intraepitelial

LP, lamina propria

LRRs, *Leucine-rich repeats*

MC, *Mast cell*

MHC, *major histocompatibility complex*

NK, *Natural killer*

NOD, *Nucleotide binding oligomerization domain*

PAMPs, Patrones moleculares asociados a patógenos

PAR2, Receptor activado por proteasa 2

PAR4, Receptor activado por proteasa 4

PP, Placa de Peyer

PPARγ, Proliferador de Peroxisoma Activados los Receptores γ

PPRs, Receptores de reconocimeinto de patrones moleculares

SIBO, Sobrecrecimiento bacteriano

SII, Síndrome del Intestino Irritable

SNA, Sistema nervioso autónomo

SNC, Sistema nervioso central

SNE, Sistema nervioso entérico

TLR, Receptor *toll-like*

VIP, *Vasoactive Intestinal Peptide*

CU, Colitis ulcerosa

5-HT Serotonina

HIPÓTESIS Y OBJETIVO

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La fisiopatología de las enfermedades funcionales gastrointestinales es poco conocida, a pesar de su elevada prevalencia en la sociedad. El síndrome del intestino irritable (SII) se caracteriza por la presencia de dolor abdominal asociado a cambios en el hábito deposicional. Aunque se desconocen los mecanismos fisiopatológicos subyacentes, destaca, como hallazgo común relacionado con la sintomatología, la alteración de la función barrera intestinal, junto con la presencia de inflamación de bajo grado en la mucosa intestinal. La activación del mastocito, célula efectora del eje cerebro-intestino, es clave en la disfunción intestinal identificada en estos pacientes, por lo que estrategias dirigidas a la modulación de su activación podrían ser beneficiosas para la recuperación de la homeostasis intestinal y la mejoría clínica de estos pacientes.

En esta tesis se postula la siguiente hipótesis: "*La inhibición de la activación del mastocito de la mucosa intestinal mediante tratamiento farmacológico, mejora la sintomatología característica del SII, como consecuencia de la regulación en la activación inmunitaria de la mucosa yeyunal*".

El objetivo de esta tesis es analizar el efecto del tratamiento oral con cromoglicato disódico, un agente estabilizador del mastocito, en la evolución de los síntomas propios del SII e identificar los mecanismos celulares y moleculares asociados a la respuesta clínica.

Debido a la función específica del mastocito en la inmunidad innata, hemos desarrollado una estrategia dirigida a la identificación de mecanismos inmunitarios relevantes en esta vertiente, como posibles marcadores biológicos de la disfunción intestinal en esta patología.

INTRODUCCIÓN

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1. FUNCIÓN Y ESTRUCTURA DEL INTESTINO

El intestino es un órgano complejo formado por diferentes tipos de tejidos, cuyas funciones principales son el soporte nutricional y energético del organismo y la vigilancia y defensa inmunológica. El desempeño adecuado de estas funciones depende del mantenimiento de la homeostasis funcional; este equilibrio, a su vez, está garantizado en gran parte por la existencia de una barrera epitelial físicamente indemne, que ejerce un control activo y selectivo del intercambio de toxinas y antígenos (Ag) entre la luz intestinal y la mucosa del tracto gastrointestinal, evitando el inicio y desarrollo de procesos de daño tisular. Además de su función de barrera, el intestino debe considerarse como un órgano sensitivo dotado de diferentes tipos celulares, particularmente células nerviosas, células endocrinas, células del sistema inmunitario (Furness *et al*., 1999) y la microbiota intestinal, con capacidad perceptiva, reguladora y ejecutiva de los cambios constantes que suceden dentro y en el entorno cercano del *milieu* de la mucosa gastrointestinal. Debe considerarse que en la mucosa gastrointestinal residen más del 60% de las células inmunitarias del organismo, que el sistema nervioso entérico (SNE) contiene alrededor de 10^8 neuronas, el sistema endocrino gastrointestinal es el mayor órgano endocrino del organismo (capaz de secretar varias decenas de hormonas y péptidos) y que la microbiota intestinal está compuesta por 10^{14} bacterias, amén de otros muchos microorganismos. Estos cuatro sistemas de detección están mucho más desarrollados en el intestino que en cualquier otro órgano del cuerpo. El buen funcionamiento de cada uno de estos sistemas también es determinante para la consecución de la homeostasis funcional intestinal y la pérdida o

alteración de sus capacidades podría ser suficiente para favorecer el inicio de patologías intestinales (Lomax *et al*., 2006; Mayer *et al*., 2006).

1.1. Anatomía de la pared intestinal:

En una sección transversal del intestino humano se distinguen, en profundidad desde la luz intestinal, diferentes tejidos organizados en capas:

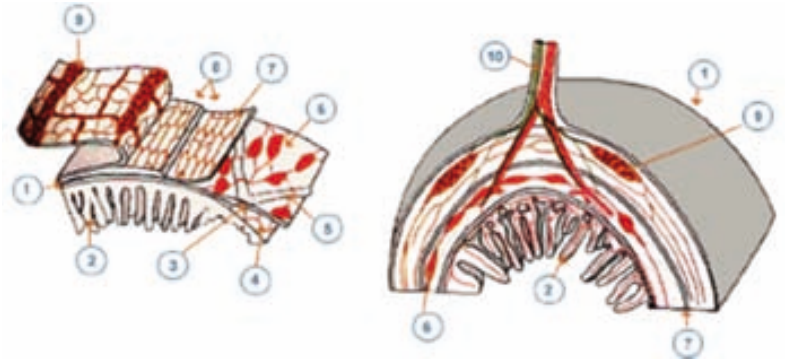
- La capa mucosa, delimitada apicalmente por una monocapa de epitelio cilíndrico en la que se intercalan diferentes estirpes celulares, sobre todo de naturaleza endocrina y mucinosa. Subyacente al epitelio se encuentra una capa de tejido conjuntivo laxo, la lámina *propria*, en la que residen numerosas y variadas estirpes de inmunocitos, así como inervación nerviosa y vascularización.

- La *Muscularis mucosae,* es una banda continua de tejido muscular liso compuesta por una capa interna circular y otra externa longitudinal.

- La submucosa, constituida por tejido conectivo en el que está inmerso el sistema linfático, los capilares y los vasos sanguíneos. En esta capa además se localiza uno de los dos plexos nerviosos del SNE, el plexo submucoso (Meissner), cuyas proyecciones nerviosas atraviesan la submucosa penetrando en la mucosa.

-La capa muscular, formada por dos estructuras, una interna que es circular y una externa que es longitudinal. Entre ambas se sitúa el plexo nervioso mientérico (Auerbach) compuesto por una red de fibras nerviosas no mielinizadas y células ganglionares.

-La serosa, compuesta por tejido conjuntivo laxo y una capa de epitelio poligonal plano.



1. Pared intestinal
2. Capa mucosa
3. Muscularis mucosae
4. Terminaciones nerviosas mucosa
5. Arteria de la submucosa
6. Plexo submucoso (Meissner)
7. Terminaciones nerviosas en capa muscular
8. Capa de fibras musculares circulares
9. Plexo mientérico (Auerbach)
10. Fibras nerviosas aferentes y eferentes junto a vasos sanguíneos

Figura 1. Representación esquemática de las capas del intestino delgado (Furness et al., 1985)

2. SISTEMA INMUNITARIO INTESTINAL

El intestino contiene el tejido linfoide asociado al intestino, *Gut-Associated Lymphoid Tissue* (GALT; Duerkop *et al*., 2005), un sistema defensivo altamente especializado que se distribuye en dos compartimentos:

- GALT organizado o inductor, constituido por diferentes estructuras linfoides tales como folículos linfoides aislados, folículos linfoides asociados o placas de Peyer (PP) y ganglios linfáticos mesentéricos. En él se inicia la respuesta inmunitaria.

- GALT difuso o efector, constituido por poblaciones leucocitarias distribuidas en el epitelio (linfocitos intraepiteliales, LIE) y en la lámina *propria* donde se encuentra la población linfoplasmocitaria. En él se ejecuta la respuesta inmunitaria.

El desarrollo de la función inmunitaria de las poblaciones leucocitarias efectoras está bajo la influencia de la flora residente intestinal y los antígenos proteicos de la dieta. Las bacterias intestinales promueven la expansión y la adquisición de la actividad citotóxica de los linfocitos intraepiteliales (LIEs). Por otra parte, la convivencia entre el intestino y la flora residente es necesaria para la digestión y absorción de nutrientes. Asimismo, la flora colabora en el desarrollo de mecanismos inmunitarios de protección frente Ag inocuos, cuya respuesta no es inflamatoria, denominada inmunotolerancia. Ciertos Ag luminales estimulan a los linfocitos de la lámina *propria* a sintetizar la inmunoglobulina más abundante en el intestino, la inmunoglobulina A (IgA). Esta inmunoglobulina atrapa en el moco diferentes antígenos procedentes de la dieta y de los microorganismos, disminuye la expresión de epítopos proinflamatorios en las bacterias comensales y así selecciona la comunidad bacteriana apropiada para cada segmento intestinal (Cerutti *et al*., 2008).

Los elementos defensivos presentes en el intestino (estructurales y no estructurales) interaccionan entre sí para el desarrollo de respuestas defensivas eficaces. La vía de entrada al organismo, el tipo de antígeno y su cantidad van a determinar el inicio de la activación de los sistemas inmunitarios innato y/o adquirido, que estimularán los mecanismos efectores implicados en la respuesta inmunológica (Mason *et al*., 2008).

2.1. Sistema inmunitario innato

El sistema inmunitario innato es el mecanismo de defensa filogenéticamente más antiguo, conservado entre animales y vegetales (Hoffmann *et* al., 1999; Jones & Dangl, 2004). El tipo de respuesta generada es de carácter inmediato

y sus mecanismos de acción no son específicos, sino independientes del antígeno o agente nocivo, no generan memoria y pretenden la destrucción del agente causal (Medzhitov & Janeway, 1997; Janeway & Medzhitov, 2002). Participan en ella células endoteliales y leucocitos, mediadores humorales como citoquinas, quimiocinas, mediadores lipídicos y proteínas del complemento y también productos derivados del propio agente infeccioso (Dahlke *et al*., 2011; Harrison & Maloy, 2011; Neher *et al.,* 2011; Cash *et al.,* 2006).

En la superficie de los agentes patógenos, habitualmente células procariotas, están presentes una serie de secuencias de moléculas, conservadas a lo largo de la evolución y ausentes en las células eucariotas, denominadas "patrones moleculares asociados a patógenos" (*Pathogen-associated molecular patterns*, PAMPs; Vance *et al.,* 2009). Para distinguir los PAMPs de otras moléculas inocuas procedentes de la flora bacteriana comensal y de los alimentos, existen receptores de reconocimiento de patrones moleculares (*Pattern recognition receptors*, PRRs). Estos receptores de inmunidad innata engloban varias familias de proteínas, entre las que destacan dos grandes grupos: aquellos localizados en la membrana celular y en el endosoma, los receptores tipo *toll* (*Toll-like receptors*, TLR), y los ubicados en el citoplasma, la familia de receptores con dominios de oligomerización de unión a nucleótidos (*Nucleotide binding oligomerization domain,* NOD). La función más importante de estos receptores es la distinción entre Ag patógenos y moléculas propias. Su activación es la clave del inicio de la respuesta defensiva frente a agentes infecciosos. El estudio de los mecanismos involucrados en esta activación no sólo ha permitido conocer y comprender mejor las interacciones entre bacterias

y huésped sino también la producción de las primeras citoquinas y los mecanismos implicados en el inicio y coordinación de la respuesta inmunitaria (Adib-Conquy & Cavaillon, 2007; McCole & Barret, 2007). Estos receptores son codificados por genes de línea germinal, muy sensibles a la detección del mínimo signo de infección.

2.1.1. Tipos celulares del sistema inmunitario innato intestinal:

- Enterocitos: son las células principales del epitelio intestinal. Aparte de su actividad enzimática y transportadora de electrolitos y macromoléculas (azúcares, lípidos y aminoácidos), estas células controlan el paso selectivo de componentes desde el lumen hacia la lámina *propria*, (Keita & Söderholm, 2010; Scow *et al*., 2011; Catalán *et al*., 2012). Por otra parte, los enterocitos tienen la capacidad de procesar y presentar a los linfocitos T los antígenos solubles que lleguen a su superficie (Hershberg *et al*., *2000*). Estas células expresan PRRs (Fusunyan *et al*., 2001; Neal *et al*., 2006) capaces de activar vías de señalización intracelular que promueven la síntesis y liberación de agentes antimicrobianos (defensinas, catelicidinas y lisozimas), quimiocinas (CCL25, CXCL12, CXCR4), citocinas (IL-1β, IL-8, IL6, TNFα, IFN-γ), factores de crecimiento (TGF-β, VEGF, IGF) y otros productos como el péptido intestinal vasoactivo (*Vasoactive Intestinal Peptide,* VIP) y la ferritina. (Markel *et al*., 2007; Wells *et al*., 2011). Estos mediadores limitan el crecimiento y acceso de las bacterias a la mucosa intestinal y reclutan células inmunitarias que complementan la función de barrera del epitelio y/o participan en la activación de la respuesta inmunitaria adaptativa (Rescigno *et al*., 2008).

- Células M: son células epiteliales especializadas y diferenciadas ubicadas sobre las PP. La vía de penetración más conocida de los antígenos luminales a la mucosa intestinal es a través de las células M (Neutra *et al*., 1996). Poseen PRRs en su superficie apical (TLR4, el factor activador de plaquetas y la integrina $\alpha5\beta1$), que favorecen su capacidad de adhesión y captación de diferentes antígenos (Kyd & Cripps, 2008). Las células M carecen de

lisosomas, por lo que captan y transportan antígenos luminales prácticamente intactos hacia las células presentadoras de Ag localizadas en las PP, principalmente linfocitos y células dendríticas, mediante un proceso de transcitosis (Mowat, 2003).

- Células de Paneth (CP): son células epiteliales especializadas en la secreción que se ubican en la base de las criptas de Lieberkühn, junto a las células madre del epitelio. En la parte apical del citoplasma se observan gránulos intensamente eosinofílicos que contienen gran variedad de péptidos antimicrobianos, como las lisozimas, defensinas α y β y fosfolipasa A2 secretora. Las CP expresan receptores NOD (Biswas *et al*., 2012), en especial NOD2/CARD15, que identifican principalmente metabolitos de las bacterias gram-positivas, cuya función es regular la expresión de defensinas. En el intestino delgado la secreción de defensinas α protege a las células madre de los agentes patógenos (Salzman *et al*., 2007). Las defensinas tienen propiedades bactericidas de amplio espectro formando poros en las membranas de los microorganismos. Además, tienen un efecto quimiotáctico sobre los leucocitos, inducen la síntesis de citocinas en diferentes tipos celulares (Niyonsaba *et al*., 2007; Ishikawa *et al*., 2009) y son capaces de activar al mastocito. (Niyonsaba *et al*., 2003)

- élulas Caliciformes (células *Goblet)*: son células intercaladas entre los enterocitos y especializadas en la producción de la capa de moco intestinal. Secretan glucopéptidos (mucinas) y otros péptidos como el “trefoil” que forman la consistencia de gel semipermeable. Existen diferentes factores que regulan la producción de mucinas, como productos bacterianos, toxinas, citocinas, hormonas, neuropéptidos y factores de crecimiento. Este gel tiene como función la protección del epitelio de la acción de las enzimas pancreáticas y la prevención del acceso de bacterias y toxinas al medio interno (Kim & Ho, 2010).

- Macrófagos: son leucocitos fagocíticos que expresan receptores de membrana para numerosas moléculas bacterianas, con una especificidad a ligandos muy amplia (Wendelsdorf *et al*., 2010). Presentan dos fenotipos:

efector o inflamatorio (m_1) y regulador (m_2). El reconocimiento de microorganismos patógenos activa dos vías de eliminación: la directa, mediante fagocitosis y liberación de productos tóxicos (como el peróxido, proteasas y radicales de oxígeno y/o nitrógeno); y la indirecta, que activa la liberación de citocinas proinflamatorias (IL-1β, IL-6, IL-23, IL12p40) y/o quimiotácticas, como el factor estimulante de colonias de granulocitos y monocitos. Estas citocinas activan y atraen otras poblaciones celulares de la inmunidad innata, amplificando así la respuesta inflamatoria. Por otra parte, el macrófago participa también en el control de la respuesta inflamatoria mediante la liberación de IL-10 que suprime la producción de citocinas proinflamatorias en las células inmunitarias (Maynard & Weaver, 2008) y mantiene la homeostasis, impidendo el desarrollo de una respuesta inflamatoria exagerada frente la flora intestinal. Los macrófagos, en estado de reposo, expresan receptores como el PPARγ (Proliferador de Peroxisoma Activados los Receptores γ) que reconocen Ag propios y procedentes de la flora comensal, lo que promueve su diferenciación hacia el fenotipo regulador (m_2) (Bassaganya-Riera *et al*., 2012; Wendelsdorf *et al*., 2010). Los macrófagos son además células presentadoras de Ag (CPA) especializadas: captan, procesan y presentan Ag en su membrana para posteriormente activar la respuesta inmunitaria adaptativa.

- Neutrófilos: son leucocitos granulocitos polimorfonucleares ausentes en la mucosa intestinal sana (Ohman & Simrén, 2010), cuyos gránulos contienen una variedad de proteínas tóxicas de acción bacteriostática (calprotectina) y bactericida (defensinas y lactoferrina), así como enzimas líticas (mieloperoxidasa, lipocalina 2). Expresan receptores de membrana TLR, receptores acoplados a proteínas G, receptores para opsoninas y citocinas, cuya activación inicia el proceso de fagocitosis y la destrucción intracelular del agente agresor. Durante la inflamación aguda, los neutrófilos liberan al medio extracelular agentes oxidantes (principalmente peróxido de hidrógeno), así como radicales libres de oxígeno, que ocasionan daño tisular (Loetscher *et al*., 2012), aunque en los últimos años, diferentes estudios otorgan también un papel reparador al neutrófilo al remodelar la matriz extracelular (Dovi *et al*., 2004).

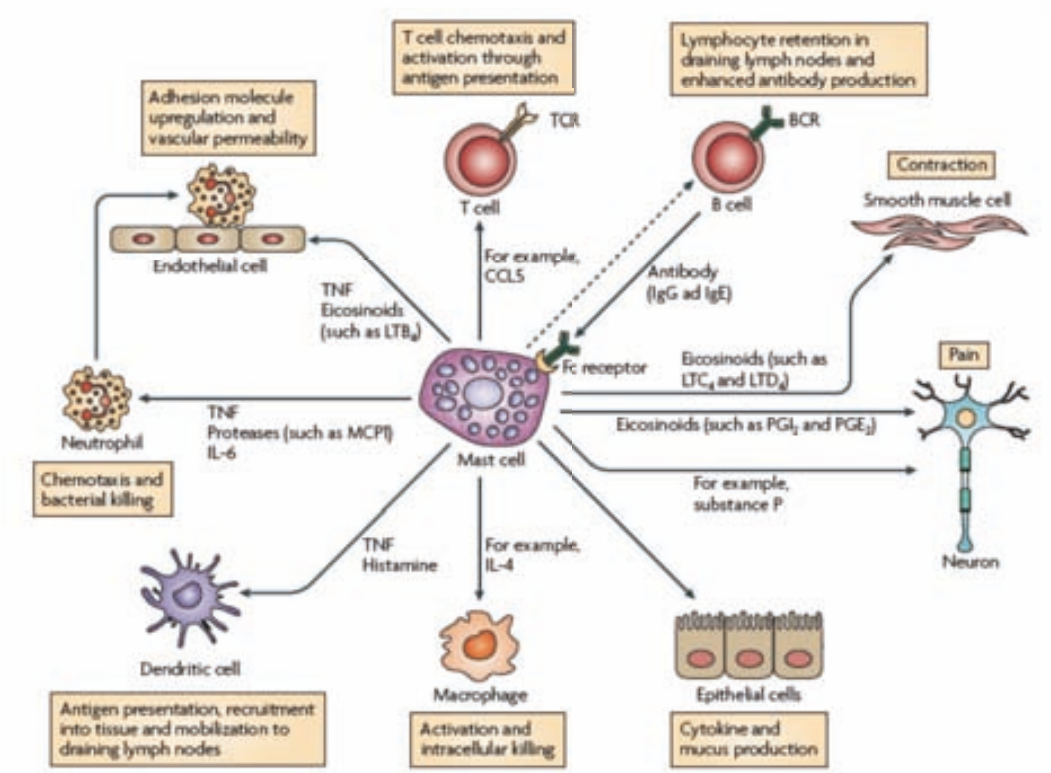
- Células dendríticas (CD): son leucocitos que constituyen una población muy heterogénea con características fenotípicas y funcionales diferentes entre las subpoblaciones de distintas localizaciones. Expresan un gran número de moléculas del complejo principal de histocompatibilidad (*major histocompatibility complex*, MHC) y moléculas co-estimuladoras. Son células fundamentales de enlace entre la inmunidad innata y adaptativa. Poseen capacidad fagocítica, de captura y procesamiento y de presentación del Ag. Sólo las CDs totalmente activadas, las APC, son competentes por su capacidad de estimular y activar linfocitos T vírgenes y conseguir una respuesta inmunológica primaria (Iwasaki & Kelsall, 1999). Existen dos fenotipos de CDs: proinflamatorio y tolerogénico, éstas últimas caracterizadas por la expresión de CD103 y su capacidad de activar linfocitos T reguladores (Coombes *et al*., 2007). Las CDs de la lámina *propria* que expresan fractalquina (CX3CL1) pueden captar antígenos luminales de forma directa extendiendo sus dendritas, sin afectar la integridad del epitelio, a través de las *uniones estrechas* (Rescigno *et al*., 2001). Cuando son estimuladas por microorganismos, las CDs migran con el antígeno a los nódulos linfáticos, donde sufren un proceso de maduración en el cual producen quimiocinas, citocinas (IL-12, IL-6) e IFN-α/β. La síntesis de estos productos les otorga un papel en el reclutamiento y amplificación de la respuesta inmunológica innata (Hart *et al*., 2005). Durante la migración las CDs procesan los Ags a nivel intracelular y presentan en la molécula MHC-II de superficie. Esto modifica el perfil de las moléculas que expresan en la superficie, presentan niveles altos de moléculas coestimuladoras (CD80, CD86) y de adhesión que favorecen la activación de las células T y aumentan la expresión de receptores para ciertas quimiquinas como el CCR7 que favorecen su migración a los ganglios linfáticos.

- **Células asesinas naturales (*natural Killer, NK*):** son leucocitos de origen linfoide, que en su superficie no expresan el complejo CD3 completo, sino que se identifican las moléculas CD16 y CD56. No son células fagocíticas y no destruyen el patógeno directamente, pero destruyen células infectadas por agentes patógenos intracelulares (virus y protozoos) o tumorales mediante apoptosis o necrosis. Por otra parte, las células NK pueden ser centinelas y

productoras de citocinas proinflamatorias (IFN-γ, IL-2, TNFα) que modulan la respuesta inmunitaria citotóxica local (Barakat *et al*., 2009).

- Mastocitos: son células leucocitarias que residen en tejidos de la interfase organismo-medio ambiente, como piel y mucosas, donde suponen el 2-3% de la población leucocitaria. Conocidas principalmente como células efectoras de la respuesta alérgica y anti-parasitaria, también promueven mecanismos de inflamación, proteólisis y mecanismos inmunitarios de regulación y reparación tisular (Bischoff, 2009). Circulan en sangre como precursores inmaduros (c-kit+, CD34+, CD13+, FcεRI), completando su proceso de maduración y diferenciación en el tejido receptor. Poseen una extensa variedad de mediadores como la histamina, la serotonina y proteasas inmersas en matrices de glicosaminoglicanos, principalmente de heparina y sulfato de condroitina. Los mastocitos humanos se clasifican, según su contenido granular (Dvorak *et al*., 2005), en tres tipos: los que contienen sólo triptasa (MCT, *mast cell tryptase*), los que contienen triptasa, quimasa, carboxipeptidasa y catepsina G (MCCT, *mast cell chymase/tryptase*) y aquellos que sólo contienen quimasa (MCC, *mast cell chymase*). Estímulos de diferente origen tanto físicos, químicos, como biológicos, factores de crecimiento, neuropéptidos y neurotransmisores, tienen la capacidad de activar y modular el fenotipo del mastocito (Ghildyal *et al*., 1992). El mastocito, una vez activado, realiza diferentes funciones vinculadas a la síntesis *de novo* de citocinas y a la liberación progresiva y selectiva del contenido granular (en segundos o minutos), mediante la formación de microvesículas, proceso conocido como degranulación selectiva o *piecemeal* (Theoharides *et al*., 1982). Otro proceso descrito es la transgranulación o transferencia de gránulos (que contienen heparina, quimasa, carboxipeptidasa) a través de los pseudópodos a otras células del entorno (Wilhelm et al., 2005). Además de las proteasas y otros mediadores anteriormente comentados, el mastocito sintetiza proteínas bactericidas (catelicidinas), citocinas y quimiocinas. Al igual que otras células del sistema inmunitario innato, es una célula presentadora de Ag con actividad fagocítica y capaz de activar la inmunidad adaptativa, en especial a los linfocitos T, principalmente a la población CD8 (Abraham & St John, 2010). Estos fenómenos permiten la interacción del mastocito con diferentes tipos

celulares y le conceden un papel clave en la coordinación de la respuesta inflamatoria, condicionada por su localización y contenido granular. Así, mediante las citocinas y quimiocinas liberadas tras su activación, pueden reclutar células inmunitarias tanto del sistema innato como adaptativo. Por otra parte, la liberación de histamina, proteasas y TNF-α, entre otros, inducen un aumento de la permeabilidad vascular y, por lo tanto, una alteración de la función de barrera epitelial. También modifica la función de las células musculares y de las neuronas a través de los mediadores liberados. Por esta capacidad de modular diversas funciones intestinales, el mastocito se considera una célula clave en el control de la respuesta defensiva frente a los patógenos.



- Figura 2. Comunicación entre el mastocito y otras células que intevienen en la defensa inmunitaria innata y la función intestinal (Abraham & St John, 2010).

El mastocito expresa diferentes receptores que reconocen distintos tipos de ligandos: TLRs para los PAMPs, receptores Fc para anticuerpos (IgG, IgE) específicos de patógenos y otros receptores para los factores inflamatorios

producidos en respuesta a una infección o agresión. Los mediadores que se liberen están vinculados al receptor responsable de la activación del mastocito. Así en ratones se ha observado que la estimulación del mastocito a través del TLR4 puede producir TNF-α, IL-6, IL-13, IL-1β sin liberar proteasas, sin embargo ambos fenómenos ocurren tas la activación a través del TLR2, y además produce IL-4, IL-5 y no IL-1β.

2.1.2. TLR en la inmunidad innata:

- Estructura y señalización de los TLRs: se trata de una familia de proteinas conservadas evolutivamente descritas por primera vez en la mosca de la fruta (*Drosophila*) (Lemaitre et al., 1996). Se han identificado 11 subtipos diferentes en humanos, presentes en diferentes células (inmunitarias y no inmunitarias) y cada uno de ellos reconoce diferentes tipos de ligandos microbianos y endógenos. Su activación inicia la transcripción de genes involucrados en la respuesta inmunitaria e inflamatoria frente a la agresión, siendo destacable su capacidad de autoregular y coordinar una adecuada respuesta inicial. Los TLR son glicoproteinas trasmembrana, compuestas por un dominio extramembrana de reconocimiento, caracterizado por repeticiones ricas en leucina (LRRs) y un dominio intracelular de señalización, compartido por los receptores Toll y la IL-1, denominado TIR (de Toll/receptor IL-1). La unión del ligando al receptor origina un cambio conformacional y activa la ruta de señalización intracelular que precisa reclutar un adaptador que interacccione con el dominio TIR para la progresión de la cascada de señalización. Existe un adaptador común, denominado MyD88, aunque evidencias recientes sugieren la presencia de vías de señalización divergentes a éste, dependientes e independientes (Abreu., 2010). La cascada de señalización finaliza con la activación del factor nuclear de transcripción kappa B (NFkB) y la producción de citoquinas inflamatorias (TNF-α, IL-6, IL-1β, IL-12) e IFN-γ, quimiocinas, o promoviendo la proliferación o la apoptosis de la célula. La mayoría de estos receptores se expresan y contactan con el Ag en la superficie celular (TLR1, 2, 4, 5, 6, 10 y 11), mientras que una minoría está presente en compartimentos endosomales (TLR3, 7, 8 y 9) que reconocen el Ag una vez fagocitado por la

célula. El dominio extracelular LRR de algunos TLR puede formar heterodímeros (por ejemplo: TLR2+TLR1 o TLR2+TLR6) lo que aumenta su capacidad de reconocimiento de diferentes ligandos.

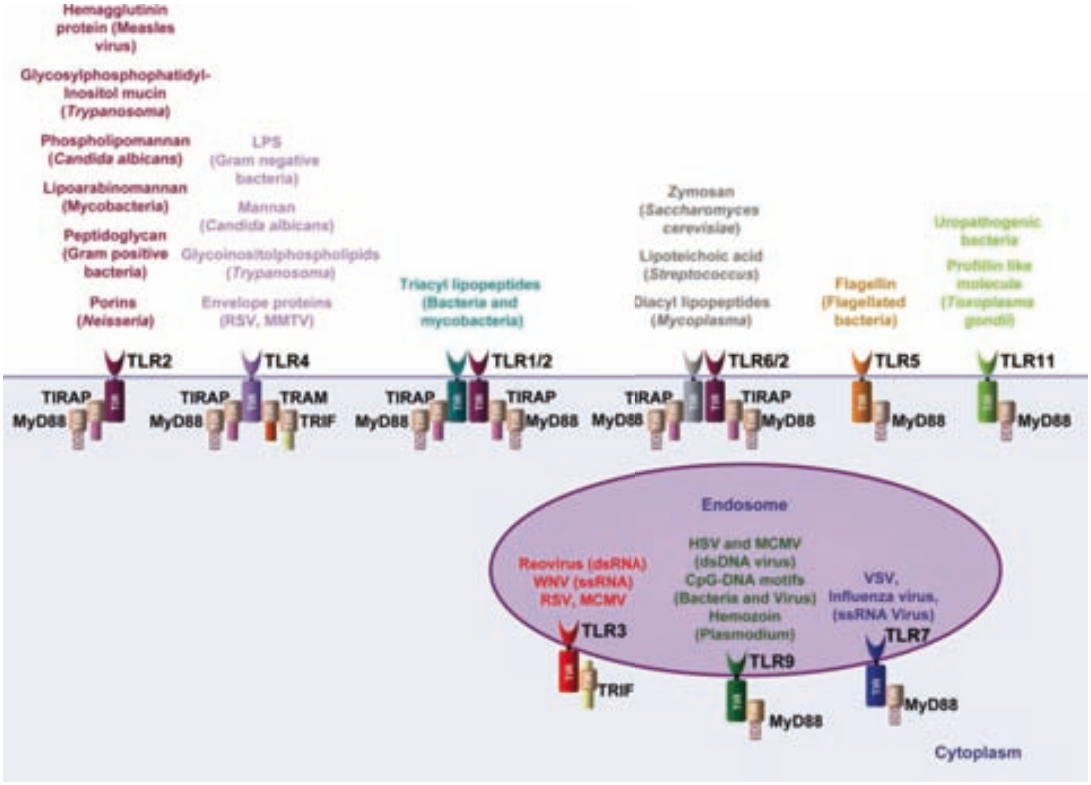


Figura 3. Ligandos (PAMPS) de los diferentes TLRs y sus adaptadores (Kumar *et al*., 2009).

- Función de los TLRs en el intestino: La expresión de estos receptores en diversas células intestinales explica su relevancia en la conexión entre la respuesta inmunitaria intestinal innata y adaptativa, así como su capacidad de coordinar una respuesta adecuada frente a una amplia variedad de PAMPs. Las células, que expresan TLRs son: DCs, macrófagos, NK, mastocitos, neutrófilos, células epiteliales, linfocitos T y B, fibroblastos, glía, neuronas y endotelio. Además del papel principal de protección frente a infecciones por agentes entéricos patógenos, mediado por la activación de las vías de señalización proinflamatorias en células inmunitarias principalmente, la señalización de los TLRs está involucrada en la migración de linfocitos B al intestino delgado y en el reclutamiento de mastocitos (Santaolalla *et al*., *2012*). El papel de la flora bacteriana en el intestino es muy importante al determinar el desarrollo y las funciones del sistema inmunitario, gracias a un proceso de selección durante la evolución de diferentes mecanismos que faciliten y

sostengan la simbiosis entre las bacterias residentes y el huesped. El intestino adquiere la flora intestinal tras el nacimiento, durante el parto; inicialmente obtiene la macrobiota de la madre y, posteriormente, durante los primeros años, la población bacteriana va aumentando en número y diversidad hasta una concentración que en el colon alcanza 10^{12} bacterias por gramo de contenido luminal. Esta flora está compuesta por cientos de especies ambientales determinadas por diferentes factores: infecciones, genética, dieta, estrés y antibióticos.

Aunque la flora sea diferente en cada individuo, ésta pertenecerá principalmente a dos filotipos: *firmicutes spp* y *bacteroides spp* (*Ley et al.,* 2008). Se ha comentado previamente la necesidad y el beneficio de la convivencia entre la flora y el huesped, los enterocitos utilizan el producto resultante de la fermentación bacteriana, los ácidos grasos, como fuente de energía. Así el intestino precisa de mecanismos de control de la actividad de los TLRs en el tracto gastrointestinal que generan tolerancia y limitan la respuesta inflamatoria exagerada frente a la flora comensal. El fallo en estos mecanismos de control puede inducir alteraciones en la función intestinal y generar procesos inflamatorios crónicos y de destrucción tisular (Salzman & Bevins, 2008). El equilibrio entre respuestas pro-inflamatorias y reguladoras en

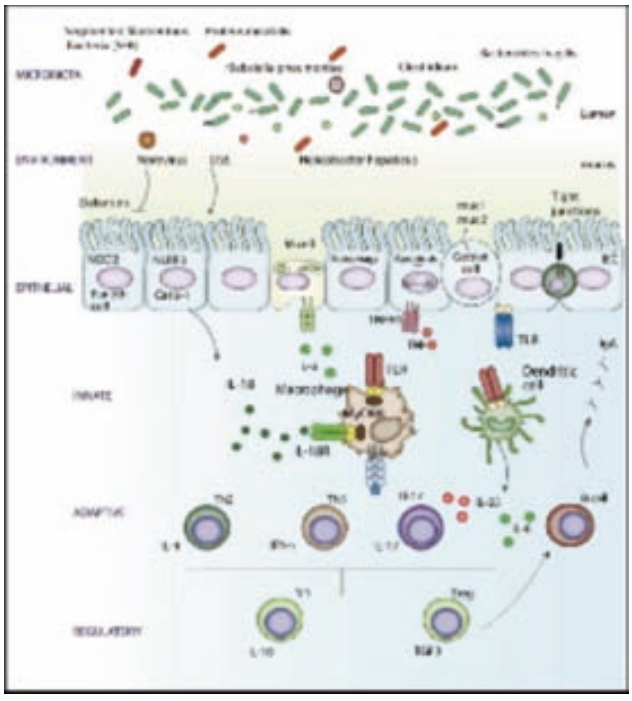


Figura 4. Ligandos (PAMPS) de los diferentes TLRs y sus adaptadores (Kumar *et al*., 2009).

el intestino se altera en situaciones de disbiosis. Diferentes estudios experimentales confirman que la estabilidad en la flora bacteriana es índice de salud, sin embargo su inestabilidad se relaciona con el SII y la enfermedad inflamatoria intestinal (EII), teniendo los TLRs un papel en el desarrollo de estas patologías (Cerf-Bensussan & Gaboriau-Routhiau, 2010; Brint *et al*., 2011; Belmonte *et al*., 2012).

La activación de las vías de señalización de los TLRs en el epitelio contribuye a la homeostasis intestinal (proliferación de células epiteliales, producción de IgA, mantenimiento de las uniones estrechas y la expresión de péptidos antimicrobianos) (Shang *et al.,* 2008*;* Cario *et al.,* 2004*,* 2007), e induce respuestas proinflamatorias (Wells *et al.,* 2010; Gibson *et al.,* 2008). Los mecanismos que controlan la activación persistente de los TLRs que se conocen hoy en día son: la internalización o alteración del tráfico intracelular de estos receptores (Gewirtz *et al*., 2001) y la expresión de moléculas que inhiben la cascada de señalización de los TLR como IRAK-M, TOLLIP, SIGIRR, A20, y PPARγ (Abreu, 2010; Miggin & O´Neill, 2006). Se ha confirmado la expresión del RNA mensajero y proteína de casi la totalidad de los TLRs en el intestino delgado y grueso, aunque nuestro conocimiento sobre su distribución y función en el epitelio, según el segmento intestinal y tipo celular, no es completo. En la literatura encontramos que a nivel del epitelio los niveles de expresión en condiciones basales de TLR2 y TLR4 son bajos y su localización principal son las células de la cripta del colon (Cario, 2008), mientras que el TLR3 se expresa en los enterocitos maduros (Furrie *et al*., 2005). Por otra parte, los TLRs tienen la capacidad de reconocer moléculas endógenas como las proteínas de choque térmico (heat shock proteins, HSP), fibrinógeno, ácido hialurónico y β-defensina, entre otras (Yu *et al*., 2010).

3. SÍNDROME DEL INTESTINO IRRITABLE

El Síndrome del Intestino Irritable (SII) pertenece al grupo de trastornos funcionales digestivos caracterizados clínicamente por la presencia de manifestaciones crónicas y recurrentes, dominadas por la presencia de dolor

y/o molestia abdominal y la alteración del hábito deposicional (cambio en el número y/o consistencia de las deposiciones). En la actualidad, según el patrón predominante del hábito deposicional, se diferencian tres subtipos clínicos: diarrea, estreñimiento o mixto. Hasta ahora no se ha establecido un sustrato orgánico universal que explique el origen y las manifestaciones clínicas de este trastorno, ni se han identificado biomarcadores útiles para establecer un diagnóstico positivo. Por ello, el diagnóstico actual del SII sigue basándose en criterios clínicos y en la exclusión de otras patologías orgánicas que cursen con sintomatología similar.

El SII constituye uno de los problemas de salud más frecuentes en el mundo, con una prevalencia en EEUU, Europa y Asia de un 10-20%. (Khan *et al*., 2010; Lee, 2010). En España, la prevalencia varía entre el 2-12%, según los criterios que se apliquen para su diagnóstico (Mearin *et al*., 2001). La prevalencia de este síndrome es mayor en el género femenino, habiendo en España de 2-4 veces más pacientes mujeres que hombres (Mearin *et al*., 2001). La inespecificidad de los criterios diagnósticos y la falta de opciones terapéuticas satisfactorias, promueve en muchos casos la realización de exploraciones innecesarias y repetidas para excluir lesiones orgánicas que justifiquen los síntomas. Al menos dos terceras partes de estos enfermos consultarán repetidamente al médico cada año y un porcentaje de éstos acudirá a diferentes especialistas. Estas particularidades explican la reducida calidad de vida que experimentan los pacientes con SII y sus familiares y la elevada y creciente repercusión social, sanitaria y económica de esta enfermedad en los países desarrollados (Camilleri *et al*., 2000; Maxion-Bergemann *et al*., 2006).

Hasta el momento, el SII se considera una patología compleja, consecuencia de la interacción entre factores psicosociales, culturales y biológicos, sin embargo, los mecanismos fisiopatológicos subyacentes no están claramente establecidos. En las últimas décadas, diferentes grupos de investigación de todo el mundo se han centrado en averiguar y entender los mecanismos implicados que justifiquen las alteraciones gastrointestinales de este síndrome.

3.1. Criterios diagnósticos

Los pacientes con SII presentan manifestaciones clínicas muy variadas y heterogéneas, por lo que generalmente su diagnóstico se realiza por exclusión de otras patologías. La falta de un marcador biológico fiable que ayude en el diagnóstico del SII, ha incentivado la búsqueda, en las últimas décadas, de criterios clínicos estandarizados que ayuden en el diagnóstico y su diferenciación de otras enfermedades orgánicas abdominales, minimizando el número de exploraciones complementarias innecesarias. Los primeros criterios que ayudaron a diferenciar el SII surgieron en 1978 por Manning y colaboradores, los cuales encontraron cuatro síntomas comunes significativos entre los pacientes de SII: dolor abdominal que alivia con la deposición, distensión abdominal objetiva, cambio de la frecuencia y cambio en la consistencia de las deposiciones con el inicio del dolor abdominal. Estos criterios se denominaron “Criterios de Manning” (Manning *et al*., 1978). Posteriormente, en 1992, un comité de expertos internacionales se reunió en Roma para la unificación de criterios diagnósticos basándose en los síntomas y en los estudios epidemiológicos. Este mismo consorcio estableció, además, la clasificación de los trastornos funcionales intestinales en diferentes subgrupos, denominándose “Criterios de Roma” (Thomson *et al*., 1992). Estos criterios tienen como fin estandarizar y aumentar la sensibilidad y la especificidad diagnóstica. También facilitan la elaboración de criterios de inclusión en ensayos clínicos y en estudios de investigación básica y, por lo tanto, permiten generalizar los resultados obtenidos a pacientes con síntomas similares, con el objetivo de contribuir al desarrollo de tratamientos más efectivos y selectivos para cada subgrupo. Estos criterios clínicos son la herramienta actual para realizar estudios poblacionales de prevalencia de las enfermedades funcionales digestivas.

Hasta la actualidad los Criterios de Roma se han revisado en diferentes ocasiones (Roma I, II y III), al incluir aspectos psicosociales, evidencias científicas y nuevos conocimientos en neuro-gastroenterologia, lo cual ha incrementado su validez potencial.

Los Criterios de Roma I (Thomson *et al*., 1992) establecían el SII como dolor abdominal que mejora o se asocia al cambio en el hábito intestinal en los últimos 3 meses y presenta además al menos otras dos características: cambio en la frecuencia, consistencia, alteración en la evacuación, distensión y/o meteorismo y presencia de moco. Posteriormente, se elaboraron los criterios de Roma II (Thomson *et al*., 1999), más estrictos, con un mayor tiempo de evolución, haciendo referencia a los síntomas en los últimos 3 meses y agregando síntomas asociados al comienzo del dolor abdominal. En esta clasificación se separa por primera a vez a los pacientes de SII en diferentes subtipos en relación a características del hábito intestinal: frecuencia, consistencia de las deposiciones, urgencia y dificultad en la evacuación. Por último, en 2006, se elaboraron los Criterios de Roma III (Longstreth *et al*., 2006), considerando conjuntamente el tiempo de evolución y la consistencia de las deposiciones para la clasificación en los diferentes subtipos clínicos.

Criterios de Roma III

Dolor o malestar abdominal recurrente**, al menos 3 días al mes en los últimos 3 meses asociado con dos o más de los siguientes síntomas:

1. Se alivia con la defecación
2. Inicio asociado con un cambio en la frecuencia de las deposiciones
3. Inicio asociado con un cambio en la consistencia de las deposiciones.

Estos criterios deben estar presentes activamente en los últimos 3 meses y los síntomas haber comenzado al menos 6 meses antes del diagnóstico. En investigación fisiopatológica y ensayos clínicos, la frecuencia del dolor/molestia debe estar presente al menos dos días por semana durante el periodo de evaluación inicial para la inclusión del sujeto.

(** Molestia significa una sensación incómoda no descrita como dolor).

En función del tipo de deposiciones, el SII se clasifica en diferentes subtipos:

- **SII estreñimiento (SII-E):** Si más del 25% de las deposiciones son duras o en bolas (Bristol 1-2) y menos del 25% son sueltas o acuosas (Bristol 6-7).

- **SII diarrea (SII-D):** Si más del 25% de las deposiciones son sueltas o acuosas (Bristol 6-7) y menos del 25% son duras o en bolas (Bristol 1-2).
- **SII mixto (SII-M):** Si las más del 25% de las deposiciones son duras o en bolas (Bristol 1-2) y más del 25% son sueltas o acuosas (Bristol 6-7).
- **SII inclasificable:** alteraciones insuficientes en la consistencia de las heces para incluirlo en cualquiera de los grupos anteriores.

El término "alternante" se reserva para los cambios a lo largo de periodos prolongados de tiempo. La consistencia de las deposiciones se evalúa en ausencia de toma de agentes antidiarreicos o laxantes.

3.2. Manifestaciones clínicas

3.2.1. Dolor abdominal

El dolor abdominal es el síntoma clínico principal que caracteriza el SII. Se relaciona con el cambio en el hábito intestinal (frecuencia y/o consistencia de las deposiciones) y mejora con la defecación. El dolor puede ser difuso o localizado a nivel infraumbilical (hemiabdomen inferior) y de tipo cólico, opresivo o punzante. Aunque se desconoce la causa que origina el dolor, uno de los mecanismos más aceptados en los últimos años es la presencia de hipersensibilidad visceral en estos pacientes, por su moderada prevalencia en diferentes estudios. Este mecanismo hace referencia a un aumento en la percepción a estímulos químicos o mecánicos en el tracto gastrointestinal de carácter fisiológico o no (Piché., 2010; Knowles & Aziz., 2009). La hipersensibilidad visceral se compone de dos fenómenos: la hiperalgesia y la alodinia. La hiperalgesia se caracteriza por un aumento de la percepción de dolor tras un estímulo doloroso o nocivo, debido a una disminución del umbral del dolor. La alodinia se caracteriza por la percepción del dolor ante un estímulo que normalmente no causa dolor. La hipersensibilidad visceral se ha propuesto como uno de los determinantes fisiopatológicos del desarrollo del SII. El aumento de la sensibilidad visceral es consecuencia de alteraciones en la interacción bidireccional entre los sistemas nerviosos entérico (SNE) y central (SNC), junto con posibles defectos en los mecanismos de modulación de la percepción. Cada vez surgen más evidencias que muestran alteraciones

en los mecanismos de control y de procesamiento del dolor a nivel del SNC y la relación entre estas anomalías y las manifestaciones clínicas del SII (Seminowicz *et al*., 2010; Ringel *et al*., 2008; Elsenbruch, 2011).

3.2.2. Patrón de deposiciones

Otro de los motivos frecuentes de consulta de los pacientes de SII al gastroenterólogo son los síntomas relacionados con la defecación. Ya se ha comentado anteriormente que el SII se divide en subtipos basado en la consistencia de las deposiciones, según predominen en el hábito deposicional. Los criterios de Roma III recomienda la escala de Bristol como herramienta para identificar siete tipos diferentes en función de la forma.

Figura 5. Escala de Bristol (Lewis & Heaton, 1997)

Esta clasificación es importante, dado que condiciona la estrategia terapéutica a adoptar, además de facilitar la evaluación de la eficacia clínica de diferentes fármacos en ensayos clínicos. La consistencia y/o forma de las deposiciones en comparación con el número se correlaciona mejor y de forma moderada con el tiempo de tránsito intestinal, por lo que se valora como posible parámetro indirecto del tiempo de tránsito intestinal (Degen & Phillips, 1996; Saad *et al*., 2010). Sin embargo, existe una variabilidad inter e intra-individual, y en aproximadamente el 80% de los pacientes se observa una fluctuación en la forma de las deposiciones a lo largo del tiempo, independiente del subtipo de

SII y del tratamiento que reciba el paciente: la consistencia varía entre los valores extremos de la escala de Bristol al menos tres veces al mes. (Palsson *et al*., 2012)

3.2.3. Meteorismo y distensión abdominal

El meteorismo es un síntoma muy común en los trastornos funcionales gastrointestinales, en especial en el SII, y hace referencia a la sensación subjetiva del incremento de la presión intra-abdominal como consecuencia de la percepción de un exceso de gas en el intestino. Generalmente este síntoma empeora tras la ingesta y a lo largo del día, desapareciendo tras el descanso nocturno (Longstreth *et al*., 2006). Su origen se desconoce y por tanto su manejo es poco gratificante. En ocasiones, el meteorismo se puede acompañar de un incremento del perímetro abdominal, denominado distensión abdominal, que se produce por una acomodación anómala al contenido de gas, mediada por una alteración en la respuesta del reflejo abdomino-frénico, cuyo resultado es la relajación de la pared abdominal (Azpiroz *et al*., 2007). Muchos de los pacientes que refieren meteorismo, también tienen hipersensibilidad visceral, aunque se desconocen los mecanismos subyacentes que lo expliquen. Entre los diferentes factores que podrían estar relacionados con el meteorismo y/o la distensión abdominal, se han propuesto: la activación inmunitaria en la mucosa intestinal (Cremon *et al*., 2009), la alteración de la flora intestinal (Simrén *et al*., 2012), las hormonas sexuales (Jiang *et al*., 2008) y los factores psicológicos (Chang *et al*., 2001), entre ellos la somatización. Sin embargo, se desconoce la interrelación entre ellos y qué papel y relevancia tienen.

3.2.4. Otros síntomas

Aunque no se ha demostrado una implicación clara de los diferentes perfiles de personalidad en el desarrollo de SII, el porcentaje de pacientes con síntomas y/o enfermedades psiquiátricas es mayor en aquellos con un grado de severidad moderada-severa del SII. Por otra parte, es muy frecuente que en pacientes con SII co-existan manifestaciones extraintestinales como fatiga crónica, cistitis intersticial (Chelimsky., *et al* 2012) y fibromialgia (Vandviket al., 2004). Así mismo, hay que tener en cuenta otras enfermedades gastrointestinales crónicas y evaluar si existen síntomas de alarma como fiebre,

síntomas gastrointestinales nocturnos, pérdida de peso, sangre en las deposiciones, y si la aparición de los síntomas se da pacientes de más de 50 años. Sin embargo, el valor predictivo positivo de la presencia de estos signos como diagnóstico de otras enfermedades no es muy elevado (Whitehead et al., 2006).

3.3. Valoración de la severidad del SII

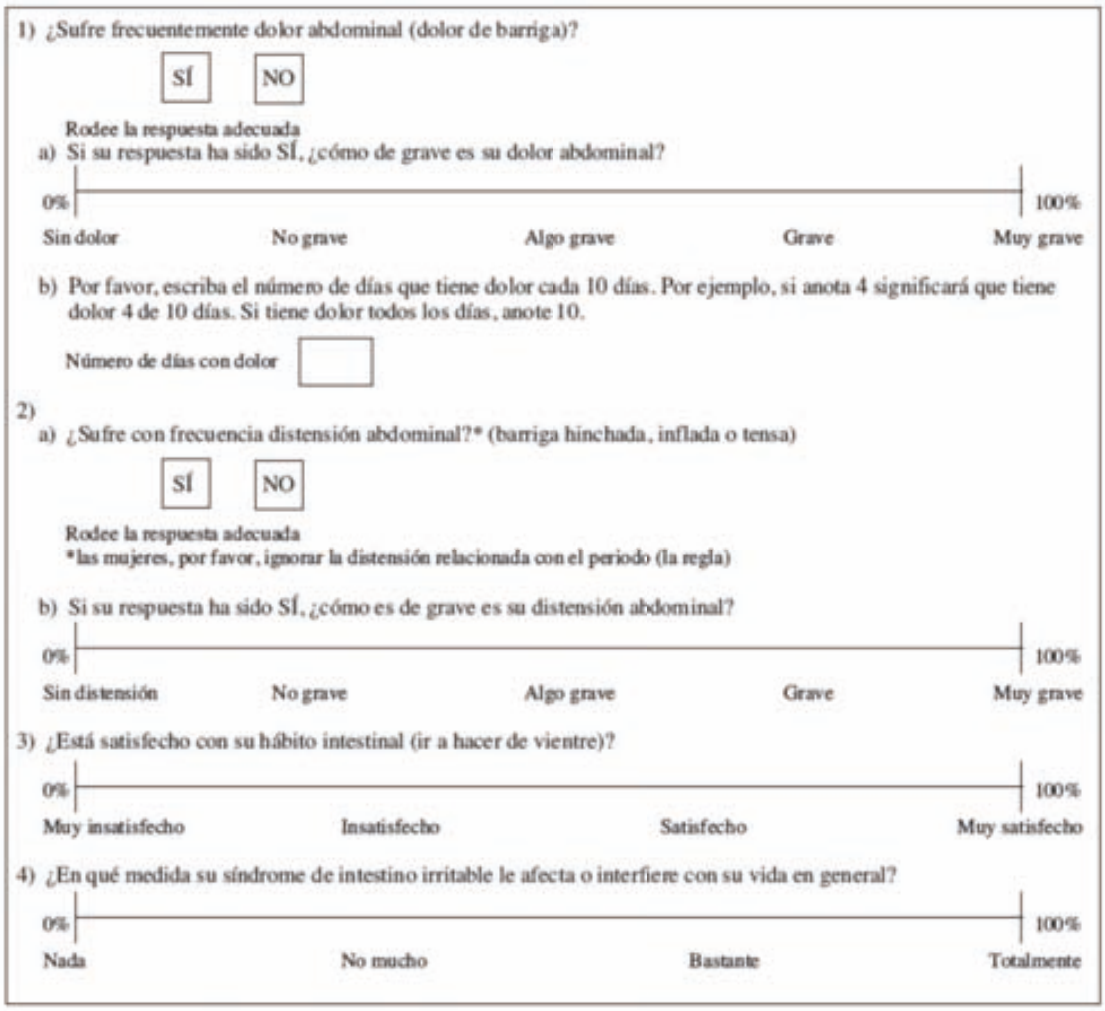
Tanto las manifestaciones clínicas del SII como su severidad pueden ser muy diversas, sin embargo, no existen muchas herramientas que ayuden en su valoración de forma estandarizada y objetiva. Este último punto es fundamental para indicar y realizar el mejor tratamiento en cada caso del SII, individualizado según el síntoma predominante. El cuestionario diseñado por Francis en 1997 (*Irritable Bowel Syndrome Severity Score;* IBSSS) permite clasificar a los pacientes en función de la severidad, así como evaluar la respuesta al tratamiento tanto desde el punto de vista clínico como en estudios de investigación (Lembo *et al*., 2005).

El IBSSS es un cuestionario sencillo en el que se evalúan los síntomas y la percepción de la enfermedad de forma retrospectiva durante los últimos 10 días. Consta de cinco preguntas: dos hacen referencia al dolor abdominal (intensidad y frecuencia), una a la intensidad de la distensión abdominal, una a la insatisfacción del paciente sobre su hábito intestinal y otra al impacto de la enfermedad en su vida diaria. La frecuencia del dolor abdominal se valora como el número de días con dolor y el resto de síntomas se evalúan mediante escalas analógicas visuales de 0 a 100. La puntuación máxima obtenida de las escalas analógicas visuales es de 100, el número de días de dolor se multiplicará por 10 y se sumará al resto, por lo que el máximo total posible es de 500 puntos.

Una vez calculada la puntuación de la severidad, este cuestionario permite también categorizar la severidad del SII en:

- **Sin enfermedad o remisión:** Puntuación menor de 75 puntos
- **Leve:** Puntuación entre 75 y 175 puntos.

- **Moderado:** Puntuación entre 175 y 300 puntos.
- **Grave:** Puntuación mayor de 300 puntos.


1) ¿Sufre frecuentemente dolor abdominal (dolor de barriga)?

SÍ NO

Rodee la respuesta adecuada

a) Si su respuesta ha sido SÍ, ¿cómo de grave es su dolor abdominal?

0% — 100%

Sin dolor | No grave | Algo grave | Grave | Muy grave

b) Por favor, escriba el número de días que tiene dolor cada 10 días. Por ejemplo, si anota 4 significará que tiene dolor 4 de 10 días. Si tiene dolor todos los días, anote 10.

Número de días con dolor

2)

a) ¿Sufre con frecuencia distensión abdominal?* (barriga hinchada, inflada o tensa)

SÍ NO

Rodee la respuesta adecuada

*las mujeres, por favor, ignorar la distensión relacionada con el periodo (la regla)

b) Si su respuesta ha sido SÍ, ¿cómo es de grave es su distensión abdominal?

0% — 100%

Sin distensión | No grave | Algo grave | Grave | Muy grave

3) ¿Está satisfecho con su hábito intestinal (ir a hacer de vientre)?

0% — 100%

Muy insatisfecho | Insatisfecho | Satisfecho | Muy satisfecho

4) ¿En qué medida su síndrome de intestino irritable le afecta o interfiere con su vida en general?

0% — 100%

Nada | No mucho | Bastante | Totalmente

Figura 6. Cuestionario IBSSS para la valoración de la severidad de los síntomas del SII (Almansa *et al*., 2011)

El cuestionario también incluye otros apartados que no tienen en cuenta el cálculo del grado de severidad, pero que, sin embargo, aportan información adicional sobre la enfermedad, como la urgencia defecatoria y el tenesmo, el número de semanas de absentismo laboral, la frecuencia de la consistencia de las deposiciones, el número máximo y mínimo de deposiciones, así como la percepción de severidad de la enfermedad. Recientemente se ha publicado la validación de la versión española de la escala de Francis que ha mostrado una excelente reproducibilidad (correlación de r=0.81 entre dos cuestionarios separados por un período de 7 a 15 días) y capacidad para detectar cambios significativos en el estado clínico de los pacientes, tales como la mejoría

clínica. Se ha establecido la variación en 50 puntos como valor estándar para detectar un cambio clínico significativo en la severidad del SII, dado que la disminución de 45 puntos en el cuestionario presenta una sensibilidad del 70,6% y una especificidad del 87,5% (Almansa *et al*., 2011). Sin embargo, existen limitaciones en cuanto a su aplicabilidad en algunos pacientes y situaciones, por ejemplo, en pacientes con discapacidades visuales o déficits motores de extremidades superiores, o su aplicación a través de encuestas telefónicas.

3.4. Origen y Fisiopatología del SII

Aunque el origen del SII es complejo y multifactorial se han identificado algunos determinantes asociados a la aparición y al desarrollo del SII incluyendo las infecciones gastrointestinales, factores socioculturales, psicológicos, factores genéticos y género-dependientes, factores dietéticos y tóxicos, antibióticos y alteraciones de la microbiota intestinal. Aunque la base fisiopatológica responsable no está claramente establecida, las alteraciones en la motilidad, la hipersensibilidad visceral y la inflamación en la mucosa, se han postulado como alguno de los principales mecanismos de disfunción intestinal.

La función gastrointestinal se modula a través del SNE y del SNC. La inervación del sistema nervioso intrínseco gastrointestinal procede del SNE, incluye los plexos mientérico y submucoso, y es capaz de controlar de forma independiente del SNC, en un 80%, las funciones fisiológicas del intestino. Sin embargo, la inervación del sistema nervioso extrínseco es bidireccional y depende principalmente del sistema nervioso autónomo (SNA) (simpático y parasimpático). El cerebro puede influir sobre la función del SNE a través del SNA y viceversa. A través de neuronas aferentes primarias intrínsecas (*intrinsic primary afferent neurons*, IPANs), el intestino responde a los estímulos luminales y regula su propia función ante la ausencia de los estímulos del SNC. Éstas se encargan de los reflejos motores y de secreción, así como de la transmisión de la nocicepción; muchos de los somas de estas neuronas se localizan a nivel de los ganglios nodosos del asta dorsal de la médula espinal. En los últimos años ha surgido un especial interés por la investigación de los

mecanismos de interacción y comunicación entre el SNC y los sistemas homeostáticos de la función intestinal, lo que se denomina el eje cerebro-intestino. La comunicación bidireccional entre el SNC, a través del SNE, y las células inmunitarias residentes en la mucosa intestinal se denomina el eje neuro-inmunológico, el cual tiene un papel clave en la fisiopatología de enfermedades gastrointestinales (Genton & Kudsk, 2003). Además de la comunicación bidireccional entre el cerebro y el intestino, existe también una relación entre los factores psicosociales y fisiológicos (Alonso *et al*., 2008) con la severidad de los síntomas funcionales gastrointestinales y con la evolución clínica en estas enfermedades, lo cual se denomina modelo biopsicosocial (Halpert & Drossman, 2005).Así, determinados factores, como el estrés, la fatiga crónica y la depresión, participan en la susceptibilidad y en el desarrollo del SII.

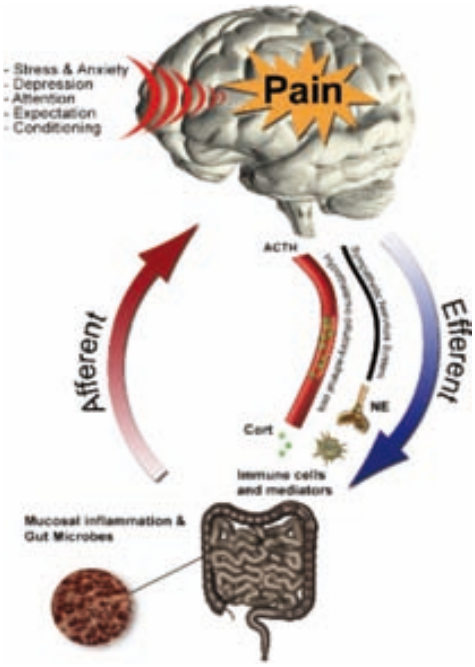


Figura 7. Vías periféricas y centrales del eje cerebro-intestino en la hiperalgesia del SII (Elsenbruch, 2011)

3.4.1. Factores relacionados con el desarrollo del SII

- Factores genéticos:

Diferentes estudios de prevalencia de trastornos funcionales en determinadas familias revelan un fenómeno de agregación familiar, siendo del 33% en pacientes del SII, mientras que en la población sana solo un 2% presenta

familiares con SII (Locke *et al*., 2000). Otros estudios han concluido que la probabilidad de padecer trastornos funcionales digestivos en gemelos se explica en un 57% por factores genéticos y en un 43% por factores ambientales (Morris-Yates *et al*., 1998) y que la probabilidad entre parejas de gemelos de que ambos hermanos tengan el SII es el doble en gemelos univitelinos respecto a bivitelinos (Levy *et al*., 2001). La posible predisposición genética en estos pacientes ha dado lugar a estudios de polimorfismos genéticos (variaciones o repeticiones en una secuencia de DNA) en los que se han descrito alteraciones en genes relacionados con los sistemas de neurotransmisión: serotinérgico (transportador de la recaptación de serotonina, SERT), adrenérgico (α2C receptores) y opiode, así como en genes involucrados en la codificación de proteinas con función inmunomoduladora y/o neuromoduladora. Existen evidencias de una mayor prevalencia de polimorfismos relacionados con el aumento de producción de TNF-α (van der Veek *et al*., 2005) y con la disminución de IL-10 (Gonsalkorale *et al*., 2003; Zucchelli *et al*., 2011) en los pacientes de SII respecto a sujetos sanos. (Barkhordari *et al*., 2010). En el caso de la fisiopatología del intestion irritalbe de origen pos-infeccioso se han identificado aquellos relacionados con el TLR9, con IL-6 y con la proteína E-cadherina (Villani *et al*., 2010).

- Factores ambientales:

En los subgrupos de SII, existen diferencias cuantitativas y cualitativas en la composición y en la estabilidad de la microbiota intestinal a lo largo del tiempo, aunque aún no se ha identificado una asociación entre especies bacterianas y el desarrollo del SII (Kassinen *et al*., 2007; Lyra *et al*., 2009). Numerosas investigaciones otorgan una gran influencia a los episodios previos de gastroenteritis agudas, tanto viral como bacteriana, en el ecosistema intestinal, y su consecuente activación del sistema inmunitario intestinal, lo que constituye uno de los factores de riesgo identificados en el desarrollo de síntomas del SII, ya que en el 10% de los pacientes el origen del SII es postinfeccioso (Spiller *et al*., 2010; Longstreth *et al*., 2001). Los factores asociados al desarrollo de síntomas, por orden de importancia, son: infección prolongada en el tiempo, virulencia de la cepa bacteriana, hábito tabáquico, existencia de marcadores de inflamación, sexo femenino, depresión, padecer hipocondria y/o un episodio de

un evento adverso vital en los tres meses previos (Spiller *et al*., 2009). Otros factores que pueden modificar la flora intestinal, y que hay que tener en cuenta por su posible papel en la generación y/o modificación de la intensidad de los síntomas en los pacientes con SII, son la dieta rica en fibra, el tratamiento con antibióticos y la ingesta de agentes prebióticos y probióticos (Dear *et al*., 2005; Shepherd *et al*., 2006; Simren *et al*., 2012).

Cómo la flora intestinal interviene en la fisiopatología del SII sigue siendo aún una incógnita, y en los últimos años además se añade el debate sobre qué papel potencial tiene el sobrecrecimiento bacteriano (*small intestinal bacterial overgrowth*, SIBO). Su presencia se asocia con diferentes síntomas del SII como el meteorismo, distensión y alteración de la función intestinal, explicados por la fermentación bacteriana de carbohidratos. Para el diagnóstico de SIBO en el intestino delgado, la herramientas que se emplea es el test de hidrógeno de lactulosa, aunque su especificidad es subóptima por el elevado porcentaje de falsos positivos. Por ello, se desconoce exactamente su prevalencia en el SII, aunque se calcula entorno al 54%. Por otra parte, cada vez surgen más evidencias que hacen referencia al posible beneficio terapéutico de antibióticos orales como la rifaximina (Pimentel *et al*., 2011) y diferentes probióticos (Moayyedi *et al*., 2010) en el SII.

- Factores psicosociales

La prevalencia de comorbilidades psiquiátricas (principalmente ansiedad y depresión) es mayor en pacientes con SII (40-50%) respecto a sujetos sanos y a pacientes con otras enfermedades gastrointestinales orgánicas (Stasi *et a*l., 2012). La existencia de estas comorbilidades conlleva una exhacerbación de los síntomas (Elsenbruch *et al*., 2010a, 2010b), una mayor demanda de atención médica y una perpetuación de las alteraciones intestinales. La mayoría de los pacientes asocian un desencadenamiento y/o exacerbación de los síntomas a la vivencia de un evento de estrés agudo psicosocial y su mejoría tras la finalización de éste (Bennett *et al*., 1998). Aunque se desconoce cuál es el factor psicológico más relevante asociado al dolor abdominal, los síntomas de ansiedad y depresión se relacionan más directamente con el dolor abdominal que con el umbral de la sensibilidad rectal (Elsenbruch *et al*.,

2010b). En los últimos años ha aumentado el interés por entender la función y los mecanismos de acción del estrés psicológico crónico, principalmente el psicosocial, en las enfermedades funcionales digestivas. Los estudios epidemiológicos en pacientes de SII destacan la relación, en un subgrupo de enfermos, entre la aparición del síndrome y antecedentes de episodios de estrés en las primeras etapas de la vida, como estrés socioeconómico en la adolescencia, padres con problemas en la relación o abusos sexuales (Irwin *et al*., 1996; Delvaux *et al*., 1997; Naliboff *et al*., 2012).

- Factores dietéticos

La dieta no está implicada en el origen del SII, pero en algunos pacientes puede desencadenar los síntomas intestinales característicos, lo que ocasiona que los pacientes retiren de la dieta alimentos que vinculan al empeoramiento de los síntomas. Esto apoya realizar, en casos concretos, una historia dietética detallada de forma prospectiva y registrar en un diario los alimentos consumidos. Una de las enfermedades a excluir para el diagnóstico del SII es la intolerancia a la lactosa, por su prevalencia en nuestro medio (Leis *et al*., 1997). Existen datos epidemiológicos que identifican una mayor prevalencia de historia familiar y personal de atopia alimentaria en el SII, principalmente en el subgrupo de diarrea, por lo que algunos estudios han evaluado la respuesta clínica a una dieta de exclusión. En algunos casos, los síntomas mejoran en asociación a la eliminación de los alimentos para los que los pacientes presentan IgG o IgE específicas (Atkinson et al Gut 2004; Drisko et al J Am College of Nutrition 2006). Aunque no existe consenso, diversas sociedades de gastroenterología europeas han elaborado guias basadas en la evidencia sobre el manejo dietético para el control de los síntomas de los pacientes con SII (McKenzie *et al*., 2012).

3.4.2. Mecanismos de disfunción intestinal en el SII

- Alteración de la motilidad intestinal

Los cambios en la forma y la frecuencia de las deposiciones, característicos del SII, sugieren alteraciones en la función motora intestinal de estos pacientes.

Existen indicios de anomalías en la motilidad intestinal en un subgrupo de enfermos (aproximadamente un 30%) con respecto a sujetos sanos, que dependen del subtipo de SII: los pacientes del subtipo mixto no presentan diferencias, sin embargo, el 26,3% de los pacientes del subgrupo SII-E y el 33,3% de los SII-D tienen aumentado y disminuido, respectivamente, el tiempo de tránsito colónico (Manabe *et al*., 2010). En estos pacientes, se observa un incremento en la respuesta motora (cualitativa y cuantitativa) respecto a sujetos sanos en respuesta a un estímulo de estrés (Fukudo *et al*., 1987; Murray *et al*., 2004). El tránsito de gas intestinal en estos pacientes también es diferente al de la población sana, lo cual sugiere una relación con la aparición de síntomas (Serra *et al*., 2002).

Estos cambios en la función motora gastrointestinal en un subgrupo de enfermos de SII, han impulsado el estudio de las diferentes vías de neurotransmisión implicadas en su control. En respuesta al estrés, el SNC modula la activación y la actividad de los sistemas SNA e hipotálamo-hipofisario-adrenal (*Hypothalamus Adrenal Pituitary Axis,* HPA). Debido al vínculo entre la respuesta al estrés y el desarrollo o la exacerbación de síntomas en el SII, una de las hipótesis sobre su patogénesis es la interacción anómala de ambos sistemas (Fukudo *et al*., 1997). Esto justifica el estudio de los diferentes neuropéptidos y hormonas gastrointestinales cuya secreción está modificada en respuesta al estrés, entre otros: Factor liberador de corticotropina (*Corticotroping Releasing Factor*, CRF), VIP y colecistoquinina (*Cholecystokinin,* CCK) (Niederau *et al*., 1992; Cassar-Malek *et al*., 1998). Sin embargo, la alteración de la motilidad gastrointestinal también está presente en respuesta a otros estímulos como la ingesta de alimento y la administración experimental de CCK. Otro de los neurotransmisores más estudiados en relación al SII es la serotonina (5-HT), por su implicación en el eje cerebro-intestino. La mayor fuente de serotonina es el intestino y su liberación se produce en respuesta a estímulos sensitivos en la luz intestinal o en la mucosa, mediando las funciones intestinales de secreción, actividad motora y percepción y, además, participa a nivel del SNC en la regulación del estado de ánimo y del apetito. Ciertas modificaciones en las vías de señalización de la serotonina podrían estar relacionados con patologías intestinales asociadas a

cambios en la función intestinal.

Existe una asociación entre la ansiedad y las anomalías intestinales con un polimorfismo en la región promotora del gen 5-HTT (Murphy *et al*., 2008), que codifica la proteína SERT para el transportador de solutos 6 (*SLC6A4).* Esta mutación origina cambios en la función del transportador y modifica las concentraciones del neurotransmisor. En pacientes con SII-D se ha observado un aumento de la concentración de serotonina y de sus metabolitos en el plasma (Atkinson *et al*., 2006) y en algunos, cambios en la expresión de transcritos SERT en la mucosa del colon (Camilleri *et al*.,2007; Stubbins *et al*., 2004; Jarrett *et al*., 2007).

Existen otros polimorfismos implicados en las vías de señalización de otros neurotransmisores, asociados a SII-E (Kim *et al*., 2004).

- Hipersensibilidad visceral

Ante la discordancia entre los síntomas y los trastornos de la motilidad, la teoría de la hipersensibilidad visceral como posible marcador biológico del SII tomó relevancia, incrementando el número de trabajos en la literatura. La hipersensibilidad se ha observado tanto en respuesta a un estímulo como a un fenómeno fisiológico (movimientos intestinales). La inervación del tracto gastrointestinal es bastante compleja, compuesta por una abundante red neuronal intrínseca y dos redes extrínsecas: vago y medular. La inervación sensitiva aferente poseen receptores, denominados nociceptores (receptores para el dolor), que se activan ante estímulos nocivos tanto físicos (distensión de la pared) como químicos (mediadores inflamatorios) y transmiten la información a través de fibras nerviosas al SNC. El resultado de este proceso es la percepción de dolor.

Los posibles mecanismos implicados en la hipersensibilidad visceral en el SII son la alteración en las vías aferentes del eje cerebro-intestino, el procesamiento del dolor a nivel del SNC e incluso una hiperrespuesta a estímulos ambientales como el estrés. Los neurotransmisores implicados en la transmisión sináptica desde las terminaciones periféricas hasta la médula a

través de las fibras C (no mielinizadas) son: glutamato, neuropéptidos como la sustancia P (SP) y el péptido relacionado con el gen de la calcitonina (*Calcitonin gene related peptide*, CGRP).

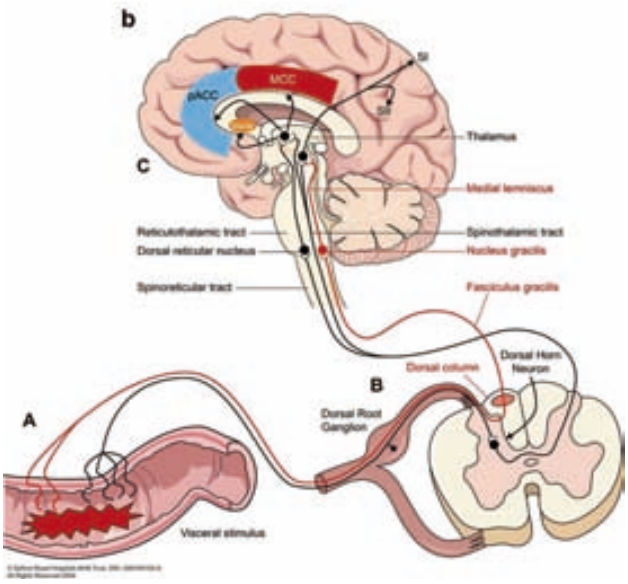


Figura 8. Esquema de las vías neuronales entre intestino y SNC de transmisión del dolor (Knowles, 2009)

Entre los receptores nociceptores de las fibras C, el más estudiado es el receptor de potencial transitorio vaniloide 1 (*transient receptor potential vanilloid 1,* TRPV1) por su relevancia en los trastornos de hipersensibilidad visceral, se distribuye a lo largo del tracto gastrointestinal (Inoue *et al*., 2006). En pacientes de SII, existe un aumento de su expresión en las fibras nerviosas intestinales, que se correlaciona con el dolor abdominal (Akbar *et al.,* 2008). La activación de estos receptores en las neuronas sensitivas primarias estimula la liberación de SP y CGRP en el tejido. Ambos neuropéptidos participan en la generación de una inflamación neurogénica y modulan la activación del mastocito, que libera proteasas que estimulan de nuevo los receptores nociceptores, lo que podría explicar la disminución del umbral de activación de los nociceptores y, por lo tanto, generar la hiperalgesia.

El procesamiento y la evaluación de la información sensitiva, en particular de aquellos estímulos desagradables o dolorosos, tiene un componente

importante cognitivo y/o emocional. Existen estudios que describen la eficacia elevada del placebo y el nocebo en la modulación de la hiperalgesia visceral en el SII (Price *et al*., 2009), lo que remarca su relevancia en los mecansimos del dolor. (Wiech & Tracey, 2009; Wiech *et al*., 2008). La introducción, en la última década, de estudios de neuroimagen, como la tomografía de emisión de positrones (*Positron Emission Tomography*, PET), ha aportado nuevas evidencias que apoyan la existencia de una alteración en el procesamiento de la información en el SNC (Mayer *et al*., 2009). En pacientes de SII y voluntarios sanos, la respuesta a estímulos viscerales, como la distensión rectal, activa diferentes áreas cerebrales (Hall *et al., 2010*), sin embargo, se desconocen los mecanismos que dan lugar a estas diferencias.

- Microinflamación intestinal.

Cada vez son más numerosas las evidencias de la existencia de alteraciones microscópicas cuyo origen se desconoce, en la mucosa intestinal, sobre todo la presencia de microinflamación y la atípica distribución de componentes de la barrera epitelial, en concreto de proteínas de las uniones estrechas (Martínez *et al*., 2012a). Estas alteraciones en la estructura de la barrera intestinal junto al aumento de la expresión del micro-RNA 29a que regula la expresión de la proteína glutamina sintetasa (Zhou *et al*., 2010), explicarían el aumento en la permeabilidad de pacientes con SII. Existen datos consistentes en el SII que muestran cambios en la permeabilidad junto a la microinflamación en la mucosa intestinal, aunque se desconoce la secuencia de los estos fenómenos.

Numerosos estudios indican un incremento en la densidad y la activación de diferentes estirpes celulares en la mucosa intestinal en, aproximadamente, el 50% de los pacientes con SII (Cremon *et al.,* 2009), principalmente en el subtipo diarrea y en aquellos de origen post-infeccioso (Guilarte *et al.,* 2007; Weston *et al.,* 1993; Spiller *et al.,* 2000). En biopsias rectales de pacientes con síntomas de SII, tras un episodio de enterocolitis aguda por *Salmonella, Campylobacter Jejuni* y *shigella,* se ha identificado un aumento del infiltrado de linfocitos T, así como un aumento de macrófagos activos e hiperplasia de células enterocromafines durante al menos los 3 meses posteriores a la

resolución de la infección (Spiller *et al.,* 2000). Este fenómeno en la mucosa intestinal podría explicar parte de los síntomas de SII, dado que la liberación de mediadores inflamatorios y citoquinas pueden sensibilizar las terminaciones nerviosas del tracto gastrointestinal, activar y promover la migración otras células inmunitarias, magnificando así el proceso inflamatorio. En la última década se han realizado múltiples estudios que han comparado la densidad de diferentes poblaciones en sangre y en la mucosa del colon de pacientes con respecto a sujetos control y la respuesta tras su estimulación (*Liebregts et al.,* 2007; Ohman *et al*., 2009).. El recuento de poblaciones de linfocitos T, con fenotipo de activación, en la mucosa del colon es mayor en pacientes con SII (Chadwick *et al*., 2002), hallazgo principal en aquellos de origen post-infección (Kim *et al*., 2010). Además, se ha descrito un aumento en la expresión de moléculas de adhesión a nivel del endotelio del colon y de integrinas a nivel de los linfocitos de sangre periférica, indicando un mayor reclutamiento de esta población al intestino de los pacientes de SII. En casos muy severos, se ha observado, en piezas quirúrgicas de intestino, infiltración de mastocitos y de linfocitos en el plexo mientérico que generan cambios neuronales en los plexos (Hiatt & Katz, *1962;* Tornblom *et al.,* 2000). Por otra parte, se han descrito diferencias en el recuento de las poblaciones celulares en función del sexo: aumento de mastocitos y disminución de linfocitos en mujeres con respecto a los hombres (Cremon *et al., 2009).*

De todas las células que componen el infiltrado inflamatorio, el mastocito parece tener un papel clave en la fisiopatología del SII. En el SII-D, la mayoría de los estudios muestran un aumento en el número de mastocitos en la mucosa intestinal (varía según la región), además, se ha constatado su activación por microscopía electrónica y en estrecho contacto con terminaciones nerviosas sensoriales en el colon de pacientes de SII (Barbara *et al.,* 2004). Además, la activación mastocitaria en el yeyuno se asocia con una mayor severidad en los síntomas en cuanto a número y forma de las deposiciones (Martinez *et al., 2012).* Estos hallazgos sugieren la interacción entre los mediadores del mastocito y los receptores neuronales y apuntan a esta célula como elemento central en la comunicación neuroinmunitaria.

En relación a la activación y la densidad de otras poblaciones inmunitarias

existen datos contradictorios, y no se han podido confirmar diferencias ni en los neutrófilos ni en las NK (Kristjánsson *et al*., 2004; Lettesjö et al., 2004; Chadwick *et al*., 2002; Motzer *et al*., 2002). Sin embargo, se ha descrito menor densidad de macrófagos en el colon de pacientes con SII, aunque la proporción de activados es mayor (Spiller *et al*., 2000; Braak *et al*., 2012).-

Así, además de las poblaciones leucocitarias, también se han evaluado los niveles de expresión de proteína y transcritos de diferentes citocinas a nivel de la mucosa del colon. Se ha descrito un incremento en la concentración de IL-1β e IL-8, sin diferencias en la de IFN-γ, IL-6 y TNFα en la mucosa intestinal de SII (Belmonte *et al.,* 2012), coincidiendo con el hallazgo previo de aumento en el nivel de expresión de RNA mensajero de IL-1βn en los pacientes de SII de origen post-infeccioso (Gwee *et al*., 2003). Por otra parte, se ha documentado un aumento en la cantidad de β-defensinas en el contenido fecal de los pacientes con respecto a controles sanos,,con niveles comparables a los de pacientes con EII (Langhorst *et al* ., 2009).

Estos hallazgos apuntan hacia un aumento en la actividad del sistema inmune innato en los pacientes con SII, y actualmente, se otorga mayor protagonismo a la activación celular que al recuento.

Por otra parte, el 33% de los pacientes con colitis ulcerosa (CU) en remisión desarrollan síntomas de tipo SII, apoyando la teoría de microinflamación como mecanismo fisiopatológico en los síntomas del SII (Burgmann *et al.,* 2006).

3.5. Interacción neuroinmunológica en el SII

El sistema nervioso es el sistema que más influye sobre el sistema inmunitario. Un buen ejemplo de ésto podrían ser las alteraciones en la respuesta inmunitaria inducidas por los estados de estrés o las diferentes evidencias científicas que sugieren una mayor severidad de los procesos inflamatorios como consecuencia de la pérdida de la inervación sensitiva.

Existen diferentes elementos que se han relacionado en la comunicación

neuroinmunitaria y el SII (Kraneveld *et al.*, 2008), se detallan a continuación:

- Neuropéptidos: Los neuropéptidos desempeñan multiples funciones a nivel gastrointestinal (Lecci et al., 2004) y son clave en esta comunicación. Por otra parte, también tienen un papel en la modulación de la respuesta inflamatoria al reclutar células inmunitarias, y participar en la activación del mastocito (Holzer *et al*., 1998). La interacción neuro-mastocito depende del microambiente local (Matsuda *et al*., 2005), el mastocito expresa receptores que reconocen diferentes neuropéptidos, entre ellos el receptor para la SP, NK1R (*neurokinin 1)* (Krumins *et al*., 1993; van der Kleij *et al*., 2003; Bischoff *et al*., 2004), liberados por las terminaciones nerviosas, lo cual explicaría la activación del mastocito por mecanismos no inmunogénicos. Además, el mastocito metaboliza y libera sustancias que modulan la respuesta neuronal entérica tanto en animales de experimentación como en humanos (Schemann *et al*., 2005). En relación a los neuropéptidos, existen cambios en los niveles de VIP, principalmente a nivel del colon, en los que se ha identificado un aumento, independientemente del subtipo de SII (Palsson et al., 2004).

- TLR: Otro de los elementos que participan en la comunicación neuroinmunológica son los TLRs, presentes en las neuronas, cuyo efecto en la neurona depende del tipo de ligando que active el TLR. Se ha documentado muerte neuronal tras la exposición de terminaciones del SNE a LPS (Arciszewski *et al*., 2007). En el intestino existen terminaciones nerviosas que coexpresan TRPV1 y TLR4, lo que explicaría la percepción de dolor ante una respuesta inflamatoria por activación directa neuronal por parte de productos bacterianos (Wadacki & Hargreaves., 2006). El TLR3 está involucrado en la producción neuronal de citoquinas y quimiocinas que participan en la atracción de leucocitos hacia las terminaciones, así como en la regulación del crecimiento neuronal. Los años de evolución de los síntomas del SII se correlacionan con los niveles de expresión génica de TLR2 y TLR4, sin embargo no existe correlación alguna con la severidad del SII (Belmonte et al., 2012). Por ahora no se conoce con detalle el papel que tienen los TLR en la microinflamación presente en el SII.

- Citoquinas: Su función es la regulación de la respuesta inmunitaria, la liberación de citoquinas desde células inmunitarias próximas a las terminaciones nerviosas aferentes en el intestino puede contribuir a la disfunción intestinal. Ciertas citoquinas se han implicado en la patogenia de la EII y en el SII (Liebregts, T., *et al* 2007). Las terminaciones nerviosas expresan citoquinas y receptores para muchas de ellas. Además diversas evidencias científicas sugieren el papel de las citoquinas en las modificaciones de las terminaciones nerviosas asociadas con la inflamación, entre ellas la neurodegeneración (Savidge *et al*., 2007). Así, el TNF-α induce la liberación de la SP, ambos relacionados con el aumento de la actividad electrofisiológica de las terminaciones nerviosas y que explicaría el dolor visceral (Ding et al., 1995; Sorkin et al., 1997). Por otra parte, la IL-1β, liberada en procesos inflamatorios crónicos, aumenta de forma indirecta la sensibilidad de las neuronas mientéricas a la bradiquinina, y a nivel submucoso actúa sobre neuronas que expresan VIP, fenómenos que están implicados en el inicio de la hipersensibilidad visceral. Además la IL-1β está actúa sobre neuronas inhibitorias motoras originando alteraciones en la actividad motora intestinal.

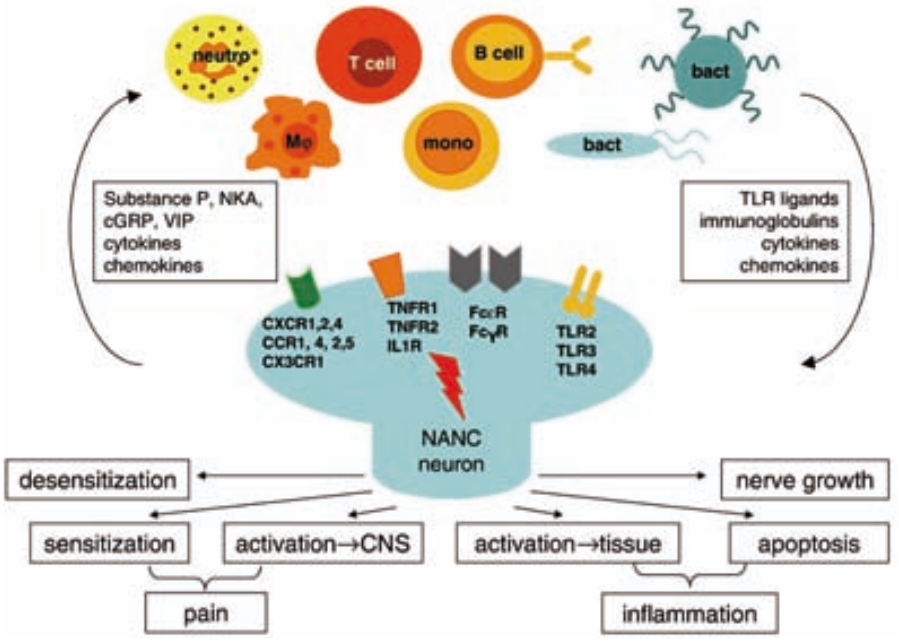


Figura 10. Esquema de los diferentes receptores neuronales para factores inmunológicos asociados en el desarrollo del SII (Kraneveld.,*et al* 2008)

3. 6. Estrés y disfunción intestinal

Diversas evidencias han confirmado que los niveles elevados de estrés crónico psicológico tienen un impacto negativo sobre la salud. Además de los efectos directos del estrés tanto a nivel psicológico, no hay que olvidar los efectos indirectos, como la modificación de los hábitos saludables: falta de ejercicio, disminución en la ingesta, cambio de las horas de sueño (Konturek.,*et al* 2011).

La maladaptación al estrés crónico tiene un papel prominente en la fisiopatología del SII. A través del eje cerebro-intestino diferentes funciones fisiológicas intestinales se ven afectadas por el estrés psicológico (Bonaz & Sabate, 2009). En los últimos años, constituye uno de los temas de mayor interés en la investigación tras conocer el papel relevante de la comunicación entre la microbiota, la inmunidad intestinal y el cerebro en la modulación de la respuesta del estrés en el intestino y en el desarrollo de diferentes patologías intestinales (Rhee *et al*., 2009). La flora es una pieza clave en esta comunicación, al modular las funciones fisiológicas del tracto gastrointestinal mediante 3 vías: directa con las células de la mucosa (vía endocrina), a través de las células inmunológicas (vía inmunológica) o a través de las terminaciones nerviosas (vía neurológica) (Rhee *et al*., 2009).

Por otra parte, el estrés altera los niveles de neurotransmisores y citoquinas inflamatorias que modifican directamente e indirectamente la composición de la flora intestinal. Aumenta la adhesión y la traslocación bacteriana secundaria al aumento de la permeabilidad bacteriana, factor que favorecería la activación del sistema inmunitario (Bailey *et al*., 2010). Además neurotransmisores como las catecolaminas alteran el crecimiento, motilidad y virulencia de la flora comensal y patogénica (Lyte *et al*., 2011).

Estudios de estrés agudo experimental en humanos han demostrado diferentes efectos en el intestino. En sujetos sanos aumenta el transporte de agua y albúmina a la luz del intestino delgado en mujeres y, en especial, en aquellas con un nivel de estrés crónico basal moderado (Alonso *et al*., 2012, 2008). Además, se ha demostrado que los pacientes de SII tienen aumento de la sensibilidad visceral en relación a sujetos sanos, tras la exposición a un evento

de estrés agudo tanto de tipo físico como psicológico (Murray *et al*., 2004).

3.7. Implicación del mastocito en el SII

En las últimas décadas, ha surgido un especial interés en estudiar en detalle la comunicación entre el SNE y las células del sistema inmunitario, sobre todo el mastocito por su función como célula efectora del eje cerebro-intestino, y su implicación en el desarrollo del SII. Se ha comentado previamente la implicación de las proteasas liberadas por el mastocito en el control de la permeabilidad epitelial y vascular (*Jacob et al., 2005*). En pacientes con SII, estudios *in vitro* y en modelos animales, se ha descrito disminución en la expresión de proteínas de las uniones estrechas, principalmente (ZO)-1 (zonula occludens-1) y ocludina, relacionadas con el aumento de la permeabilidad paracelular intestinal (Martinez *et al*., 2012a; Piché *et al*., 2009; Gecse *et al*., 2008).

Además, un ligando de la triptasa, el receptor activado por proteasa 2 (protease-activated receptor 2, PAR2), controla funciones intestinales fundamentales (Vergnolle, 2005). Este receptor se localiza a lo largo del tracto gastrointestinal, en el epitelio, en células musculares y en células inmunitarias, entre ellas en el mastocito. Por otra parte, estudios *in vitro* han demostrado que la triptasa, a través de un mecanismo dependiente de PAR2, induce cambios en la distribución de las proteinas estructurales de las uniones estrechas (Jacob et al., 2005; Bueno et al., 2008). Los sobrenadantes precedentes de biopsias de pacientes de SII, a diferencia del procedente de los sujetos sanos, aumentan la permeabilidad en cultivo Caco-2. Esto apunta hacia la relación entre el aumento de activación mastocitaria en pacientes de SII y los cambios en la permeabilidad intestinal. Además, la participación del mastocito en la alteración de la función de la barrera epitelial se ha confirmado a través de estudios ex-vivo con el uso de agentes estabilizadores del mastocito (doxantrazol o cromoglicato), al disminuir la hipersecreción del epitelio en respuesta a agentes luminales (Crowe et al., 1990; Perdue & Gall, 1985).

La degranulación (tipo piecemeal) de la triptasa por parte del mastocito también, mediante un mecanismo dependiente de PAR2, aumenta la activación

de las neuronas entéricas (Gao *et al*.,2002; Reed *et al*., 2003). Además en comparación con los sujetos sanos, la concentración de histamina y triptasa es mayor en los sobrenandantes de colon de pacientes de SII y originan una mayor activación de las neuronas sensoriales del SNE tanto en rata (Barbara *et al*., 2007) como en humano (Buhner *et al*., 2009*),* y cuyo efecto se bloquea con un agente antagonista del receptor H1(Barbara et al., 2007).

Estos hallazgos han motivado la evaluación de la relación entre la expresión de PAR2 y PAR4 en el tejido intestinal y el dolor abdominal, principalmente en el grupo de SII post-infeccioso con relación a los sujetos sanos (Han et al., 2011). Sorprendentemente, los resultados de esta comparación no muestran diferencias en la expresión de PAR2, ni a nivel de la mucosa intestinal ni de los mastocitos, y sí una disminución de PAR4 en pacientes. Además la administración de un agonista de PAR4 reduce la respuesta a la distensión colónica, lo que confirma su papel en la percepción de dolor.

Se ha comentado previamente que hormonas como el CRF en respuesta al estrés crónico tiene un papel muy importante en la fiosopatología del SII. Por otra parte, los mastocitos humanos expresan en su superficie receptores de CRF (subtipos CRF1 y CRF2)(Kempuraj *et al*., 2004) y que degranularían al interaccionar éstos con la CRF(Tache *et al*., 1993), y en consecuencia originar los efectos descritos previamente.

Además, diferentes de los mediadores liberados tienen la capacidad de activar las terminaciones nerviosas aferentes sensoriales del SNE (Gue *et al*.,1997; van den Wijngaard *et al*.,2010; Vicario *et al*., 2012), que constituyen una fuente importante de SP en el intestino, lo que sugiere otro mecanismo más como responsable de la activación persistente de mastocito mediado por el estrés.

En resumen, la activación de los mastocitos intestinales, tras una infección entérica previa o un agente agresor, y el estrés podría jugar un papel central en las manifestaciones tanto a nivel de la respuesta motora como de la percepción visceral características de esta enfermedad.

3.8. Tratamiento del SII

3.8.1 Aspectos generales

A pesar de los esfuerzos realizados en las últimas décadas, actualmente no existe un tratamiento específico para el SII y los que se utilizan están destinados estrictamente al control de los síntomas. Debido a la complejidad del síndrome, en cuanto a diversidad de manifestaciones clínicas y al fracaso en la identificación de un único factor biológico implicado, en ocasiones el tratamiento administrado es poco eficaz y frustrante para ambos: médico y paciente. Sin embargo, el avance en el conocimiento de las bases moleculares del SII ha identificado elementos clave sobre los que actuar para el restablecimiento de la homeostasis intestinal: el epitelio, las células inmunitarias, las neuronas sensitivas aferentes y el sistema nervioso central.

Las dificultades y limitaciones existentes en el manejo terapéutico de estos pacientes son:
- La necesidad de un enfoque multifactorial terapéutico.
- La gran variabilidad individual en la manifestación de síntomas y respuesta al tratamiento.
- La dificultad para realizar ensayos clínicos en el que participe un número amplio de enfermos que permita obtener conclusiones.
- La identificación de variables que ayuden en la cuantificación de la severidad del SII y en la valoración de la respuesta al tratamiento (Drossman *et al*., 2012).

El principal objetivo del tratamiento del SII es el control de los síntomas intestinales, sin olvidar identificar si existen factores psicológicos asociados a los mismos y actuar sobre ellos. Datos procedentes de diferentes trabajos describen el dolor abdominal como el síntoma de mayor relevancia en la severidad del SII comparado con las alteraciones del tránsito intestinal. Sin embargo, falta aún información más precisa sobre los mecanismos del SNC en el control de la función gastrointestinal y de la modulación del dolor. Por eso, no hay que olvidar el elevado efecto del placebo y del nocebo en la modificación de la hiperalgesia tanto visceral, como somática. Los datos procedentes de

diferentes ensayos clínicos doble ciego muestran un 30-40% de éxito en el control de los síntomas, incluso se ha demostrado una respuesta mayor (59%) en un ensayo clínico abierto con placebo durante 3 semanas (Kaptchuk *et al*., 2010).

Por lo tanto, en el óptimo tratamiento de estos pacientes, es fundamental excluir otras enfermedades intestinales graves que justifiquen los síntomas y establecer una buena y fluida relación médico-paciente, basada en la confianza y la empatía. Se ha demostrado que si ésta es positiva conlleva una mejor evolución del paciente e incluso existe una mayor respuesta al efecto placebo (Kelley *et al*., 2009). Una vez realizado el diagnóstico del SII el profesional debe proporcionar una información adecuada e individualizada: es muy importante que el paciente entienda que es una enfermedad crónica y que es preciso controlar los factores biopsicosociales que lo pueden originar, agravar o perpetuar (Kevin *et al*., 2002). Además, es conveniente explicar que el paciente mantenga unos hábitos diarios saludables como mantener un horario fijo de comidas, realizar ejercicio con frecuencia (Johannesson *et al*; 2011) y dormir las horas suficientes. También se debe aconsejar excluir o evitar aquellos alimentos que con frecuencia empeoren sus síntomas. En aquellos casos de aumento de dolor postprandial se sugiere una dieta pobre en grasas, lo cual evita el estímulo de motilidad colónica (reflejo gastrocólico).

Dado que el subtipo de pacientes objeto de estudio en este trabajo son SII-D, se detallan a continuación los diferentes tratamientos que se emplean en el tratamiento de este subgrupo de pacientes.

3.8.2. Tratamiento farmacológico, en función de la sintomatología

Dolor abdominal

Generalmente se utilizan espasmolíticos de tipo anticolinérgico (bromuro de otilinio, bromuro de pinaverio/ de cimetropio, bromuro de trimebutina/de escopolamina) o no anticolinérgicos (mebeverina y papaverina), la posología recomendada es su adminstración en tres dosis al día, ante los episodios de dolor. Hay que tener en cuenta los posibles efectos adversos, entre ellos el

estreñimiento, con el uso de los anticolinérgicos.

Diarrea

Se utilizan principalmente agonistas de los opiodes, entre ellos la loperamida y la codeína. La recomendación es utilizar la loperamid durante periodos cortos y a dosis bajas tan sólo tiene efecto beneficioso sobre la diarrea con respecto al placebo, sin mejorar los síntomas generales. No existen ensayos clínicos que avalen la eficacia de la codeína en el SII-D.

También se puede administrar de forma empírica resinas de intercambio aniónico tales como la colestiramina y el colestipol, aunque no existen ensayos clínicos que evalúen su eficacia.

- Ansiedad y depresión

Los psicofármacos son útiles cuando co-existe ansiedad y/o depresión junto a los síntomas de SII. El control de la intensidad de ambas condiciones puede romper el círculo vicioso que perpetúan los síntomas. En el caso de ansiedad se puede administrar benzodiacepinas durante periodos breves de tiempo. En aquellos pacientes en los que se asocie depresión se aconseja tratamiento con antidepresivos, por sus efectos antimuscarínicos y de modulación del dolor. La eficacia de estos fármacos para esta entidad es dudosa.

- Antidepresivos tricíclicos: La dosis recomendada es menor que la utilizada para el tratamiento de la depresión, tiene una posible indicación beneficiosa en los pacientes de SII-D, dado que su efecto indeseado más frecuente es el estreñimiento.
- Inhibidores de la recaptación de la Serotonina: Su uso se justifica por los el posible papel de la proteína SERT en la fisiopatología del SII, aunque ninguno de los ensayos clínicos realizados con fluoxetina y paroxetina han demostrado mejoría en los síntomas intestinales en relación al placebo; sólo la paroxetina mejora el bienestar general de los pacientes.

2.8.3 Otras perspectivas terapeúticas:

- Tratamientos psicológicos

En los últimos años se está evaluando la eficacia de terapias cognitivas-

conductuales, técnicas de relajación y sesiones de hipnosis, existen datos que muestran mejoría para afrontar la enfermedad, aunque los resultados no son concluyentes.

- Tratamiento dietético

Se han realizado estudios con dietas de exclusión basadas en la presencia de anticuerpos IgG frente a determinados alimentos en los que se ha observado una mejoría sintomática, aunque los datos no son concluyentes por limitaciones en la metodología empleada.

4. Nuevas dianas terapeúticas basadas en mecanismos fisiopatológicos del SII.

4.1- Receptores CRF

Su implicación en la motilidad, permeabilidad intestinal y activación mastocitaria justifica el estudio de agentes antagonistas de receptores CRF. Sin embargo, hasta ahora, los estudios realizados en pacientes con SII-D no muestran beneficios frente al placebo, ni en el tránsito colónico, ni en el dolor abdominal (Sweetser S et al., 2009).

4.2.- Receptores de la serotonina

Dada la relevancia de la 5-HT en el control de las funciones del tracto gastrointestinal, se han estudiado los receptores responsables de su acción en el intestino (subtipos y distribución). Entre los cuales destaca el 5-HT3 como posible diana terapéutica, en especial en el SII-D, por su distribución en las neuronas sensitivas y su implicación en la secreción, peristaltismo, nocicepción y vasodilatación en el intestino. De hecho, existen ensayos clínicos realizados con agentes terapéuticos antagonistas de receptores 5-HT3 para evaluar sus efectos en el SII en relación a la motilidad intestinal y la sensibilidad visceral.

- Alosetrón : Fármaco antagonista de los receptores 5-HT3, no comercializado en España, prolonga el tiempo de tránsito colónico, con mínimos efectos en la percepción visceral en sujetos sanos y con acción sobre el SNC en relación a la ansiedad (Berman *et al*., 2002). En ensayos clínicos controlados en mujeres con SII-D se ha demostrado la mejoría en la consistencia y el dolor abdominal,

valorado por el aumento del umbral de percepción ante la distensión colónica (Mayer *et al*., 2003). El estreñimiento es el efecto adverso más frecuente con este tratamiento, aunque el más severo es la aparición de colitis isquémica, lo cual originó su retirada del mercado estadounidense por la FDA. En el año 2002, se aprobó de nuevo su uso, aunque está restringido a casos de SII con diarrea severa que no respondan al tratamiento convencional, y a dosis de 2mg/día.

4.3.- Terapias antiinflamatorias.

En base a los antecedentes anteriormente expuestos, la existencia de la microinflamación y la activación del mastocito en el intestino como posible mecanismo en la fisiopatología del SII, en particular en aquellos de origen post-infeccioso. Se han evaluado tres agentes terapeúticos dirigidos al tratamiento de la activación mastocitaria y la microinflamación.

4.3.1 Estabilizadores del mastocito

En los últimos años se han desarrollado e implementado estrategias terapéuticas dirigidas al bloqueo de la activación mastocitaria y, en modelos experimentales de estrés en animales, se ha confirmado la mejoría de la permeabilidad intestinal y la hipersensibilidad visceral. A continuación se detallan estudios clínicos realizados en humanos:

- **Ketotifeno:** Fármaco que bloquea la liberación de mediadores por parte del mastocito al ser un antagonista no competitivo del receptor de la histamina H1, empleado en el tratamiento del asma. Un estudio controlado con placebo, realizado en pacientes de SII durante 8 semanas, mostró el aumento en el umbral de sensibilidad visceral, evaluada mediante distensión colorectal, sólo en aquellos pacientes con hipersensibilidad visceral. Curiosamente, en este estudio la liberación de triptasa basal determinada en los sobrenadantes procedentes de las biopsias rectales fue menor en los pacientes, sin cambios tras el tratamiento (Klooker *et al*., 2010).

- **Cromoglicato disódico:** fármaco cuyo mecanismo de acción no conocido en detalle, interfiere con el transportador de calcio en la membrana del mastocito,

bloqueando así la liberación de sus mediadores. Se emplea en el tratamiento de la mastocitosis sistémica. En estudios realizados en pacientes con SII durante 4 y 8 semanas se observó un control más efectivo que el placebo en el control de síntomas, principalmente en pacientes con test cutáneos positivos a alergenos alimentarios, aunque sin diferencias con respecto a una dieta de exclusión (Lunardi et al., 1991, Stefanini et al., 1995).

4.3.2. Otros antiinflamatorios

- **Mesalazina**: Reduce el infiltrado leucocitario, incluidos los mastocitos, así como los niveles de triptasa, histamina e IL-1β en la mucosa intestinal en los pacientes con SII-D. Sin embargo, el único estudio que existe frente a placebo no mostró diferencias, debido a que el número de pacientes estudiados fue reducido, lo cual limita la interpretación de los resultados. Será necesario ampliar el número de estudios para determinar si este fármaco podrá ser incluirlo de forma rutinaria como opción terapeútica.

CAPÍTULO 1

Assessment of clinical evolution and intestinal innate immunity after long-term stabilization of mucosal mast cells in diarrhea-irritable bowel syndrome: a pilot study.

Short Title: Effect of cromoglicate on IBS-D.

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Abbreviations used in this paper: BSFS, Bristol stool form scale; DSCG, disodium cromoglycate; **I**BS-D, diarrhea-predominant irritable bowel syndrome; IELs, intraepithelial lymphocytes; hpf, high power field; HSP, heat shock protein; Q-RT-PCR, quantitative real-time polymerase chain reaction; MC, Mast cell; MyD88, Myeloid differentiation primary response gene (88); NOD1, Nucleotide-binding oligomerization domain-containing protein 1; SIGIRR, Single Ig IL-1-related receptor; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; TOLLIP, Toll interacting protein.

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Abstract

Background & aim: Increased mast cell activity has been related to the severity of clinical manifestations in diarrhea-predominant irritable bowel syndrome (IBS-D) patients. We examined the effect of pharmacological inhibition of mast cells on the clinical response and the mucosal innate immunity, in IBS-D patients. **Material&Methods**: We recruited healthy participants (H, n= 10) and 24 naïve, non-allergic, non-celiac IBS-D patients. Abdominal pain, the number of bowel movements and the stool form were recorded. A jejunal mucosal biopsy was obtained in H and at 6 months, after natural evolution with no treatment (IBS-D, n=13), or after oral disodium cromoglycate treatment (DSCG, 200 mg/8h; n=11). Mast cells and innate immune activity were assessed by quantification of gene expression, and by confocal microscopy analysis. **Results**: DSCG treatment showed significant improvement in abdominal pain, bowel movements and stool form. Mucosal mast cell density was higher in IBS-D than that in H participants, regardless DSCG treatment. Tryptase expression was significantly increased in IBS-D (0.7-fold, $P<0.05$) and restored to H values after DSCG treatment ($P<0.01$). The expression of both TLR2 and TLR4 was higher in IBS-D by 0.6-fold, respect to H, and restored after DSCG. TLR2 and TLR4 localized in the epithelium and mucosal residents, but not mat cells. Furthermore, down-regulation of innate immunity genes paralleled clinical improvement ($P<0.05$). **Conclusion**: Oral DSCG induces sustained clinical benefit in IBS-D patients, associated with mast cell stabilization and modulation of innate immunity. The potential clinical benefit of this treatment needs to be established in future double-blinded placebo-controlled trials.

Introduction

The irritable bowel syndrome (IBS) is characterized by abdominal pain or discomfort, and altered bowel habit. IBS is one of the most common gastrointestinal disorders in western societies, affecting up to 20% of the population (Saito YA, 2002; Wilson S, 2004). Yet its pathophysiology is poorly understood, its aetiology is believed to be multifactorial, being the result of the interaction of several factors such as dysfunction of the brain-gut axis, gut flora alterations, low-grade mucosal inflammation, and epithelial barrier dysfunction (Dunlop SP, 2006; Piche T, 2009; Camilleri M, 2012; Matricon J, 2012; Stasi C et al 2012; Magnus S, 2013). The lack of reliable biomarkers and the heterogeneous symptoms observed in IBS increase the difficulty of the management of these patients (Barbara G, 2009). Nowadays, IBS therapy is quite diverse including both pharmacologic and non-pharmacologic options, mainly directed towards relieving bowel symptoms, based on the severity, frequency and nature of only one specific symptom (Chey WD, 2011). Over the last years, treatment strategies have been directed towards the underlying pathophysiologic mechanisms involved in IBS, specially those one with anti-inflammatory effects, which could relieve multiple rather than single symptoms.

Most of the diarrhea-predominant IBS (IBS-D) patients show inflammatory infiltrate in the intestinal mucosa characterized by activated mast cells, located in close proximity to nerve endings (Barbara G, 2004; Martinez C, 2012). Mast cells are part of the intestinal innate immune system and are well-recognized end effectors of the brain-gut axis. Different stimuli trigger intestinal mast cell activation, leading to piecemeal degranulation with secretion of large variety of

mediators that activate mucosal immune cells (antigen presenting cells, T, B, and NK lymphocytes), sensitise nerve terminals and act on epithelial barrier (Rijnierse, 2007; Keita AV, 2010). As key players of the innate immunity, mast cells express toll-like receptors (TLRs), which recognize microbial antigens and promote inflammatory responses through the NF-κB canonical pathway. TLR signalling has been implicated in the intestinal balance between tolerance and inflammation in response to commensal flora and dietary antigens (Harrison OJ, 2011). Mast cells contribute to intestinal homeostasis and its activation may, therefore, be of importance to IBS pathophysiology by dysregulation of this physiological balance.

Several clinical and experimental studies have focused on mast cell-mediated disturbances in gut sensori-motor function and intestinal barrier integrity, alterations associated with clinical symptoms in IBS-D patients (Bueno L, 2008; Martinez, 2012; Barbara G, 2004). Mast cell stabilizer agents have been proposed as possible therapeutic agents useful to treat IBS and abrogate gastrointestinal dysfunction. In fact, ketotifen (non competitive histamine (H1) receptor antagonist and mast cell stabilizer) has proven to in increase visceral sensory threshold, leading to improved visceral perception especially in hypersensitivity IBS group, (Klooker TK, Gut 2010). Disodium cromoglicate (DSCG) has clinical benefits in preventing food allergy and also improving intestinal symptoms after 2-months treatment in IBS-D patients, especially in those one with positive skin prick test to dietary antigens (Stefanini et al 1995). However, pitfalls in the design studies along with the short duration of those

trials may have well prevented wider clinical acceptance of DSCG in the management of IBS.

Therefore, the main aim of the present study was to study the clinical outcome and the effects on mucosal gene and protein expression profile linked to innate immune system after the long-term administration of oral DSCG in a selective subgroup of diarrhea-predominant IBS (D-IBS) patients.

Material and Methods

Participants

We recruited newly diagnosed IBS-D patients meeting the Rome II criteria (Talley et al, 1999) from the outpatient gastroenterology clinic, from September 2005 through July 2008 (A complete medical history and physical examination was carried out and compliance with functional dyspepsia according to Rome II criteria checked in all candidates, healthy volunteers (group H) were also recruited by public advertisement. Food and respiratory allergy was ruled out using a battery of skin prick tests (Leti SA, Barcelona, Spain) including 32 common foodstuffs and 24 inhalants, with histamine and saline as positive and negative controls, respectively. Reasonable exclusion of gastrointestinal comorbidities was achieved by means of a broad biochemical and serological profile including anti-transglutaminase antibodies; upper and lower fiberoptic and small bowel capsule endoscopy, abdominal sonography and barium studies were performed in some participants, as requested by the responsible physician. Previous history of acute gastroenteritis and its relationship to the initiation of IBS symptoms was specifically recorded. Patients were taking no

regular medication within 3 months of the study inclusion, including steroids, anti-allergic or immunosuppressive and related drugs, and did not receive radiotherapy or chemotherapy within prior 6 months. Other exclusion criteria included age <18 or >65 years, active smoking, major psychiatric and organic diseases. No diet restrictions, other than overnight fasting, alcohol or caffeine intake, were indicated.

Procedure and Study design

The study was prospective, open-labelled and no placebo-controlled. Patients were randomly allocated (age-matched) into two groups: group IBS-D and, group DSCG to assess the effect of oral treatment. Clinical evaluation was carried out in the last two weeks prior to inclusion and at month 6, after completing the non-treatment (IBS-D) or treatment (DSCG) period. A single mucosal biopsy was obtained *per* participant, at inclusion in the H group, and six months after inclusion in IBS-D and DSCG groups. Potential adverse effects of DSCG were recorded at follow up using daily charts. All patients provided written informed consent and the study protocol was approved by the Hospital Vall d´Hebron Ethics Committee (PR(AG) 76/2006).

Drugs

DSCG (Sigma-Tau, Madrid, Spain) was prepared by the hospital pharmacy as a powder mix on a starch base containing no lactose, and delivered in opaque gelatine capsules to be ingested at a dose of 200 mg/t.i.d./10 min before meals for 6 months. Dosage and intervals for administration derive from previous studies (Edwards A.M, 2010)

Clinical Evaluation

At inclusion and at the end of the treatment period, patients were asked to complete daily questionnaires over a 10-day period of the following parameters: a) severity of abdominal pain by a 100-point visual analogue scale; b) frequency of abdominal pain (number of days with pain); c) stool frequency (number of bowel movements per day); and d) stool consistency assessed by the Bristol stool form score (BSFS; Heaton et al., 1992). If more than one bowel movement per day, the BSFS was averaged, and the mean over the 10-day period was calculated).

Jejunal Biopsy

One intestinal mucosal biopsy was obtained in each participant using a Watson capsule with an attached aspiration tube (3 mm diameter) as previously described (Guilarte M, 2007). After overnight fast, patients were orally intubated between 08:00 and 10:00 h, and the capsule was positioned 10 cm distal to the angle of Treitz's under fluoroscopic control. Tissue samples were immediately split into two similar pieces with a sterile scalpel. One fragment was fixed in formalin and embedded in paraffin for further microscopic examination (histology, immunohistochemistry and immunofluorescence). The other fragment was placed in RNAse free tubes containing 500 μL of RNA Later Solution (Ambion, Madrid, Spain) for RNA isolation and analysis of gene expression by Q-RT-PCR.

Tissue Processing and Analyses

Histology and immunohistochemistry

Specimens were processed for routine hematoxylin and eosin (H&E) staining to assess epithelial morphology. In addition, the number of mast cells and T lymphocytes were determined at 400x magnification using anti-human *c-kit* (CD117) or CD3 (Dako, Barcelona, Spain), respectively, as previously described (Guilarte et al., 2007). Slides were coded and blindly examined by one experienced pathologist.

Immunofluorescence

Tissue sections were deparaffinised and hydrated following general procedures and blocked with Dako Blocking Solution (Dako, Barcelona, Spain) for 10 minutes followed by 60-minute room temperature incubation with the primary antibody for tryptase (mouse monoclonal; Dako, Madrid, Spain), for TLR2 and TLR4 (rabbit polyclonal antibody; abcam, Cambridge, UK). Secondary antibodies were Alexa Fluor 594 goat anti-rabbit IgG and 488 goat anti-mouse IgG (Molecular Probes, Madrid, Spain). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) before mounting in Prolong antifade reagent (Molecular Probes, Madrid, Spain). Negative control stainings were set by exposing serial sections, under similar conditions, avoiding the primary antibody. Images were acquired in a blinded manner using the FV-10-ASW Olympus software (Olympus, Barcelona, Spain).

RNA isolation and quantitative Real-Time Polymerase Chain Reaction (Q-RT-PCR)

Biopsies were homogenized in the FastPrep mixer (Bio101) in RLT cell lysis buffer (Qiagen, Madrid, Spain) followed by RNA isolation (RNeasy Mini Kit, Qiagen) and on-column DNase treatment (Qiagen). Prior to gene array analysis, RNA quantity and quality were confirmed by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, Calif., USA). Synthesis of cDNA was performed using 1 µg of total RNA with the High Capacity Reverse Transcription Reagents Kit (Applied Biosystems, Madrid, Spain). Q-RT-PCR was performed on an ABI PRISM® 7500 FAST Sequence Detection System (Applied Biosystems, Madrid, Spain) using validated TaqMan Gene Expression Assays (GEA) and the human 18S subunit ribosomal RNA gene as an endogenous control (Applied Biosystems, Madrid, Spain). Transcript quantification in each sample, including distilled water as negative control, was processed in triplicate. Gene expression was normalized to that of 18S rRNA gene, and quantified using the comparative Ct method (relative quantification) and the Sequence Detector Software SDS v2.2 (Applied Biosystems). Pooled jejunal biopsies from control D-IBS patients were used as the calibrator for comparison of the relative gene expression level across samples in the study, ($\Delta\Delta$Ct= (ΔCtsample-ΔCtcalibrator). The resulting values were converted and expressed as fold difference (=2-$\Delta\Delta$Ct). Approximately 4% of the samples were repeated for quality control purposes. The following assays were applied: tryptase (Hs02576518_gH), TLR2 (Hs00610101), TLR4 (Hs00152939_m1), SIGIRR (Hs 00222347), TOLLIP (Hs01553188), MyD88

(Hs00182082_m1), NOD1 (Hs01036720_m1), HSP70 (Hs01651017), HSP 27 (Hs01108167), and the control gene 18S (Hs99999901_s1).

Statistical Analysis

Data are expressed as median (range) or mean ± standard error of the mean (SEM), unless otherwise stated. Two-tailed parametric or nonparametric tests were used when appropriate (unpaired Student's *t*-test or Mann-Whitney *U* test) Comparison of data between groups were analyzed by one-way ANOVA (analysis of variance) followed by Bonferroni post tests using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Clinical variables were assessed by score comparison at different time points by two-way repeated measures ANOVA followed by Bonferroni post-tests. Relationships between clinical and biological variables were assessed by Spearman's rho correlation. *P* values of <0.05 were considered significant.

Results

Demographics of study population

Twenty-four patients and 10 healthy volunteers completed the study. Healthy controls were significantly younger compared to the groups of patients. Other characteristics of participants are also shown in table 1.

	IBS-D	DSCG	H	*P* value
Number of subjects	13	11	10	
Gender (F:M)	9:4	7:4	5:5	0.63
Age (years)	40.2±3.9	48.8±5.8†	30.4±1.7	0.003*
Previous gastroenteritis	1/13	1/11	NA	
Food Allergy	No	No	No	
Other inflammatory disorders	No	No	No	

Table1. Clinical and demographic characteristics of participants. Data are expressed as mean ± SEM. F, female. M, male. NA, non applicable.**P* value according to one-way ANOVA analysis; † Represents *P*<0.05 CGD vs H using Bonferroni post-test for multiple testing.

Effect of DSCG on clinical variables

Overall, DSCG was well tolerated and no major adverse effects were recorded. At baseline, IBS-D and DSCG groups displayed similar average values for the severity of abdominal pain, the frequency of bowel movements, and the stool consistency.

Patients taking DSCG reported a significant improvement of the abdominal pain at the end of the treatment period, compared to those one without any treatment (-4.4±0.66 vs -0.06±0.7; *P*=0.0051). Similar clinical benefit of DSCG was observed on bowel frequency, as indicated by the reduced number of bowel movements (-2.41±0.46 vs 0.44±0.34; *P*=0.0004). Moreover, the stool consistency was also significantly improved in the DSCG group (-2.78±0.44 vs -0.44±0.38; *P*=0.012) panels a, b, c

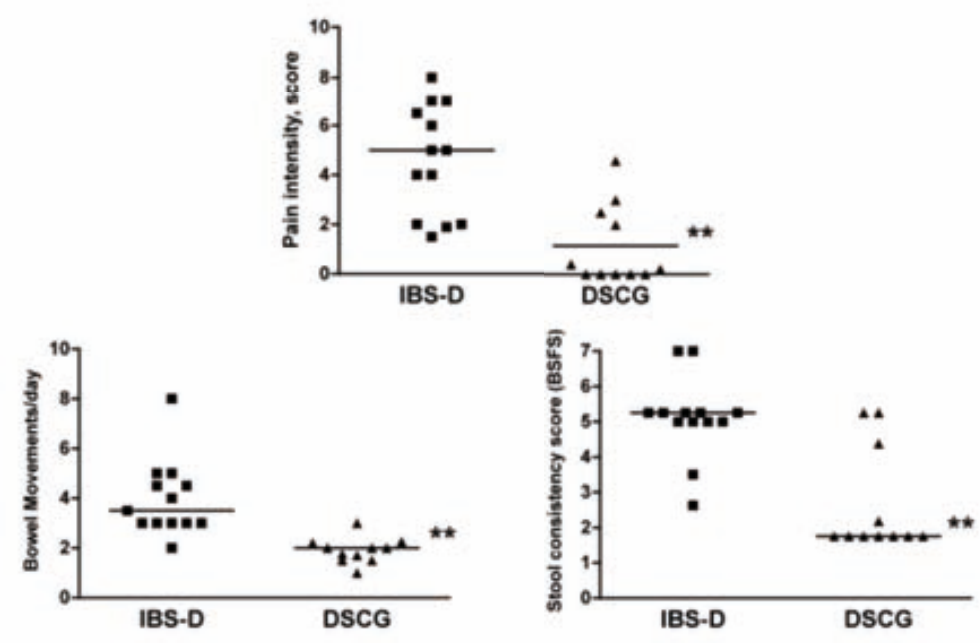

Figure 1. Clinical characteristics at 6-month follow up. This figure represents abdominal pain intensity, bowel movements and stool consistency after 6-month follow up period in untreated group (IBS-D) and DSCG treated group. A significant reduction in abdominal pain and bowel movements per day, and increase in stool consistency in DSCG group compared to IBS-D was observed. Data are expressed as median; **Represents *P*<0.01 using *U* Mann-Witney.

Jejunal histology

On routine histology, all biopsies disclosed no abnormalities as to their epithelial architecture, inflammatory infiltrate and the presence of eosinophils parasites, microbial and viral inclusions. Specific stainings revealed no statistically difference in IELs counts between groups. Remarkably mucosal mast cell infiltration was significantly higher in IBS population than in H, and was not modified by DSGC treatment, as shown in **table 2**.

	H	IBS-D	DSCG	*P* value
CD-117$^+$	13.6 ±2.2	21.8±2.3	24.2±3.6 †	0.03*
CD3$^+$	24.0 ± 3.3	20.9± 2.7	22.6±4.0	0.82

Table 2. Mucosal leukocyte population in the jejunum. The number of mast cells is expressed per high power field (400x) and the number of lymphocytes per 100 epithelial cells. Data are expressed as mean ± SEM. * Represents *P*<0.05 using one way ANOVA, † Represents *P*<0.05 CGD vs H using Bonferroni post-test for multiple testing.

Effect of DSCG on Mast cell activation

Tryptase is the most common serine protease contained in mast cells granules and a marker of mast cell activation. Thus, we analyzed the effect of DSCG on tryptase transcripts profile, by measuring gene-expression levels by Q-RT-PCR in the small intestinal mucosa of all subjects. As previously described, IBS patients exhibited significant increased levels of tryptase compared to the H group. Interestingly, DSCG reversed triptase expression to that obtained in healthy participants, suggesting a direct effect of DSCG on mucosal mast cell activation.

Furthermore, we studied the relationship between tryptase expression and clinical features in IBS-D and DSCG group. No statistically significant correlation was observed in any group, as represented in **figure 2**.

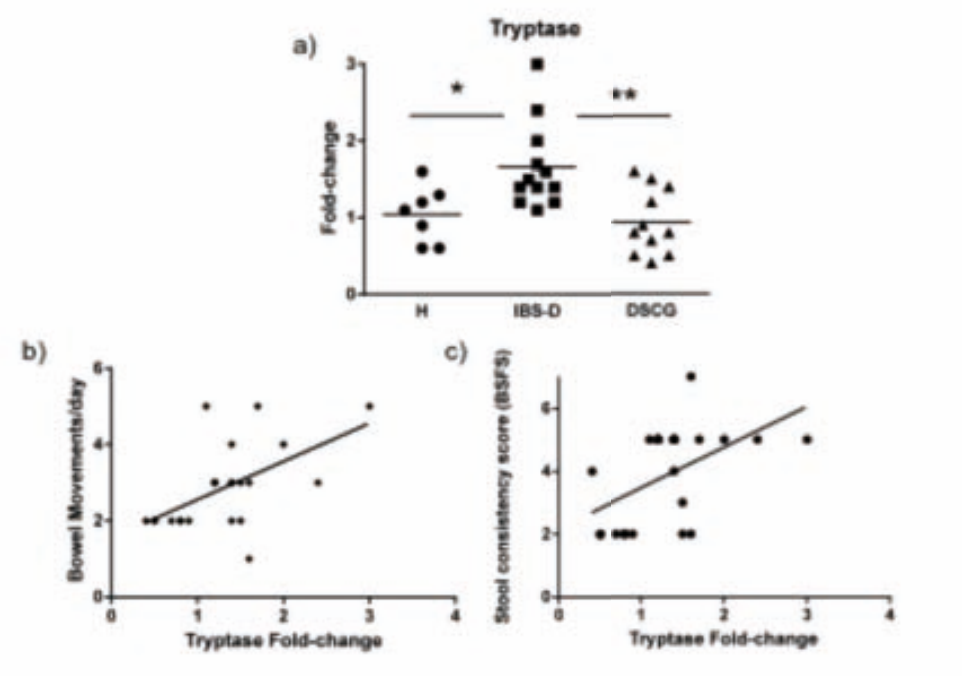

Figure 2. a) Tryptase gene expression (H=7; IBS-D= 12; DSCGD=11) measured by Q-RT-PCR the fold-change value for each sample was obtained after calculating the target gene / 18S mRNA ratio for each sample and then normalized to the average of the healthy group. A 0.7-fold increase was observed in IBS-D compared to healthy subjects. b) Correlation between pain intensity and IBS-D (squares) fold-change tryptase transcripts and DSCG (triangle) was not statistically significant. Groups were compared using Kruskal-Wallis and * represents $P<0.05$, ** indicates $P<0.01$ and *** $P<0.0001$ using Dunn´s multiple comparison test. Spearman rank correlation was used.

Effect of DSCG on Mast cell activation and innate immunity

Mast cells, as innate immune cells, express TLRs and interact with other immune cell types within the intestine. Recognition of specific ligands by TRLs leads to downstream signalling that stimulates innate immune responses. We assessed in the jejunal mucosa of H, IBS-D and CGD participants the

expression of TLR and genes involved in its signalling pathway, represented in **figure 3**. A statistically significant increase in the expression of TLR2 and TLR4 (0.5 and 0.9 fold-change, respectively) was observed in IBS-D group in comparison with H. Interestingly, the expression in DSCG-treated group was restored towards that in healthy subjects. The same profile was observed in another innate immune receptor, NOD1. Moreover, the expression of an adaptor molecule as MyD88, essential for overall TLR signalling pathway, was significantly increased by 0.6-fold in IBS-D compared to healthy subjects, and again, in DSCG-treated patients, the expression was similar to that in healthy controls. Likewise, gene expression of negative regulators of TLR signalling as TOLLIP and SIGIRR, IBS-D exhibited a significant up-regulation compared to H and DSCG groups.

Over the last years, it has been described the relationship between heat shock proteins (HSPs) and TLRs as possible endogenous ligands. Hence, we analyzed the expression profile of HSP27 and HSP70 genes in the jejunal mucosa of the participants in this study. The same gene expression profile was observed as before, with increased fold-change in IBS-D group respect to H and down-regulation in the DSCG group, as illustrated in **figure 4.**

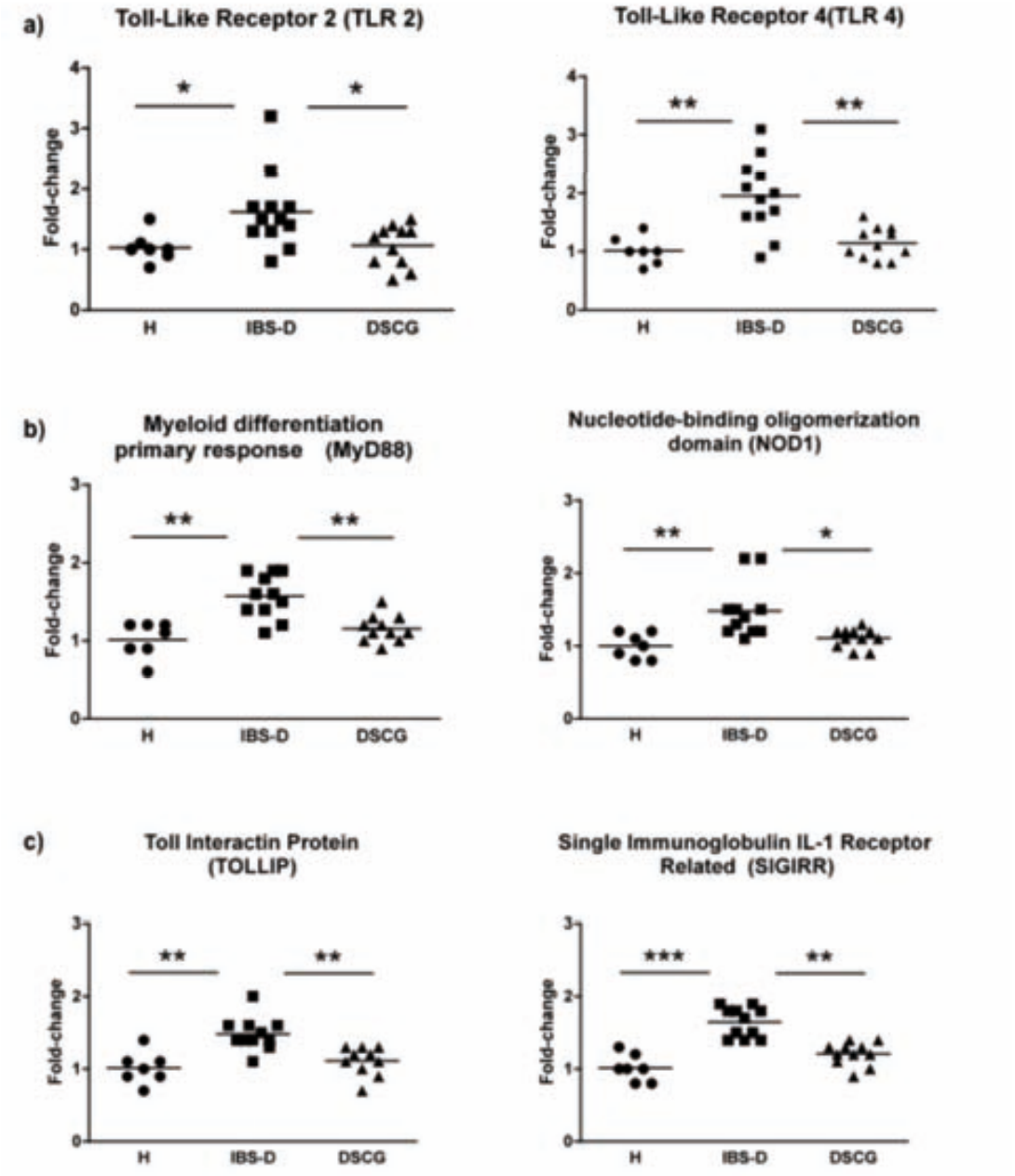

Figure 3. Profile expression of innate immunity-related genes. a) mRNA expression of TLR2 and TLR4 in H, IBS-D and DSCG assessed by Q-RT-PCR, values are expressed as mean. Gene expression was significantly increased in IBS-D mucosa compared to the other groups (H and DSCG-treated participants). b) NOD1 and MyD-88 expression profile showed significant up-regulation in IBS-D mucosa compared to H and DSCG group. c) TLR signalling inhibitors expression was significantly increased in IBS-D mucosa and reduced in DSCG mucosa. * Represents $P<0.05$, ** $P<0.01$, and *** $P<0.0001$ using Dunn´s multiple comparison test.

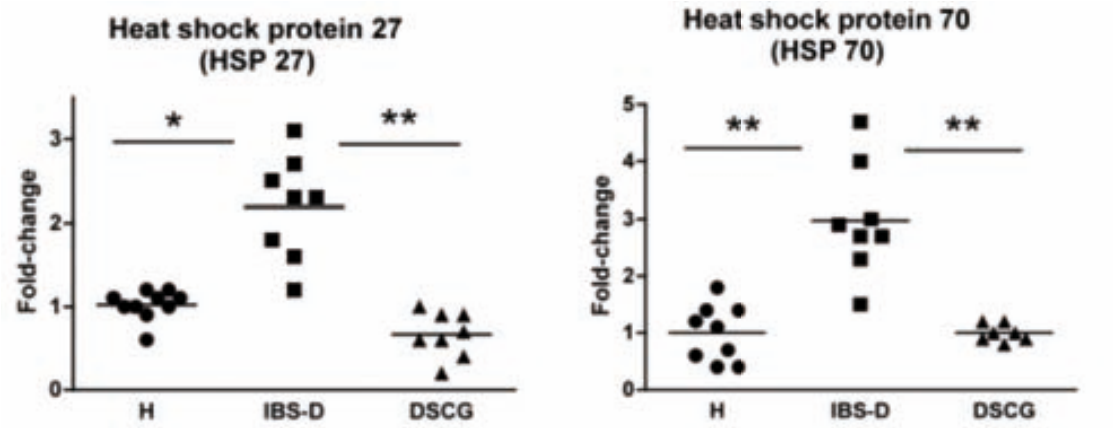

Figure 4. Profile expression of heat shock proteins. HSP27 and HSP70 gene expression in H, IBS-D, and DSCG. Data are expressed as mean. A significant up-regulation was observed in IBS-D group, while DSCG values were similar to H. *Represents *P*<0.05*, **P* <0.01, and ****P* <0.0001 using Dunn´s multiple comparison test.

Localization of TLR expression in the immune infiltrate

Differences in TLR-signaling pathway genes among groups suggest that mast cells play a role. We next evaluated co-localization of both TLRs and tryptase protein with double-labelling technique in small intestine mucosa of all participants of the study.

Immunofluorescence staining with TLR2 showed expression in the intestinal epithelium that tended to be more intense in IBS-D as compared to H and DSCG patients. Also, we found TLR2-positive cells in the lamina propria in all groups. In contrast to H and DSCG-treated group, untreated patients exhibited higher density of TLR2-positive cells, although no mast cells co-localized with TLR2 in any of the groups, as represented in **figure 5**.

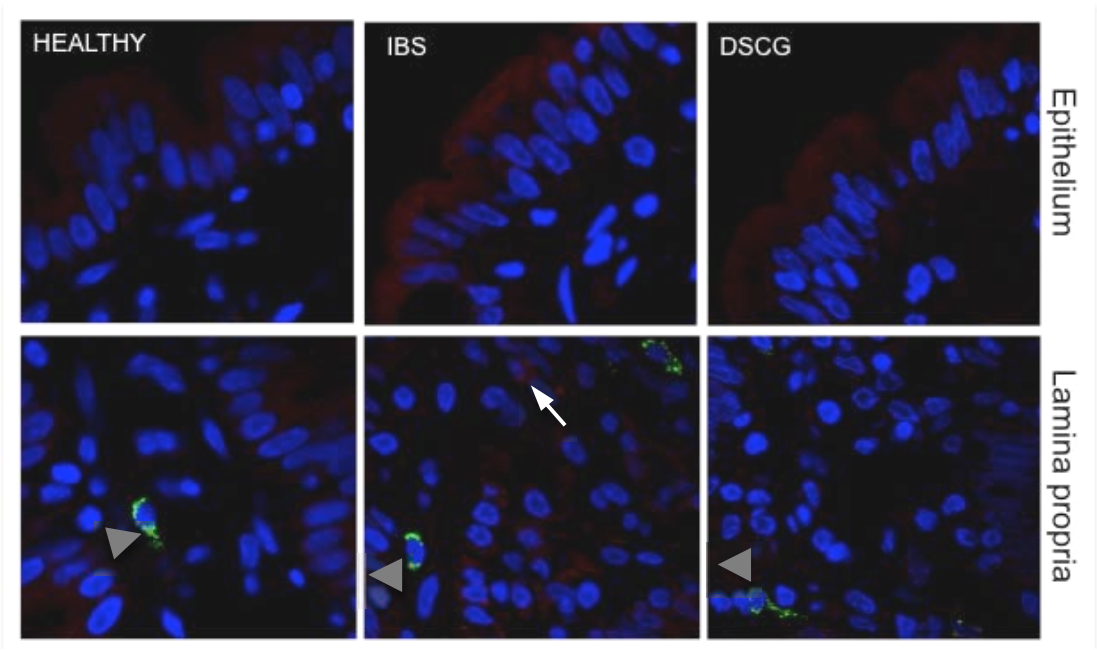

Figure 5. Representative images of double immunostaining of tryptase (green) and TLR2 (red), cells were counterstained with DAPI for nuclei (blue), in the epithelium and in the lamina propria from subjects of the 3 experimental groups. Mast cells are indicated by arrowheads and $TLR2^{+}$ cells by arrows. Original magnification x 100.

TLR4 protein distribution within the small intestinal epithelium was essentially at the apical border in most of the specimens analysed. Interestingly some of the IBS-D biopsies displayed basolateral location. In the lamina propria, the density of positive cells for TLR4 was higher in IBS-D than in the other groups, and again no mast cell was positive for TLR4, as shown in **figure 6**.

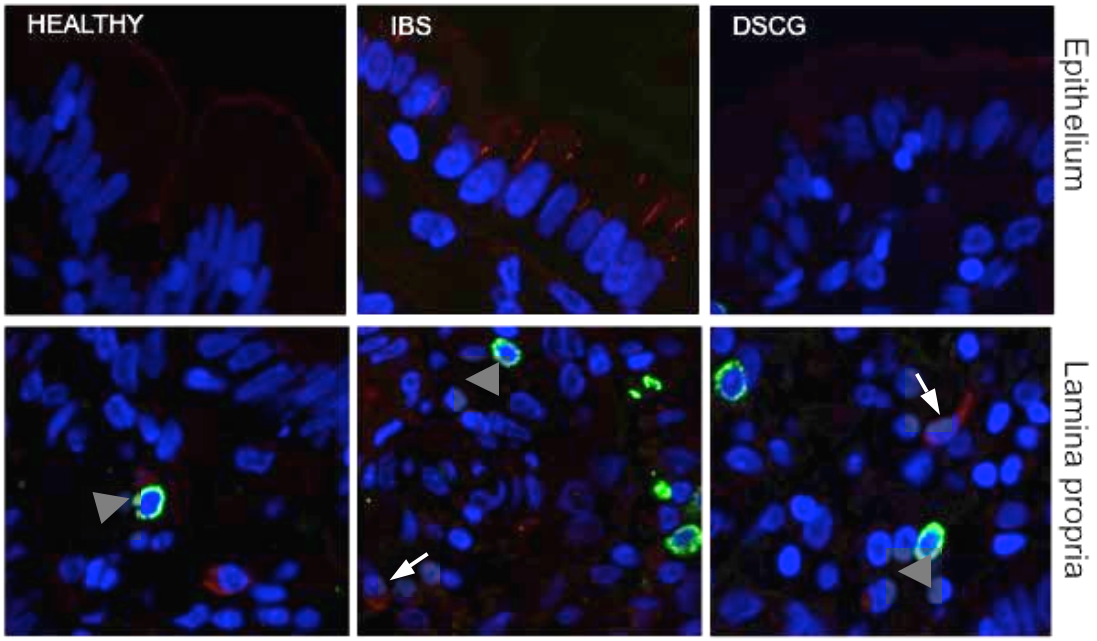

Figure 6. Representative images of double immunostaining of tryptase (green) and TLR4 (red) in the epithelium and in the lamina propria from subjects from the 3 experimental groups. Mast cells are indicated by arrowheads and $TLR4^{+}$ cells by arrows. Original magnification x 100

Link between mucosal mast cell activation and innate immunity

As shown above, DSCG had an effect on gene expression involved in TLRs signaling, to get further insight into the role of mast cell activation and innate immune profile we evaluated the relationship, based on gene expression, between tryptase and the studied innate immunity genes, in all participants. We observed a significant correlation with tryptase and TLR2, SIGIRR, HSP27 and HSP70, shown in **table 3.**

mRNA expression	Tryptase	
	r	p
TLR4	0.094	0.627
TLR2	**0.44**	**0.017**
MyD88	0.22	0.259
NOD1	0.33	0.083
TOLLIP	0.25	0.193
SIGIRR	**0.39**	**0.038**
HSP27	**0.57**	**0.005**
HSP70	**0.63**	**0.028**

Table 3. Correlation between Innate immunity-related genes and tryptase expression. Mast cell activity values of $P<0.05$ according Spearman rank correlation in bold.

Clinical-biological correlations

As shown above, we observed a significant clinical improvement in IBS symptoms after treatment with DSCG. We examined the relationship between clinical symptoms and gene expression in IBS-D untreated patients and no significant association was found. In contrast, the DSCG group showed positive correlation between MyD88 and TOLLIP expression and abdominal pain (r=0.71, P= 0.014; r=0.64, P= 0.048, respectively).

Discussion

This pilot study demonstrates the resolution of the principal manifestations of IBS-D after long-term oral DSCG treatment, suggesting the benefit of mast cell stabilization in the management of these patients. Indeed, the great majority of patients experienced at least a 50% improvement on their abdominal pain and bowel movements, and over 75% normalized the consistency of stools. In contrast, the analysis of the natural history of symptoms in IBS-D patients that remained untreated for the same six-month period indicates the stability of the severity of symptoms throughout time. Importantly, this clinical improvement is associated with modulation of innate immunity in the intestinal mucosa towards the homeostasis condition. Furthermore, tolerance to DSCG was excellent, as no significant adverse events were recorded during follow up.

Enhancement of our understanding on IBS pathophysiology could improve the accuracy in diagnosis and help to provide new and better strategies for treatment. Recent studies highlight the potential link of immune activation and low-grade mucosal inflammation in the development of IBS manifestations, yielding new perspectives to this field (Ohman L, 2010). Among several other drugs, DSCG is a mast cell membrane stabilizer introduced for the treatment of asthma and allergic symptoms based on the ability to prevent mast cell degranulation after exposure to substances that trigger its activation. However the exact mechanism of action remains unknown, the inhibition of chloride channel activity that leads to reduction of calcium influx into cells may be involved. *In vitro*, DSCG has been shown to be an effective inhibitor of the

release of histamine and prostaglandin D2 from intestinal human mast cells (Okayama Y, 1992). Indeed, the maximally effective *in vitro* dose of DSCG (~10^{-5} M) is smaller than the dose used by us herein (200 mg/t.i.d.) as a result of its high hydrophobicity, the intestinal absorption rate is vey low (≤1%) what explains its poor oral bioavailability. The estimated concentration reached into the human jejunum after oral administration of 200 mg, in normal conditions, is in the rank of 10^{-3} M (Selbekk BH, 1979). Therefore, and due to its short half-life (Deshmukh DD, 2008), this compound requires oral administration of, at least, there times a day. DSCG exhibits an excellent safety profile, here reflected by the absence of significant side effects, primarily due to its pharmacological activity confined to the mucosa (Ding X, 2004).

Oral administration of DSCG has been used in a number of gastrointestinal conditions (Soter NA, 1979; Moots RJ, 1988) with inconsistent results. The first evidence of improvement on gastrointestinal symptoms with oral DSCG administration was in the 70s for the treatment of systemic mastocytosis (Dolovich J., 1974). More specific has been its use in the control of food-induced clinical manifestations (Ortolani C, 1983; Businco L, 1986) and chronic diarrhea (Bolin TD, 1980). Heretofore, several studies suggested that DSCG might be particularly effective in IBS subgroups with food hypersensitivity/intolerance and diarrhea (Lunardi C, 1991; Stefanini GF, 1992; Stefanini GF, 1995; Leri O, 1997). However, pitfalls in the design studies along with the short duration of those trials may have well prevented wider clinical acceptance of DSCG in the management of IBS. Another mast cell stabilizer, ketotifen, has been recently shown to increase the threshold for discomfort in

patients with IBS with visceral hypersensitivity and to ameliorate clinical manifestations in a mixed population of IBS. However, the authors did not show mast cell hyperplasia or changes in tryptase or histamine release in rectal biopsies after treatment with ketotifen, and failed to correlate the clinical benefit of ketotifen and mast cell activation (Klooker TK, 2010). While those previous studies focused on the clinical outcome we now extend and highlight the potential therapeutic value of oral DSCG by showing the association between biological and clinical parameters.

Our findings corroborate the effectiveness of oral DSCG to promote the stabilization of gut mucosal mast cells by down-regulation of tryptase, as well as innate immunity molecules to levels comparables the to healthy population. Nowadays, different evidences suggest that dysregulation of intestinal TLRs signaling is involved in the development and perpetuation of intestinal inflammation processes (Abreu AT, 2010; Kubinak JL, 2012). The cross-talk between immune- and nervous-system along the whole gastrointestinal tract is bidirectional. Immune activation can sensitize nerve endings and modulate intestinal sensory and motor function, what suggests its implication in the development of gastrointestinal diseases. Under physiological conditions, the epithelial barrier allows selective pass of luminal content as dietary antigens and microbial agents, which develops a tolerogenic immune response. TLRs signalling are an important innate immune component, which play a key role in intestinal tolerance. The increase in intestinal permeability, described in a subgroup of IBS patients (especially IBS-D), could explain the activation of mucosal immunity though TLR activation. Regarding the role of TLRs in IBS

there is few data available. Two studies, focused in colonic tissue, have shown significant increase in gene and protein expression of TLR4 in IBS population as compared to healthy subjects (Brint EK, 2010). Even RNA expression of TLR2 and TLR4 were significantly increased in IBS-M subtype and positively correlated with a long history of IBS (Belmonte L, 2012).

In this study, we focused on the expression of TLR4 and TLR2 signalling in the small intestine mucosa and we demonstrated that innate immune-related genes are significantly up-regulated in IBS-D, as compared to the healthy population. These findings suggest an immune activation in the small intestinal mucosa as has been reported in the colonic mucosa. Moreover, mast cell stabilization modulated this gene expression towards homeostasis, and this fact is linked to abdominal pain reduction after long-term treatment with a mast cell stabilizer agent.

On the other hand, HSPs are intracellular proteins, which are constitutively expressed and increased under oxidative stress, toxins and glucose deprivation. Growing evidences implicate HSPs in innate and adaptive immune response and their possible regulatory role. Furthermore, a murine allergic contact hypersensitivity model has already proved that HSP27 and HSP70 are key participants in the induction of immune responses through TLR4 in antigen processing and presentation. In our study we found increased HSPs expression in IBS that may suggest their role as endogenous ligands for TLRs signalling and collaborate in the local mucosal immune activation in this disorder. Our results related to the expression of two inhibitors of TLRs (TOLLIP and

SIGIRR), involved in limiting TLR2 and TLR4 signaling and inhibiting chronic inflammatory response, showed an up-regulation in IBS patients. In contrast to previous published data in inflammatory bowel disease, the mechanisms controling inflammatory responses are conserved, limiting cytokines production and tissue injury (Michelsen KS, 2007). Since treatment with DSCG reduces the expression of innate immune genes, in association with tryptase expression, we speculate that mast cells have a key role in orchestrating mucosal innate immune activity in IBS-D.

The increase in tryptase protein and mRNA expression has previously been identified in the intestinal mucosa of IBS and has been directly related to altered hypersensitivity (Barbara G, 2004; Cenac N, 2007) and epithelial permeability (Roka R, 2007), key mechanisms in the pathophysiology of IBS. However, these effects may be mediated by the activation of PAR-2 receptors, the lack of effect of DSCG on the expression of this receptor (data not shown), suggests a limited contribution to this response.

As mentioned above, evidences support enhanced epithelial permeability in IBS-D (Dunlop SP, 2006), allowing the passage of dietary and microbial antigens that activate innate immune system. Although not studied here, the effect of DSCG on intestinal innate immunity could imply an effect on epithelial barrier, through epithelial cells or/and mast cell stabilization, therefore reducing intestinal permeability and limiting the penetration of antigens that trigger TLRs. Since DSCG can also modulate eosinophil responses and neuronal activity (Dixon M, 1980; Vieira Dos SR, 2010), we cannot rule out that the clinical

benefit also involves DSCG-mediated modulation of other cell types in the vicinity of gut mucosa. Previous studies have demonstrated effects of this compound on epithelial cells (Andre C, 1987; Weangsripanaval T, 2006). In this study we also showed that TLR2 and TLR4 are expressed at intestinal epithelium and higher density of cells within the lamina propria from IBS-D expressed both TLRs, as compared to H and DSCG groups, although mast cells did not express any of them. Further studies are needed in order to clarify the identity of TLR-bearing cells in the intestinal mucosa of IBS-D, and help to understand the mechanisms leading to immune activation in the intestine of this patients.

We acknowledge that our study has several limitations that may prevent at this time more solid conclusions to be taken; therefore, results should be interpreted with caution. Specifically, pitfalls of the study are the relatively small sample size and the lack of placebo-controlled responses. The consistency of clinical response along with positive biological readouts, although preliminary, precluded us to perform a double blind, placebo-controlled clinical assay.

In conclusion, our study findings support the use of the mast cell stabilizer DSCG in the management of IBS-D. The role of DSGC should be further explored as a promising therapy for the remission and maintenance of the clinical manifestations of this disorder.

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CAPÍTULO 2

Clinical benefit after long-term mast cell stabilization with oral disodium cromoglycate in diarrhea-predominant irritable bowel syndrome patients.

Short Title: Cromoglycate treatment in IBS-D.

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Abbreviations used in this paper: BDI, Beck Depression Inventory; BSFS, Bristol Stool Form Scale; D-IBS, diarrhea-predominant irritable bowel syndrome; DSCG, disodium cromoglycate; MC, mast cell; SRRS, Social Readjustment Rating Scale; PSS, perceived stress score; t.i.d, three times a day; IBS-SS, IBS Severity Scale.

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Abstract

Background: IBS pathophysiology and etiology are unclear. A subset of IBS–D patients display an inflammatory infiltrate in gut mucosa characterized by activated mast cells and increased intestinal permeability, and these alterations have been associated with clinical symptoms. Preliminary data from our group shows significant symptom improvement in IBS-D patients after open oral disodium cromoglycate (DSCG) treatment. **Aim:** To determine clinical effectiveness of oral DSCG treatment in IBS-D. **Methods:** A double-blind randomized placebo-controlled trial was carried out in 44 IBS-D patients meeting the Rome III diagnostic criteria. Patients received oral DSCG (200mg t.i.d) or placebo (n=21) for 6 months. Outcome measures included psychological and clinical IBS manifestations recorded by specific validated questionnaires and diary cards at baseline and–throughout the study (months 1, 3 and 6). Primary aim was to assess changes in abdominal pain (severity and frequency) and stool consistency (Bristol stool chart) at 1, 3 and 6-month follow-up, compared to baseline, and to evaluate differences between groups over time, based on the intention-to-treat analysis. **Results:** At baseline, demographics, psychological variables and IBS manifestations were similar between randomized groups, though subjects assigned to placebo were significantly younger. Baseline IBS severity score was associated with depression (r=0.64 P<0.0001) and significantly decreased in both groups. The percentage of patients showing significant clinical improvement in IBS severity (≥50-point) over the whole follow-up period in DSCG-treated group did not differ from placebo group (59% vs. 48%; P>0.05). Rates of adequate relief of abdominal pain (intensity and frequency reduction) at 1 and 6-month were not different between DSCG and placebo groups (30% vs. 11% and 45% vs. 22%, respectively, P>0.05). at the end of 6-month visit 20% of patients in the DSCG group

reported complete pain relief vs. 6% of patients in the placebo group (*P*=0.34). Stool form increased at 1 and 6-month visits in both groups, although improvement of stool pattern was superior in the DSCG arm (-1.6 vs. -0.7, *P*<0.01) and average values of stool form became normal. Interestingly, the proportion of patients that improved stool consistency at least during 50% of the study was significantly greater in DSCG group when compared to placebo (78% vs. 28, *P*<0.05). **Conclusion:** This double-blind, placebo-controlled clinical trial, shows several advantages of long-term treatment with DSCG over placebo in IBS-D clinical manifestations.

Introduction

Irritable Bowel Syndrome (IBS) is one of the most prevalent chronic gastrointestinal disorders in our society. IBS is a heterogeneous condition characterized by abdominal pain or discomfort associated with altered bowel habit, that often associates increased levels of anxiety and depression, significantly reducing quality of life, work productivity and increasing healthcare costs (Whitehead WE,. 1996; Drossman DA,. 2000). As its pathophysiology and etiology are unclear, satisfactory treatments are still not available and most can be described as symptomatic.

Different factors have been associated with the risk of developing IBS, but mucosal immune activation and psychological factors seem of great relevance, particularly mast cells (MC) and their activation, in diarrhea-predominant irritable bowel syndrome (IBS-D) developments. In addition, MC tryptase release and expression have been associated with stool consistency (Martinez C,. 2012). On the other hand, psychological disturbances such as chronic stress and anxiety may trigger and modulate clinical course in IBS (Stasi C., 2012). Pre-clinical investigations have demonstrated that acute and chronic stress induce intestinal MC activation, supporting the role of brain-MC-gut axis in IBS development. Over the last years,

different compounds with local anti-inflammatory properties have been tested including mesalamine (Tuteja AK,. 2012), disodium cromoglycate (DSCG) (Ramos L,. 2008; Stefanini GF,. 1995) and ketotifen (Klooker TK., 2010), as potential therapies for IBS-D patients. DSCG is a MC stabilizer, although its precise mechanism of action is unknown. Several studies have shown promising data regarding the benefit of oral DSCG in treating diarrhea associated or not with abdominal pain. However, clinical applicability has been limited by pitfalls of study design such as short period of therapy. Preliminary data from our group in an open study also suggest significant clinical improvement in D-IBS after long-term administration of oral DSCG.

Therefore, we aimed to evaluate, in a double-blind placebo-controlled trial, the efficacy of long-term administration of oral DSCG on IBS-D symptoms.

Material and Methods

Patients

IBS-D patients meeting the Rome III criteria were recruited from the Department of Gastroenterology of Vall d'Hebron University Hospital from August 2008 through July 2011. All subjects underwent a complete medical history and physical examination to confirm diagnosis and to exclude other gastrointestinal comorbidities by means of a broad biochemical and serological profile including anti-transglutaminase antibodies; upper and lower fiberoptic and small bowel capsule endoscopy, abdominal sonography and barium studies were performed in some participants, as requested by the responsible physician. In addition, at the time of recruitment food allergy was excluded in all candidates by clinical history and skin prick testing using a battery of

22 common foodstuffs and 12 inhalants (Laboratorios Leti, Barcelona, Spain) with histamine and saline as positive and negative controls, respectively. Previous history of acute gastroenteritis and its relationship to the initiation of IBS symptoms were also recorded. Exclusion criteria were age <18 years and > 65 years, pregnancy, a history of organic systemic or gastrointesintal disorders, abdominal surgery excluding appendectomy, radiotherapy or chemotherapy or treatment within 3 months of the study inclusion with steroids, anti-allergic or immunosuppressant drugs.

The study protocol was approved by the Institutional Review Board of the University Hospital Vall d'Hebron (PR (AG) 169/2007(2169)) and all subjects gave written informed consent to participate in the study. The study was registered in the European Community Clinical Trial System as study number EudraCT 2007-005914-38.

Study design

A double-blind randomized placebo-controlled trial was carried out. Randomization by hospital pharmacist in a 1:1 ratio was performed. Patients were assigned to receive either oral DSCG (200 mg/dose; 3 times/day) or placebo (3 times/day), before meals, during 6 months. No concomitant medication with known effects on gastrointestinal function was allowed during the treatment period, with the exception of antispasmodics or antidiarrheal agents (1-3 days/month) if abdominal symptoms worse. At inclusion, and prior to 1, 3 and 6 months follow-up visits, all participants filled in validated questionnaires to evaluate primary outcome variables (bowel

symptom severity) and levels of stress and depression. Also, clinical course was assessed with diary cards.

Drugs

The medication was prepared by the hospital pharmacy as a powder mix on a starch-base, containing no lactose, delivered in 2 opaque gelatin capsules (100 mg, each) and coded containers, taken orally 10min before meals *t.i.d*.. The active drug, DSCG was supplied by Sigma-Tau, Madrid, Spain; the placebo was physically identical to DSCG and contained only glucose.

Clinical assessment

IBS Severity Scale (IBS-SS).

The questionnaire of symptom severity evaluates changes in abdominal pain, bloating, degree of "dissatisfaction" with bowel habit, and interference of symptoms with daily life activities, through four visual analogue scales (rated on a 0-100 numerical scale, with 0 indicating "not at all" and 100 "the most intense") over the last 10-day period. Patients recorded the number of days suffering abdominal pain (this value was multiplied by 10, to give this variable the same weight as the other variables). The total maximum possible score is 500, categorizing as mild disease those subjects with a total score between 75- < 175, moderate 175- <300 and as severe >300. This tool is a well-validated illness severity assessment where higher scores in IBS-SS questionnaire mean greater severity symptoms. Also, this scale is sensitive to assess changes over time and has been used to evaluate response to

treatment. Indeed, a 50 points reduction score is suitable to detect a significant clinical improvement (Francis C.Y. 1997). Finally, to estimate severity of diarrhoea, the maximum and the minimum number of bowel movements and the stool consistency frequency were recorded within the last 10 days.

Diary Cards

Symptoms and clinical course were also assessed trough daily record of symptoms including: a) The severity of abdominal pain by a 10-point visual analogue scale; b) The frequency of abdominal pain (number of days with pain); c) The stool frequency (number of bowel movements per day); and d) The stool consistency, assessed by the Bristol Stool Form Scale (1-7) (BSFS; Heaton et al., 1992). Patients were also asked to report any concomitant medications used during the treatment period.

Psychological assessment

All participants underwent evaluation of psychological stress and depression at baseline and throughout the study, using 3 validated questionnaires:

(1) The Modified Social Readjustment Scale of Holmes-Rahe, to evaluate significant life events in the last year; (2) The Perceived Stress Scale of Cohen to assess levels of stress in the last month; and (3) Beck's inventory for depression to assess depression levels in the last week. Based on Holmes–Rahe scores, participants were stratified into baseline stress groups: low stress (<150 points), moderate stress (151–300 points) or severe stress (>300).

Beck's Depression Inventory scoring scale was as follows: low depression (10-18), moderate (19-29) and severe (>30) (Beck A, 1961; Kendall, 1986).

Efficacy of Treatment

Clinical benefit was defined as a significant improvement in primary and and/or secondary endpoints from baseline.

Primary endpoint was reduction in IBS severity, (defined as ≥ 50-point decrease in IBS-SS at any time point relative to inclusion), and specific symptoms including abdominal pain and stool consistency

Secondary endpoint was the percentage of patients reporting at least a ≥30% reduction in pain intensity score and a ≥ 50% reduction in pain frequency.

In addition, other secondary endpoint was the rate of weekly responder patients according to the FDA definition for IBS-D trials. A responder was defined as a patient who met, at the same week, responder criteria for pain and stool consistency, for at least 50 percent of the therapy period. Weekly responder criteria for pain intensity and stool consistency were defined as improvement of weekly intensity average of at least 30% and a reduction of at least 50% in frequency (days/ week) with at least one stool that has a consistency 6 or 7 compared to baseline, respectively, for at least 50 percent of the weeks of the study.

Furthermore, other secondary end points were changes in IBS-SS analogue subscales questionnaire: severity of bloating, dissatisfaction with bowel habits and interference of symptoms with daily activities of life, Finally, relationship between clinical symptoms and psychological variables were analysed.

Statistical Analysis

Sample size: We estimated a total sample size of 64 patients, based on 2-tailed analysis with an alpha a level of 5% providing 80% power to detect a 40% mean difference in clinical response rates between the active treatment arm and placebo arm, an expected response rate of 40% among patients treated with placebo and possible drop out rate of 15%.

The primary efficacy analysis was based on the intention-to-treat (ITT) population, which includes all randomized patients who received at least one dose of study medication and had at least one post-baseline efficacy measurement. Missing data were imputed using the last observation carried forward principle. The responder rates between the DSCG and the placebo treatment group were compared by the Fisher´s exact test. Analysis of change score from baseline at each time point was analyzed using repeated measures ANOVA model (Time effect of RMANOVA). All data are reported as median (range) or mean ± standard deviation, unless otherwise stated. Continuous data were compared using the Student *t*-test or the Mann-Whitney *U*-test, depending on distribution of the population in each variable. All statistical test performed were two-tailed and *P*-values <0.05 were considered significant.

RESULTS

Patient characteristics and adherence to treatment

Forty-four subjects were prospective recruited, two of them were excluded as one had <75 IBS severity score and the other one showed a *Giardia Lamblia* infection. Finally, forty-two were randomized (21 patients *per* group) Demographic,

psychological and clinical characteristics of the patients were comparable at baseline among the treatment groups, as summarized in table 1. The placebo group population was significantly younger than DSCG-treated group. The proportion of patients that exhibited moderate and severe chronic stress levels was not different between the randomised arms. The incidence of depression across the experimental groups was similar, 62% (13/21) of the patients in DSCG-treated group and 48% (10/21) in the placebo group.

Both groups showed similar baseline IBS severity score, scored as moderate severity, with moderate and severe IBS symptoms were reported in 57% (12/21) and 29% (6/21) of the patients in the DSCG group, respectively, and 52% (11/21) and 29 % (6/21) in those in the placebo group.

A total of 25 patients (60%) completed the 6-months period study, being the adherence rate of 67% (14/21) to the active therapy, and of 52% (11/21) to the placebo. Six patients (14%) discontinued the study prematurely within the first month, most of them 4 (67%) belonged to the placebo arm being lack of adherence the most common reason for premature withdrawal (figure 1).

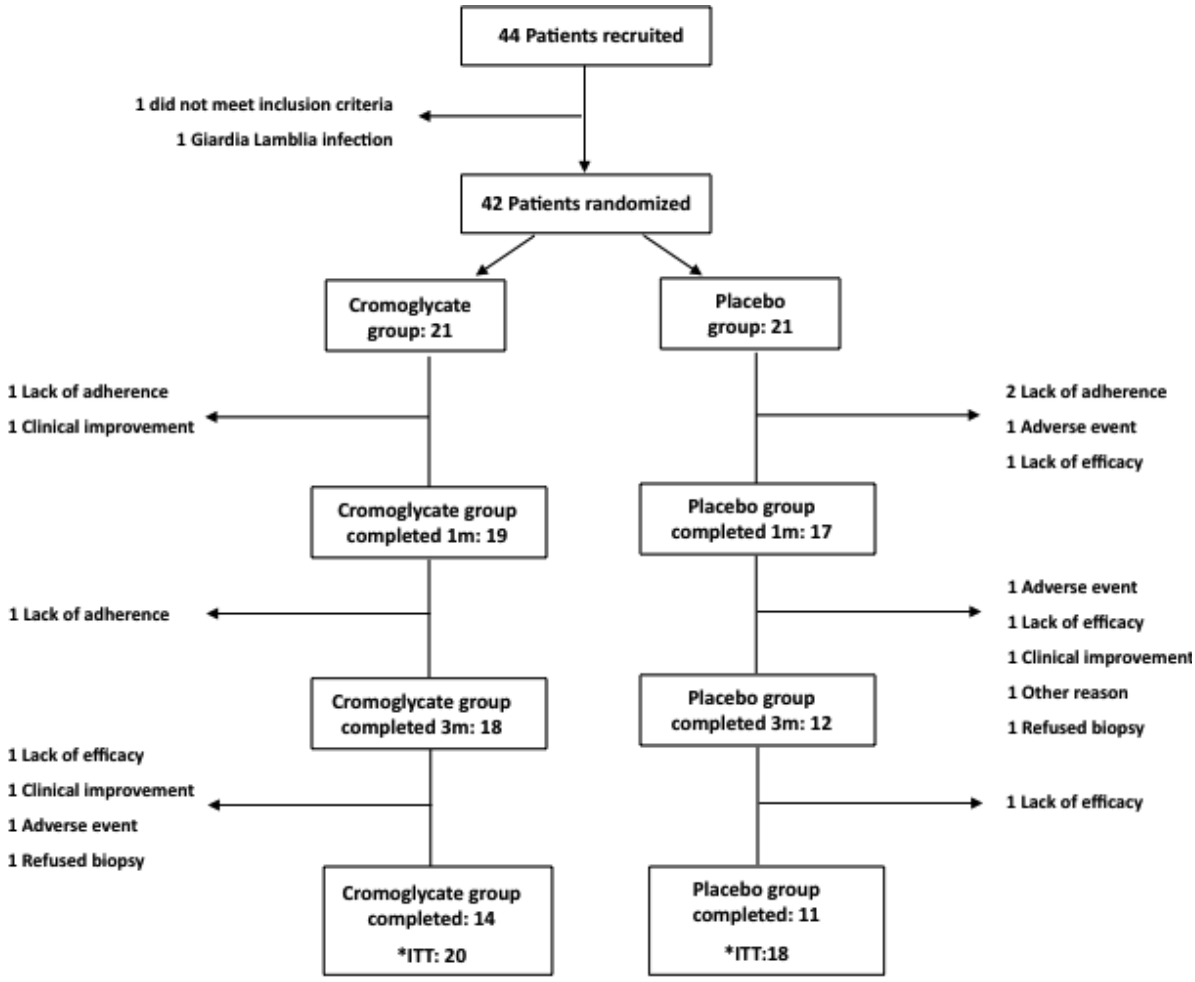

Figure1. Flow chart of inclusion and randomization of patients in the study. Note: *ITT, intent to treat.

Variable	Cromoglycate	Placebo	*P* value
Age (Years), mean (sd)	40.8 (11.7)	29.6 (9.0)	<0.01
Sex, n (%)			
Female	15 (71.4%)	11 (52.4%)	0.34
Male	6 (28.6%)	10 (47.6%)	
BMI (Kg/m^2), mean (sd)	23.9 (4.1)	22.7 (3.0)	0.29
Holmes, median (range)	150 (25-614)	138 (16-337)	0.50
Cohen, mean (sd)	25.5 (7.2)	24.4 (7.4)	0.64
Beck, median (range)	10 (1-24)	9 (0-32)	0.24
Duration of disease (%)			
< 5 years	14 (66.7%)	15 (71.4%)	1.00
≥ 5 years	7 (33.3%)	6 (28.6%)	
IBS-SSS, mean (sd)	276.3 (86.9)	258.1 (71.0)	0.49
Abdominal pain severity, mean (sd)	4.5 (2.8)	4.2 (2.5)	0.73
Abdominal pain frequency, mean (sd)	5.3 (3.1)	4.9 (2.7)	0.71
Stool consistency, mean (sd)	6 (0.6)	5.7 (0.7)	0.15
Bowel movements per day, mean (sd)	3.2 (1.7)	3 (1.4)	0.63

Table 1. Demographic and baseline characteristics of randomized patients. Holmes-Rahe scale: 0-150, low stress; 151-300 moderate stress; >300 severe stress. Perceived Stress Scale of Cohen, Beck´s depression inventory: low 10-18, moderate 19-29, severe >30. Note: BMI, Body mass index; IBS-SS, Irritable bowel syndrome severity score system, Stool consistency: 1 (hard) to 7 (entirely liquid).

Analysis of clinical correlations

At inclusion there was a weak, though significant correlation between abdominal pain severity and stool consistency (BSFS) (r= 0.32; P=0.04). The analysis of clinical and psychological variables revealed several interesting associations, as shown in table

2. In particular, a positive correlation was found between depression symptoms and IBS-SS (r=0.64, P<0.01), and abdominal pain (r=0.35, P=0.02).

	IBS-SS[a]		Abdominal pain[b]		Bowel movement		Stool consistency[c]	
	r	p	r	p	r	p	r	p
Holmes-Rahe test	0.21	0.17	0.24	0.12	-0.12	0.44	0.03	0.84
Cohen Scale (PSS)	0.22	0.16	-0.07	0.68	-0.06	0.70	-0.18	0.25
Beck´s Depression Inventory	**0.64**	**<0.0001**	**0.35**	**0.02**	0.06	0.75	0.12	0.44

Table 2. Clinical-psychological correlations at baseline in randomized IBS patients. [a]Irritable bowel syndrome severity score. [b]Severity score. [c]Bristol scale score. Values of p<0.05 according Spearman rank correlation in bold

Changes in IBS-SS, abdominal pain and stool consistency

The severity score was significant reduced in both treatment groups after the first month and at the end of the study in comparison to baseline values, as shown in figure 2. The magnitude of IBS score decrease was similar between DSCG and placebo at 1, 3 and 6-month follow-up (-53.1±79.1 vs. -39.6±56.5, P=0.55; -67.4±108.8 vs. -61.9±81.7 P= 0.86; and -71.5±91.7 vs. -68.4±66.6, P=0.91, respectively).

Overall, the proportion of patients who had significant clinical improvement (at least 50-point reduction on IBS-SS) at 1-month follow-up was of 55% (11/20) in the DSCG group, and of 38% (7/18) in the placebo group, although not reaching statistical significance between groups. Over 3 and 6 months of treatment these values were increased up to 55% (11/20) and 65% (13/20), respectively, in the DSCG group, and 50% (9/18) and 55% (10/18), respectively, in the placebo group. At these time

points, no statistical difference was detected between groups and respect to baseline values, as represented in figure 4.

The analysis of pain intensity and frequency reported the last 10 days of each follow-up visit, and based on IBS-SS questionnaire, revealed in both therapy arms a significant decreased at the end of the treatment compared to baseline. Although no significant differences were observed between groups; time had a significant effect across the treatment period.

The overall change in mean pain intensity improved significantly from baseline to the last visit in the study within DSCG group, whereas this improvement was only achieved at the final visit in the placebo group.

Abdominal pain frequency reduction within each group was statistically significant for the entire study period.

The stool consistency increased significantly at the end of the study compared to baseline in both arms, though no statistically significant difference was found, as shown in figure 3.

Notably, DSCG-treated subjects showed a trend toward higher stool consistency along the whole study period (P=0.34) relative to baseline scores. Highlighting the significant greater reduction from baseline of stool consistency score in DSCG group compared to placebo intake, especially at 1-month and the final visit (-1.6 vs. -0.7, P<0.01). Despite of stool consistency became normal in those who received DSCG, did not differ with placebo (4.4±0.26 vs. 5.0 ±0.24 score, P= 0.16).

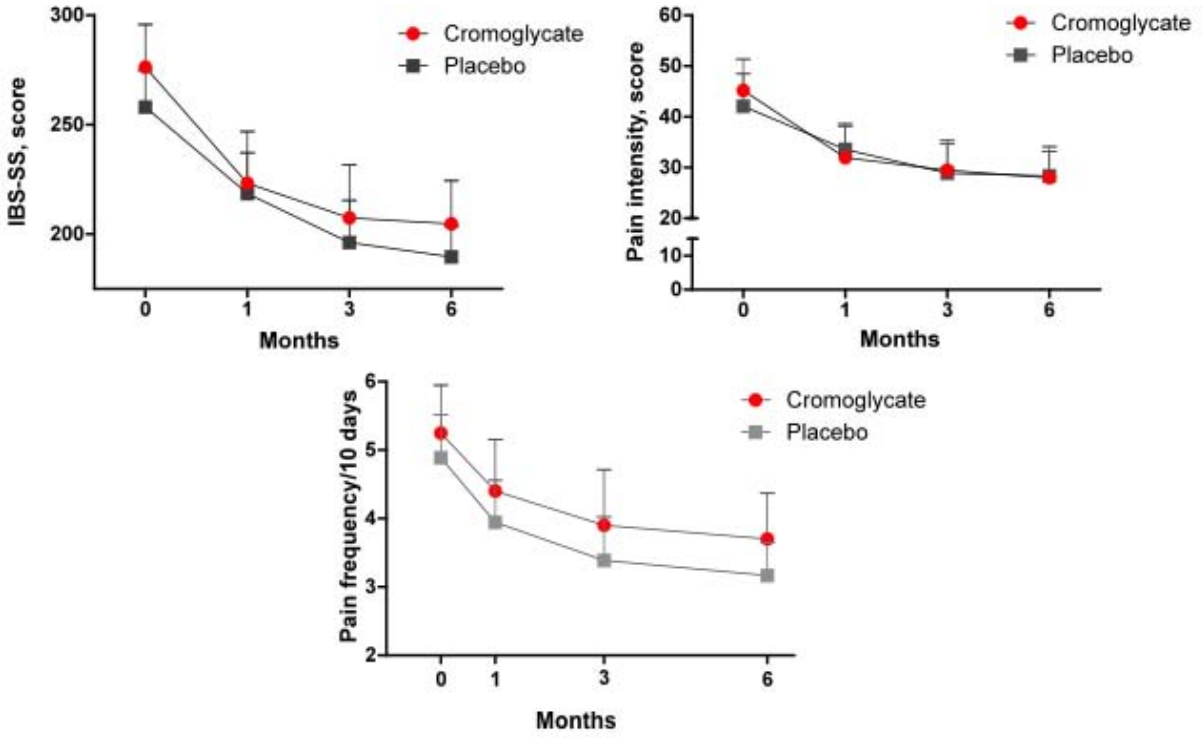

Figure 2. Changes in clinical outcomes of IBS symptoms (IBS-SS score, abdominal pain intensity and frequency) in both treatment groups. The figure is based on ITT analysis of clinical variables assessments IBS patients of treatment arms, DSCG-treated group (red dot) and placebo (grey square), over 6-month follow-up visits. Data are expressed as mean ± SEM. Analysis of groups in the repeated-measure ANOVAs revealed no significant difference in any variable. Time had overall effect on IBS-SS [F (3,108) = 15.12; $P < 0.0001$]; on abdominal pain intensity [F (3,108) = 7.48; $P = 0.0001$]; on abdominal pain frequency [F (3,108) = 7.48; $P = 0.0001$].

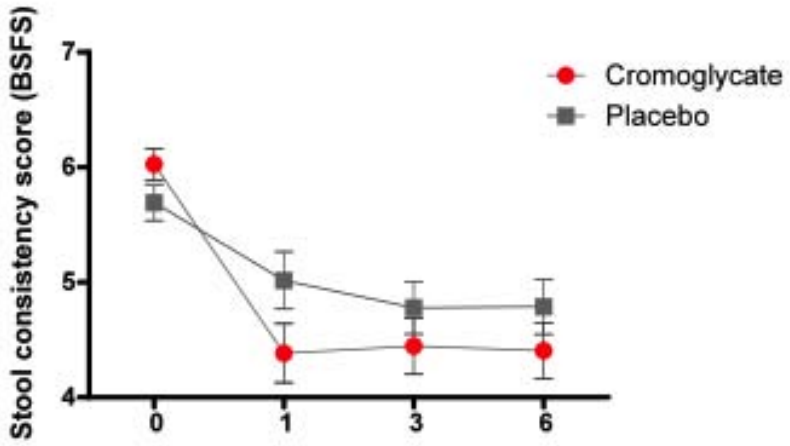

Figure 3. Influence of 6-month treatment with DSCG or Placebo on stool consistency. Stool consistency data from patients (ITT population) along the study. No significant difference was observed between the two treatment arms over time. At the end of the study stool consistency significantly increase compared to baseline in both groups (-1.6; *P*<0.0001 DSCG group; -0.7 *P*= 0.002 placebo). Stool consistency improvement in DSCG-treated patients was greater (*P*= 0.02). All P values were derived from paired and unpaired t test, respectively.

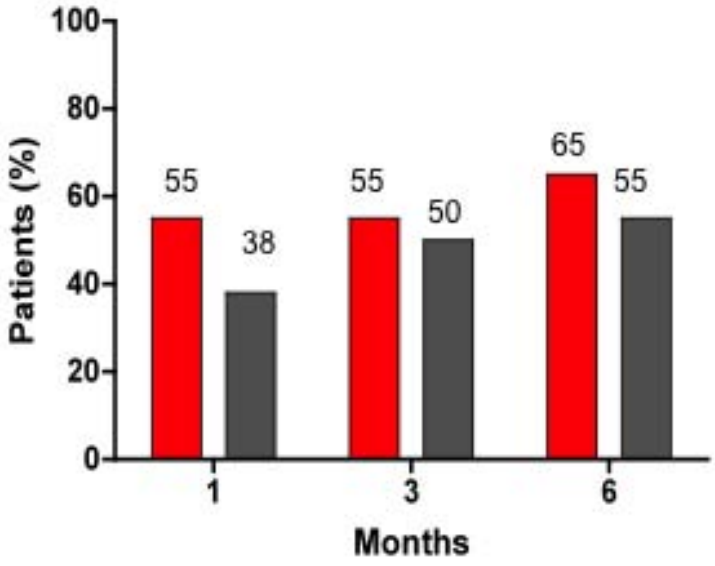

Figure 4. Proportion of patients with clinically significant decreased in IBS severity. Comparison along the study period of the percentage of patients who decreased 50-point in IBS severity score between DSCG group (red bar) and placebo (grey bar), no significant differences were observed. Data analysis according Fisher's exact test.

Secondary endpoint outcomes

To complement the analysis of the effect of DSCG on pain, we evaluated the proportion of patients who exhibited adequate relief of pain (based on IBS-SS questionnaire) over time. The proportion of patients who exhibited ≥30% reduction in intensity score and ≥ 50% reduction in frequency increased over time in both groups, being always higher in the DSCG-treated arm than in the placebo, without statistical significance. At the three scheduled visits the rates were 30, 40 and 45%, respectively, in the DSCG group and 11, 28 and 22%, respectively, in the placebo group (P>0.05 for all comparisons). It is noteworthy that at the end of the treatment 20% of the participants in the DSCG group reported no pain in the IBS-SS scale compared with 6% in the placebo treatment arm (P=0.34).

According to daily records and FDA responder criteria, and considering the whole follow-up, the proportion of responders (patients who reported at the same week pain relief and reduction in the frequency of watery stools) was significantly higher in the DSCG group than the placebo group (45% vs. 17%, P=0.09). Analysing only individual components in responders criteria, in the active treatment group the proportion of patients who reported a decrease of ≥ 30% in abdominal pain over half of the weeks of the treatment period was greater than placebo, although without significant difference among treatments (65% vs. 39%, P=0.19), as shown in figure XA. With regard to stool consistency, the percentage of patients who showed a decrease in frequency of watery stools was significantly higher in (78%) DSCG group compared with (28%) placebo group (P=0.02), as shown in figure 5B.

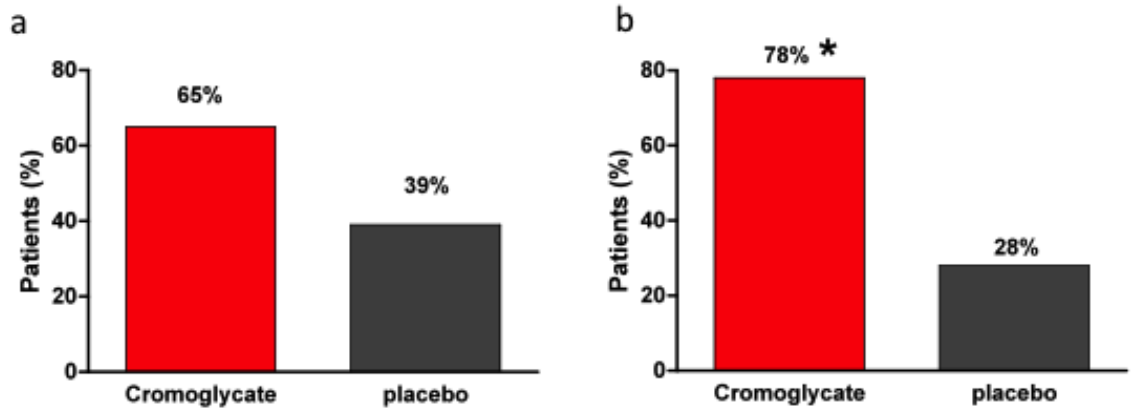

Figure 5. Responder patients rate according to FDA components. (**a**) ≤ 30% abdominal pain reduction from baseline at least ≥12/24 weeks and (**b**) ≤50% days per week with at least one stool consistency 6-7 according to Bristol scale. *P value <0.05, for cromoglycate vs. placebo were derived from Fisher's exact test; FDA, Food and Drug Administration

Other subscales from IBS-SS questionnaire were analyzed over the whole follow-up including: abdominal bloating, degree of "dissatisfaction" with bowel habit, and interference of symptoms with daily life activities, no treatment had significant effect on any of those variables.

Regarding the psychological profile over the therapy period, changes in Holmes, Cohen and Beck scores did not differ among treatments. Time had overall effect only on Beck´s inventory score, as represented in figure 6.

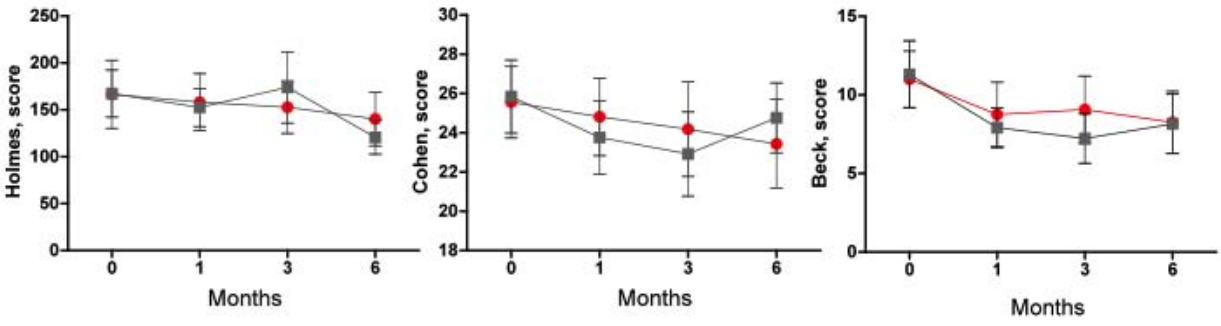

Figure 6. Psychological profile evolution over therapy period. Evolution of psychological variables over 6-month follow-up for DSCG-treated (red dot) and placebo-treated (grey square). Data are expressed as mean ± SEM. Analysis of groups in the repeated-measure ANOVAs revealed no significant difference in any variable. Time had overall effect on beck´s inventory [F (3,181) = 6.78; P= 0.0004].

Within group analysis change from baseline score in mean Beck Depression Inventory score for the entire study period showed a significant reduction in the score in the placebo-treated group, in contrast to the decrease in DSCG group that was only significant at 1-month visit.

No relationship was found between BDI score and IBS severity at the final visit. Surprisingly, a positive and significant correlation was observed between depressive symptoms score and pain severity (r=0.43, P=0.02) at the end of the study. When considering whether treatment group can be associated, only placebo-treated patients showed a significant positive relationship (r=0.55, P=0.05).

Safety

Overall, the multiple oral dose of cromoglycate used in this study was well tolerated. In the intent-to-treat population, the percentage of patients who reported at least one treatment-emergent adverse event (TEAE) was 5% (1/20) in cromoglycate-treated group and 11.1% (2/18) in placebo group along the whole treatment period. The AE reported in cromoglycate group was mild heartburn controlled with proton pump inhibitor and those one reported in the placebo group were back pain and psychotic episode. None of the patients had serious adverse events.

Discussion

This double-blind, placebo-controlled clinical trial, shows several advantages of long-term treatment with DSCG over placebo in IBS-D clinical manifestations. IBS severity decreased significantly following both treatments, however, despite the lack of statistically difference among them, the proportion of patients who reported clinical significant improvement in IBS severity and pain across the study was higher in DSCG-treated subjects than in placebo. Likewise, stool consistency significantly decreased in both groups during the whole follow-up, however in those who took DSCG the magnitude of improvement from baseline was superior as compared to placebo, especially after the first month and at the end of treatment. Moreover, the average of stool form became to be normal following treatment with DSCG in contrast to the placebo group.

Moreover, daily data from DSCG-treated group showed greater improvement than the placebo group on reducing the frequency of watery stools during at least 50% of the length study. The rate of responders for stool consistency within DSCG was significantly greater than placebo, what means that cromoglycate had a significant influence on the frequency of loose and watery stools during the whole follow-up.

Regarding the benefit of treatment with DSCG in stool consistency, our trial is in agreement with other studies. Clinical benefit with DSCG has been reported in systemic mastocytosis with GI manifestations as diarrhea (Soter NA, 1979). Likewise, other work has demonstrated effects on stool frequency and consistency in 40% of patients with persistent diarrhea without organic cause after four-week period of treatment with oral DSCG, (Bolin TD, 1980). In IBS-D, treatment with DSCG (1500mg/day) decreased significantly the global symptom score after 4-week

treatment period, effect that was especially marked in the subgroup of patients with food intolerance (Stefanini GF, 1995).

Additionally, several factors should be taken into account in DSCG individual efficacy, as its mucosal availability and effect depend on variations on intestinal permeability, the dose adherence, as well as the tachyphylaxis phenomenon described in *in vitro* studies with cromolyn and mast cells (Wells E., 1983).

As current pharmacologic therapy remains unsatisfactory, several efforts have been performed to enhance our knowledge about the mechanisms underlying IBS. Over the last years, new therapeutic approaches to this condition, focused on modifying pathophysiological factors have been developed: pharmacologic interventions and psychological treatments. Among pharmacologic compounds, there is growing interest on anti-inflammatory and non-absorbable molecules with local action and non-systemic effects. Intestinal disorders as inflammatory bowel disease (IBD) and IBS, where intestinal permeability is increased may contribute to enhance their availability in intestinal mucosa and reverse local immune activation. Several studies have shown mast cell activation within intestinal mucosa in IBS-D patients, and its association with IBS symptoms (Vivinus-Nebot *et al*., 2012;.Martinez *et al*., 2012). Mast cells release several mediators upon activation with effects on the recruitment and activation of numerous immune cells. Mast cell stabilizers can prevent all these phenomena, which justifies the use of disodium cromoglycate in IBS-D. This idea is supported by the use of another mast cell stabilizer for IBS treatment, Ketotifen, which has been showed improvement in visceral hypersensitivity in a 8-week trial (Klooker T.K., 2010).

Results observed in the present study in the placebo group are consistent with the high placebo response rates in IBS that are reported in the literature, which varies from 30% to 60% of response rate (Patel SM, 2005). It has been documented in a randomized and open-label placebo trial a meaningful symptomatic improvement from baseline in IBS-SS after treatment (Kaptchuk TJ, 2010). Moreover, there is an additional component, called “pre-cebo”, which is the expectation for improvement in symptoms before starting to take medication (Kim SE, 2012), and enhances, therefore, the likelihood of placebo effect.

The fluctuating nature of IBS symptoms, the lack of biological markers and the high placebo response rate in this condition, makes the assessment of therapeutical response difficult to perform, as not true objective outcome measures are available. All the tools that assess symptoms severity are based on patients’ perception, therefore, over the last years a debate about which are the suitable symptom measures outcome to determine the efficacy in trials has been present. This task is not easy, and most of the studies are based on the measurement of a single symptom relief. Nowadays, it seems that multi-item symptom scales questionnaire as IBS-SSS, could be useful for detecting clinical significant response to therapy (Spiegel B., 2009), although still not fulfilling our clinical requirements (Whitehead WE., 2006). Recently, the US Food and Drug Administration (FDA) requested to evaluate the 2 cardinal IBS symptoms: abdominal pain and stool consistency in IBS-D trials (FDA, 2012). These reasons justify the tools we have used as outcome measurements in our study.

In addition to immune activation and impairment of intestinal epithelial barrier, abnormal brain-gut axis interactions have been documented in IBS patients. Indeed, psychological risk factors are associated with IBS and the prevalence of psychiatric

comorbidities as anxiety and depression disorders have been found over 40-50% of IBS patients (Stasi *et al*., 2012). Even more, psychological factors have a greater impact on visceral pain modulation in IBS patients than in control subjects (Elsenbruch S, 2010). Patients frequently link symptoms relapse with life events stressors, though we did not observe any association between symptoms severity and stress scores in this study. Interestingly, our data showed that IBS patients at baseline exhibited a weak and moderate association between depression symptoms score and IBS abdominal pain and severity, respectively. Besides, we observed a reduction from baseline values on depression symptoms in both therapy arms at the end of the study, being statistically significant within the placebo group. Furthermore, the average of pain intensity reported by this group at the final visit had a positive correlation with depression symptoms score. This finding could explain the improvement of pain in the placebo group. Previous evidences in the literature suggest that psychological and neurobiological mechanisms underlie placebo effect (Finniss DG., 2010). Probably the findings in neurobiological research with functional brain imaging may allow explaining brain circuits involved in symptom and affective changes.

During the present study, we found different obstacles that probably affected the interpretation of the data and limited the assessment of significance of our negative results. Because of slower than anticipated enrolment, the sample size was recalculated. With 42 patients, the study would have had an 80% of power to detect an absolute difference of 30% in the response rates between both treatments. To avoid the placebo effect, we carried out a 6-month clinical trial, although this choice may have increased the dropout rates among the two treatment arms and may have lead to biased samples. Another potential limitation of the present study was

baseline imbalance in age between treatment arms, even though the process of random allocation, we are aware of a possible effect on our results. On the other hand, across the whole treatment period, both treatment groups were treated in a warm environment patient-physician relationship, what might have had a positive benefit effect in placebo-treated patients. The lack of significance may be due to a sample size problem, leading to an underpowered study. As these results were obtained in a relatively small sample of patients, our findings need replication in a larger randomised controlled trial before a final conclusion.

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CAPÍTULO 3

Long-term stabilization with oral disodium cromoglycate treatment modulates intestinal mucosal transcriptome and mast cell activity in diarrhea-predominant irritable bowel syndrome patients.

Short Title: Effect of cromoglycate on mucosal IBS-D transcriptome.

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Abbreviations used in this paper: BMI, body mass index DSCG, disodium cromoglycate; FC, fold-change; IBS-D, diarrhea-predominant irritable bowel syndrome; IELs, intraepithelial lymphocytes; hpf, high power field; FDA, Food and Drug Administration Q-RT-PCR, quantitative real-time polymerase chain reaction; SRRS, Modified Social Readjustment Rating Scale; PSS, Perceived Stress Scale; BDI, Beck's Depression Inventory; TEM, transmission electron microscopy; H&E, hematoxylin and eosin; MC, mast cells; IPA, Ingenuity Pathway Analysis, CPA3, carboxypeptidase.

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Abstract

Background & objective: Mucosal mast cell (MC) activation is associated with intestinal dysfunction in irritable bowel syndrome (IBS); we have reported clinical benefit and innate immune signaling modulation after oral disodium cromoglycate (DSCG) treatment, a MC stabilizer. However, its effects on gene expression profiles and immune activity in the intestinal mucosa and its relation to clinical improvement are unknown. We performed such studies in a double-blind placebo-controlled clinical trial.

Material & Methods: Forty-two diarrhea-prone IBS (IBS-D) patients meeting the Rome III criteria were randomized to receive placebo or DSCG for 6 months. Clinical and psychological variables were analyzed throughout the study. Jejunal mucosal biopsies were obtained from each patient pre- and post-treatment and studied for histology, gene expression (microarrays),, or MC ultrastructure (transmission electron microscopy). Differential transcript expression regulation was assessed in responders and non-responders (FDA criterion). **Results:** DSCG treatment provided higher proportion of responders to pain as compared to placebo treatment. Immune cell infiltration and mucosal architecture was similar in both groups, but stabilization of MC ultrastructure featured the DSCG group. Functional analysis revealed networks associated with cell-to-cell signaling and interaction, and cell death and survival, In responders, core clock genes expression was associated only with changes in depression score within the placebo group, while DSCG induced differentially regulation of an anti-inflammatory and antioxidant genes, which correlated with pain improvement.

Conclusion: DSCG stabilizes mucosal MC and modulates intestinal gene profiling in association with clinical improvement. This study supports the use of MC stabilizing agents for the treatment of IBS-D.

Introduction

Increased antigen penetration through impaired intestinal barrier leads to mucosal immune activation and, presumably, to perpetuation of altered barrier function (Matricon *et al*., 2012). The intestinal epithelial layer in Irritable Bowel Syndrome (IBS) patients has been identified to display structural alterations, specifically in tight junction proteins, in association with mast cells (MC) activation (Piche T., 2009; Martinez C., 2012), supporting the view of IBS as an organic disease rather than a functional disorder.

MCs have a key role in innate immunity, as they recognize and respond against a variety of infectious agents (bacterial and parasitic) via secretion of their mediators. MC are located in close apposition to nerve terminals in the lamina propria within the mucosa (Park CH., 2003), or beneath the epithelial surfaces, close by the external environment, which facilitates its activation by secreted neuropeptides or by antigens, respectively (Al-Khatib & Lin, 2009). Although controversial, increased infiltration of MC has been found in the mucosa of the cecum, terminal ileum, and jejunum of IBS patients (Weston AP., 1993; O'Sullivan M., 2000; Guilarte M., 2007). However, it is the activation and not the number of MC what has been identified to correlate with stool form and the number of bowel movements in diarrhea-predominant IBS (IBS-D) patients (Martinez C., 2012). Therefore, strategies addressed at inhibiting MC activation seem a promising therapy in this disease.

Disodium cromoglycate (DSCG) is a MC stabilizer that reduces calcium influx into cells, therefore, avoiding degranulation (Hemmerich S., 1991). Our group has performed a pilot study on IBS-D, in which a significant improvement in

clinical symptoms, as well as down-regulation of innate immunity, after a 6-months treatment period has been identified (Ramos L., 2007; Lobo B., 2009). Moreover, a double blind placebo-controlled clinical trial has recently allowed us to better assess the clinical response in these patients.

The aim of the present study was to gain further insight into the underlying MC-dependent mechanisms associated with the clinical improvement in IBS-D. We investigated the effect of long-term DSCG oral treatment on mucosal molecular and cellular basis of immune activity, by assessing differential transcriptional profile, the immune infiltrate and its association with clinical response.

Material and Methods

Patients

IBS-D patients meeting the Rome III criteria were recruited from the Department of Gastroenterology of University Hospital Vall d'Hebron from August 2008 through July 2011. All subjects underwent a complete medical history and physical examination to confirm diagnosis and to exclude other gastrointestinal comorbidities by means of a broad biochemical and serological profile including anti-transglutaminase antibodies; upper and lower fiberoptic and small bowel capsule endoscopy. Abdominal sonography and barium studies were performed in some participants, as requested by the responsible physician. Previous history of acute gastroenteritis and its relationship to the initiation of IBS symptoms were also recorded. Exclusion criteria were age younger than 18 years and older than 65 years, pregnancy, a history of organic GI disorders, chronic organic diseases, abdominal surgery excluding appendectomy, treatment within the 3 months prior to inclusion with steroids, anti-allergic or immunosuppressive and related drugs, and radiotherapy or chemotherapy within the 6 months prior to inclusion. In addition, at the time of recruitment, food allergy was excluded in all candidates by clinical history and skin prick testing using a battery of 22 common foodstuffs (Laboratorios Leti, Barcelona, Spain) with histamine and saline as positive and negative controls, respectively.

Study design

A double-blind randomized placebo-controlled trial was carried out. The hospital pharmacist performed randomization in a 1:1 ratio, and supplied the medication in a numbered container. Patients were assigned to receive either oral disodium cromoglycate (DSCG), or placebo *t.i.d* before meals during 6 months. No

concomitant medication with known effects on gastrointestinal function was permitted during the treatment period, and patients agreed to take occasionally antispasmodics or antidiarrheal agents only if abdominal symptoms get worse. Two jejunal biopsies were obtained per participant: one at inclusion, before the administration of the treatment, and another one at the end of the experimental period, within 72h after the last dose. All participants completed validated questionnaires to evaluate their bowel symptoms and their psychological level of stress and depression. The study analyzed the effect of DSCG/placebo on differential mucosal gene expression and immune cell populations, and the association between clinical and biological variables. All participants received a written informed consent form before any study procedure was performed, and all agreed to participate. The Institutional Review Board of the University Hospital Vall d´Hebron had previously approved the protocol for the study (PR(AG) 169/2007(2169)). The study was registered in the European Community Clinical Trial System as study number EudraCT 2007-005914-38.

Drugs

The medication was prepared by the hospital pharmacy as a powder mix on a starch-base, containing no lactose, and delivered in opaque gelatine capsules. The active therapy contained 100 mg of DSCG (Sigma-Tau, Madrid, Spain) *per* capsule; the placebo was physically identical to the active therapy and contained only glucose. All patients ingested 2 capsules before meals, 3 times/day (600 mg/day).

Clinical assessment

Each enrolled patient was asked to fill the IBS-SS questionnaire (as detailed in chapter 2) prior to the biopsy procedure, where the average of severity and frequency of abdominal pain and the stool frequency during the last 10 days were recorded. This questionnaire categorizes different degrees of IBS severity according to the score: low (75-175), moderate (175-300) and severe (>300). Moreover, diary cards details related to abdominal pain intensity (rated on a 0-10 scale), bowel movements and stool consistency (rated the 7-point Bristol Stool Scale) were daily recorded over the study period.

The present study was designed to identify the relationship between the clinical response and changes in the intestinal mucosal transcriptome. Since diagnosis criteria for IBS is based on two major clinical symptoms (abdominal pain associated with altered bowel movements), our outcome measures were focused on FDA´s weekly responders criteria, as previously defined in chapter 2. Therefore, in order to get further insight into treatment clinical benefit, we analyzed the percentage of patients who met responder criteria for pain or stool consistency in each group, for subsequent studies.

Psychological assessment

All participants underwent psychological stress and depression evaluation by 3 validated tests (as detailed in chapter 2) at inclusion and after completing the study: the Spanish version of the Social Readjustment Rating Scale (SRRS) of Holmes-Rahe (Holmes and Rahe, 1967), Perceived Stress Scale of Cohen (PSS) (Cohen, 1983) and Beck's Depression Inventory (BDI; Beck, 1961).

Jejunal biopsy

Two jejunal biopsies were obtained in each eligible patient: at inclusion (T_0) and after completing the period of 6-months treatment (T_6) between 9:30-10:30 A.M. No diet restrictions, other than overnight fasting, alcohol or caffeine intake in the previous 24 hours, were indicated. The mucosal biopsy was obtained from the proximal jejunum, 10 cm distal to the Treitz's angle, using a Watson's capsule with an attached aspiration tube (3 mm diameter) as previously described (Guilarte M., 2007). Tissue samples were immediately split into two similar pieces with a sterile scalpel. One fragment was fixed in formalin, embedded in paraffin, and processed for routine histology and immunohistochemistry, to assess jejunal mucosa integrity and to characterize mucosal immune cells subtypes. The remaining fragment was placed, in a randomized fashion, in RNA later solution (Ambion, Madrid, Spain) for gene expression study by microarrays, or fixed in glutharaldehide/paraformaldehyde solution for ultrastructure studies by transmission electron microscopy (TEM).

Tissue Processing and Analyses

Histology and Immunohistochemistry

Tissue sections were processed for routine hematoxylin and eosin (H&E) staining to assess intestinal and epithelial morphology and eosinophil counts. In addition, antibodies against CD3 (Dako, Barcelona, Spain) were used to assess the number of intraepithelial lymphocytes (IELs), or against *c-kit* (CD117), to quantify mucosal MC. The eosinophil and MC density throughout the lamina propria in each jejunal biopsy was expressed as the average number of cells

per high power field (hpf), and IELs were expressed per 100 epithelial cells, as previously described (Guilarte M., 2007), under light microscope (400x). An experienced pathologist, who was unaware of the experimental groups, evaluated all intestinal biopsies.

Transmission electron microscopy

Samples were immediately immersed in fixative buffer (2.5% glutaraldehyde and 2% paraformaldehyde) for at least 48 hours. Tissues were post-fixed in 1% (w/v) osmium tetroxide containing 0.8% (w/v) of potassium hexacyanoferrate (III) (Sigma) and infltrated in Epon's resin and polymerised at 60ºC. Ultrathin sections were mounted in copper grids, contrasted with standard uranyle acetate and lead citrate double-staining and observed in a Jeol JEM-1400 (Jeol LTD, Tokyo, Japan) transmission electron microscope equipped with a Gatan Ultrascan ES1000 CCD camera. Mucosal MCs were identified based on morphological characteristics and images were taken at 10,000x magnification. Tissue examination was performed in a blinded manner. Apart from exploring the general ultrastructure, each individual MC was assessed for signs of degranulation according to previously described criteria (Dvorak AM, 2005). Piecemeal degranulation pattern is characterized by extrusion of granule content with the lack of intergranule fusions or granule union to the plasma membrane, observing partially and completely empty granules containers.

RNA isolation

Biopsies were individually homogenized in the FastPrep mixer (Bio101) in RLT cell lysis buffer (Qiagen, Madrid, Spain) followed by RNA isolation (RNeasy Mini Kit, Qiagen) and on-column DNase treatment (Qiagen). Prior to gene array

analysis, RNA quantity and quality were confirmed by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, Calif., USA). Synthesis of cDNA was performed using 1 µg of total RNA with the High Capacity Reverse Transcription Reagents Kit (Applied Biosystems, Madrid, Spain), following manufacturer's instructions.

Microarray data analysis

Microarray technology (Affymetrix human gene 1.1 ST 96-array plate) and pathway analysis were performed on mucosal biopsies collected pre- and post-therapy. To identify the underlying differential profile in gene expression, we applied hierarchical clustering on the complete set of differentially expressed genes using average linkage and correlation as measures of similarity. To further select differentially expressed genes, a comparative analysis and linear models for microarray data (LIMMA) were performed for the entire probe set. Probe's fold change (FC) expression was obtained as log-2 scale with an associated *P*-value. To favor discovery of a wider set of genes a false discovery rate (FDR) adjustment was not utilized at this point. We considered a probe set as differentially expressed within a comparison group if have a *P*-value < 0.025 after LIMMA and a FC in either direction ≥1.5. Only genes that filled the filtering criteria as differentially expressed were further analyzed, therefore selected transcripts could be confirmed by quantitative real PCR (Q-RT-PCR). In this analysis there is more than one comparison, thus it is interesting to look for common patterns of regulation between different experimental conditions. All transcripts were clustered with the group of comparison after non-supervision

analysis. Every clustering is represented in a two-dimensional heatmap with one endogram, indicating the similarity between groups of genes.

Functional and pathway analysis

Changes in transcript levels, assessed by microarray analysis results in long lists of genes and most of these changes are not independent events. To help understanding the biological meaning of these changes we used the Ingenuity Pathway Analysis software (IPA Software 7.0, Ingenuity Systems; http://www.ingenuity.com), which analyses gene expression profile and integrate data from different experimental platforms to provide insight into the molecular interaction. Also, it helps to identify gene function pattern obtained from microarray and how different genes are biologically related. In addition, this tool provides information on the causes of observed gene expression changes and indicates the predicted downstream biological effects of those changes. «Downstream Effects Analysis» compares the direction of the differently expressed genes with expectations based on the literature and predicts for each function a direction change using the «regulation z-score algorithm» this procedure ensures these significant predictions and not due to chance. *Z*-scores ≥ 2 and *Z*-scores ≤ -2, indicate that the function is significantly increased and decreased, respectively. For network analysis, IPA provided a score according to the connectivity of supplied genes and the list of biological functions involved. The provided score indicates the probability that the focus genes are together in a network could be achieved by chance alone. The probability for a network to be selected by chance (score<3) decreases when corresponding score value increases. Therefore, scores of 3 or higher represent a >99.9% confidence level.

Moreover, IPA identified the top differentially expressed genes (up-regulated and down-regulated), which were further validated by quantitative gene analysis. In functional analyses, the most significant biological functions to the dataset were highlighted. The significance value designated to the functions has into account the chances that the genes of interest participate in the biological functions. The IPA software calculates a *P*-value by comparing the number of genes-of-interest in a particular function with their occurrences in all the functions in the IPA knowledge database.

Synthesis of cDNA and Q-RT-PCR analysis

Synthesis of cDNA was performed using 1 µg of total RNA with the High Capacity Reverse Transcription Reagents Kit (Applied Biosystems, Madrid, Spain). Q-RT-PCR was performed on an ABI PRISM® 7500 FAST Sequence Detection System (Applied Biosystems, Madrid, Spain) using validated TaqMan Gene Expression Assays (GEA) and the human 18S subunit ribosomal RNA and cyclophilin (PPIA) genes as endogenous control (Applied Biosystems, Madrid, Spain). Transcript quantification in each sample, including distilled water as negative control, was processed in triplicate. Gene expression was normalized to endogenous genes, and quantified using the comparative Ct method (relative quantification) and the Sequence Detector Software SDS v2.2 (Applied Biosystems). To compare gene expression differences between therapies, Ct values for each experimental group was determined involving a target gene and a housekeeping gene (PPIA) that were subtracted to obtain the ΔΔCt (ΔΔCt = ΔCt T_6—ΔCt T_0 = (Ct target gene T_6—Ct PPIA T_6)-(Ct target

gene T_0—Ct PPIA T_0)). Q-RT-PCR was used to validate microarray analysis and to further study the expression of specific genes, detailed in **table 1.**

Cromoglycate T_6 vs.T_0		Placebo T_6 vs.T_0		Cromoglycate vs. Placebo (T_6 vs.T_0)	
Probe ID	*Gene name*	*Probe ID*	*Gene name*	*Probe ID*	*Gene name*
Hs00197519_m1	SLC34A2	Hs00899658_m1	**MMP1**	Designed primer	IGHG
Hs00426592_m1	UGT2B7	Hs00271467_m1	**IFI27***	Designed primer	IGHM
Hs01557155_m1	LRRC19	Hs00268113_m1	CCL18	Designed primer	IGHA
Hs01551078_m1	TLR3	Hs01092603_m1	**PER1***	Hs00899658_m1	**MMP1***
Hs01573162_m1	FABP2			Hs00271467_m1	**IFI27***
Hs00159357_m1	MUC1			Hs01092603_m1	**PER1***
Hs00386811_m1	CD27			Hs00253876_m1	NR1D1
Hs00998119_m1	**CD79A***			Hs00998119_m1	**CD79A***
Hs01030003_m1	**MPV17L***			Hs01030003_m1	**MPV17L***

Table 1. Selection of genes for quantitative RNA expression evaluation using Q-RT-PCR. The 3 columns indicate the top gene list obtained in the 3 comparisons performed on the differential gene profile assessed by microarray technology followed by IPA analysis. Genes selected in DSCG vs Placebo comparison common in other comparison were shown in bold.

Statistical Analysis

Sample size was calculated as detailed in chapter 2. Data are reported as median (range) or mean ± standard deviation, unless otherwise stated; all statistical test performed were two-tailed and *P*-values <0.05 were considered significant. Continuous data were compared among treatment groups using Student *t*-test or the Mann-Whitney *U*-test, depending on the distribution of the population in each variable. Clinical and histological changes within each group before and after treatment were analyzed by paired *t*-test. The relationship between clinical and biological variables was evaluated by the Spearman's rho correlation.

For the gene expression study, randomization of patients (microarray study) and genes (validation of microarray data) were performed using a standard random number generator: a linear congruential generator was used to generate uniform random values, followed by a transformation to obtain integer values in a set with the same size as the number of patients/genes. Non-adjusted *P*-value was used to select a statistical significant cut-off for detecting the genes differentially expressed. P-value for each comparison was calculated by applying the linear empirical Bayesian methodology developed by Smyth (Smyth K., 2004). Association between changes in pain severity score from baseline to 6 months and individual absolute log2 expression values obtained by microarray analysis or AACt values obtained by Q-RT-PCR was evaluated by the Spearman's rho correlation.

Results

Demographic and clinical characteristics of participants

Forty-two patients were invited to participate in this study, and an appropriate intestinal biopsy was obtained at inclusion visit in 41 of them. A total of 25 patients completed the 6-months treatment period (14 in the DSCG arm and 11 in the placebo arm), of these, 1 patient (DSCG group) refused to undergo the second biopsy. Moreover, from the biopsies already collected at post-treatment visit, one from the placebo group and 3 from the CGD group, were not suitable for histological analysis due to small biopsy specimens. Finally, 10-paired matched biopsies within each treatment arm were evaluated for histology

The number of participants with adequate biological material, suitable for transcriptional profiling or MC ultrastructure study, was of 24. Demographic baseline characteristics of selected participants are detailed in **table 2**. The two groups did not differ in demographic characteristics at baseline, except in the age, being significantly lower in the placebo group than in the DSCG group.

	DSCG			Placebo			
Characteristics	rt-PCR	TEM	Total	rt-PCR	TEM	Total	p Value
Number of subjects	9	4	**13**	7	4	**11**	
Gender (F:M)	6:3	3:1	**9:4**	4:3	3:1	**7:4**	1.000
Age (years)	42.9±4.0	37.5 (27-60)	**42.2±3.3**	31.3±3.0	21.5 (21-43)	**29.6±2.6**	0.009 [a]
BMI (Kg/m²)	22.2±1.1	28.3 (17.2-31.5)	**23.5±1.3**	22.5±1.3	20.7 (16.8-25.8)	**22.0±1.1**	0.371
Previous gastroenteritis	1/9	0/4	**1/13**	0/7	0/4	**0/11**	1.0
Dyspepsia	6/9	2/4	**8/13**	5/7	1/4	**6/11**	1.000
Food Allergy	NA	NA	**NA**	NA	NA	**NA**	
Other inflammatory disorders			**No**			**No**	

Table 2. Clinical and demographic characteristics of participants. Data are expressed as mean ± SEM or (range). F, female. M, male. BMI, body mass index. [a]The mean age in DSCG group was statistically different from the Placebo group**.**

Clinical evolution and psychological status

Patients selected for evaluating mucosal gene expression profile and ultrastructure MC features, intra- and inter-groups, displayed similar demographic and clinical characteristics. To gain further insight into potential molecular mechanisms underlying clinical benefit after oral treatment, we

focused on those patients subjected to RNA analysis for gene expression (a total of 16 subjects).

Clinical outcomes and psychological profile were assessed in the two treatment arms before and after treatment. Comparison of clinical characteristics at baseline among treatment groups gave no statistically significant differences (data not shown). Moreover, both groups showed similar clinically significant decrease in IBS severity (>50-point), abdominal pain and bowel movements (**Table 3**), from baseline to the final visit.

Clinical outcome assessment within-group revealed significant differences at 6 months compared to baseline: in the placebo group, the severity of disease decreased by 97.3±20.9 points (*P*=0.02), and the number of bowel movements declined by 1.0±0.3 (*P*=0.01). The stool consistency significantly improved in both groups.

The percentage of responders in each treatment arm was also calculated. In the DSCG-treated group, 7/9 patients (78%) met the responder criteria for pain, as compared to 3/7 patients (57%) in the placebo-treated group. However, this difference did not reach statistical significance. Remarkably, 3 patients who took DSCG reported complete relief for pain (33%) at the end of the treatment, while such outcome was recorded only in one (14%) of the placebo-treated group.

In both groups the percentage of stool responders were similar, being of 44% (4/9) in the DSCG group and of 43% (3/7) in the Placebo group.

Clinical outcomes	DSCG-treated	*P*-value	Placebo-treated	*P*-value	Difference between groups (DSCG vs. Placebo)	*P*-value
IBS-SS[a]	-60.9 ±29.1	0.07	**-97.3±20.9**	**0.02**	36.4±38.0	0.35
Abdominal pain[b]	-4.3±6.6	0.53	-16.2±10.4	0.17	11.9±11.8	0.33
Bowel movement	-0.4±0.3	0.33	**-1.0±0.3**	**0.01**	0.7±0.5	0.17
Stool consistency[c]	**-1.7±0.5**	**0.01**	**-1.3±0.3**	**0.03**	-0.5 ±0.6	0.43
Abdominal distention	1.7±13.0	0.9	-1.8±10.4	0.86	3.5±17.4	0.84
Psychological outcomes						
Holmes-Rahe test	6.4±26.6	0.81	-39.1±26.1	0.30	45.6±38.0	0.25
Cohen Scale (PSS)	-3.2±2.9	0.30	-1.4±2.9	0.66	-1.8±4.3	0.70
Beck´s Depression Inventory	-2,4±2.1	0.17	**-4,1±1.6**	**0.04**	1.7±2.8	0.55

The relationship between depression score (BDI) and IBS-SS or abdominal pain, has been previously shown in chapter 2. At baseline, depression score in this subset of patients showed a significant positive relationship with IBS-SS and with abdominal pain intensity (r=0.70, P<0.01 and r=0.61, P=0.01; respectively). These correlations were strong and significant just in those who were going to receive placebo (r=0.86, P=0.01 and r=0.89, P<0.01).

No baseline differences in psychological variables, including SRRS, PSS and BDI, were observed among treatment arms. After the 6-month follow-up, patients from both groups reported reduction in depression score without statistical differences between groups. No significant association was neither observed with clinical outcomes.

Effect of treatment on mucosal histology

In paired tissue sections, similar histological features, with normal epithelial architecture, no eosinophil increase, and no microbial or viral inclusions, were observed before and after the experimental treatment. Only in one mucosal biopsy specimen *Giardia Lamblia* infection was found, and this patient was excluded from the study. At baseline, cell counts were comparable between groups. **Figure 1** shows representative images of the immune cells counted in the jejunal mucosa. There were no effect of any treatment in IELs or eosinohphil counts. Although not significant, the number of intestinal MC in the Placebo group decreased at the end of the 6-months experimental period (P=0.07). We further analyzed the distribution of leukocytes based on gender and did not find any statistical difference between males and females in any of the cell populations studied. No significant association was found either

between the number of MC or IELs and the clinical variables (pain intensity and frequency, bowel movements or stool consistency) or the stress and depression scores (data not shown). No differences at pre- or post-treatment were identified within and between group in patients selected for gene expression analysis. Results are summarized in **table 4**.

	DSCG		Placebo	
	T0	T6	T0	T6
CD117$^+$	**25.6**	23.0	**25.4**	14.9 *
(SD)	(9.3)	(7.6)	(10.6)	(11-42.7)
CD3$^+$	**18.9**	17.5	**28.8**	19.5
Median (range)	(8.5-28.5)	(10-98)	(15-64)	(12-81)
Eosinophils	**2.6**	2.8	**3.0**	3.4
(SD)	(1.6)	(1.9)	(1.7)	(1.7)

Table 4. Mucosal leukocyte counts in the jejunal mucosa at inclusion (T_0) and at the end of the therapy period (T_6). The number of MC and eosinophils is expressed per hpf (400x) and the number of IELs per 100 epithelial cells, measured in 10 DSCG-treated samples and 10 Placebo-treated samples. SD: standard deviation. **P*=0.07 respect to baseline.

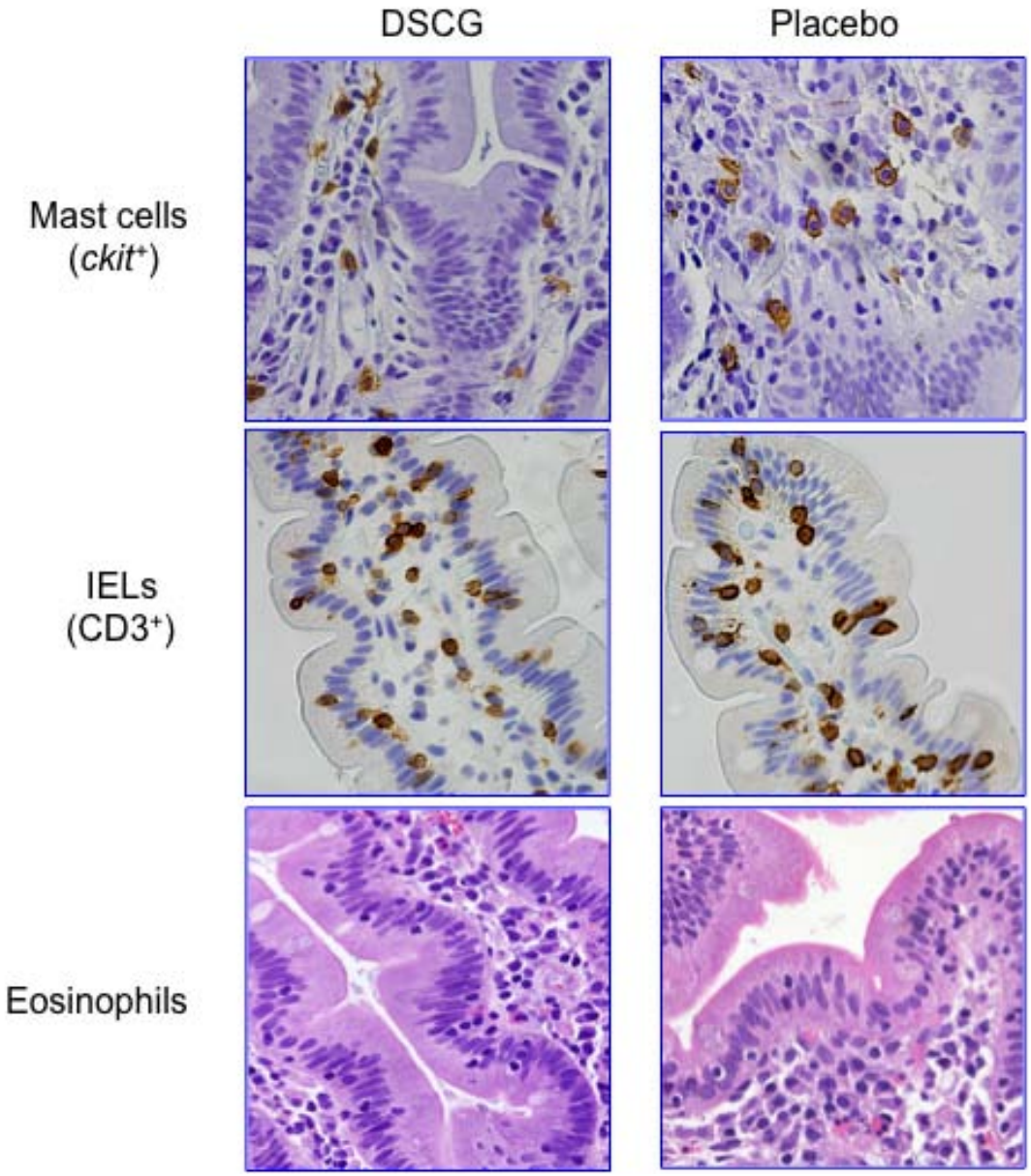

Figure 1. Representative images of CD117, CD3 and H&E stainings obtained from the jejunal mucosa of DSCG and Placebo groups at inclusion.

Effect of treatment on MC ultrastructure

At baseline, jejunal mucosal MC from both groups displayed distinctive signs of activation, such as irregular plasma membrane, and different granular electro-density with clear signs of a specific degranulation pattern known as piece-meal. Interestingly, after the DSCG treatment, ultrastructure of mucosal MC showed signs of reduced activated-type morphology, as indicated by lower degree of degranulation along with regular plasma membrane and less

presence of membrane pseudopods. In contrast, in the MC observed from the placebo group, the degree of activation was similar to that observed at baseline.

These morphological alterations can be identified in **figure 2**.

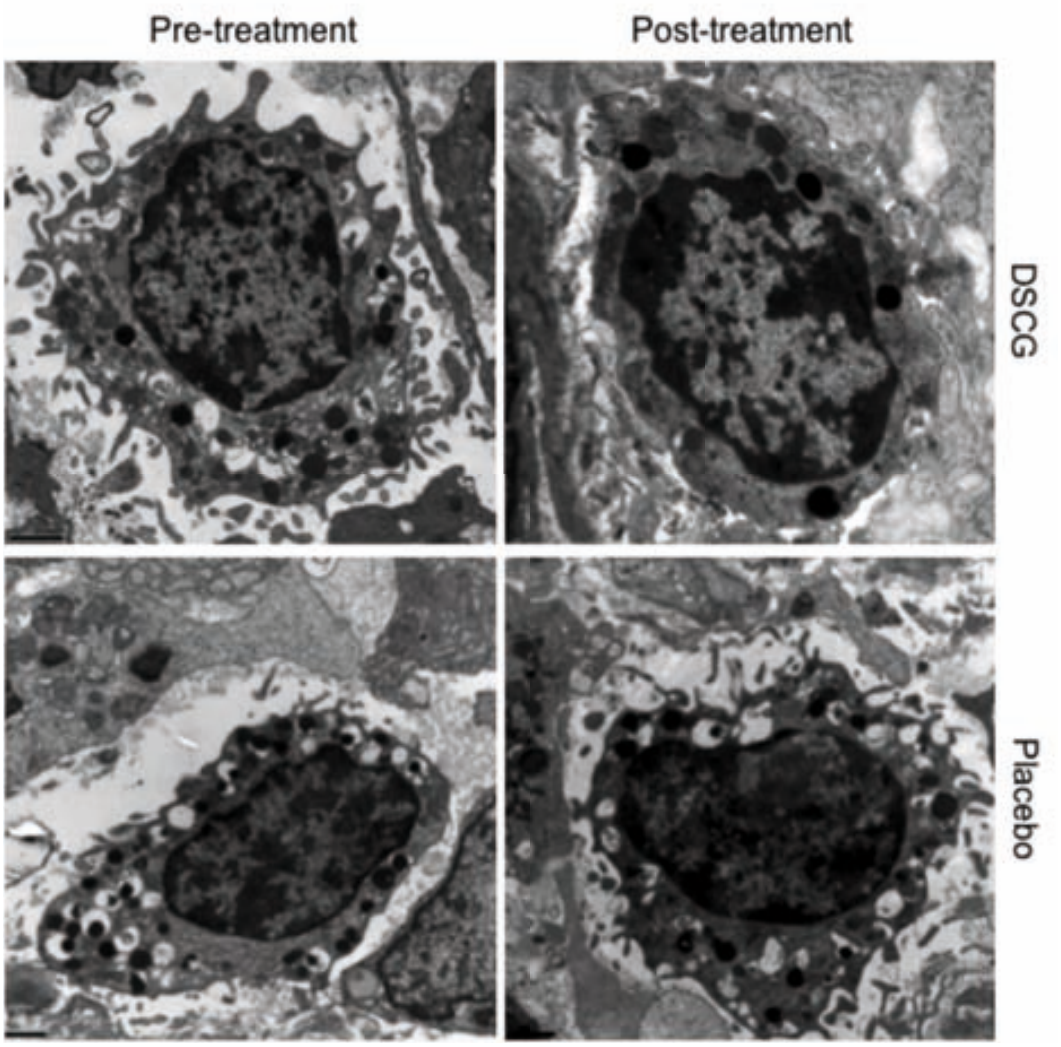

Figure 2. Representative micrographs of ultrastructure of mucosal MC, (from the same patients), before and after DSCG or Placebo treatment.

Effect of treatment on jejunal mucosa transcriptome

- Quality control and number of samples: 16-paired small intestinal samples from 8 patients were randomly selected within each group for microarray analysis. After quality checks had been performed, 4 samples were identified as outliers and excluded from the final analysis (to allow paired comparison, a total

of 8 samples were therefore discarded). The final analysis was performed on 12 samples distributed as follows: 14 DSCG (T_0=7 and 7 T_6=7) and 10 Placebo (T_0=5 and T_6=5) groups.

A number of 5,754 probe sets were included in the LIMMA analysis for each experimental comparison. After applying the previous criteria established to identify differentially expressed transcripts in the mucosal mRNA microarray screening (described in methods), we obtained the corresponding results from the different comparisons between groups which were submitted to biological category enrichment study, as follows.

Analysis of gene expression:

Hierarchical clustering analysis was applied to the differential transcriptome (T_6 *vs.* T_0 comparison) in the two groups, and represented in a heatmap (figure 2). The behavior of differentially expressed genes is indicated on a color gradient scale ranging from blue (low expression) to red (high expression), being the magnitude of the gene change proportional to the darkness of the color. Each column represents a separate subject (7 DSCG; 5 Placebo) and each row represents a separate gene. Genes with similar expression profiles across the subjects were grouped together (Y-axis) in a hierarchical way.

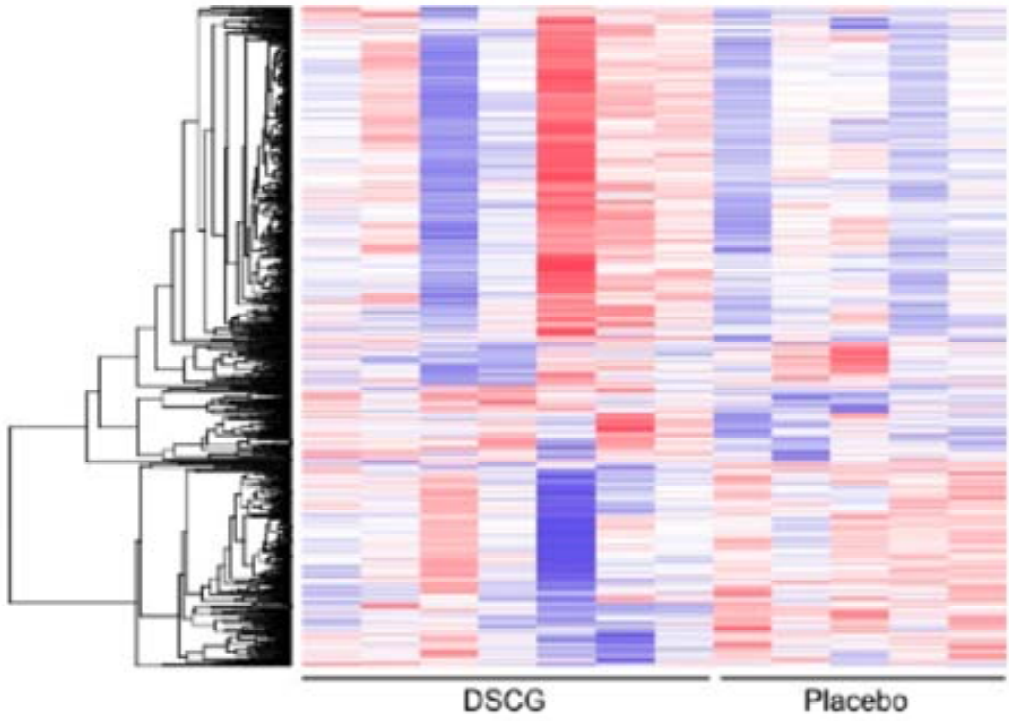

Figure 3. Heatmap of DSCG and Placebo groups representing the differential gene expression profile in the jejunal mucosal of each subject, respect to baseline. Individual samples are shown in columns and genes are represented in rows. The expression level for each gene is indicated by colour grading, blue indicates low level of and red indicates high level of expression. The dendogram on the left side indicates the distance at which the clusters of genes merge.

At inclusion, microarray analysis of intestinal biopsies between patients randomized to DSCG or placebo, yielded similar profile (data non shown). In order to assess differences in gene expression associated with the experimental treatments, 3 analysis were separately performed:

1- Effect of DSCG on mucosal gene expression:

A total number of 42 genes were identified as differentially expressed. Of these genes, 27 were defined as up-regulated and 15 were defined as down-regulated genes after DSCG treatment. Of the 27 up-regulated gene sets, 21 code for proteins with known function, most of them involved in cell proliferation, immune response and molecular transport. The 15 down-

regulated genes code for proteins, with known function related to humoral immune activity and cell proliferation. Top 20 differentially expressed genes are indicated in **table 5**. The biological processes and cellular functions modulated by DSCG treatment, are shown in t**able 6**.

	Entrez gene	Gene Symbol	Gene name	Type	Active Location	Fold-change	*P*-Value
Up-regulated	406969	MIR-194-1	microRNA 194-1			1.74	0.0014
	7364	UGT2B7	UDP-Glucuronosyltransferase-2B7	Enzyme	Cytoplasm	1.69	0.0006
	8142452	TFEC	Micropthalmia (MiT) family of basic helix-loop-helix leucine zipper transcription factors	Transcription	Nucleus	1.67	0.0003
	8129666	SLC2A12	Solute carrier family 2 (facilitated glucose transporter), member 12	Transporter	Plasma membrane	1.64	0.0002
	8160504	LRRC19	leucine rich repeat containing 19	Other	Cytoplasm	1.63	0.0010
	8001149	VPS35	vacuolar protein sorting 35 homolog (S. cerevisiae)	Transporter	Cytoplasm	1.60	0.0014
	8098611	TLR3	toll-like receptor 3	Transmembrane receptor	Plasma membrane	1.58	0.0007
	7972239	SLITRK6	SLIT and NTRK-like family, member 6	Other	Plasma membrane	1.56	0.0012
	8090715	ACAD11	acyl-CoA dehydrogenase family, member 11	Enzyme	Cytoplasm	1.54	0.0013
	8068168	SOD1	superoxide dismutase 1, soluble	Enzyme	Cytoplasm	1.54	0.0012
Down-regulated	973	CD79A	CD79a molecule, immunoglobulin-associated alpha	Transmembrane receptor	Plasma membrane	-1.77	8.84E-05
	5347	PLK1	Polo-like kinase 1	Kinase	Cytoplasm	-1.67	0.0013
	4605	MYBL2	Myb-related protein B	Transcription	Nucleus	-1.62	0.0010
	4582	MUC1	Mucin 1, cell surface associated	Transcriptor regulator	Plasma membrane	-1.61	0.0023
	939	CD27	CD27 molecule	Transmembrane receptor	Plasma membrane	-1.61	0.0005
	8340	HIST1H2BL	Hstone cluster 1, H2bl	Transcription	Nucleus	-1.56	0.0013
	255027	MPV17L	MPV17 mitochondrial membrane protein-like	Other	Cytoplasm	-1.55	0.0004
	10568	SLC34A2	Solute carrier family 34 (sodium phosphate), member 2	Transporter	Plasma membrane	-1.55	0.0007
	7960340	FOXM1	Forkhead box M1	Transcription	Nucleus	-1.51	0.0045
	8016159	DCAKD	Dephospho-CoA kinase domain containing	Other	Cytoplasm	-1.51	0.0005

Table 5. Top up-regulated and down-regulated genes modulated by DSCG treatment (T_6 *vs.* T_0). For each gene symbol, name, type, location and fold change, expressed as absolute log2 ratio, are indicated. *P*-Value according to multiple comparison (LIMMA). *P*-value according to multiple comparison (LIMMA).

Molecular and Cellular Functions	*P*-Value	Gene n	Gene Symbol
Cell Cycle	6.30E-06 – 3.93E-02	6	AKAP9, FOXM1, mir-192, MYBL2, PLK1, SOD1
Cellular Development	2.51E-05 – 4.07E-02	11	FABP2, FOXM1, mir-192, mir-194, MUC1, PLK1, SOD1, CD27, CD79A
Cellular Growth and Proliferation	2.51E-05 – 4.07E-02	11	CD27, CD79A, FABP2, FOXM1, mir-192, mir-194, MUC1, MYBL2, PLK1, SOD1, TLR3
Cellular Compromise	2.92E-05 – 4.07E-02	5	MUC1, PON2, SOD1, PLK1,CD27
Free Radical Scavenging	2.18E-04 – 4.07E-02	3	MUC1, PON2, SOD1

Table 6. List of the statistically relevant top five biological functions modulated by DSCG. *P*-value, the number and the symbol of genes involved within each function are reported.

Investigation of biological interaction among the 36 differentially expressed genes revealed 4 distinctive significant functional networks in the jejunal mucosa. Here, top 2 relevant and high-scoring networks (score >10) with their focus molecules and functions are represented in **table 7**.

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	APEX1,ASS1,CBX4,COL6A3,CSF3R,CYP3A4,EIF2AK3,ELAVL4,EP400,FANCC,GCLC,GSTA5,HSPB1,HTT,JUN,LHX1,LIN9,**LRRC19**,MAD1L1,**mir192**,**mir194**,**MPV17L**,**MYBL2**,**MYL1**,OAS1,**PON2**,PPP3CA,**SLC34A2**,**SNRNP70**,**TFEC**,TP53,**UGT2B7**,VAPA,VDR,**VPS35**	26	**12**	Cell Cycle, Connective Tissue Development and Function, Free Radical Scavenging
2	**AKAP9**,ASL,ASS1,**BCR(complex)**,CC2D1A,**CD27**,**CD79A**,ECT2,**FABP2**,FOXF1,**FOXM1**,GBP2,**GOLIM4**,HADH,HIVEP3,IKBKB,KIF23,KIF20A,KIF2C,LGALS4,MBL2,**MUC1**,NFkB(complex),NUP62,**PLK1**,PPARA,PRC1,PRKCE,ProinflammatoryCytokine,PTGR1,SLC11A2,**SOD1**,**TLR3** TRIM38,ZBTB32	20	**10**	Cell Signaling, Cell Cycle, Cellular Movement

Table 7. The most relevant functional networks in the intestinal mucosa associated with DSCG treatment. Each network contains direct and indirect interactions scored by significance. The genes differentially expressed within each one are shown in bold, up-regulated genes are indicated in red and down-regulated genes are indicated in green. ID, identification.

The most significant canonical pathway signaling altered by DSCG was Altered T cell and B cell signaling in Rheumatoid Arthritis (P=7.21 10^{-3}). Within this comparison, IPA did not identify any biological functions or upstream regulators that were predicted to be activated or inactivated.

2- Effect of placebo on mucosal gene expression:

A total number of 16 genes were found as differentially expressed after the placebo treatment. Of these, 6 were defined as up-regulated and 10 were defined as down-regulated. The overall up-regulated transcripts were small nuclear non-coding RNA (4), and only 2 genes code for protein, one is involved in gene regulation in mitosis, and the other one is implicated in the extracellular matrix remodeling. Of the 10 down-regulated genes, 8 code for proteins with known functions, most of them were interferon-induced genes that regulate interferon-mediated apoptosis. The top differentially expressed transcripts are indicated in **table 8,** and the biological molecular and cellular functions identified by this analysis are shown in **table 9**.

	Entrez gene	Gene Symbol	Gene name	Type	Active Location	Fold-change	*P*-value
Up-regulated	4312	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	Peptidase	Extracellular	1.98	0.016
Down-regulated	9572	NR1D1	Nuclear receptor subfamily 1, group D, member 1	Ligand-dependent nuclear receptor	Nucleus	-1.86	0.004
	3429	IFI27	Interferon, alpha-inducible protein 27	Other	Cytoplasm	-1.84	0.007
	6362	CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	Cytoquine	Extracellular	-1.83	0.006
	5621	PRNP	prion protein	Other	Plasma membrane	-1.59	0.023
	5187	PER1	Period circadian clock 1	Other	Nucleus	-1.55	0007

Table 8. Top up-regulated and down-regulated genes modulated by placebo treatment (T_6 *vs.* T_0). For each gene symbol, name, type, location and fold change expressed as absolute log2 ratio are indicated. *P*-Value according to multiple comparison (LIMMA).

Molecular and Cellular Functions	*P*-Value	Gene n	Gene Symbol
Cellular Development	8.25E-05 – 1.80E-02	2	PRNP, NR1D1
Cell Cycle	3.87E-04 – 1.16E-03	2	PRNP, PER1
Cell-To-Cell Signaling and Interaction	3.87E-04 – 1.80E-02	4	CCL18,MMP1, PRNP, PER1
Cellular Growth and Proliferation	3.87E-04 – 1.50E-02	3	PRNP, NR1D1, PER1
Cellular Movement	3.87E-04 – 2.87E-02	5	CCL18,MMP1, PRNP, PER1, NR1DR1

Table 9. List of the statistically relevant top five of placebo-treated data biological functions. *P*-value, the number and the symbol of genes involved within each function are reported.

Only one network was identified as relevant in the placebo-treated group following gene interaction analysis. This network displayed Behavior, Nervous System Development and Function and Cellular Movement as top functions (**table 10**).

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	ABL2,ADAM8,ADAM9,ARNTL,ATP1A2,AXL,BHLHE41,C1QA,**CCL18**,DBP,EHF,ERK1/,glutathione peroxidase,GPX4,GSR,**IFI27**,IFNG,Ikb,LAMA3, lymphotoxin-alpha1beta2, **MMP1**,NAMPT,**NR1D1**,OLR1,**PER1**,PER2, Pkc(s),**PRNP**,RBP3,S100A10,SAA,SP100,TACR1,TIRAP,TNF	16	**6**	Behavior, Nervous System Development and Function, Cellular Movement

Table 10 The relevant functional network in intestinal mucosa associated with the placebo treatment. The network contains direct and indirect interactions scored by significance. The genes differentially expressed within each one are shown in bold, up-regulated genes are indicated in red and down-regulated genes are indicated in green. ID, identification.

The most significant canonical pathway signaling altered by the placebo treatment was Circadian Rhythm signaling (P=6.54 10^{-5}). Within this comparison, IPA did not identify any biological functions or upstream regulators that were predicted to be activated or inactivated.

3- Comparison between DSCG and Placebo differential transcriptomes:

To identify a differentially gene signature linked with DSCG treatment in IBS-D, we compared mucosal transcripts modulated following 6 months of DSCG treatment with those altered after placebo treatment.

This comparative analysis of DSCG vs. Placebo yielded 83 differentially expressed gene data sets. Of these gene sets, 58 were identified as up-regulated (39 code for proteins), with functions related to free radicals

scavenging and neuronal survival, and 25 were identified as down-regulated genes (16 code for proteins), mainly involved in immune response regulation. Top 20 differentially expressed transcripts are indicated in **table 11**.

	Entrez gene	Gene Symbol	Gene name	Type	Active Location	Fold-change	*P*-value
Up-regulated	116159	CYYR1	cysteine/tyrosine-rich 1	Other	Unknown	2.17	0.0012
	3429	IFI27	Interferon, alpha-inducible protein 27	Other	Cytoplasm	2.11	0.0106
	9572	NR1D1	Nuclear receptor subfamily 1, group D, member	Ligand-dependent nuclear receptor	Nucleus	2.05	0.0106
	406969	mir-194	MicroRNA mir-194	microRNA	Cytoplasm	2.04	0.0072
	6647	SOD1	Superoxide dismutase 1, soluble	Enzyme	Cytoplasm	1.97	0.0011
	6625	SNRNP70	Small nuclear ribonucleoprotein 70kDa	Other	Nucleus	1.92	0.0089
	4632	MYL1	Myosin, light chain 1, alkali	Other	Cytoplasm	1.87	0.0036
	5594	MAPK1	Mitogen-activated protein kinase 1	Kinase	Cytoplasm	1.74	0.0084
	5187	PER1	Period circadian clock 1	Other	Nucleus	1.72	0.0101
	4637	MYL6	Myosin, light chain 6, alkali, smooth muscle and non-muscle	Other	Cytoplasm	1.72	0.0101
Down-regulated	4312	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	Peptidase	Extracellular	1.92	0.0239
	9837	GINS1	GINS complex subunit 1	Other	Nucleus	-1.89	0.0015
	973	CD79A	CD79a molecule, immunoglobulin-associated alpha	Transmembrane receptor	Plasma membrane	-1.73	0.0085
	6637	SNRPG	small nuclear ribonucleoprotein polypeptide G	Other	Nucleus	-1.71	0.0085
	6502	SKP2	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase	Other	Nucleus	-1.61	0.0061
	79877	DCAKD	Dephospho-CoA kinase domain containing	Other	Cytoplasm	-1.59	0.0084
	4862	NPAS2	Neuronal PAS domain protein 2	Transcriptional	Nucleus	-1.59	0.0107
	999	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	Other	Plasma membrane	-1.55	0.0248
	399	RHOH	Ras homolog family member H	Other	Cytoplasm	-1.52	0.0214
	84152	PPP1R1B	protein phosphatase 1, regulatory (inhibitor) subunit 1B	Phosphatase	Cytoplasm	-1.52	0.0192

Table 11. Top up-regulated and down-regulated genes obtained from comparison between DSCG and placebo treatments. For each gene symbol, name, type, location and fold change expressed as absolute log2 ratio are indicated. *P*-Value according to multiple comparison (LIMMA).

Interestingly, 4 differentially expressed genes (*IFI27, PER1, NR1D1* and *MPP1*) already identified in the placebo-treatment comparison (T_6 *vs.* T_0) had an opposite trend when analyzing the "global" DSCG effect (DSCG *vs.* Placebo).

These changes may suggest that DSCG modulate gene expression associated with the course of the disease in IBS-D.

To identify biological processes specifically altered by DSCG treatment in contrast to IBS-D evolution, the dataset obtained from comparing treatments was subjected to gene enrichment analysis. **Table 12** indicates the biological molecular and cellular functions identified by this analysis.

Molecular and Cellular Functions	*P*-Value	Gene n	Gene Symbol
Cell-To-Cell Signaling and Interaction	1.28E-04 – 3.26E-02	13	MAPK1, CDH1, SOD1, ANXA1, CD3D,CD79A, CD8A,Gh,MMP1, PPP1R1B, MT1H, PER1, CDK5
Cell Death and Survival	1.37E-04 – 3.30E-02	19	CDH1,Gh,MAPK1,MT1H,PLRG1,SKP2, CDK5, FAM134B,NR1D1,SOD1,ANXA1,CD2AP, CD79A,CD8A,CDK5, COX8A, mir-194, MT1H, PER1
Cellular Development	1.91E-04 – 2.93E-02	14	ANXA1,CD3D,CD79A,CD8A, MAPK1, SKP2, SOCS5, SOD1, Gh, CDH1,NR1D1, CDK5, mir-194
Cellular Growth and Proliferation	1.91E-04 – 3.26E-02	14	ANXA1,CDK5,MAPK1,mir-194,SKP2,SOD1, CD8A, Gh,MT1H, NR1D1, PPP1R1B, CDH1, PER1, CD79A
Cellular Function and Maintenance	2.00E-04 – 3.21E-02	14	ANXA1,CD3D,CD79A,CD8A,MAPK1,SKP2,SOCS5,SOD1, VPS35, MMP1, MT1H, CDH1 MAPK1, PPP1R1B

Table 12. List of the statistically relevant top five DSCG-treated *vs.* placebo-treated biological functions. *P*-value, the number and the symbol of genes involved within each function are reported.

Moreover, the top 5 canonical pathways identified were related to signaling and metabolic functions:

- Systemic Lupus Erythematosus Signaling (P=4.4 10^{-5})
- Gα12/13 Signaling (P=3.9 10^{-4})
- Primary Immunodeficiency Signaling (P=4.9 10^{-4})
- Phospholipase C Signaling (P=5.8 10^{-4})
- Growth Hormone Signaling (P=1.1 10^{-3})

Pathway analysis displayed 9 networks of gene interaction. There were 3 top high-scoring networks (score>10), with functions related to cell signaling and interaction, nervous system development and function, and cell biology, as represented in **table 13.**

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	Akt,ANXA1,Ap1,CD2AP,CD3D,CD79A,CDH1,CDK5,Cg,Creb,ERK,ERK1/2 ,estrogen receptor, FAM132A,IFI27,IgG,Igm, Immunoglobulin,Interferon alpha,Lh,MAPK1,Mapk,MMP1, MT1H,NFkB (complex), NR1D1,P38MAPK, PDGF BB, PER1 ,PI3K (complex), Pkc(s),PPP1R1B,Ras,SKP2, SOCS5	32	**16**	Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Cell Morphology
2	ACTN1,AHR,ANXA1,ARNT,BCAS2,BTG2,CCND1,COMMD6,COX8A ,DDX60L,EHD1,G6PD,GSTM5,IFI35,IQGAP2,mir-194,mir-373,MPV17L, MYL1,MYL6,NUPR1,PARM1, PDE3A, PER2,PLRG1, PRDX6,PRL, RELA,SNRNP70, TGM2, TMSB10/ TMSB4X,TP53,TRIM22,UBE2C,YY1	20	**11**	Cellular Compromise, Cell Death and Survival, Cellular Development
3	ASS1,CA3,CRABP2,Csn1s1,Csn1s2a,CTNNB1,CYP8B1,DCC,E2F1, EHD1, EHF,ENO3,EP300,Ferritin,Gh,GINS1,GSTM1,Histone h3, H I V E P 3 , HN1,HOXA11,IL1B,KDM5B,LSM3,LSM5,MAPK9,MUC3A,MYLPF, NAT8, NPAS2,PSMA2,RNA polymerase II,SNRPG,SOD1,Sod	15	**9**	Cancer, Cell Death and Survival, Cell Cycle

Table 13. The relevant functional networks in intestinal mucosa associated with DSCG treatment. Each network contains direct and indirect interactions scored by significance. Differentially expressed genes within each network are shown in bold, up-regulated genes are indicated in red and down-regulated genes are indicated in green. ID, identification.

Biologically relevant functional relationships associated with DSCG therapy:

Preliminary data (chapter 1) showed modulation of innate immune response following DSCG administration. We focused our analysis on those biological functions, canonical pathways, and their modulated genes associated with immune response regulation, obtained from the comparison between therapies.

Many of the transcripts of the dataset participate in immune-related subcategories of biological functions such as T cell development (*ANXA1, CD3D, CD79A, CD8A, MAPK1, SKP2, SOCS5*; P=6.8 10^{-4}) and homeostasis of leukocytes (*ANXA1, CD3D, CD79A, CD8A, MAPK1, SKP2, SOCS5, SOD1*; P=2.0 10^{-4}). Moreover, other function categories including humoral immune

response (*CD79A, MT1H; P*=8.9 10^{-3}), immune cell trafficking (CD3D, CD79A, CD8A, Gh; *P*=2.8 10^{-2}) and flux of Ca^{2+} (*ANXA1, CD79A, CD8A, MMP1; P*=5.7 10^{-3}) were also significant, although with reduced number of genes involved.

In the top 15 significant canonical pathways altered, other pathways of interest included: Cdc42 Signaling (*MAPK1,MYL6,CD3D,MYL1*) involved in the regulation of intestinal epithelial barrier functions, CDK5 Signaling (*CDK5, MAPK1, PPP1R1B*) involved in T cell activation, T Cell Receptor Signaling (*MAPK1, CD8A, CD3D*), and Circadian Rhythm Signaling (*PER1, NR1D1*).

Interestingly, network 1 contained direct and direct interactions of genes mainly involved in immune responses, as represented in **figure 4.**

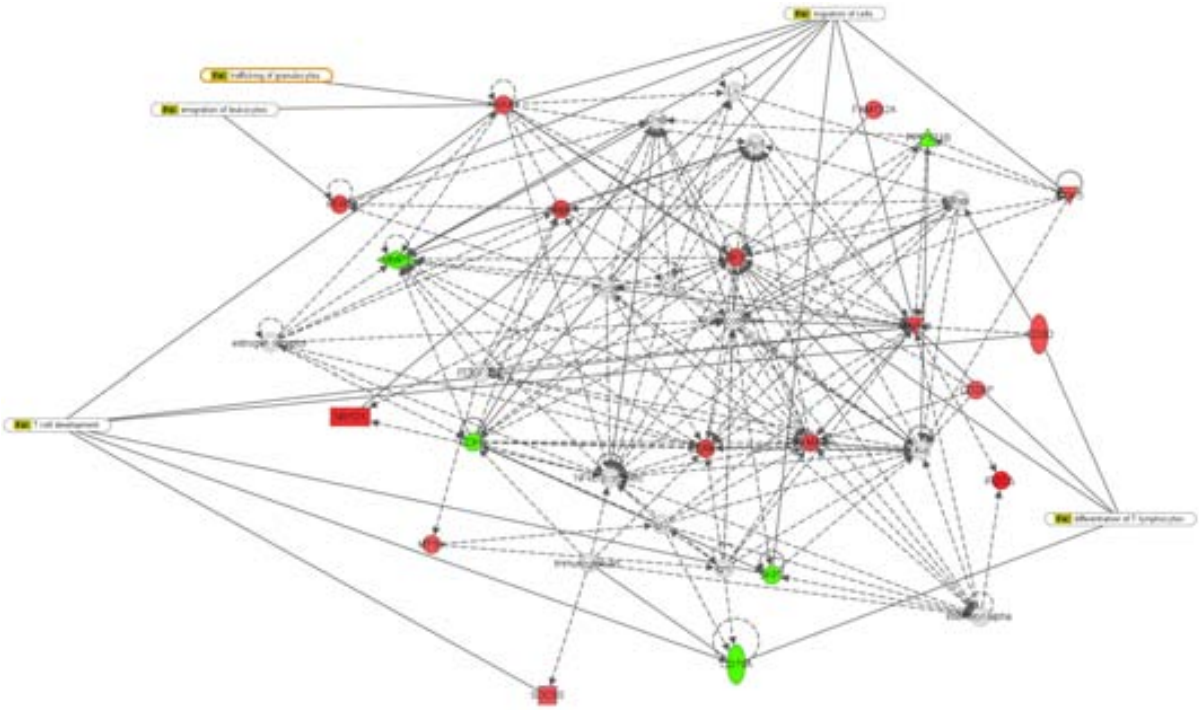

Figure 4. Network 1 obtained from comparison of the differential transcriptomes from DSCG and placebo-treated groups. Genes involved in biological functions associated with immune system are indicated. Direct and indirect interactions between molecules are indicated with continuous or discontinuous arrows, respectively. The magnitude of the gene change is proportional to the darkness of the color. Up-regulated genes are shown in red and down-regulated genes are shown in green.

Additionally, many of the differentially expressed genes that generate the

network 2 had numerous (significant?) direct and indirect interactions with TP53 or p53 gene. Of importance for the understanding of the molecular mechanisms underlying DSGC effect, several of these genes are upstream regulators of p53; furthermore, due to gene interaction, IPA predicted its activation, as shown in **figure 5**. TP53 is one of the major cellular stress response pathways, and also an inhibitor of inflammation (Gudkov *et al*., 2011). Most of the upstream regulators of p53 had lipid metabolism and anti oxidant functions.

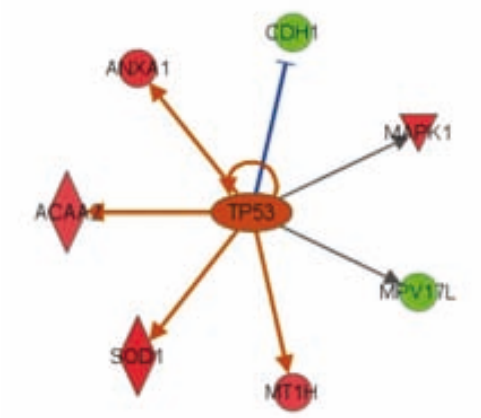

Figure 5. Genes involved in upstream regulatory pathway of TP53. Up-regulated genes are indicated in red and down-regulated genes are indicated in green.

Interestingly, IPA was able to predict decreased Neuronal Apoptosis (z-score -2.18; 6 molecules; P=7.3 10^{-4}) that belonged to Cellular Death and Survival function, involving: *CDK5, FAM134B, MT1H, NR1D1, PLRG1* and *SOD1*.

Among the differentially transcripts obtained, it was noteworthy the increase of *ANXA-1* expression, also present in several biological functions modulated by DSCG. Indeed, *ANXA-1* belongs to the top 2 high-scoring networks and is identified as p53 target gene. The importance of *ANXA-1* gene relies on its coding function of the calcium-dependent phospholipid-binding protein annexin A1, which displays anti-inflammatory, antioxidant and immunomodulatory properties (D'Acquisto *et al*., 2008; Buckingham *et al*., 2006).

Validation of microarray data by Q-RT-PCR.

In order to verify our microarray findings, mRNA expression of selected genes was assessed using Q-RT-PCR. We obtained significant correlation coefficients between RNA expression measured by using Q-RT-PCR, and RNA expression obtained from microarray analysis, from the same gene-probe location (**table 11).**

DSCG T_6 vs.T_0			Placebo T_6 vs.T_0			DSCG vs Placebo (T_6 vs.T_0)		
Gene name	Correlation	p	Gene name	Correlation	p	Gene name	Correlation	p
SLC34A2	0.43	0.34	MMP1	0.60	0.29	IGHG		
UGT2B7	*0.75*	*0.07*	IFI27	0.70	0.19	IGHM		
LRRC19	**0.93**	**0.03**	CCL18	0.50	0.39	IGHA		
TLR3	0.57	0.18	***PER1***	**0.90**	**0.04**	***MMP1***	**0.77**	**0.04**
FABP2	**0.96**	**<0.01**				***IFI27***	**0.95**	**<0.01**
MUC1	0.54	0.24				***PER1***	**0.90**	**<0.01**
CD27	0.39	0.40				***NR1D1***	**0.92**	**<0.01**
CD79A	0.57	0.18				CD79A	0.45	0.14
MPV17L	0.11	0.83				MPV17L	0.11	0.73

Table 11. Correlation between absolute log2 fold-change obtained by microarray and fold-change by Q-RT-PCR of selected genes. Values of *P*<0.05 according Spearman rank correlation are indicated in bold.

Within the DSCG-treated group, Q-RT-PCR confirmed the significant over-expression of *FABP2,* and gene-expression changes for *LRRC19*, *TLR3, UGT2B7, CD27* and *CD79A* mRNA follow the same trend to increased levels after treatment (**table 12**).

Following genes, did not show any changes in expression between pre- and post-treatment: *MUC1, NR1D1* and *CCL18*

Statistically significance (*P*<0.05) was confirmed for *PER1* expression between pre- and post-treatment with DSCG *vs.* placebo.

□

Cromoglycate T_6 vs.T_0			Placebo T_6 vs.T_0			Cromoglycate vs. Placebo (T_6 vs.T_0)	
Gene name	FC	*P*-value	*Gene name*	FC	*P*-value	*Gene name*	*P*-value
SLC34A2	0.9	0.25	MMP1	3.6	0.16	IGHG	
UGT2B7	1.6	0.11	IFI27	0.7	0.44	IGHM	
LRRC19	2.1	0.14	CCL18	1.0	0.69	IGHA	
TLR3	2.1	0.14	PER1	0.5	0.06*	MMP1	0.24
FABP2	2.9	0.04	NR1D1	1.0	0.31	IFI27	0.15
MUC1	1.0	0.63	CD79A	0.5	0.03	PER1	0.01
CD27	0.8	0.12	MPV17L	1.1	1.00	NR1D1	0.11
CD79A	0.6	0.12				CD79A	0.61
MPV17L	1.2	0.85				MPV17L	0.46
NR1D1	1.4	0.15					
IFI27	1.7	0.62					
PER1	1.5	0.20					
MMP1	1.9	0.27					
Tryptase	0.9	0.46	Tryptase	0.7	0.03	Tryptase	0.41
CPA3	1.7	0.38	CPA3	1.2	1.00	CPA3	0.66

Table 12. Expression values obtained by Q-RT-PCR of selected genes. Matched gene regulation microarray data are highlighted Up-regulated are indicated in red, down-regulated are indicated in green mismatched are indicated in bold. Values of $P<0.05$ according paired and unpaired *t*-test are in bold. FC, Fold-change.

Similar reduction in CD79A expression was observed after both treatments, although within placebo-treated this difference was significant (P=0.03).

Genes encoding MC proteases

While in the present study no differentially expressed mast cell-specific gene was identified by microarray analysis, we assessed by Q-RT-PCR the mRNA expression levels of two MC proteases (tryptase and carboxypeptidase (CPA3)) in paired samples from both treatment arms. Tryptase expression was down regulated by both treatments, without statistical difference between them. This reduction compared to baseline levels was significant only in those patients who took placebo ($P<0.05$). Furthermore, carboxypeptidase expression increased following DSCG and placebo, however no significant difference between treatments was identified (**table 12**).

Clinical and biological correlations

- MC specific proteases

We determined baseline tryptase RNA expression in jejunal biopsies of 14 IBS-D patients. No significant correlation was found between tryptase expression and IBS-SS score, abdominal pain intensity and stool consistency. We observed a negative correlation between baseline mucosal tryptase expression and the number of $CD117^{+}$ cells in the jejunal mucosa (Pearson r= -0.6, P=0.02). No association between CPA3 expression level and MC density was identified.

- Differential transcriptome profile.

Despite no statistically significance, the percentage of responder patients, following the FDA criteria for pain, was higher in the DSCG-treated group as compared to the Placebo group. Few genes showed different regulation pattern in microarray analysis when comparing responders *vs*. non-responders for pain within each experimental group, not allowing us to identify biological mechanisms underlying clinical response.

In the DSCG-treated group, only *PON2* was differentially regulated in microarrays as follows: up regulated in responders *vs*. down-regulated in non-responders. Interestingly, the expression changes, showed a strong negative and significant correlation with changes in pain score from baseline to the end of treatment (r= -0.94, P=0.002).

In the Placebo group, clock genes expression (*NR1D1* and *PER1*) was down-regulated in responders and up-regulated in non-responders. No association

between expression and changes in pain score was found. Given their importance in mood disorders, its association with BDI score (T_6 *vs.* T_0) was assessed. A significant positive correlation was found in both regulated genes (NR1D1: r=-0.90, P=0.037; PER1: r=-0.90, P=0.037), for NR1D1 this correlation was confirmed with Q-RT- PCR data.

Gene regulation patterns in treatment-comparison were different for clock genes (N1RD1, PER1 and N1RD2), and were regulated in the opposite direction in pain responders only in placebo-treated. Of these genes, only N1RD2 correlated with changes in pain intensity (r=0.90, P=0.037). Likewise, CD79A gene was down-regulated in all samples from responders of both groups and up-regulated in samples from placebo and most of the DSCG-treated, without significant correlation with changes in pain intensity.

Discussion

This study provides the first analysis of differential jejunal mucosal gene expression profile in IBS-D after oral treatment with a MC stabilizer (DSCG), in a placebo-controlled trial. Moreover, the modulation of gene expression at the mucosal level, revealed potential association with clinical evolution. MC has been associated in IBS pathophysiology and is thought to play a key role on IBS symptoms (Martinez C., 2012). Therefore, targeting MC as a potential therapeutic strategy has been proposed, however limited clinical data have been available. Unfortunately, no controlled clinical trial had studied yet the effects of pharmacological intervention directed at MC stabilization and its association with intestinal gene expression profiling in IBS-D. We have addressed these questions, in order to gain insight into MC-related molecular mechanisms associated to DSCG-treatment clinical evolution.

In this study, a clinical improvement following both treatments was observed, whereas there was a trend towards higher rate of responders to pain among DSCG-treated group than within the placebo group, with similar percentages of responders to stool consistency in both groups.

Psychological co-morbidities have been documented in IBS (Hillilä MT., 2007). In the present study, baseline depression scale was associated with IBS and pain severity score, especially in patients allocated to receive placebo. Moreover, within this group, a significant reduction in depression score after treatment was observed. Depression may have a potential role in clinical improvement, since several studies have documented a higher impact of psychological status on visceral pain modulation in IBS patients than in healthy

controls (Elsenbruch S., 2010). However, despite targeting MC in the intestinal mucosa, this study did not identify any baseline correlation between intestinal or psychological symptoms and histological features. In agreement with our observations, no relationship was observed between MC number and IBS symptoms or stress (Braak B., 2012), although previous studies from our laboratory described an association between MC density and stool consistency in a larger sample size (Martinez C., 2012b).

DSCG had no effect on immune cell infiltration (IELs, MC or eosinophils) in the mucosa of the small intestine, however, a more stable phenotype was observed in the DSGC-treated group. Growing evidence suggests a higher impact on IBS pathophysiology of MC activation or its proximity to nerve fibers rather than the number of infiltrated MC. A previous clinical trial with ketotifen failed to show changes in rectal mucosal MC numbers and their mediators, despite decreasing visceral hypersensitivity (Klooker T.K., 2010). Mesalazine, other anti-inflammatory agent, was shown to reduce mucosal immune cells, including MC, however, with no significant effect on abdominal pain in IBS (Corinaldesi R., 2009).

As our target is MC stabilization, we assessed the expression of two specific MC proteases (tryptase and carboxypeptidase) in paired samples of both groups. At baseline, jejunal tryptase mRNA expression levels are negatively related to MC density. Once therapies were finished, we failed to find differences in mRNA expression of the two MC proteases between them. Tryptase levels decreased in both treatments from baseline, and this reduction was significant only within the placebo group. The same effect was also seen in

MC density within the placebo group, which could be related with tryptase decrease. While we theorize that long-term administration of DSCG did not completely inhibit MC degranulation, all our findings show the ability of DSCG to modulate immune response and oxidative stress through MC stabilization; although we cannot exclude its impact on other leukocytes or on enterocytes as additional mechanism.

Study of changes in mucosal transcriptome profile after DSCG treatment in IBS-D patients improved our understanding of underlying mechanisms at molecular level after MC stabilization. One of the potential pathophysiological mechanisms in IBS, especially in the IBS-D subtype, is the enhancement of intestinal permeability as a consequence of epithelial barrier disruption. Even more, an increase in the density of epithelial gap in the terminal ileum of IBS patients has recently been described (Turcotte JF., 2013). Indeed, our group has previously described disrupted apical junctional complex in association with MC activation in IBS-D patients (Martínez C., 2012). We observed a significant up-regulation of fatty acid binding protein 2 (FABP2) in the jejunum after DSCG treatment. This protein is expressed in the cytoplasm of fully differentiated enterocytes and displays a key function in the intestine as participates in the absorption and intracellular trafficking of fatty acids. Reduced expression of FABP2 has been found in intestinal diseases and experimental conditions where epithelial damage is present (Simula MP., 2010; Reiff C., 2009). Together, these data indicate that DSCG may impact on enterocytes turnover in the small intestine, however whether through a direct or indirect mechanism is unknown.

Mucosal differential gene expression resulting from treatment with placebo identified few genes, most of them were down-regulated and they generated only one network. The unique up regulated gene was *MMP1*, which is involved in the pathophysiology of several intestinal inflammatory disorders. This metalloproteinase has a role in antibacterial response, inflammation regulation, apoptosis, and extracellular matrix remodelling (Parks WC., 2004). MC-derived mediators are capable to induce MMP1 up-regulation. Even more, MCs express and contain MMP1, which is contained in the same granules as the tryptase (Milne SA., 2001; Di Girolamo N., 2000). Therefore, MC activation is related to tissue remodeling through MMPs release and activation. Importantly, DSCG treatment induced MMP1 downregulation, suggesting that DSCG contributes to attenuate the matrix remodelling and the inflammatory reactions through MC stabilization.

Remarkably, our microarray analysis showed that DSCG, in contrast to placebo, had greater effect on mucosa gene expression modulation in IBS-D. Most of the gene sets identified were involved in key functions to intestinal mucosa homeostasis maintenance, as modulation of mucosal immune response or host defense to intraluminal antigen related to T and B cells signaling, superoxide degradation, and epithelium maturation or turn-over. These results support the hypothesis of mucosal increased immune activation in IBS (Matricon J., 2012; Öhman L., 2010) and the key role of MC in immune modulation, as its stabilization directly or indirectly affect these pathways at a molecular level.

Interestingly, paraoxonase-2 (*PON2*) gene came out from the sub analysis of responders vs. non-responders in DSCG group and showed a positive

correlation with changes in pain following treatment. PONs are a family of Ca^{2+}-dependent esterases that have been linked to the pathogenesis of several inflammatory diseases. All of them possess antioxidant property and protect cells from oxidative stress. Recently, it has been shown reduced *PON1* expression in intestinal biopsies from IBD and celiac disease (Rothem L., 2007). PON2 expression is directly modulated by corticoids as dexamethasone increases its transcription. All these findings suggest an anti-inflammatory and antioxidant effect of DSCG on the intestine that might lead to pain improvement.

Pathway analysis of our data revealed several biologically relevant events, IPA predicted neuronal apoptosis in the jejunum and this finding could suggest a reestablishment of homeostatic conditions with DSCG treatment. The enteric nervous system is crucial in the regulation of intestinal key functions such as absorption, secretion and motility, and its disruption can lead to altered bowel movements.

Moreover, IPA predicted activation of upstream regulators, the transcription factor Tumor suppressor p53, whose expression increases in response to a range of stressors as oxidative damage. p53 modulates immune responses, therefore, probiotics have shown an increase in the transcription of p53 in healthy small bowel mucosa (Di Caro S., 2005). Recently, it has been demonstrated its role as a negative regulator of MC activation (Suzuki K., 2011). Nowadays, there is no data available on p53 expression in IBS, however p53 could mediate MC stabilization and therefore immune response modulation, as observed with DSCG treatment.

Notably, in our data one specific gene identified as upstream regulator of p53 was *ANXA1*, which played a central role in two regulatory networks and in multiple biological functions. ANXA1 participates in the anti-inflammatory mechanism of action of glucorticoids, although its expression can be modulated by other anti-inflammatory drugs. It is highly expressed in immune cells including neutrophils, MC, and epithelial cells, what makes possible its capacity to modulate innate and adaptive immune response and to facilitate epithelial barrier recovery (Leoni G., 2013). ANXA1 possesses an inhibitory effect on MC histamine release (Bandeira-Melo C., 2005). Our data showed intestinal ANXA1 up-regulation which may be influenced by DSCG, and is in agreement with the data reported in other cromones study, where the effect on leukocytes recruitment inhibition was an ANXA-1 mediated event (Yazid S., 2010). These findings provide a possible mechanism by which DSCG stabilizes mucosal MC and exerts different modulatory effects on immune response.

Interestingly, DSGC therapy induced clock genes (*PER1, NR1D1*) up-regulation. These transcriptional factors regulate circadian rhythmicity of behaviour and physiology at the molecular level in the SNC and peripheral tissues (eg; gastrointestinal (GI) tract and myenteric plexus) (Pardini L., 2005; Sládek M., 2007). Circadian rhythm disruption can be both the cause and consequence of many diseases. Different factors can potentially regulate peripheral clock gene expression, such as central clock (through autonomic nervous system innervation and HPA axis), light/dark cycle, food, stress (Zhang J., 2011), immune challenges, and certain hormones. These genes control circadian physiological processes, including rhythmic changes in GI functions

such as motility or the expression of several nutrients transport proteins (Hoogerwerf WA., 2010). Importantly, pain has circadian variations and clock genes interact with genes associated with pain and analgesia (Zhang J., 2012). This result suggests other potential mechanism by which DSCG reestablishes the intestinal homeostatic conditions and could be linked to clinical improvement, although we are unaware of the underlying mechanisms.

Interestingly, *PER1*, *NR1DR1* and *NR1DR2* genes were down-regulated in non-responders, and the opposite in responders group among the patients treated with placebo. Clock gene alterations have been linked to psychological disorders and pain, therefore we explore whether they were linked to changes in pain and depression score. Even though, the unique gene expression that showed relation with changes in pain intensity was *NR1D2,* changes in *PER1*, and *NR1DR1* expression had a strong association with changes in depression scale. Our findings suggest that clock genes are potential inductors at the molecular level of dysregulation brain-gut axis in the intestine, and they might display a pathophysiological role in IBS development and placebo effect.

The microarray analysis based on the subset of responders to stool consistency did not identify any different regulation pattern in any group, not allowing us to determine which genes are involved in clinical benefit. It could be explained by the lack of differences among treatments in the responder's rate.

The current study had several limitations that make it difficult to draw conclusions. It was not possible to analyze all biopsies obtained from all study patients. This led to reduced sample size for pair-wise comparison, which decreases the chance to explore more genomic and clinical associations and

find robust correlations, especially since IBS is a heterogeneous condition. Only samples from those patients who finished the treatment period were collected. In relation to this, we missed biological information from patients who did not respond to medication and in consequence dropped out of the study before.

In summary, this study identifies different pathways related to DSCG treatment in IBS-D, which involve anti-inflammatory and antioxidant activities. Our study provides a new approach to underlying molecular processes and signs in IBS, to further investigate therapies directed to modulate oxidative and inflammatory mucosal features. Additional studies are needed to address the impact of circadian rhythm in IBS and GI function. Partial clinical response after MC stabilization was observed, probably due to the complexity of underlying mechanisms that contribute to the pathology of IBS symptoms.

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DISCUSIÓN GENERAL

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El síndrome del intestino irritable (SII) es uno de los trastornos funcionales gastrointestinales más prevalentes en la consulta de gastroenterología (Khan S., 2010). Actualmente carecemos de marcadores biológicos para su diagnóstico, el cual se basa, principalmente, en criterios clínicos y en la exclusión de otras entidades orgánicas. El desconocimiento actual sobre la fisiopatología del SII deriva en la dificultad de su manejo terapéutico, lo cual se traduce en un elevado coste sanitario y en el deterioro en la calidad de vida de estos pacientes. Es necesario, por lo tanto, investigar los mecanismos fisiopatológicos que subyacen a la disfunción intestinal, así como el desarrollo de estrategias terapéuticas eficaces.

Aunque su fisiopatología no está establecida, sus características cardinales, la alteración de la motilidad gastrointestinal y la hipersensibilidad visceral, podrían ser el resultado de una respuesta neuro-inmunológica inadecuada frente al estrés psicológico crónico y las infecciones gastrointestinales (Drossman DA., 2006). Uno de los hallazgos comunes es la presencia de microinflamación en la mucosa intestinal y colónica de algunos de estos pacientes y, recientemente, la asociación entre la activación mastocitaria y las alteraciones estructurales de la barrera epitelial y su relación con las manifestaciones clínicas (Zhou *et al*., 2010; Vivinus-Nébot *et al*., 2012: Martinez *et al*., 2012a). Por ello todo, la hipótesis del presente trabajo propone el uso de un estabilizador del mastocito como tratamiento del SII. Los objetivos derivados de este planteamiento han pretendido, por lo tanto, identificar la eficacia del tratamiento con cromoglicato disódico (CGDS), al

mismo tiempo que estudiar los mecanismos fisiopatológicos del SII. Ello ha permitido obtener un conocimiento más profundo a nivel molecular de los fenómenos inmunológicos que acontecen en la mucosa intestinal de estos pacientes, lo cual ha proporcionado un substrato biológico sobre el que plantear nuevos estudios para la identificación de posibles marcadores biológicos de la enfermedad.

Para ello, realizamos un primer estudio de carácter exploratorio que nos permitió identificar el efecto del tratamiento oral con CGDS sobre las manifestaciones clínicas del SII. El CGDS presenta escasa absorción sistémica, y por lo tanto, se espera un efecto local sobre el intestino. Este tratamiento logró disminuir el dolor abdominal en la mitad de los pacientes y en dos terceras partes normalizó la consistencia de las deposiciones. La administración oral de ketotifeno ha demostrado también la disminución en la hipersensibilidad visceral en pacientes de SII, sin cambios en el número de mastocitos (Klooker T.K., 2010). Ambos estudios refuerzan la importancia de la activación, y no del número de mastocitos, sobre la disfunción intestinal característica en esta enfermedad.

Así, en nuestro estudio, determinamos la expresión génica de triptasa, una de las proteasas más abundantes en el mastocito, y de genes específicos de respuesta inmunitaria innata, por el papel clave del mastocito en esta vertiente inmunitaria. Debido a la mayor permeabilidad del epitelio intestinal, la mucosa intestinal está, presumiblemente, más expuesta a antígenos luminales, lo cual explica su estado de inflamación. Así, de forma similar a

nuestro estudio, en el colon de pacientes de SII, se ha identificado una mayor expresión de TLR, asociado al tiempo de evolución de la enfermedad, aunque no a su gravedad (Brint EK., 2011; Belmonte L., 2012). La disminución significativa en la expresión de triptasa, *TLR2*, *TLR4*, *NOD1*, *MyD88, SIGIRR* y *TOLLIP* por el tratamiento con CGDS sugiere que la estabilización del mastocito reduce la activación del sistema inmunitario a través de la señalización mediante TLR. Esta disminución parece ser un efecto indirecto, ya que son otras células (enterocitos y otros leucocitos) y no el mastocito, las que expresan TLR 2 y 4 en la mucosa intestinal. La implicación, además, de las proteínas de choque térmico (HSP), como ligandos endógenos implicados en su activación, sugiere un elevado estado oxidativo en la mucosa intestinal de SII-D, por lo que moléculas con actividad antioxidante, como el CGDS, podrían ser utilizadas para restablecer la homeostasis intestinal, por su efecto neutralizador de radicales libres (Sadeghi-Hashjin *et al*., 2002). Otro factor determinante en la regulación de las HSP es la flora bacteriana, por lo que su expresión diferencial respecto a sujetos sanos podría estar influenciada por la inestabilidad y disminución en la diversidad de la flora intestinal, descrita en pacientes con SII (Kassinen AM *et al*.2007; Mättö J *et al*., 2005). Sin embargo, es importante destacar que, a diferencia de otras entidades inflamatorias crónicas intestinales, como la enfermedad inflamatoria intestinal (EII) y la enfermedad celíaca, en el SII-D, los mecanismos de control de la inflamación a través de la señalización de los TLRs parecen preservados, gracias al aumento en la expresión de *TOLLIP*, y *SIGIRR*, inhibidores de su señalización (Brint EK., 2011)

A pesar de los resultados prometedores sobre los síntomas cardinales del SII y la modulación de la expresión génica en la mucosa intestinal tras el tratamiento con CGDS, la ausencia un grupo placebo no nos permite evaluar su eficacia terapéutica real. Otros estudios han identificado la disminución del dolor y la mejora en el hábito deposicional tras el tratamiento oral con CGDS (estudios no controlados con placebo) empleando diferentes dosis (800-2.000 mg/día), y durante un período de tiempo menor (máximo dos meses). Sin embargo, dadas las limitaciones en su diseño no han permitido establecer los mecanismos por los cuales el CGDS mejora los síntomas.

Las herramientas que evaluaron el curso clínico de los pacientes en este estudio fueron: el cuestionario de gravedad del SII y un diario de registro de síntomas. La puntuación basal de severidad de la enfermedad en la mayoría de los enfermos de ambos grupos estaba en el rango de enfermedad moderada. Aunque el valor promedio de severidad disminuyó en ambos tratamientos a lo largo del seguimiento, el efecto fue ligeramente mayor en el grupo de pacientes tratados con CGDS tanto en la severidad del SII como en el dolor abdominal. A pesar de que el tratamiento con CGDS no mostró un beneficio significativamente superior sobre el dolor al del placebo, un 20% de los pacientes del grupo CGDS en la visita final manifestaron una desaparición del dolor frente al 6% del grupo placebo. En relación al efecto sobre la consistencia de las deposiciones, aunque ambos grupos refirieron mejoría, el efecto fue mayor en el grupo tratado con CGDS, ya que, además de normalizar la consistencia a lo largo del seguimiento, en una proporción significativamente mayor de pacientes, el CGDS redujo la frecuencia de las

deposiciones acuosas por un período superior al 50% del total del cumplimiento terapéutico. Estos resultados están en concordancia con otras observaciones que demuestran la eficacia del CGDS en el control de la diarrea en entidades con aumento de mastocitos en el intestino, como la mastocitosis sistémica (Dolovich *et al*., 1974).

Además de la microinflamación intestinal, uno de los mecanismos más relevantes implicado en el desarrollo del SII es la comunicación neuro-inmunológica. En este sentido, del estudio de la relación entre los factores psicosociales y biológicos en el desarrollo del SII surge el modelo bio-psicosocial (Halpert A., 2005), que ha cobrado especial importancia por cuanto supone su comprensión y abordaje. Así, en el presente estudio, evaluamos las posibles relaciones entre el perfil psicológico de estos pacientes y sus síntomas, así como con el perfil transcripcional en la mucosa intestinal.

Es interesante destacar la asociación entre la depresión y la intensidad del dolor abdominal al final del tratamiento en el subgrupo de enfermos que recibieron placebo, lo que sugiere que la relación con el terapeuta y el seguimiento constante de estos pacientes tiene un papel relevante en el efecto placebo sobre el estado de ánimo y el dolor. Sin embargo, a pesar de que se ha descrito el estrés como factor importante en el desarrollo y el curso de los síntomas en esta entidad, no encontramos asociación entre la severidad de los síntomas y las escalas de estrés en nuestro estudio. Este resultado sugiere que la percepción del dolor es un fenómeno complejo, y no sólo es una experiencia perceptiva o sensorial sino también afectiva, y que

factores psicológicos como el estado de ánimo actúan como mediadores de la percepción. Aunque el origen del dolor esté asociado a procesos biológicos, las variables psicológicas contribuyen en la modulación de su percepción, y explican la variabilidad interindividual de la misma y su tolerancia. Hay que destacar, así mismo, que en la respuesta al placebo influyen múltiples factores: los relativos al medicamento (si el fármaco activo posee más acción el placebo será más eficaz), los factores relativos a la relación médico-paciente y los factores relativos al estado de ánimo del paciente, así como la expectativa que se deposite en el tratamiento.

La interpretación de los resultados obtenidos en este estudio debe realizarse con cautela ante las limitaciones que presenta. Es destacable el desafortunado e inesperado elevado número de abandonos y que redujo el número de pacientes que realizaron el seguimiento hasta el final. Creemos que uno de los factores que contribuyeron fue la posología del tratamiento, que dificultó el cumplimiento del tratamiento durante los 6 meses. Por causas ajenas al estudio, el contenido de cada cápsula dispensada fue de 100 mg, lo cual elevó a un total de 6 el número de cápsulas diarias a ingerir para cumplir con la dosis establecida. Así, los datos se analizaron según “intención de tratar” para disminuir la probabilidad de sesgo en la evaluación de la magnitud del efecto del tratamiento, aunque no es perfecto, ya que altera la realidad de la evolución al perder información.

La obtención de dos biopsias intestinales, antes y después del tratamiento, permitió asociar la respuesta clínica a la expresión génica diferencial en la

mucosa intestinal e identificar mecanismos celulares y moleculares locales vinculados a esta respuesta clínica. Existen pocos estudios que hayan efectuado dos biopsias intestinales a un mismo sujeto para evaluar el efecto de un tratamiento en el SII, siendo el intervalo máximo de tiempo transcurrido entre ambas de 8 semanas (Corinaldesi *et al*., 2009; Klooker *et al*., 2010). Tras este período de tiempo, la activación del mastocito, identificada mediante su degranulación, parece ser más relevante en la fisiopatología del SII que el número de células infiltradas (Martinez *et al*., 2012a). En este sentido, la evaluación de la ultraestructura del mastocito mostró signos de estabilización tras el tratamiento activo, mientras en el grupo placebo solo se observó disminución en la densidad celular, que no alcanzó significación estadística.

Con el propósito de identificar mecanismos de transcripción asociados al beneficio clínico del CGDS, comparamos los patrones de expresión génica intestinal entre ambos tratamientos. Hay que resaltar que es la primera vez que se evalúa el perfil transcripcional en la mucosa intestinal en respuesta a un tratamiento en el SII. Utilizamos como técnica exploratoria *microarrays* de expresión génica, seguido del análisis de la expresión diferencial mediante el programa IPA, que permitió conocer, analizar y profundizar en las vías funcionales en las que interaccionan y participan los genes diferencialmente expresados. El tratamiento con CGDS generó un impacto mayor sobre el transcriptoma de la mucosa intestinal con respecto al placebo, asociado a mecanismos de control del balance de la respuesta inmunológica, la homeostasis del epitelio y el estrés oxidativo. Aunque no se conoce en detalle

la fisiopatología del SII, estas funciones se han asociado a la disfunción intestinal presente en estos pacientes. Por ejemplo, alteraciones estructurales epiteliales justifican el aumento de la permeabilidad intestinal y, por lo tanto, el aumento de la activación mastocitaria que se relacionan principalmente con el hábito deposicional (Martinez *et al*., 2012b). Así mismo, el aumento del estrés oxidativo se relaciona con la activación de diferentes células inmunológicas, entre ellas, el mastocito, cuyos mediadores activan neuronas sensoriales y dan lugar a la hipersensibilidad visceral (Han *et al*., 2012). Ello sugiere que el aumento del estrés oxidativo en la mucosa intestinal podría ser un mecanismo fisiopatológico en esta entidad, por lo que podría esperarse un efecto del DSCG sobre la reducción del dolor.

Otro de los hallazgos de este trabajo es la modulación del tratamiento con CGDS sobre la regulación de genes implicados en el ritmo circadiano intestinal. La disrupción del ritmo circadiano está relacionada con el desarrollo de diferentes patologías, aunque en la actualidad se desconocen los mecanismos implicados. Hoy en día conocemos que la expresión de diferentes genes implicados en la absorción y secreción intestinal tiene una regulación circadiana (Pardini *et al*., 2005), los cuales, además se asocian con el balance del estrés oxidativo. Así, en estudios preclínicos, se ha demostrado que la carencia del gen *PER* aumenta el nivel de radicales libres y eleva la susceptibilidad a la neurodegeneración mediada por el estrés oxidativo (Xu YQ., 2012). El CGDS originó cambios en la expresión de un grupo de genes que evidencian una disminución en la activación de la vía de señalización p53 y la apoptosis neuronal. Estos cambios están involucrados

en el mantenimiento de la homeostasis intestinal. Así, el gen p53 desempeña un papel importante en la respuesta antiinflamatoria (Gudkov AV., 2011), en la apoptosis y en el control del ciclo celular.

La modulación en la expresión génica, inducida por el tratamiento con CGDS, revela la asociación entre la disminución de la intensidad del dolor abdominal en los pacientes y el patrón de regulación de expresión del gen *PON2*. Este gen codifica una proteína con propiedades antioxidantes, protegiendo a las células del estrés oxidativo, y antiinflamatorias. A nivel del tracto gastrointestinal se localiza en la parte apical del epitelio, aunque se desconoce su papel, se ha sugerido su función antibacteriana (Shamir *et al*., 2005; Levy *et al*., 2007). Otro agente que inhibe la activación del mastocito y con elevada actividad antioxidante es la quercetina, en la que se ha descrito su capacidad de incrementar la expresión de *PON2* a nivel de los macrófagos (Boesch-Saadatmandi *et al*., 2009). En nuestro estudio desconocemos cual es el mecanismo y la célula responsable de este incremento en la expresión.

En resumen, esta tesis contribuye a ampliar el conocimiento actual sobre los efectos clínicos y biológicos derivados de la estabilización del mastocito. El cromoglicato disódico mejora principalmente la diarrea, consistencia de las deposiciones, entre los síntomas principales del SII-D. Efecto asociado al restablecimiento de la homeostasis intestinal por su acción antiinflamatoria y antioxidante.

CONCLUSIONES

CONCLUSIONES:

Los resultados obtenidos en esta tesis doctoral han dado lugar a las siguientes conclusiones:

1.- Los pacientes con SII-D presentan mayor actividad inmunitaria innata en la mucosa intestinal, que recuperan las características de los sujetos sanos mediante el tratamiento oral con CGDS durante 6 meses, confirmando la relevancia del mastocito en la inmunidad inespecífica en esta enfermedad.

2.- La sintomatología característica intestinal (dolor abdominal y diarrea) en pacientes con SII-D mejora tras el tratamiento oral con CGDS durante 6 meses.

3.- El ensayo clínico con doble ciego, controlado con placebo, para evaluar el tratamiento con CGDS como terapia en el SII-D:

- Muestra el beneficio clínico, con respecto al placebo, sobre la diarrea, la consistencia de las deposiciones, y no sobre la severidad del síndrome y el dolor abdominal.
- Revela que el placebo reduce la severidad del síndrome y la intensidad del dolor abdominal.
- Demuestra el cambio en el perfil de expresión génica de la mucosa intestinal en el contexto de la respuesta clínica, por su modulación de vías de señalización asociadas al balance de la respuesta inmunológica, la homeostasis del epitelio y el estrés oxidativo, sugiriendo éstos como posibles mecanismos de su efecto clínico en el SII-D.

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