Universidad de Navarra

Facultad de Farmacia y Nutrición

The pro-inflammatory cytokine TNF-α reduces intestinal sugar transport: Its relation with obesity and the blocking effects of EPA, DHA and DHA-derived pro-resolving lipid mediators

---

La citoquina pro-inflamatoria TNF-α reduce el transporte intestinal de azúcar: su relación con obesidad y el efecto bloqueante del EPA, DHA y mediadores lipídicos pro-resolutivos derivados del DHA

Rosa Mª Castilla Madrigal

Pamplona, 2017

Universidad de Navarra

Facultad de Farmacia y Nutrición

Memoria presentada por Dña. Rosa María Castilla Madrigal para aspirar al grado de Doctor por la Universidad de Navarra.

Fdo. Rosa Mª Castilla Madrigal

El presente trabajo ha sido realizado bajo mi dirección en el **Departamento de Ciencias de la Alimentación y Fisiología** de la Facultad de Farmacia y Nutrición de la Universidad de Navarra y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Vº Bº Directora

María Pilar Lostao Crespo

Este trabajo ha sido posible gracias a la financiación de diversas entidades: Gobierno de España (Ministerio de Economía, Industria y Competitividad) [BFU2012-36089 y BFU2015-65937-R], Gobierno de Navarra (Departamento de Salud) [67-2015], Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBERObn) Instituto de Salud Carlos III (ISCIII) [CB12/03/30002], Centro de Investigación en Nutrición (Universidad de Navarra). Beca predoctoral 2013-2017 de la "Asociación de Amigos de la Universidad de Navarra".

Deseo agradecer a todas las personas e instituciones que han hecho posible llevar a cabo este trabajo y que me han apoyado durante todos estos años para poder llegar hasta aquí.

En primer lugar, me gustaría agradecer a la *Universidad de Navarra* por tantos años de formación. A la *Facultad de Ciencias* por proporcionarme el primer atisbo del trabajo de laboratorio. A la *Facultad de Farmacia y Nutrición* por la formación recibida tanto profesional como personal durante estos cuatro años de doctorado. Y en especial, al departamento de *Ciencias de la Alimentación y Fisiología*, por la formación obtenida como alumna interna y de máster, y posteriormente por darme la oportunidad de desarrollar este trabajo de investigación e iniciar mi camino en la carrera investigadora y docente.

Agradecer también a la *Asociación de Amigos* de la Universidad de Navarra, la confianza depositada en mi persona a través de una beca predoctoral.

A mi directora *María Pilar Lostao*, por su apoyo durante estos años, tanto a nivel profesional como personal, por toda su dedicación, tiempo, paciencia, y buenos consejos, que me han ayudado a crecer como persona y profesional. Y por todas las tardes de verano sacrificadas en pro de la ciencia.

A la Profesora *María Jesús Moreno* por sus consejos, muestras de apoyo y cercanía siempre que lo he necesitado. Y por su ayuda en la organización de los experimentos de laboratorio.

A la directora del departamento *Diana Ansorena* por todas sus muestras de cariño y preocupación a lo largo de estos años. A la Profesora *Marian Zulet*, por sus ánimos y conversaciones camino a casa. Al Profesor *Alfredo Martínez* por tener siempre un buen consejo o chiste a mano para animarme.

Al Dr. *Pedro González*, por toda la ayuda y consejos recibidos. Incluso por recordarme las reuniones del colegio de mi peque cuando las cartas no me llegaban. A la Dra. *Silvia Lorente Cebrián* por su apoyo y conversaciones.

Al resto de profesores del Departamento, porque todos, de una u otra forma, me habéis ayudado en mi camino. Gracias por vuestros consejos y sinceridad.

A la Dra. *Jaione Barrenetxe*, por su cariño, alegría, sinceridad y su continua ayuda cada vez que la he necesitado. Por ser capaz de sacarme siempre una sonrisa y sus

siempre ingeniosos comentarios a la hora de la comida. A *Asun Redín* por tu alegría contagiosa, buen humor y todos los momentos que hemos compartido. Porque junto a Jaione, fuisteis mis mentoras en el laboratorio, todavía tengo el protocolo de cuando me enseñaste la técnica de Western blot.

A *Neira*, que siempre ha estado disponible para ayudarme y me ha enseñado mucho de PCR, no pierdas esa energía.

A *Xavier*, por tener siempre para echarme una mano con cualquier problema del laboratorio.

A *Paula, Bea y Alba* no solo por vuestra labor administrativa sino por la de veces que nos hemos reído juntas y por escucharme. A *Vero, Ana y Mery*, porque siempre que he pedido algo me habéis ayudado.

A mis compis de despacho, Eva y Laura. *Eva*, comenzaste siendo mi "aprendiza" pero en seguida pasaste a ser una amiga. *Laura*, comenzamos juntas esta etapa y has estado a mi lado en todos los momentos del camino. Habéis sido un gran punto de apoyo tanto dentro como fuera del laboratorio.

A todos mis compañeros. Mis veteranos, *Jonai, Carmen, Miguel, Usune, Idoia, Aurora, Rocio, Paúl, Ana, Marta, Ana Laura, Ana Elsa* por acogerme en el departamento. Mis contemporáneos, *Rodrigo, Laura, Leyre, Leticia, Miriam, Amaya, Irene* y las nuevas adquisiciones que han ido llegando durante estos años, *Eva, Marcos, Elisa, Ana, Lydia, Ana*, *Vanesa, Claudia, Araceli*. A *Rodrigo* por siempre estar dispuesto a escuchar y ayudar. A Miguel porque siempre formaras parte del "protein team". A *Leyre* por poner siempre una nota de humor. A *Marcos*, por estar siempre dispuesto a ayudarme.

A *Sandra* del departamento de Farmacología, por las veces que me has ayudado y los paseos que nos hemos dado al contador de centelleo.

I would like to thank Dr. *Pradeep Dudeja* and all the members of his lab (Division of Gastroenterology & Hepatology, Dept. of Medicine, University of Illinois in Chicago), specially to *Ishita* and *Dulari*, for the support, lessons and affection received during my three months of pre-doctoral stay.

A mis chic@s de Ciencias, porque un chiste de átomos nunca viene mal, y siempre habéis estado disponibles. En especial a *Susana* porque, aunque tuvieras mil proyectos en marcha siempre has acudido a mi llamada. A mis compañeras de piso *Raquel,*

Apellaniz y Sheila, porque sin vosotras los años de carrera no habrían sido los mismos. Hemos vivido juntas mil y una aventuras, esas cosas unen para siempre.

A mi cuadrilla de Haro, *Ibeas, Berta, Isa, Mery, Borja, Bea, Diana, Nagore, Alex*, y a *Alexandra* porque, aunque muchas veces no entendierais una palabra, siempre me habéis escuchado.

Gracias a toda mi familia, por vuestro esfuerzo y apoyo. A mi abuela *Angelines* y mi amama *Jesu* por vuestro cariño. A mis tíos *Ana, Ángela, Fernando y Juan Carlos*, y a mi prima *Ángela*, por vuestro cariño. A mis padres, *Alberto y Rosa*, porque siempre me habéis apoyado y ayudado en este largo camino, porque me habéis convertido en la persona que soy ahora. A mi hermana *Elena*, porque siempre me has hecho feliz, y aunque no siempre podamos vernos sé que estás ahí. A mi marido *Rubén*, que es capaz de estar media noche despierto solo para hacerme compañía mientras trabajo, por todo tu apoyo, amor y confianza, por creer más en mí que yo misma. A mi hija *Rosa* porque eres mi alegría, mi niña. Os quiero mucho a ambos. Por toda la paciencia, cariño y amor recibidos, gracias.

A mi familia

ABBREVIATIONS

A

AA:	Arachidonic acid
AHA:	American Heart Association
AKT:	Serine/threonine protein kinase
ALA:	α-linolenic acid
AMPK:	Adenosine monophosphate-activated protein kinase
ASCT2:	Alanine, serine, cysteine transporter 2
ATB^0:	Amino acid transporter B^0
ATP:	Adenosine triphosphate

B

B^0AT1:	Broad neutral amino acid transporter 1
BBMV:	Brush border membrane vesicles
BMI:	Body mass index
BSA:	Bovine serum albumin

C

CC:	Compound C
CT-1:	Cardiotrophin-1

D

DHA:	Docosahexaenoic acid
DIO:	Diet induced obesity
DIO-MaR1:	Diet induced obesity treated with Maresin 1
DMEM:	Dulbecco's Modified Eagle's Medium
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DNAc:	Complementary deoxyribonucleic acid
DPA:	Docosapentaenoic acid
DTT:	Dithiothreitol

E

EDTA:	Ethylenediaminetetraacetic acid
EII:	Enfermedad Infamatoria Intestinal
ERK:	Extracellular signal-regulated kinase 1/2
EPA:	Eicosapentaenoic acid

F

FAO:	Food and Agriculture Organization
FBS:	Fetal bovine serum
FFA:	Free fatty acids
FFAR:	Free fatty acid receptor

G

Gln:	Glutamine
GLUT:	Facilitative glucose transporter
GPR:	G-protein coupled receptor

H

HFD:	High fat diet
hMSC:	Human mesenchymal stem cells
hMSC-DA	Human mesenchymal stem cells derived adipocytes

I

IBD:	Intestinal bowel disease
IBMX:	Isobutylmethylxanthine
IL:	Interleukin

K

KRT:	Krebs Ringer Tris

L

LPS: Lipopolysacharide

M

αMG: Alpha-Methyl-D-Glucoside

MAPK: Mitogen-activated protein kinases

MaR: Maresin

M-MLV: Moloney Murine Leukemia Virus

mTNFα: transmembrane TNFα

N

Na^+/K^+ ATPase: Sodium-potassium adenosine triphosphatase

n-3: Omega-3

n-6: Omega-6

NEAA: Non-esencial amino acids

NF-κB: Nuclear factor kappa b

NOD2: Nucleotide-binding oligomerization domain-containing protein 2

P

PAT1: H^+-neutral amino acid co-transporter 1

PBS: Phosphate buffered saline

PD: Protectin D

PK: Protein kinase

PMA: Phorbol myristate

PUFA: Polyunsaturated fatty acids

Q

Q-PCR: Quantitative polymerase chain reaction

R

mRNA:	Messenger ribonucleic acid
RvD:	Resolvin D series

S

SCD1:	Stearoyl-CoA desaturase-1
SGLT1:	Sodium glucose cotransporter 1
SPMs:	Specialized pro-resolving mediators
STNF-α:	Soluble TNF-α

T

TACE:	Tumor necrosis-α-converting enzyme
TBS-T:	Tris-Buffered Saline -Tween
TER:	Transepithelial resistance
THP-1:	Transformed human mononuclear cell lines
TLR4:	Toll-like receptor 4
TNF-α:	Tumor necrosis factor alpha
TNFR:	Tumor necrosis factor receptor

W

WAT:	White adipose tissue
WHO:	World Health Organization

I. INTRODUCTION

1. Tumor Necrosis Factor alpha (TNF-α)

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine implicated in the innate immune system and inflammation. It was identified in 1975 as an endotoxin-induced glyco-protein, which caused necrosis of tumors *in vitro* (Carswell *et al.*, 1975). In 1985, human TNF-α was cloned (Pennica *et al*., 1985).

1.1. Synthesis and function

Human TNF-α is codified by a single copy gene, with 4 exons and 3 introns, located in chromosome 6 (murine chromosome 17). TNF-α is mainly produce by lymphocytes, monocyte/macrophage lineage, but it also can be produced by many other cell types during inflammatory processes (Bradley, 2008). At the transcriptional level, TNF-α is regulated by several factors including nuclear factor kappa B (NF-κB) (Parameswaran & Patial, 2010). Human cells synthesize TNF-α as 233 amino acids transmembrane protein (mTNF) of 27 kDa (Parameswaran & Patial, 2010). The metalloproteinase tumor necrosis-α-converting enzyme (TACE), cleaves mTNF to release the 17 kDa soluble TNF (sTNF). Both mTNF and sTNF are biologically active and show autocrine/paracrine and endocrine effects, through the binding to Types 1 and 2 TNF receptors (TNFR1 and TNFR2) (Black *et al.*, 1997; Cabal-Hierro & Lazo, 2012; Horiuchi *et al.*, 2010; Parameswaran & Patial, 2010).

The TNFRs have a similar extracellular ligand-binding domain, with cysteine rich subdomains. However, the intracellular domains of each TNFRs show no sequence homology and activate distinct signal transduction pathways (Bradley, 2008). Activation of TNFR1 and TNFR2 by TNF-α induces apoptosis, cell proliferation and cytokine production, including TNF-α itself, related with inflammation. TNF-α signaling pathway involves MAPKs phosphorylation and NF-κB activation (Cabal-Hierro & Lazo, 2012; Horiuchi *et al.*, 2010; Rossol *et al.*, 2007) (Fig. 1).

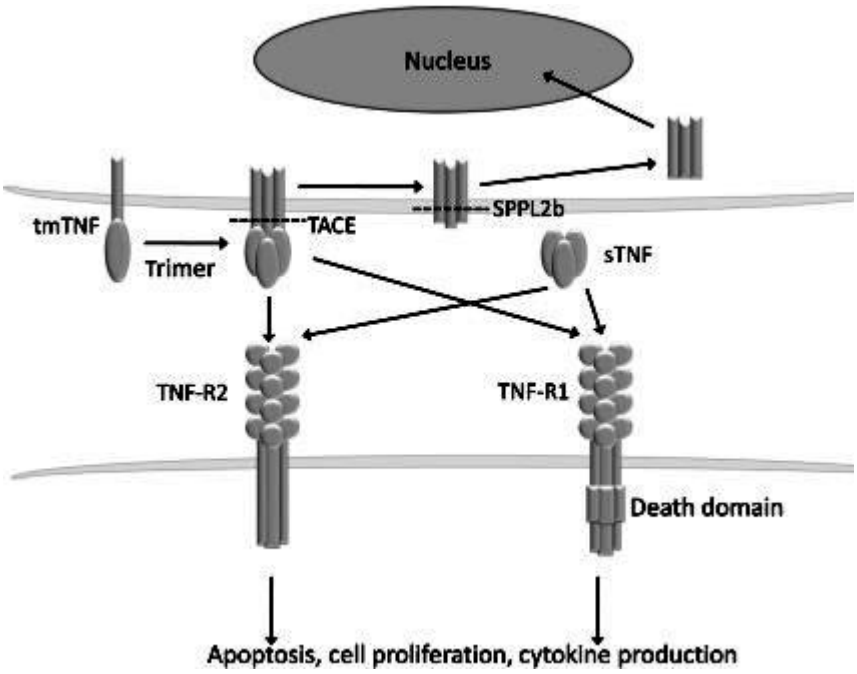


Figure 1. **Biology of transmembrane TNF-α (tmTNF) and soluble TNF-α (sTNF).** Transmembrane TNF-α is a precursor form of soluble TNF-α that is expressed on TNF-α-producing cells as a homotrimer. After processing by TACE, soluble TNF-α is generated and binds to TNFR1 or R2. Transmembrane TNF-α also binds to TNFR1 and R2. Upon binding to TNF receptors, both transmembrane and soluble TNF-α mediate pleiotropic effects (apoptosis, cell proliferation and cytokine production). The remaining transmembrane TNF-α after cleavage with TACE is further processed by SPPL2b and the intracellular domain is translocated into the nucleus and is supposed to mediate cytokine production. (From Horiuchi *et al.*, 2010).

After trauma, infection or exposure to bacterial-derived LPS, TNF-α is rapidly released and activates pro-inflammatory cytokine cascade to induce inflammatory cells activation and recruitment. One of the most important cells attracted to the inflammatory site are macrophages, which destroy microbes and clean up cellular debris. TNF-α is implicated in the long-term survival, proliferation and differentiation of macrophages (Parameswaran & Patial, 2010). Macrophages also secrete pro-inflammatory cytokines including TNF-α (Parameswaran & Patial, 2010). Resolution phase of inflammation implicates apoptosis of inflammatory cells and their efferocytosis by phagocytes. At that point, the presence of TNF-α reduces efferocytosis delaying the resolution phase of inflammation and exacerbating chronic inflammatory conditions (Michlewska *et al.,* 2009).

1.2. TNF-α and related pathologies

As mentioned, TNF-α is critical in the normal physiological response to infection, injury, burn, etc. In the situations in which the inflammatory response is not properly controlled and therefore, the resolutive phase does not start, chronic inflammation appears with high production of TNF-α (Bradley, 2008). Thus, rheumatoid arthritis (RA) is characterized by synovial inflammation of joints accompanied with joint destruction and functional disability. TNF-α is an important mediator in local joint damage and systemic bone loss in RA (Manara & Sinigaglia, 2015). Psoriasis is a skin disorder produced by inflammatory cell infiltrate in which high levels of TNF-α, TNFR1 and TNFR2 are found. TNF-α is involved in both the initial phase and the chronic phase of psoriasis (Bradley, 2008; Grine *et al.*, 2015). Heart contains resident macrophages which produce TNF-α in response to infection, endotoxemia, ischemia and reperfusion, burn trauma, clinical myocardial infarction and cardiopulmonary bypass. The cardiovascular effects of TNF-α are characterized by decreased myocardial contraction and reduced ejection fraction. Patients with inflammatory disease show higher risk of cardiovascular disease development (Bradley, 2008; Ferrari, 1999). TNF-α is also implicated in chronic bronchitis, chronic obstructive pulmonary disease, acute respiratory distress syndrome and asthma (Bradley, 2008).

Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the gastrointestinal mucosa due to a dysregulation of the immune system (Tabas & Glass, 2013). During intestinal inflammation, TNF-α is produced by invading immune cells in the intestinal epithelium (Roulis *et al.*, 2011). This cytokine modulates mucus secretion; tight-junction disruption; and survival, death, and proliferation intracellular signaling cascades; epithelial wound healing and interaction between enterocytes and immune cells (Leppkes *et al.*, 2014; Fig. 2). TNF-α also affects the expression and activity of many nutrients and electrolytes transporters (Barrenetxe *et al.*, 2013; Bertolo *et al.*, 2002; Bradley, 2008; Foley *et al.*, 2007; Sharma *et al*., 2005; Tabas & Glass, 2013).

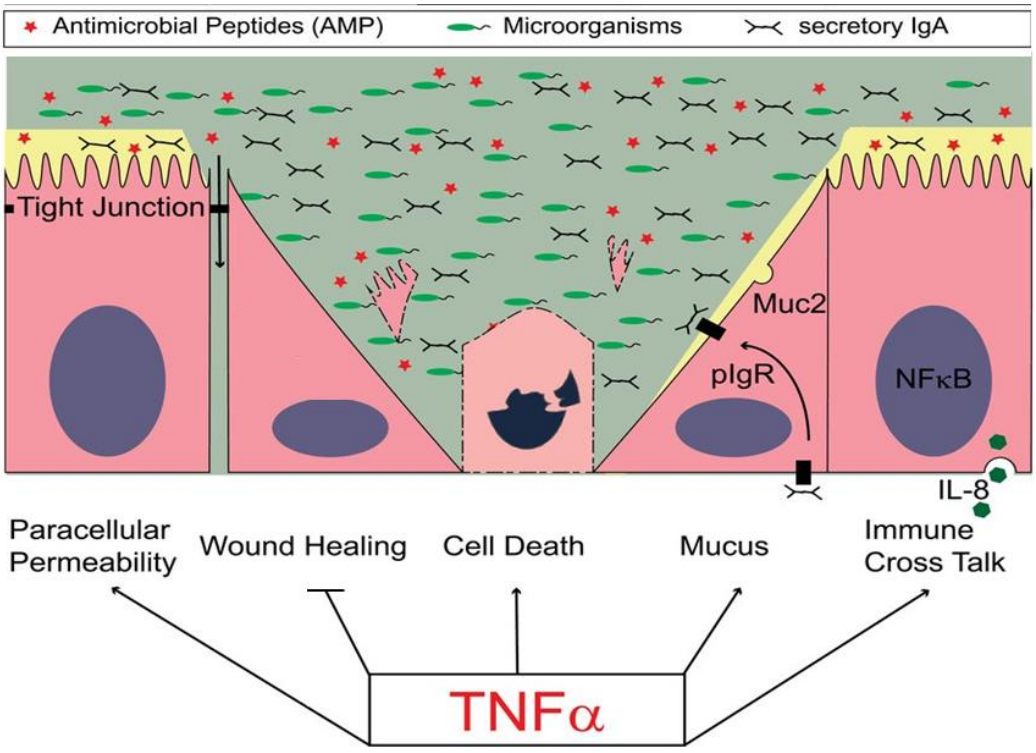


Figure 2. **TNF-α role on intestinal inflammation.** The intestinal barrier is breached in the context of inflammation. Cellular mechanisms targeted by TNF-α are depicted including alteration of paracellular permeability and TNF-α-induced cell death. Immune cell attraction is actively controlled by cytokine and chemokine cross-talk. TNF-α contributes to modified mucus secretion by increasing expression of polymeric immunoglobulin receptor (pIgR) and Muc2 gene, which is necessary for the transcytosis of secretory IgA into the mucus. (Modified from Leppkes *et al.* 2014).

1.3. Anti-TNF-α drugs

The blockage of TNF-α has been proved to be effective in the control of different inflammatory diseases. Therefore, biological drugs such as monoclonal antibodies have been developed to target TNF-α (Table 1). Nevertheless, studies show that around 30-40% of the patients do not improve after the therapy. In other cases, loss of response may occur over time (Argollo *et al.*, 2017; Bradley, 2008).

Table 1. **Anti-TNF-α drugs (Argollo et al. 2017).**

Anti-TNF-α drug	Active principle	Disease treatment
Infliximab	Chimeric monoclonal antibody IgG1 (mouse/human)	IBD, psoriasis, ankylosing spondylitis, RA
Adalimumab	Human monoclonal antibody IgG1	RA, IBD, psoriasis, ankylosing spondylitis
Certolizumab	Human Fab fragment conjugated with polyethylene glycol	RA, psoriasis, ankylosing spondylitis
Golimumab	Human monoclonal antibody IgG1	RA, ankylosing spondylitis
Etanercept	Fusion protein of TNFR2 and IgG1 Fc component	RA, psoriasis, ankylosing spondylitis
Rituximab	Monoclonal antibody IgG1 (mouse/human)	RA

2. Intestine

The intestine is the largest physical and biochemical barrier between the organism and the external environment. It is particularly adapted to maintain the homeostasis between microbial colonization and the risk of infection and inflammation (Peterson & Artis, 2014). Special structural features of the small intestine facilitate the process of digestion and absorption. These features include circular folds, villi and microvilli. The villi are finger-like projections of the mucosa that are surrounded by opening of glandular structures called crypts of Lieberkuhn. The cells lining the villi are responsible for nutrients and electrolytes absorption, whereas the crypt cells participate in secretion. The intestinal cells are continuously renewed by pluripotent intestinal epithelial stem cells allocated in the crypts. The most abundant cells on the villi are the absorptive cells called enterocytes (Peterson & Artis, 2014; Shaker *et al.*, 2010). Enterocytes are attached to their neighboring cells by tight junctions and placed on the lamina propria. They are polarized cells with two specialized and differentiated membranes, the apical membrane with numerous microvilli forming the "brush border", and the basolateral membrane. Each membrane contains a different population of transporters to allow the absorption process from the lumen to the blood.

2.1. Intestinal absorption

Intestinal absorption of sugars and amino acids from the diet occurs through the transcellular pathway. This implies the existence of a variety of membrane transporters with different location (apical or basolateral), kinetic characteristics, substrate selectivity and Na^+ dependence.

2.1.1. Sugar absorption

SGLT1

The sodium glucose cotransporter SGLT1 is the major glucose transporter in the apical membrane of enterocytes. It was the first eukaryote cotransporter to be cloned (Hediger *et al.*, 1989). It belongs to the SLC5 gen family of membrane transporters (member SLC5A1). SGLT1 contains 664 amino acid residues arranged in 14 trans-

membrane α-helices (Wright *et al.*, 2004) with a molecular weight of 75 kDa (Wright *et al.*, 2011). It is a high affinity ($K_{0.5}$ ~0.5-1 mM) glucose and galactose transporter, specifically inhibited by phlorizin (Díez-Sampedro *et al.*, 2000). SGLT1 uses the electrochemical gradient of Na^+ to transport glucose and galactose, with a 2:1 ratio, into the enterocytes against its concentration gradient. SGLT1 is also able to transport water and urea, coupled to Na^+ and glucose during the transport cycle, suggesting and alternative function for SGLT1 in small intestine as water and urea transporter (Wright *et al.*, 2011; Wright *et al.*, 2004).

GLUT2

The facilitative glucose transporter member 2 (GLUT2) is a low affinity ($K_{0.5}$ ~15-20 mM) transporter for glucose, galactose, mannose and fructose, but a high affinity transporter for glucosamine ($K_{0.5}$ ~0.8 mM) (Uldry & Thorens, 2004). GLUT2 belongs to the facilitative membrane transporter family SLC2 (member SLC2A2); it comprises ~500 amino acid residues arranged in 12 transmembrane domains, and shows a molecular weight of ~60 kDa (Mueckler & Thorens, 2013). GLUT2 is expressed in the basolateral membrane of the enterocytes and is responsible of the downhill transport of glucose, galactose and fructose from the enterocytes to the blood. During postprandial periods, in which glucose concentration in the intestinal lumen increases, GLUT2 is recruited to the brush border membrane to facilitate the transport of sugars into the enterocytes without energy expenditure (Karasov, 2017; Kellett & Helliwell, 2000).

GLUT5

The facilitative glucose transporter member 5, GLUT5 (SLC2A5) is a high affinity ($K_{0.5}$ ~1.4 mM) fructose transporter, the major transporter for fructose across membrane. It does not transport glucose or galactose (Douard & Ferraris, 2008). GLUT5 highest expression is found the small intestine. It is located in the brush border membrane where is in charge of fructose transport from the intestinal lumen into the enterocytes (Douard & Ferraris, 2008; Uldry & Thorens, 2004).

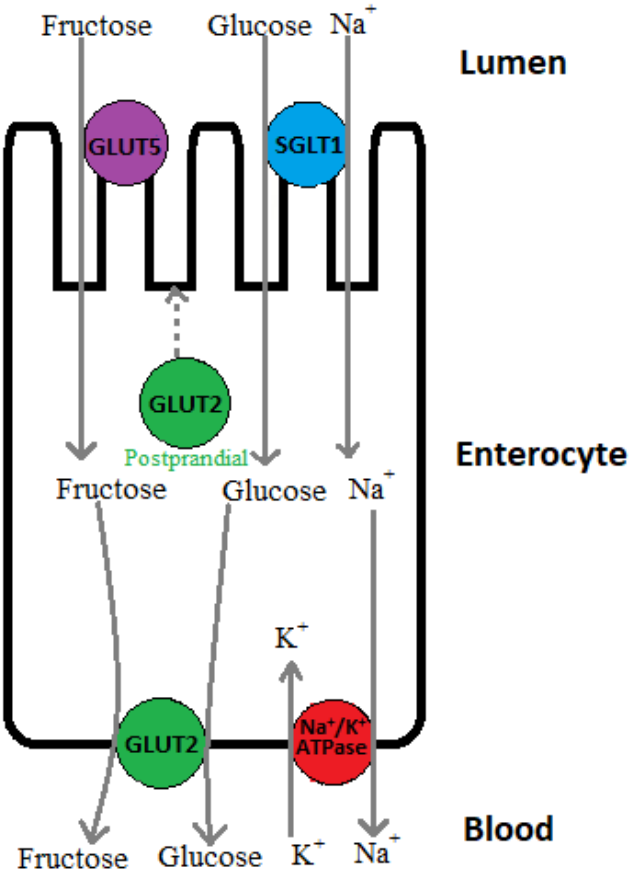


Figure 4. **Diagram of sugar transport across the enterocyte**.

2.1.2. Glutamine absorption

Glutamine (Gln) is the main energy source for the intestine, necessary for the maintenance of the gut barrier function and intestinal cell proliferation and differentiation (Chun *et al.*, 1997). Gln is considered a semi-essential amino acid due to its high requirement in some physiopathological conditions as physical trauma, intense exercise, immune deficiencies or cancer. (Lenaerts *et al.*, 2005). Over-expression of TNF-α in mice reduced intestinal barrier integrity, which was recovered by Gln supplementation (Noth *et al.*, 2013).

The main Gln transporters in the brush border of the small intestine are B^0AT1 and ASCT2 (Romeo *et al.*, 2006; Bröer, 2008).

<u>B^0AT1</u>

The broad neutral amino acid transporter 1 (B^0AT1) belongs to the SLC6 gene family of membrane transporters (member SLC6A19). It comprises 630 amino acids arranged in 12 transmembrane helices domains, with intracellular N- and C-termini,

and a molecular weight of 72 kDa (Margheritis *et al.*, 2016). Human B^0AT1 show a $K_{0.5}$ of 0.25 mM (Souba *et al.*, 1992). It is a Na^+-dependent transporter with a stoichiometry of 1Na:1Gln (Bröer et al., 2004). B^0AT1 needs the carboxypeptidase angiotensin-converting enzyme 2 (ACE2) and collectrin (non-peptidase homolog of ACE2) as key regulators for the insertion in the membrane and function (Camargo *et al.*, 2009; Fairweather *et al.*, 2012; Kowalczuk *et al.*, 2008). PKB/Akt up-regulates B^0AT1 increasing its expression in the membrane (Bogatikov *et al.*, 2012).

ASCT2

ASCT2 is a Na^+-dependent small neutral amino acid exchanger, whose three preferred substrates are alanine, serine and cysteine. Its expression is higher in the jejunum that in the duodenum (Bröer, 2008). It belongs to the SLC1 gene family of membrane transporters (member SLC1A5). ASCT2 transports small amino acids with $K_{0.5}$ of 20 µM. Glutamine transport and ASCT2 mRNA are upregulated by phorbol myristate (PMA) in Caco-2 cells involving stimulation of MAPK and PKC (Bröer, 2008).

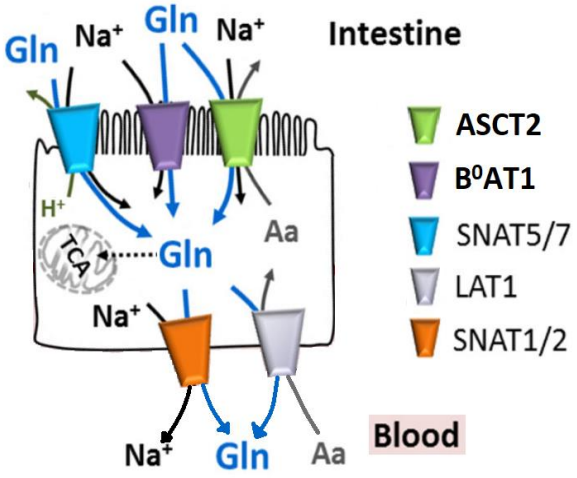


Figure 4. Diagram of glutamine transport in enterocytes (modified from Pochini *et al.*, 2014). ASCT2: Na^+-glutamine/neutral amino acids antiporter; B0AT1: Na^+-glutamine co-transporter (electrogenic); SNAT5/7: Na^+-glutamine/H^+ antiporter (electroneutral); LAT1: Glutamine/large neutral amino acids antiporter; SNAT1/2: Na^+-glutamine co-transporter (electrogenic).

ASCT2 could also help to equalize neutral amino acid composition in epithelial cells, but ASC-like activity is only about 1/10 of system B^0 activity in the intestine (Munck & Munck, 1999).

2.2. Intestine and inflammation

Inflammation is a prototypical reaction of the immune system in response to tissue or cell damage, microbial attack or invasion, radiation, hypoxia, pH changes and other threats to tissue homoeostasis. However, the cause of inflammatory bowel disease (IBD) is diverse and not clearly identified. IBD is characterized by chronic inflammation of the gastrointestinal mucosa, due to dysregulation of the immune system, with high concentrations of TNF-α (Tabas & Glass, 2013; Vitale *et al.*, 2017). The major forms of IBD are ulcerative colitis (UC) and Chron's disease (CD) (Tabas & Glass, 2013). Genome, diet and intestinal microbiota are implicated in the pathogenesis of IBD (Albenberg *et al.*, 2012). Cytokines play a crucial role in this pathogenesis as they orchestrate many aspects of intestinal inflammation (Soufli *et al.*, 2016). Usually, after the inflammatory process, that includes neutrophils and macrophages infiltration, the resolution phase starts. In IBD that resolution phase fails. Moreover, there are more than 250 genetic susceptibility factors that increase the risk of IBD (Rogler, 2017). During intestinal inflammation, the expression and activity of many nutrients and electrolytes transporters can be modified (Barrenetxe *et al.*, 2013; Bertolo *et al.*, 2002b; Foley *et al.*, 2007; Sharma *et al.*, 2005), which may explain the characteristic malabsorption and diarrhea found in IBD patients. As a consequence, patients may lose weight and suffer from vitamins, micronutrients and protein deficiency (Hébuterne *et al.,* 2009). In that line, we have previously demonstrated, in the human intestinal epithelial cell line Caco-2, that TNF-α inhibits αMG (a glucose analog) uptake by decreasing SGLT1 expression in the brush border membrane (Barrenetxe *et al.*, 2013).

3. Adipose tissue

Adipose tissue contains adipocytes as the major cell type, together with pre-adipocytes, mesenchymal stem cells, macrophages and other immune cells. Adipocytes are classified in white adipocytes, which store energy in the form of triglicerydes, and brown adipocytes, which produce heat by uncoupling the respiratory chain. There is a third adipocyte category, the beige or brite adipocytes which can be considered inducible brown-like cells with thermogenic properties, found in white fat depots. White and beige adipocytes proceed from the same mesenchymal stem cells (Rodríguez *et al.*, 2015).

3.1. White adipose tissue (WAT)

Mature white adipocytes (~70%), cells of the stromal vascular fraction (pre-adipocytes, fibroblast, endothelial cells) and immune cells, such as macrophages or lymphocytes, conform white adipose tissue (WAT) (Scherberich *et al.*, 2013). White adipocytes are cells with a peripheral nucleus, few mitochondria, thin cytoplasm and a big unilocular lipid droplet (Le Lay *et al.*, 2009). The main function of white adipocytes is energy storage, lipolysis and secretion (Rodríguez *et al.*, 2015). Thus, WAT is considered the main energy storage organ of the body, and also an endocrine organ since it secretes important regulatory molecules, the adipokines (Scherer, 2006). Adipokines are involved in the regulation of multiple metabolic functions including appetite, satiety, energy expenditure, insulin sensitivity and secretion, glucose and lipid metabolism and fat distribution, among others (Blüher, 2013). WAT is the main adipose tissue in adulthood and one of the largest organs. This organ has a highly variable weight between individuals. Changes on size (hypertrophy) and/or number (hyperplasia) of adipocytes are the main pathways to increase body fat accumulation (Rutkowski *et al.*, 2015; Virtue & Vidal-Puig, 2010). However, body fat distribution is important. Central adiposity increases the risk of mortality, type 2 diabetes and cardiovascular disease (Farb & Gokce, 2015).

3.2. Obesity and intestinal inflammation

Obesity is a low grade chronic inflammatory state where inflammation results in secondary diseases in the long run, and impacts the progression of other illnesses (Rocha & Folco, 2011). It was recognized as a disease in 1948 by the World Health Organization (WHO) (James, 2008). Actually, obesity has gained pandemic proportions (Rodríguez *et al.*, 2015). Obesity is characterized by increase of body fat, defined as an excess of adiposity, associated with the onset of other pathologies, such as type 2 diabetes, cardiovascular disease, rheumatoid arthritis and IBD (Rodríguez *et al.*, 2015).

During obesity, the excessive energy intake is accumulated in the adipocytes through hypertrophy and hyperplasia. In obesity, there is an altered secretory pattern with an increase in pro-inflammatory protein secretion, including monocyte chemoattractant protein-1 (MCP-1), IL-6 and TNF-α (Moreno-Aliaga *et al.*, 2010; Weisberg *et al.*, 2003). Moreover, T cells and macrophages activation and infiltration into the adipose tissue occurs (Kraakman *et al.*, 2014). These activated macrophages form a crown-like structure surrounding the adipocytes (Cinti, 2002). Finally, this pathological condition can develop a chronic systemic inflammation (Moller, 2000).

Obesity is associated with a worst diagnosis and development of IBD (Gonçalves *et al.*, 2015; Max *et al.*, 2013). Patients with IBD exhibit ectopic adipose tissue extending from the mesenteric depot along the intestinal surface. Dysfunctional inflamed mesenteric adipose tissue in obesity could also contribute to the chronic intestinal inflammation in IBD (Sideri *et al.*, 2015). Independently of body weight, a characteristic hyperplasia of the mesenteric fat-tissue, called creeping fat, appear in IBD patients (Kredel & Siegmund, 2014). In normal to mildly overweight healthy women, it was found a positive association between visceral adiposity and intestinal permeability (Gummesson *et al.*, 2011). Moreover, in obesity, high amount of pro-inflammatory adipokines, such as TNF-α, are secreted from the adipocytes and activates macrophages that produce intestinal inflammation (Moller, 2000). Also, it has been observed that TNF-α acts as link between obesity, insulin resistance and intestinal inflammation (Ding & Lund, 2011).

4. Macrophages

Macrophages are derived from monocytes, a type of white blood cells (WBCs). Monocytes are characterized by small cytoplasmic granules, kidney-shape nucleus and a blue-gray cytoplasm due to azurophilic lysosomes. There are tissue resident macrophages but, under a pathogen stimulus, monocytes migrate from the blood into the tissues, where they enlarge and differentiate into macrophages. Macrophages destroy microbes and clean up cellular debris by phagocytosis after infection.

Macrophages are classified as pro-inflammatory M1 macrophages and immuno-modulatory M2 macrophages (Mantovani *et al.*, 2013). During inflammation, M2 macrophages are activated and switch to M1, which produces high amount of pro-inflammatory cytokines.

4.1. Macrophages and intestinal inflammation

Macrophages are an important part of the innate immune system. Their role on intestinal mucosa is to remove debris, but they are also important in the pathophysiology of IBD (Bain & Mowat, 2014). In the small intestine, there are tissue resident M2 macrophages underneath the lamina propria (Mahida *et al.*, 1989) that can be activated and recruited to the intestinal lumen in the inflammatory process. Also, M1 macrophages are recruited from blood circulation (Peluso & Palmery, 2016; Weisberg *et al.*, 2003). The monocyte chemoattractant protein-1 (MCP-1) is related with the invading and differentiation of macrophages during IBD (Spoettl *et al.*, 2006). Moreover, during intestinal inflammation disease, there is increased secretion of pro-inflammatory cytokines such as TNF-α (Kamada *et al.*, 2008; Schenk *et al.*, 2007; Zareie *et al*., 2001). By contrast, in healthy intestinal mucosa, there is high number of M2 type macrophages with tolerogenic and inflammation-resolving properties (Lissner *et al.*, 2015; Zhu *et al.*, 2014).

5. Omega-3 polyunsaturated fatty acids (n-3 PUFAs)

The fatty acids contained in the diet are classified, attending to the number of double bound in the hydrocarbon chain, into saturated and unsaturated fatty acids. Saturated fatty acids do not contain double bonds in its chain. They are present in animal sources like meat. Unsaturated fatty acids are divided into monounsaturated (one double bond) and poly-unsaturated (more than one double bond) fatty acids. They are predominantly found in food derived from fish or plants such as olive oil, nuts and seeds.

Long chain omega-6 polyunsaturated fatty acids (n-6 PUFAs) have the first double bound in the 6th carbon of their chains. They are considered to have pro-inflammatory properties. Arachidonic acid (AA, 20:4, n-6) is a precursor of pro-inflammatory eicosanoids in the body (Marventano *et al.*, 2015).

Long chain omega-3 polyunsaturated fatty acids (n-3 PUFAs) receive this name because the first double bond is allocated in the 3rd carbon of their chains. The major n-3 PUFAs contained in fish oil (marine origin) are eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). They can be found in salmon, tuna, mackerel and anchovy. Alpha-linolenic acid (ALA, 18:3) is the most relevant n-3 PUFA from vegetal source (linseed, flaxseed oil, nuts). Since mammals cannot produce ALA, this is considered an essential fatty acid. After ALA is obtained from the diet, mammals can metabolize it to EPA and DHA. However, this rate of conversion is low and not enough to obtain the necessary levels of EPA and DHA for the organism; for that reason, it is required to obtain them from the diet (Burdge *et al.*, 2002; Burdge & Wootton, 2002).

Marine n-3 PUFAs have been widely recognized to exert favorable anti-inflammatory actions on inflammatory-related pathologies such as cardiovascular diseases, atherosclerosis, Alzheimer disease, asthma, arthritis, obesity and intestinal inflammation (Calder, 2015; Lorente-Cebrián et al., 2013; 2015; Martinez-Fernandez *et al.*, 2015). Nowadays, industrialized countries have increased the consumption of n-6 PUFAs contained in vegetable oils, such as maize and sunflower oils (Blasbalg *et al.*, 2011). However, the increase n-3 PUFA:n-6 PUFA, ratio protects against the development of IBD (Ananthakrishnan *et al.*, 2014; Pai *et al.*, 2014; Tjonneland *et al.*,

2009). Different international organizations have recognized n-3 PUFAs beneficial actions of reducing hypertriglyceridemia and cardiovascular risk (Kris-Etherton *et al.,* 2003; Lavie *et al.*, 2009; Mozaffarian & Wu, 2011). Based on that, the Food and Agriculture Organization (FAO) recommends a daily intake of 250 to 500 mg of EPA and DHA in healthy adults to prevent diseases (European Food Safety Authority, 2009; FAO, 2010); and the American Heart Association recommends 2-4 g/day of EPA and DHA supplements for hypertriglyceridemia treatment (Bradberry & Hilleman, 2013; Kris-Etherton *et al.*, 2003).

5.1. EPA

Eicosapentaenoic acid (EPA) is a marine n-3 PUFA (20:5, n-3) (Fig. 5) (Brinton & Mason, 2017) that was identified in 1952 (Klenk & Bongard, 1952). EPA has beneficial actions in obesity and insulin resistance by reducing TNF-α gene expression and increasing adiponectin secretion by adipocytes (Pérez-Matute *et al.*, 2007; Pérez-Echarri et al., 2008; 2009). In humans, EPA supplementation also regulates the production of important adipokines, such as leptin, and adipose tissue gene expression (Huerta et al., 2015; 2016; 2017). In a rat IBD model, PUFA-rich diet ameliorates inflammation (Hassan et al., 2010; Mbodji et al., 2013). Also, EPA has beneficial effects in other pathologies, such as cardiovascular disease, hypertriglyceridemia, gestational diabetes and osteoporosis (Breslow, 2006; Kruger *et al.*, 1998; Patel & Budoff, 2016; Yessoufou *et al.*, 2015).

5.2. DHA

Docohexaenoic acid (DHA) is a marine n-3 PUFA (22:6, n-3) (Fig. 5) (Wright *et al.*, 1987). In 1953, Hammond and Lundberg firstly purified DHA and determined its structure. Later, in 1960, the metabolic pathway of DHA conversion from ALA was determined (Klenk & Mohrhauer, 1960).

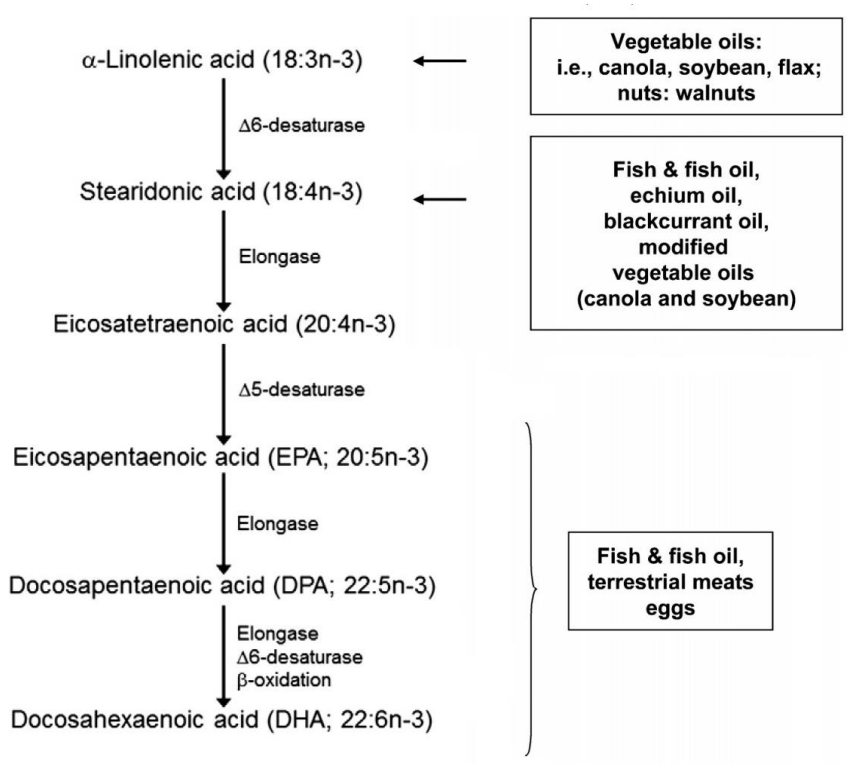


Figure 5. **Pathway of the biosynthesis of EPA and DHA (modified from Serham 2013).**

Several beneficial actions has also been reported for DHA on inflammatory diseases such as obesity, intestinal inflammation, cardiovascular disease and Alzheimer (Horrocks & Yeo, 1999; Swanson *et al.*, 2012; Yurko-Mauro *et al.*, 2010). DHA is the main structural fatty acid of the membranes in the central nervous system (Lauritzen et al. 2016). Moreover, DHA increase on membrane fluidity is higher than that produced by EPA (Hashimoto *et al.,* 1999). In this sense, it has been hypothesized that the incorporation of DHA-rich domains into the plasma membrane would be responsible, in part, of the health benefits from dietary consumption of DHA (Wassall & Stillwell 2008)

5.3. GPR120

GPR120, also known as free fatty acid receptor 4, is a cell-surface receptor, member of the G protein-coupled receptors (GPCRs) superfamily, which can bind unsaturated medium-to long chain fatty acids (Hirasawa *et al.*, 2005; Ichimura, Hara & Hirasawa, 2014). GPR120 is widely expressed in several organs, including intestine, and it is involved in several homeostatic functions such as inflammation, glucose homeostasis and insulin sensitivity (Ichimura, Hara & Hirasawa, 2014). It has been

observed that the anti-inflammatory effects of EPA and DHA are mediated by GPR120 acting as a sensor/receptor (Oh *et al.*, 2010). Thus, it has been proved that GPR120 stimulation by n-3 PUFAs inhibits inflammatory responses mediated by TNF-α (Calder, 2015; Oh & Lagakos, 2011). Nevertheless, EPA and DHA might act through three possible alternative mechanisms: 1) binding and activation of GPR120; 2) interfering with early membrane events involved in other receptors activation; 3) diffusing through the membrane and activation of PPAR-γ (Calder, 2015).

5.4. n-3 PUFAs and inflammation

There are strong evidences that n-3 PUFAs have anti-inflammatory properties by decreasing anti-inflammatory cytokines synthesis, stimulating M1 switch into M2 macrophages, reducing n-6 derived metabolites and promoting the formation of pro-resolutive lipid mediators (Barbalho *et al.,* 2016; Calder, 2013; Martínez-Fernández *et al.*, 2015).

In adipocytes, n-3 PUFAs down-regulate the production of pro-inflammatory adipocytokines including MCP-1, IL-6 and TNF-α, and induce the release of anti-inflammatory ones (Ding *et al.*, 2014; Figueras *et al.*, 2011; Kalupahana *et al.*, 2010; Kang & Weylandt, 2008; Pérez-Echarri *et al.*, 2008; Rossmeisl *et al.*, 2012; Sato *et al.*, 2010). Also, n-3 PUFAs reduce leukocyte chemotaxis (Todoric *et al.*, 2006) and decrease macrophage infiltration and activation. In Caco-2 cells and a mice colitis model, it was observed that DHA decreased NF-κB activation, which is involved in TNF-α signaling pathway (Anbazhagan *et al.*, 2016; Zhao *et al.*, 2017). In IL-10 KO mice (spontaneous model of colitis) feeding with fish oil significantly reduced colonic inflammation (Chapkin *et al.*, 2007). EPA and DHA reduce production of arachidonic acid-derived pro-inflammatory lipid mediators (Corey *et al.*, 1983; González-Périz & Clària, 2010; Hudert *et al.*, 2006; Needleman *et al.*, 1979). Furthermore, n-3 PUFAs can be enzymatically converted into specialized pro-resolving lipid mediators (SPMs) within the organism (Poulsen *et al.*, 2008; Serhan & Chiang, 2009; Serhan, 2007).

6. Specialized Pro-Resolving Lipid Mediators (SPMs)

EPA and DHA serve as substrates for the formation of specialized pro-resolving lipid mediators (SPMs), with potent anti-inflammatory and pro-resolving properties, namely resolvins (Rv), maresins (MaR) and protectins (PD). These bioactive lipid mediators are classified depending on their precursor. The E-series Resolvins (RvE1-3) derive from EPA and the D-series Resolvins (RvD1-6), Protectins (NPD1 and PDX), and Maresins (MaR1-2) derive from DHA (Serhan, 2014). These SPMs are produced by oxidation, epoxidation and peroxidation through 5-, 12- and 15- lipoxigenase (LOX) and the cyclooxygenase-2 (COX2) (Serhan, 2007) (see Fig. 6). T-series Resolvins derive from the n-3 PUFA docosapentaenoic acid (DPA), after aspirin or atorvastatin stimulation (Dalli *et al.*, 2015; Dalli *et al.*, 2013). Interestingly, the n-6 PUFA arachidonic acid, which is known to have pro-inflammatory actions, also serves as substrate for lipoxins, that promote resolution of inflammation (Börgeson *et al.*, 2015).

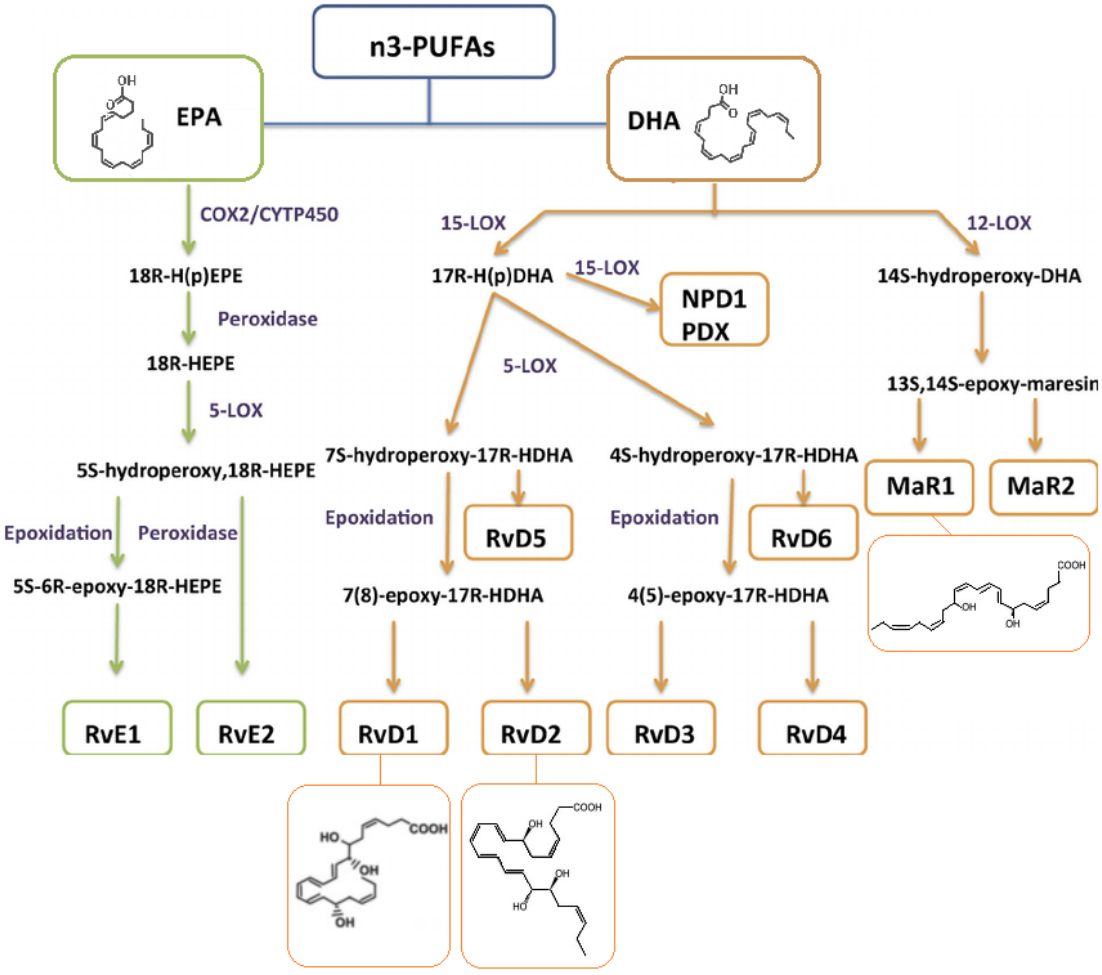


Figure 6. Human SPM biosynthetic routes from EPA and DHA (modified from Martínez-Fernández *et al.* 2015).

6.1. MaR1

"**Ma**crophage mediator in **res**olving **in**flammation" 1 or MaR1, derives from DHA via 12-LOX through macrophages participation (Fig. 7). MaR1 is the first member of the maresin family identified. It was discovered in mouse peritonitis exudates during the resolution of the inflammation (Serhan *et al.*, 2009). Maresins have been predominantly detected as products synthetized by monocytes/macrophages upon acute inflammatory response and related with strong anti-inflammatory effects and potent pro-resolutive actions (Serhan *et al.*, 2009), although they have pro-healing properties too (Serhan *et al.*, 2012; Tang *et al.,* 2013). MaR1 has also been found in joints synovial fluid from rheumatoid arthritis patients (Serhan & Chiang, 2013) and in a murine model of acute respiratory distress syndrome (Abdulnour et al., 2014).

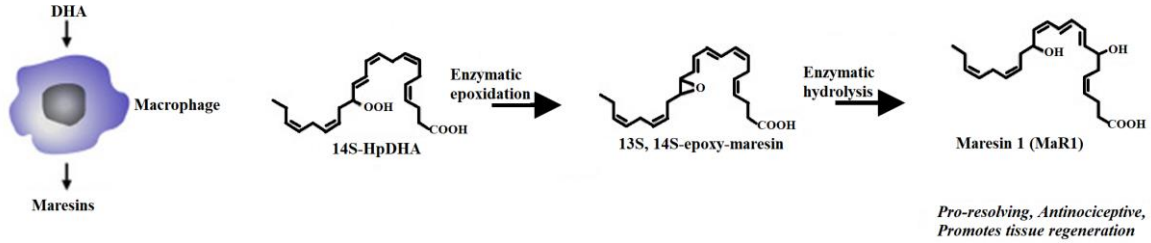


Figure 7. Maresin 1 biosynthetic pathway (modified from Serhan, 2017).

6.1.1. MaR1 and inflammation

Preclinical studies have reported important beneficial effects of MaR1 on inflammatory processes in endothelial cells, lung, brain, gut, kidney, liver and adipose tissue (Abdulnour *et al.*, 2014; Chatterjee *et al.*, 2014; Gong *et al.*, 2014, 2015; Laiglesia et al., 2017-J Cell Physiol; 2017-Int J. Obesity; Li *et al.*, 2016; Martínez-Fernández *et al.*, 2017; Nordgren *et al.,* 2015; Serhan *et al.*, 2012; Viola *et al*., 2016; Wang *et al.*, 2016). MaR1 mechanism of action includes decreasing neutrophil infiltration, increasing macrophage phagocytosis, inhibiting NF-κB activation as well as limiting pro-inflammatory cytokines production, among others (Chatterjee *et al.*, 2014; Dalli *et al*., 2016).

Interestingly, MaR1 seems to have a role in hemostasis, since a recent study has proved that it enhances the spreading and aggregation of platelets. These effects suggest that MaR1 promotes a favorable scenario for wound healing (Lannan *et al*., 2017). In relation with these data, it has been demonstrated that the production of

MaR1 by macrophages reduce the regeneration time and pain in mice (Serhan *et al.*, 2012). Given the importance of tissue regeneration in different diseases, it may be hypothesized that MaR1 would have an important function in wound healing as other SPMs such as RvD1-2 (Spite *et al.*, 2014; Tang *et al.*, 2013).

A recent study from our group has shown that MaR1 improve insulin sensitivity and attenuate WAT inflammation in obese mice (Martínez-Fernández *et al.*, 2017), suggesting that MaR1 might be an attractive therapeutic approach to counteract the dysfunctional inflamed WAT and the subsequent insulin resistance associated to obesity. In this context, MaR1 was able to ameliorate TNF-α-induced alterations on lipolysis and to reduce autophagy in 3T3-L1 adipocytes and liver steatosis (Laiglesia *et al.,* 2017a, 2017b). In a mice model of colitis, MaR1 treatment improved the mucosa barrier due to reduction on pro-inflammatory markers (including TNF-α) and inhibition of NF-κB pathway (Marcon *et al*., 2013). Remarkably, Caco-2 cells exposed to LPS can also produce MaR1 (Le Faouder *et al*., 2013).

6.2. Resolvins (RvD1 and RvD2)

Resolvins are defined by their potent actions as bioactive local mediators (autacoids). RvD1 and RvD2 received the name from resolvins of the D series 1 or 2 (Serhan, 2007). Resolvins means resolution-phase interaction products. The name makes reference to biomolecules that are endogenous mediators, synthesized during the resolution phase of inflammation, that have potent anti-inflammatory and immunoregulatory actions (Serhan *et al.*, 2002). Their functions include reduction of neutrophil traffic, cytokine synthesis and reactive oxygen species production, and induction of M1 macrophages differentiation towards M2 phenotype,(Serhan, 2007). Efferocytosis increases SPM biosynthesis, including RvD1 and RvD2 (Norling *et al.*, 2012). RvD1 and RvD2 modulate inflammatory pain (Xu *et al.*, 2010). GPR32 acts as RvD1 receptor (Spite *et al.*, 2014) and GPR18 as RvD2 receptor (Figure 8, Chiang *et al.*, 2017).

6.2.1. Resolvins and inflammation

Resolvins and SPMs are locally produced and act as autacoids to terminate acute inflammatory responses, but circulating levels of these molecules at the concentration ranges for anti-inflammatory and pro-resolving actions, have been found (Mas *et al.*, 2012). RvD1 reduces neuroinflammation produced in Alzheimer's disease through GPR32 (Mizwicki *et al*., 2013). It also reduces immune cells infiltration and promotes M1 macrophages switch to M2 in smoke-induced lung inflammation mice (Titos *et al*. 2011; Hsiao *et al.*, 2013). Intravenous administration of RvD1 and RvD2 to a mice model of colitis, reduces pro-inflammatory cytokines secretion in the gut (Bento *et al.*, 2011). Interestingly, Lee *et al*. (2017) found, in intestinal mucosa of mice with induced-colitis, increased levels of RvD1, together with decreased levels of its precursor DHA, suggesting initiation of healing by endogenous lipids.

RvD1 promotes the secretion of anti-inflammatory cytokines in human visceral adipose tissue from obese subjects (Titos *et al*., 2016). In obese mice with non-alcoholic steatohepatitis, RvD1 increases the hepatic resolution process by reducing the inflammatory component (Rius *et al*., 2014). RvD1 and RvD2 also counteract both local pro-inflammatory adipokine production and monocyte accumulation in obesity-induced adipose tissue inflammation (Clària *et al.,* 2012).

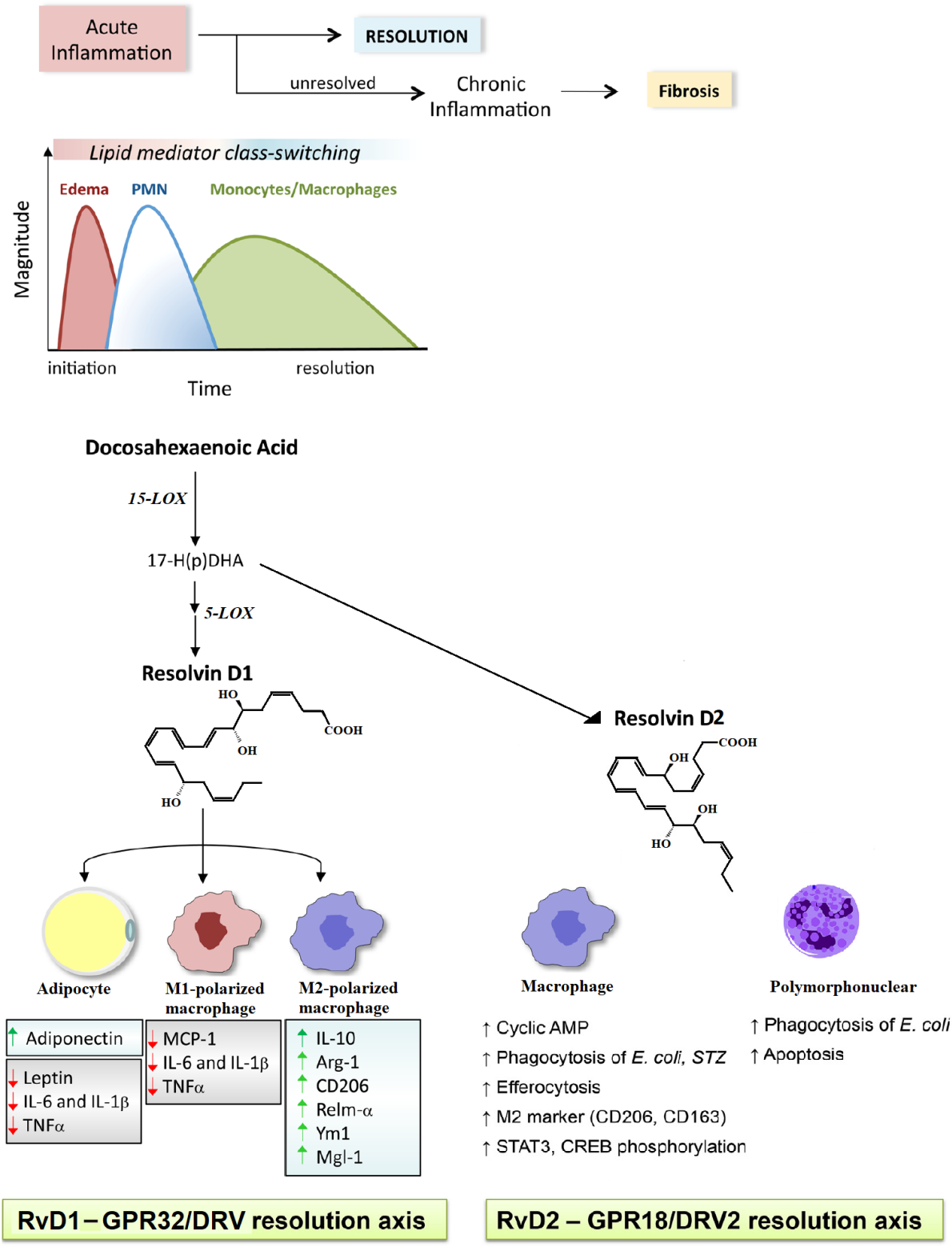


Figure 8. RvD1 and RvD2 in inflammatory resolution (modified from Chiang & Serhan, 2017; Spite *et al.*, 2014).

References

A

Abdulnour, R.E., Dalli, J., Colby, J. K., Krishnamoorthy, N., Timmons, J. Y., Tan, S. H., Colas, R.A., Petasis, N.A., Serhan, C.N. & Levy, B. D. (2014). Maresin 1 biosynthesis during platelet–neutrophil interactions is organ-protective. *Proceedings of the National Academy of Sciences* ***111*** (46), 16526–16531.

Albenberg, L. G., Lewis, J. D. & Wu, G. D. (2012). Food and Gut Microbiota in IBD: A Critical Connection. *Current Opinion in Gastroenterology* ***28*** (4), 314–320.

Ananthakrishnan, A. N., Khalili, H., Konijeti, G. G., Higuchi, L. M., de Silva, P., Fuchs, C. S., Willett, W.C., Richter, J.M. & Chan, A. T. (2014). Long-term intake of dietary fat and risk of ulcerative colitis and Crohn's disease. *Gut* ***63*** (5), 776–784.

Anbazhagan, A. N., Priyamvada, S., Gujral, T., Bhattacharyya, S., Alrefai, W. A., Dudeja, P. K. & Borthakur, A. (2016). A Novel Anti-inflammatory Role of GPR120 in Intestinal Epithelial Cells. *American Journal of Physiology - Cell Physiology* **310** (7), C612-21.

Argollo, M., Fiorino, G., Hindryck, P., Peyrin-Biroulet, L. & Danese, S. (2017). Novel therapeutic targets for inflammatory bowel disease. *Journal of Autoimmunity*.

B

Bain, C. C. & Mowat, A. M. (2014). Macrophages in intestinal homeostasis and inflammation. *Immunological Reviews* **260** (1), 102-17.

Barbalho, S.M., de Alvares Goulart, R., Quesada, K., Dib Bechara, M. & de Cássio Alves de Carvalho, A. (2016). Inflammatory bowel disease : can omega-3 fatty acids really help? *Annals of Gastroenterology* ***29*** (1), 37–43.

Barrenetxe, J., Sánchez, O., Barber, A., Gascón, S., Rodríguez-Yoldi, M. J. & Lostao, M. P. (2013). TNFα regulates sugar transporters in the human intestinal epithelial cell line Caco-2. *Cytokine* ***64*** (1), 181–187.

Bento, A. F., Claudino, R. F., Dutra, R. C., Marcon, R. & Calixto, J. B. (2011). Omega-3 Fatty Acid-Derived Mediators 17(R)-Hydroxy Docosahexaenoic Acid, Aspirin-Triggered Resolvin D1 and Resolvin D2 Prevent Experimental Colitis in Mice. *The Journal of Immunology* ***187*** (4), 1957–1969.

Bertolo, C., Barrenetxe, J., Barber, A. & Lostao, M.P. (2002). Cytokine effect on intestinal galactose absorption. *Journal of Physiology and Biochemistry* ***58*** (1), 61–62.

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J. & Cerretti, D. P. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature* ***385*** (6618), 729–733.

Blasbalg, T. L., Hibbeln, J. R., Ramsden, C. E., Majchrzak, S. F. & Rawlings, R. R. (2011). Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. *American Journal of Clinical Nutrition* ***93*** (5), 950–962.

Blüher, M. (2013). Adipose tissue dysfunction contributes to obesity related metabolic diseases. *Best Practice & Research Clinical Endocrinology & Metabolism* ***27*** (2), 163–77.

Bogatikov, E., Munoz, C., Pakladok, T., Alesutan, I., Shojaiefard, M., Seebohm, G., Föller, M., Palmada, M., Böhmer, C., Bröer, S. & Lang, F. (2012). Up-regulation of amino acid transporter SLC6A19 activity and surface protein abundance by PKB/Akt and PIKfyve. *Cellular Physiology and Biochemistry* ***30*** (6), 1538–1546.

Börgeson, E., Johnson, A. M. F., Lee, Y. S., Till, A., Syed, G. H., Ali-Shah, S. T., Guiry, P.J., Dalli, J., Colas, R.A., Serhan, C.N. & Sharma K Godson, C. (2015). Lipoxin A4 Attenuates Obesity-Induced Adipose Inflammation and Associated Liver and Kidney Disease. *Cell Metabolism* ***22*** (1), 125–37.

Bradberry, J. C. & Hilleman, D. E. (2013). Overview of omega-3 Fatty Acid therapies. *P & T : A Peer-Reviewed Journal for Formulary Management* ***38*** (11), 681–91.

Bradley, J. R. (2008). TNF-mediated inflammatory disease. *The Journal of Pathology* ***214***, 149–160.

Breslow, J. L. (2006). N-3 Fatty Acids and Cardiovascular Disease. *The American Journal of Clinical Nutrition* ***83*** (6 Suppl), 1477S–1482S.

Brinton, E. A. & Mason, R. P. (2017). Prescription omega-3 fatty acid products containing highly purified eicosapentaenoic acid (EPA). *Lipids in Health and Disease* ***16*** (1), 23.

Bröer, A., Klingel, K., Kowalczuk, S., Rasko, J. E. J., Cavanaugh, J. & Bröer, S. (2004). Molecular cloning of mouse amino acid transport system B0, a neutral amino acid transporter related to Hartnup disorder. *Journal of Biological Chemistry* ***279*** (23), 24467–24476.

Bröer, S. (2008). Amino acid transport across mammalian intestinal and renal epithelia. *Physiological Reviews* ***88*** (1), 249–86.

Burdge, G. C., Jones, A. E. & Wootton, S. A. (2002). Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men*. *British Journal of Nutrition* ***88*** (4), 355–363.

Burdge, G. C. & Wootton, S. A. (2002). Conversion of α-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. *British Journal of Nutrition* **88** (4), 411.

C

Cabal-Hierro, L. & Lazo, P. S. (2012). Signal transduction by tumor necrosis factor receptors. *Cellular Signalling* **24** (6), 1297-305.

Calder, P. C. (2013). Omega-3 polyunsaturated fatty acids and inflammatory processes: Nutrition or pharmacology? *British Journal of Clinical Pharmacology* ***75*** (3), 645–662.

Calder, P. C. (2015). Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. *BBA - Molecular and Cell Biology of Lipids* ***1851*** (4), 469–484.

Camargo, S. M. R., Singer, D., Makrides, V., Huggel, K., Pos, K. M., Wagner, C. A., Kuba, K., Danilczyk, U., Skovby, F., Kleta, R., Penninger, J.M. & Verrey, F. (2009). Tissue-Specific Amino Acid Transporter Partners ACE2 and Collectrin Differentially Interact With Hartnup Mutations. *Gastroenterology* ***136*** (3).

Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. & Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors (activated macrophage). *Immunology* ***72*** (9), 3666–3670.

Chapkin, R. S., Davidson, L. a, Ly, L., Weeks, B. R., Lupton, J. R. & McMurray, D. N. (2007). Immunomodulatory effects of (n-3) fatty acids: putative link to inflammation and colon cancer. *The Journal of Nutrition* ***137*** (1 Suppl), 200S–204S.

Chatterjee, A., Sharma, A., Chen, M., Toy, R., Mottola, G. & Conte, M. S. (2014). The pro-resolving lipid mediator maresin 1 (MaR1) attenuates inflammatory signaling pathways in vascular smooth muscle and endothelial cells. *PLoS ONE* ***9*** (11).

Chiang, N., de la Rosa, X., Libreros, S. & Serhan, C. N. (2017). Novel Resolvin D2 Receptor Axis in Infectious Inflammation. *The Journal of Immunology* ***198*** (2), 842–851.

Chiang, N. & Serhan, C. N. (2017). Structural elucidation and physiologic functions of specialized pro-resolving mediators and their receptors. *Molecular Aspects of Medicine*. https://doi.org/10.1016/j.mam.2017.03.005

Chun, H., Sasaki, M., Fujiyama, Y. & Bamba, T. (1997). Effect of enteral glutamine on intestinal permeability and bacterial translocation after abdominal radiation injury in rats. *Journal of Gastroenterology* ***32*** (2), 189–195.

Cinti, S. (2002). Adipocyte differentiation and transdifferentiation: Plasticity of the adipose organ. *Journal of Endocrinological Investigation* **25** (10):823-35.

Corey, E. J., Shih, C. & Cashman, J. R. (1983). Docosahexaenoic acid is a strong inhibitor of prostaglandin but not leukotriene biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* ***80*** (12), 3581–3584.

Croasdell, A., Thatcher, T. H., Kottmann, R. M., Colas, R. a, Dalli, J., Serhan, C. N., Sime, P.J. & Phipps, R. P. (2015). Resolvins attenuate inflammation and promote resolution in cigarette smoke-exposed human macrophages. *American Journal of Physiology - Lung Cellular and Molecular Physiology* ***309*** (1), L888–L901.

D

Dalli, J., Chiang, N. & Serhan, C. N. (2015). Elucidation of novel 13-series resolvins that increase with atorvastatin and clear infections. *Nature Medicine* ***21*** (9), 1071–5.

Dalli, J., Colas, R. A. & Serhan, C. N. (2013). Novel n-3 Immunoresolvents: Structures and Actions. *Scientific Reports* ***3*** (1), 1940.

Dalli, J., Vlasakov, I., Riley, I. R., Rodriguez, A. R., Spur, B. W., Petasis, N. A., Chiang, N. & Serhan, C. N. (2016). Maresin conjugates in tissue regeneration biosynthesis enzymes in human macrophages. *Proceedings of the National Academy of Sciences* ***113*** (43), 12232–12237.

Díez-Sampedro, A., Lostao, M. P., Wright, E. M. & Hirayama, B. A. (2000). Glycoside binding and translocation in Na^+-dependent glucose cotransporters: Comparison of SGLT1 and SGLT3. *Journal of Membrane Biology* ***176*** (2), 111–117.

Ding, S. & Lund, P. K. (2011). Role of intestinal inflammation as an early event in obesity and insulin resistance. *Current Opinion in Clinical Nutrition and Metabolism Care* ***14*** (4), 328–333.

Ding, W. J., Wang, Y. & Fan, J. G. (2014). Regulation of adipokines by polyunsaturated fatty acids in a rat model of non-alcoholic steatohepatitis. *Archives of Iranian Medicine* ***17*** (8), 563–568.

Douard, V. & Ferraris, R. P. (2008). Regulation of the fructose transporter GLUT5 in health and disease. *AJP: Endocrinology and Metabolism* ***295*** (2), E227–E237.

E

European Food Safety Authority. (2009). Scientific Opinion on the substantiation of health claims related to EPA , DHA , DPA and maintenance of normal blood pressure (ID 502), maintenance of normal HDL-cholesterol concentrations (ID 515), maintenance of normal (fasting) blood concentratio. *EFSA Journal **7*** (1924), 1–26.

F

Fairweather, S. J., Bröer, A., O'Mara, M. L. & Bröer, S. (2012). Intestinal peptidases form functional complexes with the neutral amino acid transporter B ^{0} AT1. *Biochemical Journal **446*** (1), 135–148.

FAO, W. (2010). Fats and fatty acids in human nutrition. Report of an expert consultation. *FAO Food Nutrition* Paper **91**: 1-166

Farb, M. G. & Gokce, N. (2015). Visceral adiposopathy: A vascular perspective. *Hormone Molecular Biology and Clinical Investigation* **21** (2): 125-136.

Ferrari, R. (1999). The role of TNF in cardiovascular disease. *Pharmacological Research **40*** (2), 97–105.

Figueras, M., Olivan, M., Busquets, S., López-soriano, F. J. & Argilés, J. M. (2011). Effects of eicosapentaenoic acid (EPA) treatment on insulin sensitivity in an animal model of diabetes: improvement of the inflammatory status. *Obesity **19*** (2), 362–9.

Foley, K. F., Pantano, C., Ciolino, A. & Mawe, G. M. (2007). IFN-gamma and TNF-αlpha decrease serotonin transporter function and expression in Caco2 cells. *American Journal of Physiology Gastrointestinal and Liver Physiology **292*** (3), G779-84.

G

Gonçalves, P., Magro, F. & Martel, F. (2015). Metabolic Inflammation in Inflammatory Bowel Disease: Crosstalk Between Adipose Tissue and Bowel. *Inflammatory Bowel Diseases **21*** (2), 453–467.

Gong, J., Liu, H., Wu, J., Qi, H., Wu, Z., Shu, H.Q., Li, H.B., Chen, L., Wang, Y.X., Li, B., Tang, M., Ji, Y.D., Yuan, S.Y., Yao, S.L. & Shang, Y. (2015). Maresin 1 Prevents Lipopolysaccharide- Induced Neutrophil Survival and Accelerates Resolution of Acute Lung Injury. *Shock* ***44*** (4), 1.

Gong, J., Wu, Z.-Y., Qi, H., Chen, L., Li, H.-B., Li, B., Yao, B., Wang, Y., Wu, J., Yuan, S., Yao, S. & Shang, Y. (2014). Maresin 1 mitigates lipopolysaccharide-induced acute lung injury in mice. *British Journal of Pharmacology* **171** (14): 3539-3550.

González-Périz, A. & Clària, J. (2010). Resolution of adipose tissue inflammation. *The Scientific World Journal*, ***10***, 832–856.

Grine, L., Dejager, L., Libert, C. & Vandenbroucke, R. E. (2015). An inflammatory triangle in psoriasis: TNF, type I IFNs and IL-17. *Cytokine and Growth Factor Reviews* ***26*** (1), 25–33.

Gummesson, A., Carlsson, L. M. S., Storlien, L. H., Bäckhed, F., Lundin, P., Löfgren, L., Stenlöf, K., Lam, Y.Y., Fagerberg, B. & Carlsson, B. (2011). Intestinal Permeability Is Associated With Visceral Adiposity in Healthy Women. *Obesity* ***19*** (11), 2280–2282.

H

Hammond, E. G. & Lundberg, W. O. (1953). A methyl docosahexaenoate: Its isolation and characterization. *Journal of the American Oil Chemists Society* ***30*** (11), 438–441.

Hashimoto, M., Hossain, S., Yamasaki, H., Yazawa, K., Masumura, S. (1999). Effects of eicosapentaenoic acid and docosahexaenoic acid on plasma membrane fluidity of aortic endothelial cells. *Lipids* **34** (12): 1297-304.

Hassan, A., Ibrahim, A., Mbodji, K., Coëffier, M., Ziegler, F., Bounoure, F., Chardigny, J.M., Skiba, M., Savoye, G., Déchelotte, P. & Marion-Letellier, R. (2010). An α-linolenic acid-rich formula reduces oxidative stress and inflammation by regulating NF-κB in rats with TNBS-induced colitis. *The Journal of Nutrition* ***140*** (10), 1714–21.

Hébuterne, X., Filippi, J., Al-Jaouni, R. & Schneider, S. (2009). Nutritional consequences and nutrition therapy in Crohn's disease. *Gastroentérologie Clinique et Biologique* **33** Suppl 3, S235-44.

Hediger, M. a, Turk, E., & Wright, E. M. (1989). Homology of the human intestinal Na^+/glucose and Escherichia coli Na^+/proline cotransporters. *Proceedings of the National Academy of Sciences of the United States of America* ***86*** (15), 5748–52.

Hirasawa, A., Tsumaya, K., Awaji, T., Katsuma, S., Adachi, T., Yamada, M., Sugimoto, Y., Miyazaki, S. & Tsujimoto, G. (2005). Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nature Medicine* ***11*** (1), 90–94.

Horiuchi, T., Mitoma, H., Harashima, S. I., Tsukamoto, H. & Shimoda, T. (2010). Transmembrane TNF-α: Structure, function and interaction with anti-TNF agents. *Rheumatology* ***49*** (7), 1215–1228.

Horrocks, L. a & Yeo, Y. K. (1999). Health benefits of docosahexaenoic acid (DHA). *Pharmacological Research : The Official Journal of the Italian Pharmacological Society* ***40*** (3), 211–225.

Hsiao, H. M., Sapinoro, R. E., Thatcher, T. H., Croasdell, A., Levy, E. P., Fulton, R. A., Olsen, K.C., Pollock, S.J., Serhan, C.N., Phipps, R.P. & Sime, P. J. (2013). A Novel Anti-Inflammatory and Pro-Resolving Role for Resolvin D1 in Acute Cigarette Smoke-Induced Lung Inflammation. *PLoS ONE* ***8*** (3).

Hudert, C. A., Weylandt, K. H., Lu, Y., Wang, J., Hong, S., Dignass, A., Serhan, C.N. & Kang, J. X. (2006). Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. *Proceedings of the National Academy of Sciences of the United States of America* ***103*** (30), 11276–81.

Huerta, A.E., Navas-Carretero, S., Prieto-Hontoria, P.L., Martínez, J.A., Moreno-Aliaga, M.J. (2015). Effects of α-lipoic acid and eicosapentaenoic acid in overweight and obese women during weight loss. *Obesity* **23** (2):313-21.

Huerta, A.E., Prieto-Hontoria, P.L., Sáinz, N., Martínez, J.A., Moreno-Aliaga, M.J. (2016). Supplementation with α-Lipoic Acid Alone or in Combination with Eicosapentaenoic Acid Modulates the Inflammatory Status of Healthy Overweight or Obese Women Consuming an Energy-Restricted Diet. *Journal of Nutrition*: jn224105.

Huerta, A.E., Prieto-Hontoria, P.L., Fernández-Galilea, M., Escoté, X., Martínez, J.A. & Moreno-Aliaga, M.J. (2017). Effects of dietary supplementation with EPA and/or α-lipoic acid on adipose tissue transcriptomic profile of healthy overweight/obese women following a hypocaloric diet. *Biofactors* **43** (1):117-131.

I

Ichimura, A., Hara, T. & Hirasawa, A. (2014). Regulation of energy homeostasis via GPR120. *Frontiers in Endocrinology **5***, 1–5.

J

James, W. P. T. (2008). WHO recognition of the global obesity epidemic. *International Journal of Obesity **32***, S120–S126.

K

Kalupahana, N. S., Claycombe, K., Newman, S. J., Stewart, T., Siriwardhana, N., Matthan, N., Lichtenstein, A.H. & Moustaid-Moussa, N. (2010). Eicosapentaenoic acid prevents and reverses insulin resistance in high-fat diet-induced obese mice via modulation of adipose tissue inflammation. *The Journal of Nutrition* **140** (11): 1915-1922.

Kamada, N., Hisamatsu, T., Okamoto, S., Chinen, H., Kobayashi, T., Sato, T., Sakuraba, A., Kitazume, M.T., Sugita, A., Koganei, K., Akagawa, K.S. & Hibi, T. (2008). Unique CD14+ intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-γ axis. *The Journal of Clinical Investigation **118*** (6), 2269–2280.

Kang, J. X. & Weylandt, K. H. (2008). Modulation of inflammatory cytokines by omega-3 fatty acids. *Sub-Cellular Biochemistry **49***, 133–143.

Karasov, W. H. (2017). Integrative physiology of transcellular and paracellular intestinal absorption. *The Journal of Experimental Biology* ***220*** (14), 2495–2501.

Kellett, G. L. & Helliwell, P. A. (2000). The diffusive component of intestinal glucose absorption is mediated by the glucose-induced recruitment of GLUT2 to the brush-border membrane. *The Biochemical Journal* ***350*** (1), 155–62.

Klenk, E. & H. Mohrhauer. 1960. Studies on the metabolism of polyenoic fatty acids in the rat. *Hoppe Seylers Zeitschrift fur physiologische Chemie*. **320**: 218 – 232.

Klenk , E. & W. Bongard . 1952. Constitution of the unsaturated C20 and C22 fatty acids of the glycophosphatides of the brain (Die constitution der ungesättigten C 20- und C 22-fettsäuren der glycerinphosphatide des gehirns [Article in German]). *Hoppe Seylers Zeitschrift fur physiologische Chemie*. **291**: 104 – 118.

Kowalczuk, S., Bröer, A., Tietze, N., Vanslambrouck, J. M., Rasko, J. E. J. & Bröer, S. (2008). A protein complex in the brush-border membrane explains a Hartnup disorder allele. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* ***22*** (8), 2880–7.

Kraakman, M. J., Murphy, A. J., Jandeleit-Dahm, K. & Kammoun, H. L. (2014). Macrophage polarization in obesity and type 2 diabetes: Weighing down our understanding of macrophage function? *Frontiers in Immunology* ***5*****,** 470.

Kredel, L. I. & Siegmund, B. (2014). Adipose-tissue and intestinal inflammation - visceral obesity and creeping fat. *Frontiers in Immunology* ***5***, 1–12.

Kris-Etherton, P. M., Harris, W.S. & Appel, L. J. (2003). Omega-3 Fatty Acids and Cardiovascular Disease: New Recommendations From the American Heart Association. *Journal of the American Heart Association* ***23*** (2), 151–152.

Kruger, M. C., Coetzer, H., de Winter, R., Gericke, G. & van Papendorp, D. H. (1998). Calcium, gamma-linolenic acid and eicosapentaenoic acid supplementation in senile osteoporosis. *Aging* ***10*** (5), 385–394.

L

Lannan, K. L., Spinelli, S. L., Blumberg, N. & Phipps, R. P. (2017). Maresin 1 induces a novel pro-resolving phenotype in human platelets. *Journal of Thrombosis and Haemostasis* ***15*** (4), 802–813.

Lavie, C. J., Milani, R. V., Mehra, M. R. & Ventura, H. O. (2009). Omega-3 Polyunsaturated Fatty Acids and Cardiovascular Diseases. *Journal of the American College of Cardiology* **54** (7), 585-594.

Lauritzen, L., Brambilla, P., Mazzocchi, A., Harsløf, L.B., Ciappolino, V. & Agostoni, C. (2016). DHA Effects in Brain Development and Function. *Nutrients* **8** (1). pii: E6.

Le Faouder, P., Baillif, V., Spreadbury, I., Motta, J. P., Rousset, P., Chêne, G., Guigné, C., Tercé, F., Vanner, S., Vergnolle, N., Bertrand-Michel, J., Dubourdeau, M. & Cenac, N. (2013). LC-MS/MS method for rapid and concomitant quantification of pro-inflammatory and pro-resolving polyunsaturated fatty acid metabolites. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* ***932***, 123–133.

Le Lay, S., M. Blouin, C., Hajduch, E. & Dugail, I. (2009). Filling up adipocytes with lipids. Lessons from caveolin-1 deficiency. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **1791** (6), 514-518.

Lee, Y., Choo, J., Kim, S. J., Heo, G., Pothoulakis, C., Kim, Y. H. & Im, E. (2017). Analysis of endogenous lipids during intestinal wound healing. *PLoS ONE* ***12*** (8), 1–23.

Lenaerts, K., Mariman, E., Bouwman, F. & Renes, J. (2005). Differentiation stage-dependent preferred uptake of basolateral (systemic) glutamine into Caco-2 cells results in its accumulation in proteins with a role in cell-cell interaction. *FEBS Journal* ***272*** (13), 3350–3364.

Leppkes, M., Roulis, M., Neurath, M. F., Kollias, G. & Becker, C. (2014). Pleiotropic functions of TNF-α in the regulation of the intestinal epithelial response to inflammation. *International Immunology* ***26*** (9), 509–515.

Li, R., Wang, Y., Zhao, E., Wu, K., Li, W., Shi, L., Wang, D., Xie, G., Yin, Y., Deng, M., Zhang, P. & Tao, K. (2016). Maresin 1, a Proresolving Lipid Mediator, Mitigates Carbon Tetrachloride-Induced Liver Injury in Mice. *Oxidative Medicine and Cellular Longevity 2016*. https://doi.org/10.1155/2016/9203716

Lissner, D., Schumann, M., Batra, A., Kredel, L.-I., Kühl, A. A., Erben, U., May, C., Schulzke, J.D. & Siegmund, B. (2015). Monocyte and M1 Macrophage-induced Barrier Defect Contributes to Chronic Intestinal Inflammation in IBD. *Inflammatory Bowel Diseases* **21** (6), 1297–13051.

Lorente-Cebrián, S., Costa, A. G. V, Navas-Carretero, S., Zabala, M., Martínez, J. A. and Moreno-Aliaga, M. J. (2013). Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: A review of the evidence. *Journal of Physiology and Biochemistry* **69** (3), 633–651.

Lorente-Cebrián, S., Costa, A. G. V, Navas-Carretero, S., Zabala, M., Laiglesia, L. M., Martínez, J. A. and Moreno-Aliaga, M. J. (2015). An update on the role of omega-3 fatty acids on inflammatory and degenerative diseases. *Journal of Physiology and Biochemistry* **71** (2), 341–349.

M

Mahida, Y. R., Patel, S., Gionchetti, P., Vaux, D. & Jewell, D. P. (1989). Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. *Gut **30*** (6), 826–834.

Manara, M. & Sinigaglia, L. (2015). Bone and TNF in rheumatoid arthritis: clinical implications. *RMD Open **1*** (Suppl 1), e000065.

Mantovani, A., Biswas, S. K., Galdiero, M. R., Sica, A. & Locati, M. (2013). Macrophage plasticity and polarization in tissue repair and remodelling. *Journal of Pathology **229*** (2), 176–185.

Marcon, R., Bento, A. F., Dutra, R. C., Bicca, M. a, Leite, D. F. P. & Calixto, J. B. (2013). Maresin 1, a proresolving lipid mediator derived from omega-3 polyunsaturated Fatty acids, exerts protective actions in murine models of colitis. *Journal of Immunology **191*** (8), 4288–98.

Margheritis, E., Imperiali, F. G., Cinquetti, R., Vollero, A., Terova, G., Rimoldi, S., Girardello, R. & Bossi, E. (2016). Amino acid transporter B^0AT1 (slc6a19) and ancillary protein: impact on function. *Pflugers Archiv European Journal of Physiology* ***468*** (8), 1363–1374.

Martínez-Fernández, L., González-Muniesa, P., Laiglesia, L.M., Sáinz, N., Prieto-Hontoria, P.L., Escoté, X., Odriozola, L., Corrales, F.J., Arbones-Mainar, J.M., Martínez, J.A. & Moreno-Aliaga, M.J. (2017). Maresin 1 improves insulin sensitivity and attenuates adipose tissue inflammation in ob/ob and diet-induced obese mice. *FASEB Journal* ***31*** (5), 2135–2145.

Martinez-Fernandez, L., Laiglesia, L. M., Huerta, A. E., Martinez, J. A. & Moreno-Aliaga, M. J. (2015). Omega-3 fatty acids and adipose tissue function in obesity and metabolic syndrome. *Prostaglandins & Other Lipid Mediators* ***121***, 1–18.

Marventano, S., Kolacz, P., Castellano, S., Galvano, F., Buscemi, S., Mistretta, A. & Grosso, G. (2015). A review of recent evidence in human studies of n-3 and n-6 PUFA intake on cardiovascular disease, cancer, and depressive disorders: does the ratio really matter? *International Journal of Food Sciences and Nutrition* ***66*** (6), 611–22.

Mas, E., Croft, K. D., Zahra, P., Barden, A. & Mori, T. A. (2012). Resolvins D1, D2, and other mediators of self-limited resolution of inflammation in human blood following n-3 fatty acid supplementation. *Clinical Chemistry* ***58*** (10), 1476–1484.

Max, J. B., Stidham, R., Su, G. L. & Waljee, A. K. (2013). Obesity and IBD: Are We Tipping the Scales Toward an Epidemic? *Gastroenterology*, ***145*** **(**2), 478–479.

Mbodji, K., Charpentier, C., Guérin, C., Querec, C., Bole-Feysot, C., Aziz, M., Savoye, G., Déchelotte, P. & Marion-Letellier, R. (2013). Adjunct therapy of n-3 fatty acids to 5-ASA ameliorates inflammatory score and decreases NF-κB in rats with TNBS-induced colitis. *Journal of Nutritional Biochemistry* ***24*** (4), 700–705.

Michlewska, S., Dransfield, I., Megson, I. L. & Rossi, A. G. (2009). Macrophage phagocytosis of apoptotic neutrophils is critically regulated by the opposing actions of pro-inflammatory and anti-inflammatory agents: key role for TNF-α. *The FASEB Journal* ***23*** (3), 844–854.

Mizwicki, M. T., Liu, G., Fiala, M., Magpantay, L., Sayre, J., Siani, A., Mahanian, M., Weitzman, R., Hayden, E.Y., Rosenthal, M.J., Nemere, I., Ringman, J. & Teplow, D. B. (2013). 1α,25-dihydroxyvitamin D3 and resolvin D1 retune the balance between amyloid-β phagocytosis and inflammation in Alzheimer's disease patients. *Journal of Alzheimer's Disease* ***34*** (1), 155–170.

Moller, D. E. (2000). Potential role of TNF-αlpha in the pathogenesis of insulin resistance and type 2 diabetes. *Trends in Endocrinology and Metabolism* ***11*** (6), 212–7.

Moreno-Aliaga, M. J., Lorente-Cebrián, S. & Martínez, J. A. (2010). Regulation of adipokine secretion by n-3 fatty acids. *The Proceedings of the Nutrition Society* ***69*** (3), 324–332.

Mozaffarian, D. & Wu, J. H. Y. (2011). Omega-3 fatty acids and cardiovascular disease: Effects on risk factors, molecular pathways, and clinical events. *Journal of the American College of Cardiology* ***58*** (20), 2047–2067.

Mueckler, M. & Thorens, B. (2013). The SLC2 (GLUT) family of membrane transporters. *Molecular Aspects of Medicine* **34** (2-3), 121-38.

Munck, B. G. & Munck, L. K. (1999). Effects of pH changes on systems ASC and B in rabbit ileum. *The American Journal of Physiology* ***276*** (1), G173-84.

N

Needleman, P., Raz, A., Minkes, M. S., Ferrendelli, J. A. & Sprecher, H. (1979). Triene prostaglandins: prostacyclin and thromboxane biosynthesis and unique biological properties. *Proceedings of the National Academy of Sciences of the United States of America* ***76*** **(**2), 944–8.

Nordgren, T. M., Bauer, C. D., Heires, A. J., Poole, J. A., Wyatt, T. A., West, W. W. & Romberger, D. J. (2015). Maresin-1 reduces airway inflammation associated with acute and repetitive exposures to organic dust. *Translational Research* ***166*** (1), 57–69.

Norling, L. V., Dalli, J., Flower, R. J., Serhan, C. N. & Perretti, M. (2012). Resolvin D1 limits polymorphonuclear leukocyte recruitment to inflammatory loci: Receptor-dependent actions. *Arteriosclerosis, Thrombosis, and Vascular Biology* ***32*** (8), 1970–1978.

Noth, R., Häsler, R., Stüber, E., Ellrichmann, M., Schäfer, H., Geismann, C., Hampe, J., Bewig, B., Wedel, T., Böttner, M., Schreiber, S., Rosenstiel, P. & Arlt, A. (2013). Oral glutamine supplementation improves intestinal permeability dysfunction in a murine acute graft-vs.-host disease model. *American Journal of Physiology. Gastrointestinal and Liver Physiology* ***304*** (7), G646-54.

O

Oh, D. Y. & Lagakos, W. S. (2011). The role of G-protein-coupled receptors in mediating the effect of fatty acids on inflammation and insulin sensitivity. *Current Opinion in Clinical Nutrition and Metabolic Care* ***14*** (4), 322–327.

Oh, D. Y., Talukdar, S., Bae, E. J., Imamura, T., Morinaga, H., Fan, W., Li, P., Lu, W.J., Watkins, S.M. & Olefsky, J. M. (2010). GPR120 Is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-inflammatory and Insulin-Sensitizing Effects. *Cell* ***142*** (5), 687–698.

P

Pai, M.-H., Liu, J.-J., Hou, Y.-C. & Yeh, C.-L. (2014). A Soybean and Fish Oil Mixture With Different ω-6/ω-3 PUFA Ratios Modulates DSS-Induced Changes in Small Intestinal Intraepithelial γδT-Lymphocyte Expression in Mice. *JPEN. Journal of Parenteral and Enteral Nutrition* **40** (3):383-391.

Parameswaran, N. & Patial, S. (2010). Tumor necrosis factor-α signaling in macrophages. *Critical Reviews in Eukaryotic Gene Expression* ***20*** (2), 87–103.

Patel, A. A. & Budoff, M. J. (2016). Effects of eicosapentaenoic acid and docosahexaenoic acid on lipoproteins in hypertriglyceridemia. *Current Opinion in Endocrinology & Diabetes and Obesity* ***23*** (2), 145–149.

Peluso, I. & Palmery, M. (2016). The relationship between body weight and inflammation: Lesson from anti-TNF-α antibody therapy. *Human Immunology* ***77*** **(**1), 47–53.

Pennica, D., Hayflick, J. S., Bringman, T. S., Palladino, M. & Goeddel, D. V. (1985). Cloning and expression in Escherichia coli of the cDNA for murine tumor necrosis factor. *Proceedings of the National Academy of Sciences of the United States of America* ***82***, 6060–6064.

Pérez-Echarri, N., Pérez-Matute, P., Marcos-Gómez, B., Baena, M. J., Marti, A., Martínez, J. A. & Moreno-Aliaga, M. J. (2008). Differential inflammatory status in rats susceptible or resistant to diet-induced obesity: Effects of EPA ethyl ester treatment. *European Journal of Nutrition* ***47*** (7), 380–386.

Pérez-Echarri, N., Pérez-Matute, P., Marcos-Gómez, B., Marti, A., Martínez, J.A. & Moreno-Aliaga, M.J. (2009). Down-regulation in muscle and liver lipogenic genes: EPA ethyl ester treatment in lean and overweight (high-fat-fed) rats. *Journal of Nutritional Biochemistry* **20** (9):705-714.

Pérez-Matute, P., Pérez-Echarri, N., Martínez, J. A., Marti, A. & Moreno-Aliaga, M. J. (2007). Eicosapentaenoic acid actions on adiposity and insulin resistance in control and high-fat-fed rats: role of apoptosis, adiponectin and tumour necrosis factor-alpha. *The British Journal of Nutrition* ***97*** (2), 389–398.

Peterson, L. W. & Artis, D. (2014). Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nature Reviews Immunology* ***14*** (3), 141–153.

Pochini, L., Scalise, M., Galluccio, M. & Indiveri, C. (2014). Membrane transporters for the special amino acid glutamine: structure/function relationships and relevance to human health. *Frontiers in Chemistry* ***2***, 1–23.

Poulsen, R. C., Gotlinger, K. H., Serhan, C. N. & Kruger, M. C. (2008). Identification of inflammatory and proresolving lipid mediators in bone marrow and their lipidomic profiles with ovariectomy and omega-3 intake. *American Journal of Hematology* ***83*** (6), 437–445.

R

Rocha, V. Z. & Folco, E. J. (2011). Inflammatory Concepts of Obesity. *International Journal of Inflammation* ***2011***, 1–14.

Rodríguez, A., Ezquerro, S., Méndez-Giménez, L., Becerril, S. & Frühbeck, G. (2015). Revisiting the adipocyte: a model for integration of cytokine signaling in the regulation of energy metabolism. *American Journal of Physiology - Endocrinology And Metabolism* ***309*** (8), E691–E714.

Rogler, G. (2017). Resolution of inflammation in inflammatory bowel disease. *The Lancet Gastroenterology & Hepatology* ***2*** (7), 521–530.

Romeo, E., Dave, M. H., Bacic, D., Ristic, Z., Camargo, S. M. R., Loffing, J., Wagner, C.A. & Verrey, F. (2006). Luminal kidney and intestine SLC6 amino acid transporters of B0AT-cluster and their tissue distribution in Mus musculus. *American Journal of Physiology. Renal Physiology* ***290*** (2), F376–F383.

Rossmeisl, M., Macek Jilkova, Z., Kuda, O., Jelenik, T., Medrikova, D., Stankova, B., Kristinsson, B., Haraldsson, G.G., Svensen, H., Stoknes, I., Sjövall, P., Magnusson, Y., Balvers, M.G., Verhoeckx, K.C., Tvrzicka, E., Bryhn, M. & Kopecky, J. (2012). Metabolic effects of n-3 PUFA as phospholipids are superior to triglycerides in mice fed a high-fat diet: Possible role of endocannabinoids. *PLoS ONE* ***7*** (6).

Rossol, M., Meusch, U., Pierer, M., Kaltenhäuser, S., Häntzschel, H., Hauschildt, S. & Wagner, U. (2007). Interaction between transmembrane TNF and TNFR1/2 mediates the activation of monocytes by contact with T cells. *Journal of Immunology* **179**, 4239–4248.

Roulis, M., Armaka, M., Manoloukos, M., Apostolaki, M. & Kollias, G. (2011). Intestinal epithelial cells as producers but not targets of chronic TNF suffice to cause murine Crohn-like pathology. *Proceedings of the National Academy of Sciences* ***108*** (13), 5396–5401.

Rutkowski, J. M., Stern, J. H. & Scherer, P. E. (2015). The cell biology of fat expansion. *Journal of Cell Biology* ***208*** (5), 501–512.

S

Saito, T., Hasegawa-Moriyama, M., Yamada, T., Kurimoto, T., Inada, E. & Kanmura, Y. (2016). Resolution of inflammation by resolvin D1 is essential for peroxisome proliferator-Activated receptor-ϒ-mediated analgesia during postincisional pain development in type 2 diabetes. *Japanese Journal of Anesthesiology* ***65***, S155–S162.

Sato, A., Kawano, H., Notsu, T., Ohta, M., Nakakuki, M., Mizuguchi, K., Itoh, M., Suganami, T. & Ogawa, Y. (2010). Antiobesity Effect of Eicosapentaenoic Acid in high-fat/high-sucrose diet-induced obesity: importance of hepatic lipogenesis. *Diabetes* ***59*** (10), 2495–504.

Schenk, M., Bouchon, A., Seibold, F. & Mueller, C. (2007). TREM-1–expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. *The Journal of Clinical Investigation* ***117*** (10), 3097–3106.

Scherberich, A., Di Maggio, N. Di & McNagny, K. M. (2013). A familiar stranger: CD34 expression and putative functions in SVF cells of adipose tissue. *World Journal of Stem Cells* ***5*** (1), 1–8.

Scherer, P. E. (2006). Adipose tissue: From lipid storage compartment to endocrine organ. In *Diabetes* **55**, 1537–1545.

Serhan, C. N. (2007). Resolution Phase of Inflammation: Novel Endogenous Anti-Inflammatory and Proresolving Lipid Mediators and Pathways. *Annual Review of Immunology* ***25*** (1), 101–137.

Serhan, C. N. (2014). Pro-resolving lipid mediators are leads for resolution physiology. *Nature* ***510*** (7503), 92–101.

Serhan, C. N. (2017). Discovery of specialized pro-resolving mediators marks the dawn of resolution physiology and pharmacology. *Molecular Aspects of Medicine*.

Serhan, C. N. & Chiang, N. (2009). Endogenous pro-resolving and anti-inflammatory lipid mediators: a new pharmacologic genus. *British Journal of Pharmacology* ***153*** (S1), S200–S215.

Serhan, C. N. & Chiang, N. (2013). Resolution phase lipid mediators of inflammation: Agonists of resolution. *Current Opinion in Pharmacology* ***13*** (4), 632–640.

Serhan, C. N., Dalli, J., Karamnov, S., Choi, A., Park, C.-K., Xu, Z.-Z., Ji, R.R., Zhu, M. & Petasis, N. A. (2012). Macrophage proresolving mediator maresin 1 stimulates tissue regeneration and controls pain. *The FASEB Journal* ***26*** (4), 1755–1765.

Serhan, C. N., Hong, S., Gronert, K., Colgan, S. P., Devchand, P. R., Mirick, G. & Moussignac, R.-L. (2002). Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *The Journal of Experimental Medicine* ***196*** (8), 1025–1037.

Serhan, C. N., Yang, R., Martinod, K., Kasuga, K., Pillai, P. S., Porter, T. F., Oh, S.F. & Spite, M. (2009). Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions. *The Journal of Experimental Medicine* ***206*** (1), 15–23.

Shaker, A., Swietlicki, E. A., Wang, L., Jiang, S., Onal, B., Bala, S., DeSchryver, K., Newberry, R., Levin, M.S. & Rubin, D. C. (2010). Epimorphin deletion protects mice from inflammation-induced colon carcinogenesis and alters stem cell niche myofibroblast secretion. *Journal of Clinical Investigation* ***120*** (6), 2081–2093.

Sharma, N., Laftah, A. H., Brookes, M. J., Cooper, B., Iqbal, T. & Tselepis, C. (2005). A role for tumour necrosis factor alpha in human small bowel iron transport. *The Biochemical Journal* ***390,*** 437–46.

Sideri, A., Stavrakis, D., Bowe, C., Shih, D. Q., Fleshner, P., Arsenescu, V., Arsenescu, R., Turner, J.R., Pothoulakis, C. & Karagiannides, I. (2015). Effects of obesity on severity of colitis and cytokine expression in mouse mesenteric fat. Potential role of adiponectin receptor 1. *American Journal of Physiology. Gastrointestinal and Liver Physiology* ***308*** (7), G591-604.

Souba, W. W., Pan, M. & Stevens, B. R. (1992). Kinetics of the sodium-dependent glutamine transporter in human intestinal cell confluent monolayers. *Biochemical and Biophysical Research Communications* ***188*** (2), 746–753.

Soufli, I., Toumi, R., Rafa, H. & Touil-Boukoffa, C. (2016). Overview of cytokines and nitric oxide involvement in immuno-pathogenesis of inflammatory bowel diseases. *World Journal of Gastrointestinal Pharmacology and Therapeutics* ***7*** (3), 353.

Spite, M., Clària, J. & Serhan, C. N. (2014). Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. *Cell Metabolism* ***19*** *(1), 21-36*.

Spoettl, T., Hausmann, M., Herlyn, M., Gunckel, M., Dirmeier, A., Falk, W., Herfarth, H., Schoelmerich, J. & Rogler, G. (2006). Monocyte chemoattractant protein-1 (MCP-1) inhibits the intestinal-like differentiation of monocytes. *Clinical and Experimental Immunology* ***145*** (1), 190–199.

Swanson, D., Block, R. & Mousa, S. A. (2012). Omega-3 fatty acids EPA and DHA: health benefits throughout life. *Advances in Nutrition* ***3*** (1), 1–7.

T

Tabas, I. & Glass, C. K. (2013). Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science* ***339*** (6116), 166–72. ç

Tang, Y., Zhang, M. J., Hellmann, J., Kosuri, M., Bhatnagar, A. & Spite, M. (2013). Proresolution therapy for the treatment of delayed healing of diabetic wounds. *Diabetes* ***62*** (2), 618–627.

Titos, E., Rius, B., González-Périz, A., López-Vicario, C., Morán-Salvador, E., Martínez-Clemente, M., Arroyo, V. & Clària, J. (2011). Resolvin D1 and Its Precursor Docosahexaenoic Acid Promote Resolution of Adipose Tissue Inflammation by Eliciting Macrophage Polarization toward an M2-Like Phenotype. *The Journal of Immunology* ***187*** (10), 5408–5418.

Tjonneland, A., Overvad, K., Bergmann, M. M., Nagel, G., Linseisen, J., Hallmans, G., Palmqvist, R., Sjodin, H., Hagglund, G., Berglund, G., Lindgren, S., Grip, O., Palli, D., Day, N.E., Khaw, K.T., Bingham, S., Riboli, E., Kennedy, H. & Hart, A. (2009). Linoleic acid, a dietary n-6 polyunsaturated fatty acid, and the aetiology of ulcerative colitis: a nested case-control study within a {European} prospective cohort study. *Gut* ***58*** (12), 1606–1611.

Todoric, J., Löffler, M., Huber, J., Bilban, M., Reimers, M., Kadl, A., Zeyda, M., Waldhäusl, W. & Stulnig, T. M. (2006). Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia* ***49*** (9), 2109–2119.

U

Uldry, M. & Thorens, B. (2004). The SLC2 family of facilitated hexose and polyol transporters. *Pflugers Archiv European Journal of Physiology* **447** (5), 480-489.

V

Viola, J. R., Lemnitzer, P., Jansen, Y., Csaba, G., Winter, C., Neideck, C., Silvestre-Roig, C., Dittmar, G., Döring, Y., Drechsler, M., Weber, C., Zimmer, R., Cenac, N. & Soehnlein, O. (2016). Resolving Lipid Mediators Maresin 1 and Resolvin D2 Prevent Atheroprogression in Mice. *Circulation Research* ***119*** (9), 1030–1038.

Virtue, S. & Vidal-Puig, A. (2010). Adipose tissue expandability, lipotoxicity and the Metabolic Syndrome - An allostatic perspective. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **1801** (3):338-49.

Vitale, S., Strisciuglio, C., Pisapia, L., Miele, E., Barba, P., Vitale, A., Cenni, S., Bassi, V., Maglio, M., Del Pozzo, G., Troncone, R., Staiano, A. & Gianfrani, C. (2017). Cytokine production profile in intestinal mucosa of paediatric inflammatory bowel disease. *PLoS ONE* ***12*** (8), 1–20.

W

Wang, H., Shi, P., Huang, C. & Liu, Q. (2016). Maresin 1 ameliorates iron-deficient anemia in IL-10-/- mice with spontaneous colitis by the inhibition of hepcidin expression though the IL-6/STAT3 pathway. *American Journal of Translational Research* ***8*** (6), 2758–2766.

Wassall, S.R. & Stillwell, W. (2008). Docosahexaenoic acid domains: the ultimate non-raft membrane domain. *Chemistry Physics Lipids* **153** (1): 57-63.

Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L. & Ferrante, A. W. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigation* ***112*** (12), 1796–1808.

Wright, S.W., Kuo, E.Y. & Corey, E. J. (1987). An effective process for the isolation of Docosahexaenoic acid in quantity from cod liver oil. The *Journal of organic chemistry* ***52*** (19), 4399–4401.

Wright, E., Loo, D. & Hirayama, B. (2011). Biology of Human Sodium Glucose Transporters. *Physiological Reviews* ***91***, 733–794..

Wright, E. M., Loo, D. D. F., Hirayama, B. A. & Turk, E. (2004). Surprising versatility of Na^+-glucose cotransporters: SLC5. *Physiology* ***19*** (6), 370–6.

X

Xu, Z.-Z., Zhang, L., Liu, T., Park, J. Y., Berta, T., Yang, R., Serhan, C.N. & Ji, R.-R. (2010). Resolvins RvE1 and RvD1 attenuate inflammatory pain via central and peripheral actions. *Nature*

Y

Yessoufou, A., Nekoua, M. P., Gbankoto, A., Mashalla, Y. & Moutairou, K. (2015). Beneficial effects of omega-3 polyunsaturated fatty acids in gestational diabetes: Consequences in macrosomia and adulthood obesity. *Journal of Diabetes Research*, **2015**; 2015:731434.

Yurko-Mauro, K., McCarthy, D., Rom, D., Nelson, E. B., Ryan, A. S., Blackwell, A., Salem, N.Jr., &Stedman, M. (2010). Beneficial effects of docosahexaenoic acid on cognition in age-related cognitive decline. *Alzheimer's & DMEMtia : The Journal of the Alzheimer's Association* ***6*** (6), 456–464.

Z

Zareie, M., Singh, P. K., Irvine, E. J., Sherman, P. M., Mckay, D. M. & Perdue, M. H. (2001). Monocyte / Macrophage Activation by Normal Bacteria and Bacterial Products Implications for Altered Epithelial Function in Crohn ' s Disease. *American Journal of Pathology* ***158*** (3), 1101–1109.

Zhao, J., Wang, H., Shi, P., Wang, W., & Sun, Y. (2017). GPR120, a potential therapeutic target for experimental colitis in IL-10 deficient mice. *Oncotarget* ***8*** (5), 8397–8405.

Zhu, L., Yang, T., Li, L., Sun, L., Hou, Y., Hu, X., Zhang, L., Tian, H., Zhao, Q., Peng, J., Zhang, H., Wang, R., Yang, Z., Zhang, L. & Zhao, Y. (2014). TSC1 controls macrophage polarization to prevent inflammatory disease. *Nature Communications* ***5***, 4696.

II. HYPOTHESIS AND AIMS

1. Justification for the study

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine produced by activated T-lymphocytes and macrophages that acts by binding to its receptors TNFR1 and TNFR2. This binding induces the activation of the nuclear factor NF-κB which, in turn, stimulates the production of other cytokines, including TNF-α itself (Rossol *et al*., 2007). This cytokine is implicated in numerous diseases with an inflammatory component, as inflammatory bowel disease (IBD) and obesity (Argollo *et al.*, 2017; Peluso & Palmery, 2016).

The Inflammatory Bowel Disease (IBD) is characterized by chronic inflammation of the gastrointestinal mucosa due to a dysregulation of the immune system (Tabas & Glass, 2013). High concentration of TNF-α is involved in the pathogenesis of intestinal inflammation (Vitale *et al*., 2017; Tabas & Glass, 2013).

Obesity is a low grade chronic inflammatory state, responsible for secondary diseases and the progression of other illnesses (Rocha & Folco, 2011). Dysfunctional inflamed mesenteric adipose tissue in obesity could also contribute to the chronic intestinal inflammation in IBD (Sideri *et al*., 2015). Increase of pro-inflammatory cytokines expression has been found in a colitis mice model supplemented with high fat diet (Teixeira *et al*., 2011).

During the triggering of intestinal inflammation, different mediators induce the switch of tissue-resident M2 macrophages into pro-inflammatory M1 macrophages (Mahida *et al.*, 1989; Peluso & Palmery, 2016; Labonte *et al*., 2014) and M1 macrophages infiltration. In turn, M1 macrophages secrete pro-inflammatory cytokines, including TNF-α (Kamada *et al*., 2008; Schenk *et al.*, 2007; Zareie et al., 2001). Obesity would impact the intestinal function through the recruitment and activation of pro-inflammatory macrophages (Neuman, 2007; Nishimura *et al*., 2009).

Marine omega-3 polyunsaturated fatty acids (n3-PUFAs) show anti-inflammatory actions on inflammatory-related pathologies such as cardiovascular diseases, atherosclerosis, obesity and colitis (Calder, 2015; Lorente-Cebrián *et al*., 2013; 2015; Martínez-Fernández *et al.*, 2015).

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the main n-3 PUFAs in fish oil (Calder, 2013). Thus, an alternative or complementary treatment for IBD therapy is the supplementation of the diet with n-3 PUFAs (Neuman & Nanau, 2012; Stewart *et al.*, 2011). Moreover, fish oil supplementation improves intestinal integrity in LPS-induced intestinal injury, by reducing TNF-α expression (Liu *et al*., 2012). N-3 PUFAs serve as substrates for the formation of specialized pro-resolving lipid mediators (SPMs) with potent anti-inflammatory and pro-resolving properties, namely maresins (MaR), resolvins (Rv) and protectins (PD) (Chatterjee *et al*., 2014; Serhan, 2007; Bento *et al.*, 2011).

In the enterocytes, glucose is actively transported by the Na^+/glucose cotransporter SGLT1. Glutamine, an essential amino acid for the maintenance of the gut barrier function and the intestinal cell proliferation (Chun *et al.*, 1997), is mainly transporter by the Na^+-dependent glutamine transporter B^0AT1 (Pochini *et al.*, 2014).

During intestinal inflammation, nutrients and electrolytes malabsorption may occur in relation to alterations on the expression and activity of their intestinal transporters (Powel, 1990; Bertolo *et al.*, 2002; Foley *et al.*, 2007; Sharma et al., 2005). We have previously demonstrated, in the human intestinal epithelial cell line Caco-2, that TNF-α inhibits αMG transport by decreasing SGLT1 expression in the brush border membrane (Barrenetxe *et al*., 2013).

2. Hypothesis

Based on the mentioned observations, the hypothesis of the present Doctoral Thesis was double: 1) That omega-3 fatty acids and its specialized pro-resolving lipid mediators can counteract TNF-α-induced inhibition of nutrients intestinal transport, acting from the apical membrane of the enterocytes; 2) That basolateral TNF-α, in relation with obesity, can inhibit nutrients transport and induce its own expression in the enterocytes.

3. Objectives

The specific objectives to demonstrate the working hypothesis were the following:

1. To study the ability of EPA to block apical TNF-α-induced inhibition of sugar uptake in Caco-2 cells, as well as to investigate the potential involvement of ERK1/2, AMPK and GPR120 in the EPA blocking effect (Chapter 1).
2. To investigate the potential blocking effect of DHA and its derived lipid mediators, MaR1, RvD1 and RvD2 on apical TNF-α-induced inhibition of sugar and glutamine uptake in Caco-2 cells (Chapter 2).
3. To investigate the possible effect of basolateral TNF-α on sugar and glutamine uptake and the regulation of pro-inflammatory protein genes in Caco-2 cells (Chapter 3).
4. To study the potential basolateral effect of secreted medium from adipocytes of lean, overweight and morbidly obese subjects and macrophages on sugar uptake in Caco-2 cells, and its relation with the regulation of pro-inflammatory protein genes (Chapter 3).
5. To investigate the effect of diet induced obesity on intestinal sugar transport and on pro-inflammatory protein genes expression in jejunal mucosa (Chapter 2 and 3).

1. Justificación del estudio

El factor de necrosis tumoral alfa (TNF-α) es una citoquina pro-inflamatoria producida por linfocitos T activados y macrófagos, que actúa uniéndose a sus receptores TNFR1 y TNFR2. Esta unión induce la activación del factor nuclear NF-κB que, a su vez, estimula la producción de otras citoquinas, incluyendo el propio TNF-α (Rossol *et al*., 2007). Esta citoquina está implicada en numerosas enfermedades con un componente inflamatorio, como la enfermedad inflamatoria intestinal (EII) y la obesidad (Argollo *et al.*, 2017; Peluso & Palmery, 2016).

La enfermedad inflamatoria intestinal se caracteriza por una inflamación crónica de la mucosa gastrointestinal debida a una desregulación del sistema inmune (Tabas & Glass, 2013). Concentraciones altas de TNF-α están implicadas en la patogénesis de la inflamación intestinal (Vitale *et al*., 2017; Tabas & Glass, 2013).

La obesidad es un estado inflamatorio crónico de bajo grado responsable de la aparición y progresión de otras enfermedades (Rocha & Folco, 2011). El tejido adiposo mesentérico inflamado disfuncional en la obesidad también podría contribuir a la inflamación intestinal crónica en la EII (Sideri *et al*., 2015). Así, en un modelo de ratón con colitis suplementado con una dieta rica en grasas se ha observado un aumento de la expresión de citoquinas pro-inflamatorias (Teixeira *et al*., 2011).

Durante la inflamación intestinal, diferentes mediadores estimulan la transformación de los macrófagos M2, residentes bajo la mucosa intestinal, a macrófagos pro-inflamatorios M1 (Mahida *et al.*, 1989; Peluso & Palmery, 2016; Labonte *et al*., 2003), así como la infiltración de macrófagos M1. Éstos, a su vez, secretan citoquinas pro-inflamatorias incluyendo TNF-α (Kamada *et al*., 2008; Schenk *te al.*, 2007; Zareie et al., 2001). La obesidad puede afectar a la función intestinal a través del reclutamiento y activación de macrófagos pro-inflamatorios (Neuman, 2007, Nishimura *et al*., 2009).

Los ácidos grasos poliinsaturados omega-3 (n-3 PUFAs) de origen marino muestran acciones antiinflamatorias sobre patologías relacionadas con la

inflamación como enfermedades cardiovasculares, aterosclerosis, obesidad y colitis (Calder, 2015, Lorente-Cebrián *et al*., 2013; 2015; Martínez-Fernández *et al.*, 2015). El ácido eicosapentaenoico (EPA) y el ácido docosahexaenoico (DHA) son los principales n-3 PUFAs en el aceite de pescado (Calder, 2013). Por lo tanto, un tratamiento alternativo o complementario para la terapia contra la EII es la suplementación de la dieta con n-3 PUFAs (Neuman & Nanau, 2012; Stewart et al., 2011). De hecho, la suplementación con aceite de pescado mejora la integridad intestinal alterada por LPS, por disminución de la expresión de TNF-α (Liu *et al*., 2012). Los n-3 PUFAs sirven como sustratos para la formación de mediadores lipídicos pro-resolución (SPMs), con potentes propiedades antiinflamatorias y pro-resolutivas llamados maresinas (MaR), resolvinas (Rv) y protectinas (PD) (Chatterjee *et al*., 2014; Serhan, 2007; Bento *et al.*, 2011).

En los enterocitos, la glucosa es transportada activamente por el cotransportador de Na^+/glucosa SGLT1. La glutamina, un aminoácido esencial para el mantenimiento de la función de barrera intestinal y la proliferación de las células intestinales (Chun *et al.*, 1997), es principalmente transportada por el transportador de glutamina dependiente de Na^+ B^0AT1 (Pochini *et al.*, 2014).

Durante la inflamación intestinal, puede producirse malabsorción de nutrientes y electrolitos en relación con alteraciones en la expresión y actividad de sus transportadores intestinales (Powel, 1990; Bertolo *et al.*, 2002; Foley *et al.*, 2007; Sharma *et al.*, 2005). En la línea de celular enterocitaria humana Caco-2, hemos demostrado previamente que el TNF-α inhibe el transporte de αMG al disminuir la expresión de SGLT1 en la membrana del borde de cepillo (Barrenetxe *et al*., 2013).

2. Hipótesis

En base a las observaciones mencionadas, la hipótesis del presente trabajo doctoral fue doble: 1) Que los ácidos grasos omega-3 y sus derivados lipídicos pro-resolutivos pueden contrarrestar la inhibición inducida por TNF-α sobre el transporte intestinal de nutrientes, actuando desde la membrana apical de los enterocitos; 2) Que el TNF-α basolateral, en relación con la obesidad, puede inhibir el transporte de nutrientes e inducir su propia expresión en los enterocitos.

3. Objetivos

Los objetivos específicos para demostrar la hipótesis de trabajo fueron los siguientes:

1. Estudiar la capacidad del EPA para bloquear la inhibición de la captación de azúcar en células Caco-2 inducida por TNF-α apical, así como investigar la posible implicación de ERK1/2, AMPK y GPR120 en los efectos bloqueantes del EPA (Capítulo 1).
2. Investigar el potencial efecto preventivo del DHA y sus derivados lipídicos, MaR1, RvD1 y RvD2, sobre la inhibición de la captación de azúcar y glutamina en células Caco-2 inducida por TNF-α apical (Capítulo 2).
3. Investigar el posible efecto del TNF-α basolateral sobre la captación de azúcar y glutamina y la regulación de genes de proteínas pro-inflamatorias en células Caco-2 (Capítulo 3).
4. Estudiar el potencial efecto basolateral de medios secretados por adipocitos provenientes de sujetos delgados, con sobrepeso y obesos mórbidos, así como de medios secretados por macrófagos, sobre la captación de azúcar en células Caco-2, y su relación con la regulación de genes de proteínas pro-inflamatorias (Capítulo 3).
5. Investigar el efecto de la obesidad inducida por la dieta sobre el transporte intestinal de azúcar y la expresión de proteínas pro-inflamatorias en la mucosa de yeyuno (Capítulos 2 y 3).

References

A

Argollo, M., Fiorino, G., Hindryck, P., Peyrin-Biroulet, L. & Danese, S. (2017). Novel therapeutic targets for inflammatory bowel disease. *Journal of Autoimmunity*.

B

Barrenetxe, J., Sánchez, O., Barber, A., Gascón, S., Rodríguez-Yoldi, M. J. & Lostao, M. P. (2013). TNFα regulates sugar transporters in the human intestinal epithelial cell line Caco-2. *Cytokine* ***64*** (1), 181–187.

Bento, A. F., Claudino, R. F., Dutra, R. C., Marcon, R. & Calixto, J. B. (2011). Omega-3 Fatty Acid-Derived Mediators 17(R)-Hydroxy Docosahexaenoic Acid, Aspirin-Triggered Resolvin D1 and Resolvin D2 Prevent Experimental Colitis in Mice. *The Journal of Immunology* ***187*** (4), 1957–1969.

Bertolo, C., Barrenetxe, J., Barber, A. & Lostao, M.P. (2002). Cytokine effect on intestinal galactose absorption. *Journal of Physiology and Biochemistry* ***58*** (1), 61–62.

C

Calder, P. C. (2013). Omega-3 polyunsaturated fatty acids and inflammatory processes: Nutrition or pharmacology? *British Journal of Clinical Pharmacology* ***75*** (3), 645–662.

Calder, P. C. (2015). Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. *BBA - Molecular and Cell Biology of Lipids* ***1851*** (4), 469–484.

Chatterjee, A., Sharma, A., Chen, M., Toy, R., Mottola, G. & Conte, M. S. (2014). The pro-resolving lipid mediator maresin 1 (MaR1) attenuates inflammatory signaling pathways in vascular smooth muscle and endothelial cells. *PLoS ONE* ***9*** (11).

Chun, H., Sasaki, M., Fujiyama, Y. & Bamba, T. (1997). Effect of enteral glutamine on intestinal permeability and bacterial translocation after abdominal radiation injury in rats. *Journal of Gastroenterology* ***32*** (2), 189–195.

F

Foley, K. F., Pantano, C., Ciolino, A. & Mawe, G. M. (2007). IFN-gamma and TNF-αlpha decrease serotonin transporter function and expression in Caco2 cells. *Am J Physiol Gastrointest Liver Physiol* ***292*** (3), G779-84.

K

Kamada, N., Hisamatsu, T., Okamoto, S., Chinen, H., Kobayashi, T., Sato, T., Sakuraba, A., Kitazume, M.T., Sugita, A., Koganei, K., Akagawa, K.S. & Hibi, T. (2008). Unique CD14+ intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-γ axis. *The Journal of Clinical Investigation* ***118*** (6), 2269–2280.

L

Labonte, A.C., Tosello-Trampont, A.C. & Hahn, Y.S. (2014). The Role of Macrophage Polarization in Infectious and Inflammatory Diseases. Molecules and Cells **37** (4): 275–285.

Liu, Y., Chen, F., Odle, J., Lin, X., Jacobi, S. K., Zhu, H., Wu, Z. & Hou, Y. (2012). Fish Oil Enhances Intestinal Integrity and Inhibits TLR4 and NOD2 Signaling Pathways in Weaned Pigs after LPS Challenge. *Journal of Nutrition* ***142*** (11), 2017–2024.

Lorente-Cebrián, S., Costa, A. G. V, Navas-Carretero, S., Zabala, M., Martínez, J. A. and Moreno-Aliaga, M. J. (2013). Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: A review of the evidence. *Journal of Physiology and Biochemistry* **69** (3), 633–651.

Lorente-Cebrián, S., Costa, A. G. V, Navas-Carretero, S., Zabala, M., Laiglesia, L. M., Martínez, J. A. and Moreno-Aliaga, M. J. (2015). An update on the role of omega-3 fatty acids on inflammatory and degenerative diseases. *Journal of Physiology and Biochemistry* **71** (2), 341–349.

M

Mahida, Y. R., Patel, S., Gionchetti, P., Vaux, D. & Jewell, D. P. (1989). Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. *Gut* ***30*** (6), 826–834.

Martinez-Fernandez, L., Laiglesia, L. M., Huerta, A. E., Martinez, J. A. & Moreno-Aliaga, M. J. (2015). Omega-3 fatty acids and adipose tissue function in obesity and metabolic syndrome. *Prostaglandins & Other Lipid Mediators* ***121***, 1–18.

N

Neuman, M. G. (2007). Immune dysfunction in inflammatory bowel disease. *Translational Research* ***149*** (4), 173–186.

Neuman, M. G. & Nanau, R. M. (2012). Inflammatory bowel disease: Role of diet, microbiota, life style. *Translational Research* **160** (1), 29-44.

Nishimura, S., Manabe, I., Nagasaki, M., Eto, K., Yamashita, H., Ohsugi, M., Otsu, M., Hara, K., Ueki, K., Sugiura, S., Yoshimura, K., Kadowaki, T. & Nagai, R. (2009). CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nature Medicine* ***15*** (8), 914–920.

P

Peluso, I. & Palmery, M. (2016). The relationship between body weight and inflammation: Lesson from anti-TNF-α antibody therapy. *Human Immunology* ***77*** (1), 47–53.

Pochini, L., Scalise, M., Galluccio, M. & Indiveri, C. (2014). Membrane transporters for the special amino acid glutamine: structure/function relationships and relevance to human health. *Frontiers in Chemistry* ***2***, 1–23.

Powel DW. (1990). Immunophysiology of intestinal electrolyte transport. In *Handbook of Physiology. The Gastrointestinal System. Intestinal Absorption and Secretion.* (pp. 591–564). Bethesda, MD: American Physiological Society

R

Rocha, V. Z. & Folco, E. J. (2011). Inflammatory Concepts of Obesity. *International Journal of Inflammation* ***2011***, 1–14.

Rossol, M., Meusch, U., Pierer, M., Kaltenhäuser, S., Häntzschel, H., Hauschildt, S., & Wagner, U. (2007). Interaction between transmembrane TNF and TNFR1/2 mediates the activation of monocytes by contact with T cells. *Journal of Immunology* ***179***, 4239–4248.

S

Schenk, M., Bouchon, A., Seibold, F. & Mueller, C. (2007). TREM-1–expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. *The Journal of Clinical Investigation* ***117*** (10), 3097–3106.

Serhan, C. N. (2007). Resolution Phase of Inflammation: Novel Endogenous Anti-Inflammatory and Proresolving Lipid Mediators and Pathways. *Annual Review of Immunology* ***25*** (1), 101–137.

Sharma, N., Laftah, A. H., Brookes, M. J., Cooper, B., Iqbal, T. & Tselepis, C. (2005). A role for tumour necrosis factor alpha in human small bowel iron transport. *The Biochemical Journal* ***390***, 437–46.

Sideri, A., Stavrakis, D., Bowe, C., Shih, D. Q., Fleshner, P., Arsenescu, V., Arsenescu, R., Turner, J.R., Pothoulakis, C. & Karagiannides, I. (2015). Effects of obesity on severity of colitis and cytokine expression in mouse mesenteric fat. Potential role of adiponectin receptor 1. *American Journal of Physiology. Gastrointestinal and Liver Physiology* ***308*** (7), G591-604.

Stewart, M., Day, A. S. & Otley, A. (2011). Physician Attitudes and Practices of Enteral Nutrition as Primary Treatment of Paediatric Crohn Disease in North America. *Journal of Pediatric Gastroenterology and Nutrition* ***52*** (1), 38–42.

T

Tabas, I., & Glass, C. K. (2013). Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science* ***339***(6116), 166–72. https://doi.org/10.1126/science.1230720

Teixeira, L. G., Leonel, A. J., Aguilar, E. C., Batista, N. V, Alves, A. C., Coimbra, C. C., Ferreira, A.V., de Faria, A.M., Cara, D.C. & Alvarez Leite, J.I. (2011). The combination of high-fat diet-induced obesity and chronic ulcerative colitis reciprocally exacerbates adipose tissue and colon inflammation. *Lipids in Health and Disease* ***10*** (1), 204.

V

Vitale, S., Strisciuglio, C., Pisapia, L., Miele, E., Barba, P., Vitale, A., Cenni, S., Bassi, V., Maglio, M., Del Pozzo, G., Troncone, R., Staiano, A. & Gianfrani, C. (2017). Cytokine production profile in intestinal mucosa of paediatric inflammatory bowel disease. *PLoS ONE* ***12*** (8), 1–20.

Z

Zareie, M., Singh, P. K., Irvine, E. J., Sherman, P. M., Mckay, D. M., & Perdue, M. H. (2001). Monocyte / Macrophage Activation by Normal Bacteria and Bacterial Products Implications for Altered Epithelial Function in Crohn ' s Disease. *American Journal of Pathology* ***158*** (3), 1101–1109.

III. EXPERIMENTAL DESIGN AND METHODS

1. Cell culture

1.1. Caco-2 cells

The epithelial colorectal adenocarcinoma cell line (Caco-2) clone PD7 was kindly donated by Dr. Brot-Laroche (INSERM, UMR S 872, Centre de Recherches de Cordeliers, Paris). These cells grow as a monolayer with enterocytes morphology and phenotype, and strong tight junctions between adjacent cells (Natoli *et al.,* 2012). Caco-2 cells present low glucose metabolism, low glycogen content and high expression of metabolic enzymes and membrane transporter, in both apical and basolateral membranes (Mahraoui *et al*., 1994; Zweibaum, 1993).

1.1.1. Passage and cell culture

Caco-2 cell line was maintained in the laboratory with Dubelcco's Modified Eagle medium (DMEM (1X) + GlutaMAX, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% non-essential amino acids (MEN eagle NEAA 100X, LONZA), 1% penicillin (10,000 U/ml)–streptomycin (10,000 µg/ml) (Gibco) and 1% amphotericin B (250 µg/ml, Gibco). For storage, cells were diluted in culture medium supplemented with 10 % dimethyl sulfoxide (DMSO) (Sigma) at a concentration of 1-1.5 million cells per tube, and frozen in an ultra-low temperature freezer (-140 °C model; Sanyo).

For the experiments, cells were rapidly thawed to avoid possible cellular damage by DMSO, and transferred into a 25 cm^2 surface plastic flask (Costar) with 5 mL of fresh culture medium. They were maintained in a CO_2 incubator (MCO-18 AIC UV; Sanyo) at 37 °C with 5 % CO_2, 95 % O_2 and 90 % of relative humidity. The medium was changed every 2-3 days until the cells reached 80 % cellular confluence in the flask (3-5 days).

Next, cells were dissociated with 0.5 mL of a trypsin-EDTA solution (Composition in g/L: trypsin 5, EDTA 2 and NaCl 8.5 (Gibco BRL) without Ca^{+2} and Mg^{+2}, previously warmed at 37°C. After 5 min in the CO_2 incubator, the reaction was stopped by adding fresh culture medium; the cellular suspension was homogenized, and the cells were counted in a Neubauer's chamber of 0.1 mm^3 depth.

The number of cells/mL was calculated applying the following formula:

$$\text{cells/mL} = n/v = n \times 10^4$$

v = Counted cell volume that is equal to 0.1 mm^3 or 10^{-4} mL.

n = Average of the cells number counted in at least four fields of the chamber.

Once this value was calculated, the cells were seeded in 25 cm^2 flasks at a density of 200,000 cells per cm^2, in order to maintain cell culture.

Uptake experiments in Caco-2 cells were performed in cells seeded, at a density of 60,000-200,000 cells per cm^2, on 24-well plates, 12-well plates or 12-well insert plates (TranswelTM Costar). The insert consists on a support contained a polycarbonate membrane (filter) of 12 mm of diameter, an area of 1 cm^2 and micropores of 0.4 µm. The insert placed in the well allows to distinguish between apical and basal sides (Fig. 9).

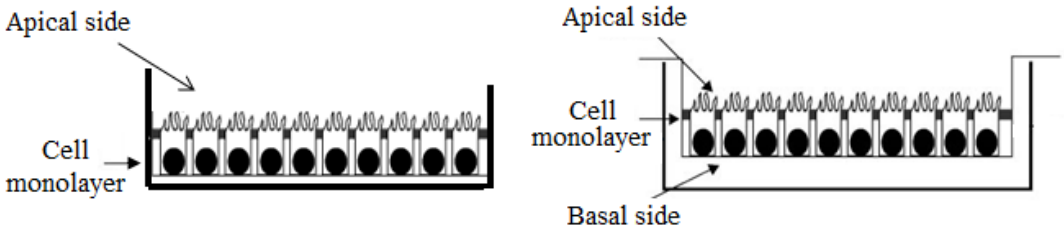


Figure 9. Caco-2 culture in well-plate and well insert-plate

Before seeding the Caco-2 cells on the filters, 1 mL of growth medium was added to each well. The cells were observed routinely with an optical microscope to control the development. After 15-21 days, the cells were ready for the experiments.

Transepithelial resistance (TER) was measured (ohms per cm^2) with a Millicel ERS electrode (Millipore) to verify the integrity of the Caco-2 cell monolayer grown on the filters. The transepithelial resistance increased as the cell monolayer grew. When it reached a maximum TER value between 350-450 ohm.cm^2, it was considered that the monolayer presented optimal conditions for the experiments (Natoli *et al.,* 2012).

1.2. Human Mesenchymal Stem Cells (hMSC)

Human mesenchymal stem cells (hMSC) were kindly provided by Dr. Arbones-Mainar (*Instituto Aragonés de Ciencias de la Salud*). They were obtained from subcutaneous abdominal and visceral adipose tissue of three subjects with different body mass index (BMI), undergoing elective laparoscopic surgery: lean (24 kg/m^2), overweight (27 kg/m^2) and morbidly obese (44.1 kg/m^2) (see Table 2 for overweight/obesity classification). None of the individuals presented chronic pathologies associated with metabolic syndrome. All donors signed the written consent, and the study was approved by the local Institutional Review Board (CEIC-A) and the Ethics Committee of the University of Navarra. Human mesenchymal stem cells were isolated as previously described (Perez-Diaz *et al*., 2017).

Table 2. Weight categories and their corresponding range of BMI (Nuttal 2015)

Weight categories	BMI (kg/m^2)
Lean/normal weight	18.5-24.9
Overweight	25-29.9
Obese	30-34.9
Severely obese	35-39.9
Morbidly obese	$\geq$40

1.2.1. Passage and cell culture

Human MSC were maintained in the laboratory using low-glucose DMEM (Lonza) supplemented with 10% FBS and 1% penicillin–streptomycin. For the differentiation, high-glucose DMEM (Gibco) was supplemented with 500 µM IBMX, 1.67 µM insulin, 1 µM dexamethasone, 1 µM rosiglitazone, and 10% FBS. Cells were stored as previously described for Caco-2 cells.

To use the cells, they were rapidly thawed and transferred into a 75 cm^2 surface plastic flask (Costar) with 10 mL of fresh culture medium. They were maintained in a CO_2 incubator (MCO-18 AIC UV; Sanyo) at 37 °C with 5 % CO_2, 95 % O_2 and 90 % of relative humidity. The medium was changed every 2-3 days until the cells reached 80 % cellular confluence in the flask (7-10 days). Next, cells were dissociated with trypsin-EDTA solution and counted as previously described for Caco-2 cells.

For the experiments, the cells were grown on 12 well plates and seeded at a density of 200,000 cells per cm^2. After confluence, hMSC were differentiated into adipocytes by adding the differentiation medium for 3 days. Then, the medium was refreshed with new differentiation medium and the cells were incubated for 3 more days. Next, 10% FBS high-glucose medium was added until cells displayed typical features of mature white adipocytes (hMSC-derived adipocytes, hMSC-DA).

1.3. THP-1 cells

Transformed human mononuclear cell line (THP-1) is an immortalized human leukemia monocytic cell line, used to study monocyte/macrophage functions, mechanisms, signaling pathways, and nutrients and drugs transport (Chanput *et al.*, 2014). This cell line presents some advantages: Reduced average doubling time, no infectivity, can be cultured *in vitro* up to passage 25 without changes of cell sensitivity and activity, can be stored for several years, and presents homogenous genetic background that minimizes phenotype variability (Chanput *et al.*, 2014).

1.3.1. Passage and cell culture

THP-1 cell line was maintained in the laboratory with RPMI-1640 medium (ATCC) supplemented with 10% FBS and 1% penicillin–streptomycin. The cells were stored, thawed and maintained as described for Caco-2 cells, although they grew in suspension. In order to change the medium, every 2 days, THP-1 cells were centrifuged, counted and diluted in the appropriate volume of fresh medium.

When the number of cells/mL necessary for the assays were reached, cells were placed in 12-well plates at a density of $2x10^5$ cells/cm^2 and differentiated into inactive macrophages by the treatment with 200 nM phorbol myristrate acetate (PMA) for 2 days. Then, inactive macrophages were activated by 4 h treatment with LPS.

2. Uptake assays

2.1. Uptake in Caco-2 cells

2.1.1. Media

For the sugar uptake assays, cells were incubated in Dulbecco's Modified Eagles Medium without glucose (DMEM, Gibco). For the glutamine assays, the medium used was a Krebs modified saline solution (5.4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 137 mM NaCl, 0.3 mM KH_2PO_4, 10 mM glucose and 10 mM HEPES/Tris, pH 7.5). Both media were supplemented with 1% BSA free fatty acids (Sigma) in the experiments that contained n-3 PUFAs (EPA or DHA). Media were supplemented with NEAA when performing the long-term experiments (24h).

Uptake solution contained 0.1 mM α-methylO-D-glucose (αMG; Sigma) with traces of [^{14}C]-α-methyl-glucoside (0.08 µCi/ml; ARC 0131), or 0.1 mM L-Glutamine (Gln; Sigma) with traces of [^{14}C]-L-Glutamine (0.1 µCi/ml; ARC 0196) in DMEM or Krebs modified buffer, respectively.

2.1.2. Procedure

Similar experimental procedure was performed with Caco-2 cells grown on plates and filters. The specific characteristics of incubations and pre-incubations of each uptake assay are described in their correspondent chapter.

Cells were incubated for 15 min at 37 °C with 500 µL of uptake solution. Then, the uptake was stopped by the addition of 500 µL ice-cold free-substrate buffer, followed by aspiration. Next, cells were again washed twice with ice-cold buffer to eliminate non-specific radioactivity. Cells were solubilized in 500 µL of 1 % Triton X-100 in NaOH 1M and kept at 37°C for 1 h and 30 min to liberate the radioactive cell content. In the assays using inserts, the membrane of every filter was cut and introduced in an eppendorf with Triton X-100 solution.

After this time, 100 µL of sample were taken for duplicate and, after the addition of 2 mL of scintillation liquid (AquaLight Hidex), the radioactivity was counted in a liquid scintillation counter (Hidex 300 SL). The protein content was measured by the standardized method of Bradford (Bio-Rad Protein Assay; Bio-Rad laboratories).

The final αMG or glutamine uptake was calculated by applying the following formula:

$$\text{Uptake}\left(\frac{\text{nmol}}{\text{mg}}\text{prot}\right) = \frac{\text{Cpm}_m}{\text{Cpm}_i}\ x\ \frac{[\text{substrate}]\ \text{x}\ \text{V}_i \text{x}\ \text{V}_d}{\text{V}_c\ \text{x mg prot}}$$

[substrate] = Substrate concentration (mM)

Cpm_m = Counts per min in the TritonX-100 medium

cpm_i = Counts per min in the initial medium

V_i = Sample volume of initial medium (0.01 mL)

V_d= Triton X-100 volume (0.5 mL)

V_c = Triton X-100 sample volume (0.1 mL)

2.2. Uptake in everted jejunal rings

The uptake assays using intestinal everted rings were performed following the procedure described by Crane & Mandelstam (1960).

2.2.1. Animal model

C57BL/6J male mice were purchased from Harlan Laboratories and fed as previously described (Martínez-Fernández *et al*., 2017) to obtain the obese phenotype. Diet-induced obesity (DIO) mice were divided into two groups (n=8), DIO-MaR1 and DIO groups, that received for 10 days a daily gavage of oral Maresin 1 (MaR1) (50 µg/kg BW) or the vehicle, respectively. The lean (control) group, received vehicle (Laiglesia *et al*., 2017).

Mice were sacrificed and jejunum (8 cm) was isolated for the uptake assays. Samples of jejunal mucosa were also extracted and kept at -80 °C for Western blot analysis. All experimental procedures were performed under protocols approved by the University of Navarra Ethics Committee for the Use of Laboratory Animals, according to the National and Institutional Guidelines for Animal Care and Use (Protocols 029-12 and 047-15).

2.2.2. Media

The incubation medium used was Krebs-Ringer-Tris (KRT) saline solution (140 mM NaCl, 5.6 mM KCl, 3 mM $CaCl_2$, 1.4 mM KH_2PO_4, 1.4 mM $MgSO_4$, 6.1 mM Tris, 4.9 mM HCl, pH 7.4). Uptake medium contained 1 mM αMG with traces of [^{14}C]-αMG (0.0025 µCi mL-1) and 10 ng/mL of recombinant mouse TNF-α (Peprotech Inc.) dissolved in KRT saline solution.

2.2.3. Experimental procedure

Isolated jejunum was rapidly soaked in physiological serum (NaCl 0.9 %) at 4 °C. Using a syringe, the jejunum was washed with serum, in order to eliminate the possible food content and, the remaining fat adhered to the serous layer was also carefully removed with tweezers. Then, the intestine was everted and cut in small fragments of approximately 0.2-0.3 cm and 5-15 mg weight. Ten intestinal rings were obtained from each of the 4 animals per experimental group (lean, DIO and DIO-MaR1), and divided into 2 groups of 5 rings each: control and plus TNF-α. Intestinal rings were distributed in tubes containing 5 mL of uptake medium (KRT).

Incubation of the rings was performed at 37 °C for 15 min, under continuous shaking and gassed with O_2. Before the beginning of the incubation period, a 200 µL sample of the uptake medium was collected to obtain the initial cpm. After the incubation, rings were washed in ice-cold KRT solution, dried on a filter paper and weighed individually in an analytical balance (Newclassic ML 104/01; Mettler Toledo). Next, the rings were incubated for 24 h in a solution containing 0.1 $HN0_3$ to denature the proteins and allow the exit of the cellular radioactivity. Finally, duplicated samples of 200 µL from each tube were collected and, after the addition of 2 mL of scintillation liquid (AquaLight Hidex), the radioactivity was counted in a liquid scintillation counter (Hidex 300 SL). The uptake in each ring was calculated by the following formula:

$$\mu mol \frac{substrate}{g\ wet\ weight} = \frac{cpm_{ts}}{cpm_i}\ x\ \frac{[substrate]\ x\ V_t\ x\ V_i}{V_{ts}\ x\ Ww}$$

[Substrate] = Substrate concentration (mM) in the incubation medium

Ww= Wet weight of the intestinal ring (g).

cpm_{ts} = Counts per minute in 0.1 HNO_3 solution.

cpm_i = Counts per minute of the initial medium.

V_t = Volume of nitric acid (0.5 mL).

V_i = Volume of initial medium sample (0.2 mL).

V_{ts} = Volume of denatured medium sample (0.2 mL).

For the calculation of the substrate accumulation in the rings, it was considered that the tissue has approximately 70 % of water content.

3. Western blot experiments

Western blot studies were performed to analyze the expression of SGLT1 and B^0AT1 in the brush border membrane, as well as to determine the intracellular expression of ERK 1/2 and AMPK.

3.1. BBMV isolation

Caco-2 cells grown on 75 cm^2 plastic flasks were incubated under the different experimental conditions described in each chapter. After the incubation period, brush border membrane vesicles (BBMV) were isolated using the method of Shirazi-Beechey (Shirazi-Beechey *et al*., 1990) with some modifications (Garriga *et al.*, 1999). BBMV were also obtained from jejunal mucosa of lean, DIO and DIO-MaR1 mice following the same procedure.

3.1.1. Media and reagents

- Buffer A: 100 mM mannitol, 0.2 mM Phenylmethyl sulfonyl fluoride (PMSF), 2 mM Hepes/TRIS, 0.41 µM sodium azide and 0.2 mM benzamidine. The buffer was adjusted at pH 7.4.
- Buffer B: 300 mM mannitol, 20 mM Hepes/TRIS, 0.1 mM $MgS0_4.7\ H_2O$, 0.41 µM sodium azide. The buffer was adjusted at pH 7.4.
- Protease inhibitor cocktail complete mini (1 pill/10 mL; Roche).
- $MgCl_2$ 1 M.

3.1.2. Experimental procedure

All the manipulations were performed at 4 °C to avoid protein degradation of the samples.

Caco-2 cells were homogenized in 5 mL of buffer A. Then, 1 mM of $MgCl_2$ was diluted in the cell homogenate until a final concentration of 20 mM $MgCl_2$. After 20 min with constant shaking, the solution was centrifuged at 5,200 rpm during 15 min to remove precipitated cellular material and organelles. The supernatant containing mainly brush border membranes was centrifuged at 15,000 rpm during 35 min. The pellet was resuspended in buffer B (100 µL). Finally, the pellet was homogenized with a tip to cycle the vesicles that were stored at -80 °C (Fig. 10).

The jejunal mucosa suffered the same experimental procedure but with 2 extra centrifugations, as indicated in Figure 10.

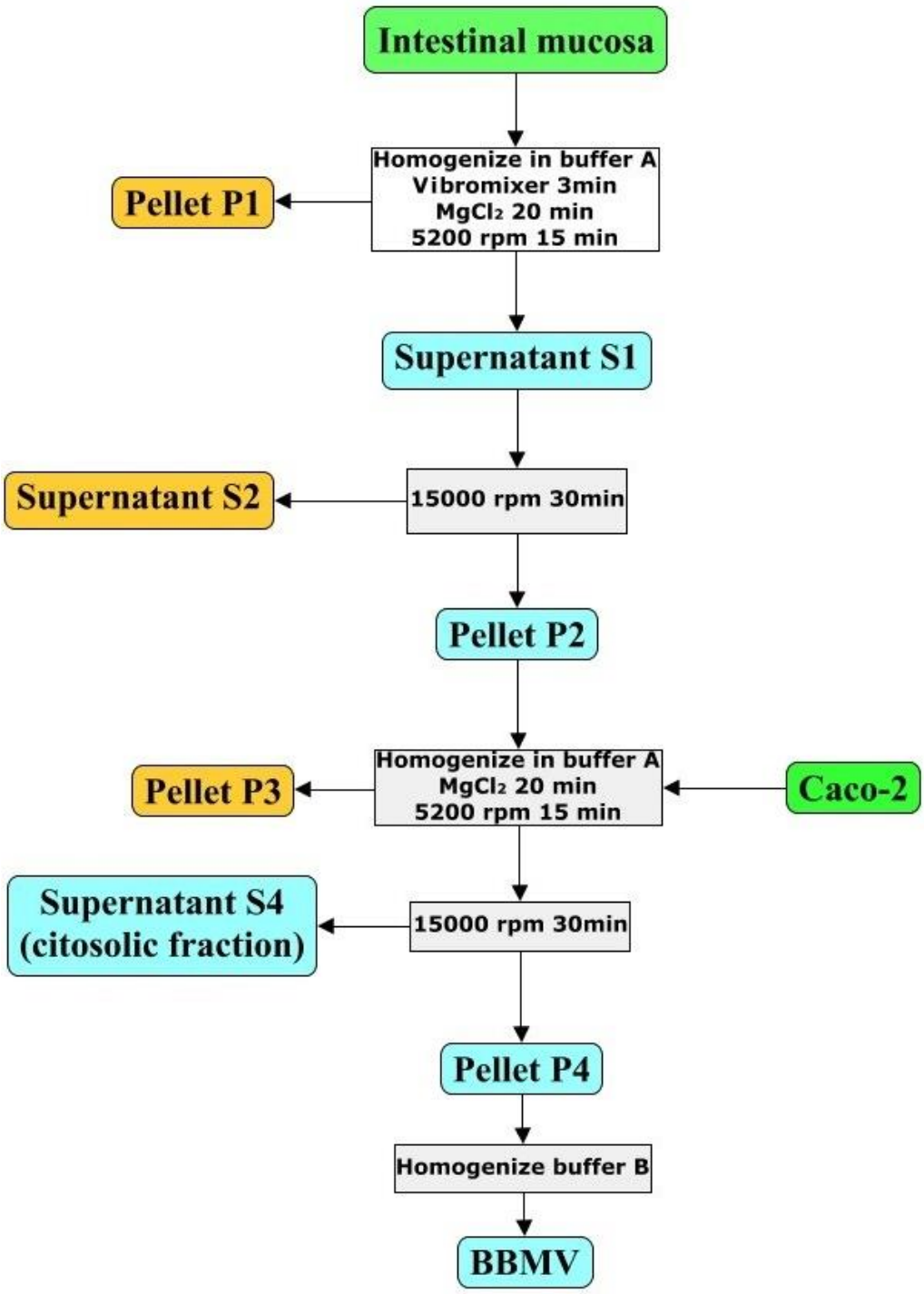


Figure 10. Scheme of brush border membrane isolation from tissue and cell culture

3.2. Whole Cell lysate

Caco-2 cells grown on 12 well plates were treated as described in the corresponding chapter.

3.2.1. Media and reagents

- Lysis buffer: 8 mM NaH_2PO_4, 42 mM Na_2HPO4, 1 % SDS, 0.1 M $NaCl_2$, 0.1 % NP4O, 1 mM NaF, 10 mM sodium orthovanadate, 2 mM PMSF, 10 mM EDTA and 1% protease inhibitor cocktail 1 (Sigma).
- PBS buffer (Gibco).

3.2.2. Experimental procedure

The cells were collected in PBS and centrifugate for 15 min at 13,000 rpm to obtain the precipitated cells. Then, the cells were resuspended in lysis buffer and homogenized using a sonicator.

3.3. Protein surface biotinylation

Caco-2 cells grown on 12-well plates were treated under the different experimental conditions as described in each chapter.

3.3.1. Media and reagents

- PBS (Gibco) with 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (PBS+Ca+Mg)
- Sulfo-NHS-SS-biotin (1.5 mg/ml; 100 µL/well; Thermo Scientific)
- Biotinylation buffer: 20 mM Hepes, 150 mM NaCl and 2 mM $CaCl_2$, pH 8.5
- Glycine buffer: 100 mM glycine diluted in PBS+Ca+Mg
- Lysis buffer: 8 mM NaH_2PO_4, 42 mM Na_2HPO4, 1 % SDS, 0.1 M $NaCl_2$, 0,1 % NP4O, 1 mM NaF, 10 mM sodium orthovanadate, 2 mM PMSF, 10 mM EDTA and 1% protease inhibitor cocktail (Sigma).
- StreptAvidin-agarose beads (100 µL/ 150 µg protein; Thermo Scientific)
- Loading buffer 2x with 100 µM dithiothreitol (DTT)

3.3.2. Experimental procedure

The cells were washed with PBS+Ca^{2+}+$Mg^{2+}$and incubated for 1 h under continuous shaking with sulfo-NHS-SS-biotin in biotinylation buffer. Next, cells were washed with glycine buffer (15 min) and PBS+Ca^{2+}+Mg^{2+}. Then, they were lysate in lysis buffer, homogenized with sonicator, and centrifuged at 13,000 rpm for 5 min. Supernatant was mixed with Streptavidin-agarose beads and incubated overnight to pull down biotinylated antigens. Next, samples were centrifuged at 13,000 rpm to allow pelleting of the beads containing the membrane surface protein bound to them. Beads were washed with 100 µL lysis buffer and centrifuged for 5 min at 13,000 rpm. This procedure was repeated three times. Biotinylated fractions were boiled in gel loading buffer containing 100 µM DTT to separate the beads from the proteins

3.4. Determination of protein concentration

The protein content of the samples was determined by the standardized method of Bradford (Bio-Rad Protein Assay; Bio-Rad laboratories).

3.5. Western blot assays

This technique involves the separation of the proteins of a sample by polyacrylamide gel electrophoresis under denaturing conditions, and subsequent transfer to a membrane in which the protein of interest is immunodetected, using specifics antibodies.

3.5.1. Electrophoresis

Solubilized proteins (30-40 µg) in Laemmli buffer were resolved by electrophoresis on 12% SDS-PAGE. The BBMV was heated at 90 °C during 5 min and then placed on ice during 1 min previously to the loading into the gel. The 12 % gels were allocated in the cassette and covered with Running Buffer 1X (1/10 dilution from Running buffer 10X Bio-Rad). Then, the samples and the Ladder (molecular weight marker) were loaded inside the wells. Samples were run for 15 min at 90 V (until the proteins ended the stacking part of the gel) and 90 min at 120 V (to separate the different proteins in the resolving gel).

3.5.2. Protein transfer

Once the proteins were separated by electrophoresis, the blot was transferred onto a polyacrylamide membrane (PVDF, needed methanol activation, Amersham) or nitrocellulose membrane (Hybond P, GE Healthcare). The gel was allocated in the negative part of the cassette and the membrane in the positive, to allow the transference of the proteins (negatively charged) from the gel to the membrane. The process was performed using transfer buffer 1X (1/10 dilution from transfer buffer 10X Bio-Rad) at 400 mA for 1 h.

3.5.3. Protein immunodetection

Immunodetection blotting provides a simple and effective method for identifying specific antigens in a complex mixture of proteins. The membrane containing the transferred proteins was blocked in TBS-Tween 1X buffer (TBS-T 1X) with 10% of milk (Sveltesse, Nestle) for 1 h at room temperature, and incubated overnight at 4 °C with the corresponding primary rabbit antibodies used at 1:1000. Then, the membranes were washed out four times in TBS-T 1X and incubated for 1 h at room temperature with the corresponding peroxidase conjugated secondary antibody at 1:10,000. Membrane stripping was performed, with re-blot Plus (Millipore), to reprobe the membrane with a different primary antibody.

The immunoreactive bands were detected by enhanced chemiluminescence (Super Signal West Dura; Thermo Scientific) and quantified by densitometry analysis (Image Studio Lite). The results are expressed in percentage of the control value, which was set to 100.

Table 3: List of antibodies used for the Western Blot studies.

Antibody	Manufacturer	Ref	Source
Actin	Sigma-Aldrich	A1978	Mouse
Phospho AMPK (Thr172)	Cell Signaling Technology	2535	Rabbit
AMPK	Cell Signaling Technology	2532	Rabbit
B^0AT1	Abcam	Ab180516	Rabbit
Phospho ERK 1/2 (Thr202/Tyr204)	Cell Signaling Technology	4370	Rabbit
MAPK (ERK 1/2)	Cell Signaling Technology	9102	Rabbit
SGLT1	Santa Cruz Biotechnologies	sc-98974	Rabbit
Goat anti-mouse	Santa Cruz Biotechnologies	sc-2005	Goat
Goat anti-rabbit	Santa Cruz Biotechnologies	sc-2004	Goat

4. Gene expression by Real-Time PCR

4.1. RNA extraction from cells and intestinal mucosa

Cells (Caco-2 or hMSC-DA from individual wells) or jejunal mucosa (10 mg) were homogenized in a phenol solution (TRIzol™ Reagent) that protects the sample from the degradation by RNAses

4.1.1. Media and Reagents

- TRIzol™ Reagent (500 µL; Sigma)
- Chloroform (200 µL; Scharlau)
- Isopropyl alcohol (250 µL; Scharlau)
- Ethanol-DEPC at 75 % (With DEPC-treated water 0.01 %; 500 µL; Scharlau)
- RNase and DNase free water (~12 µL; Ambion)
- Spray anti-RNases (RNAse Zap, Ambion)

4.1.2. Experimental procedure

Cells or homogenized tissues were incubated for 10 min with Trizol on ice under continuous agitation. Then, the samples were incubated for 5 min at room temperature to allow the complete dissociation of the nucleoprotein complex. After that, 200 µL of chloroform was added to separate the RNA from the proteins and the DNA. Samples were shaken for 20 seconds, maintained during 2-3 min at room temperature and centrifuged for 15 min at 13,000 rpm and 4 °C. The colorless upper aqueous phase that contained the RNA was carefully transferred to a new tube. Then, isopropyl alcohol (250 µL) was added and the samples kept at -80 °C for 24-48 h to allow the RNA precipitation. Next, RNA samples were maintained at room temperature for 10 min and centrifuged for 10 min at 13,000 rpm (4 °C) to separate RNA from the alcohol. The white pellet appearing after the centrifugation was washed 2 times with 75 % ethanol-DEPC and precipitated by centrifugation for 10 min at 13,000 rpm (4 °C). Finally, the samples were dried and resuspended in 20 µL of molecular water.

4.2. DNAse Treatment

This process is performed to remove traces of DNA from the RNA preparations.

4.2.1. Media and Reagents

The kit DNAfree (Ambion) contained the following reagents:

- rDNase I (1 µL/sample)
- 10X DNase I Buffer (2 µL/sample)
- DNase inactivation reagent (1 µL/sample)
- Nuclease-free Water (5 µL/sample)

4.2.2. Experimental procedure

The rDNase I and the 10x DNase I buffer were added to the RNA samples and incubated for 30 min at 37 °C to eliminate the possible contamination of the samples with DNA. Then, DNase activity was stopped by the addition of DNase inactivation reagent (2 min at room temperature). Next, samples were centrifuged at 13,000 rpm for 1.5 min and the supernatant containing the clean RNA was transferred to a new tube.

4.3. Retrotranscription

The reverse transcription is the synthesis of complementary DNA (cDNA) from RNA samples.

4.3.1. Media and Reagents

- dNTP (10 mM of dATP, dCTP, dGTP, and dTTP at neutral pH; 1 µL/sample; Bioline)
- Oligo dT (hexamers, 1 µL/sample; Invitrogen)
- Buffer 5X (4 µL/sample; Invitrogen)
- 0.1 M DTT (2 µL/sample; Invitrogen)
- RNasin® Plus RNase Inhibitor (1 µL/sample; Takara)
- M-MLV Reverse Transcriptase (1 µL/sample; Invitrogen)

4.3.2 Experimental procedure

RNA was mixed with dNTP and different hexamers and incubated for 5 min at 37 °C. Then, buffer 5X, DTT, RNasin and M-MLV were added to the sample and incubated for 50 min at 37 °C and for additional 15 min at 70 °C to finally obtain the cDNA.

4.4. Real-time PCR

Real-time PCR is a quantitative PCR used to detect relative or absolute gene expression level. All qPCR involves the use of fluorescence to detect the threshold cycle (Ct) during PCR, the level of fluorescence gives a signal over the background and this signal is in the linear portion of the amplified curve. This Ct value is the reference for the accurate quantization of qPCR. In this study, we have used the TaqMan® Master Mix containing ROX fluorescent dye that penetrates within the double-stranded DNA. The qPCR machine (ABI PRISM 7900HT Fast Sequence Detection System) detects the fluorescence level and the corresponding software calculates Ct values related to fluorescence.

4.4.1. Media and Reagents

- Master mix (5 µL/sample; TaqMan® Applied Biosystems)
- Probes (0.5 µL/sample; TaqMan® Applied Biosystems)
- RNase and DNase free water (0.5 µL/sample; Ambion)
- cDNA diluted 1:20

4.4.2. Experimental procedure

Each PCR plate well was loaded with 4.5 µL/well of the sample and 5.5 µL/well of a mix containing master mix, probe and water. Then, a gently vortex was given to the PCR plate to mix all the reagents, before placing the plate into the qPCR machine. The qPCR cycles were: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min.

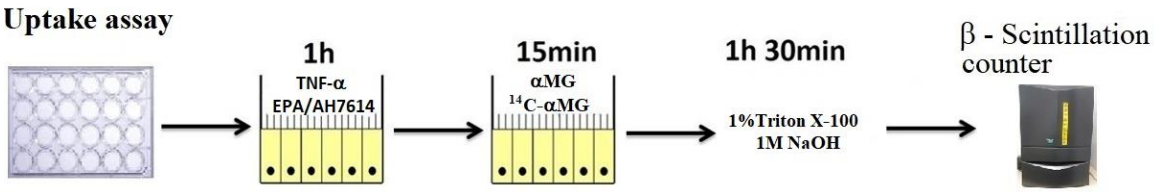
Table 4: List of Taqman probes used for RT-PCR.

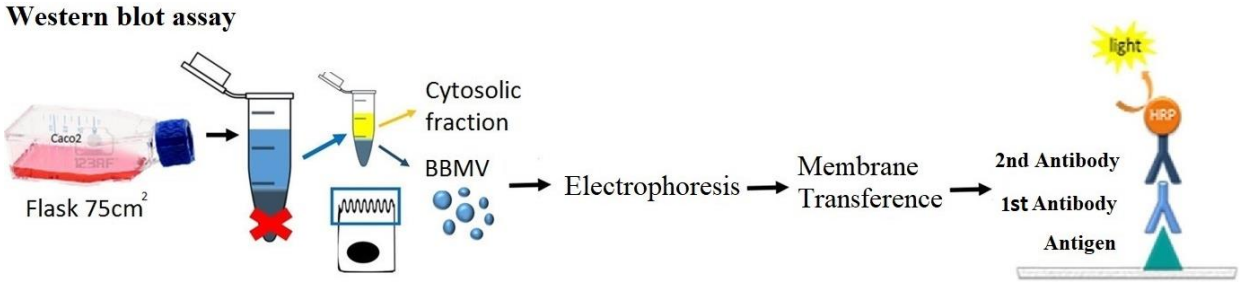
Name	Ref	Source
IL-1β	Hs00174097_m1	Human
IL-1β	Mm00434228_m1	Mus musculus
IL-6	Hs00985639_m1	Human
IL-6	Mm00446190_m1	Mus musculus
MCP1	Hs00234140_m1	Human
MCP1	Mm00441242_m1	Mus musculus
TNF-α	Hs00174128_m1	Human
TNF-α	Mm00443258_m1	Mus musculus
18S	Hs03003631_g1	Human

5. Statistical analysis

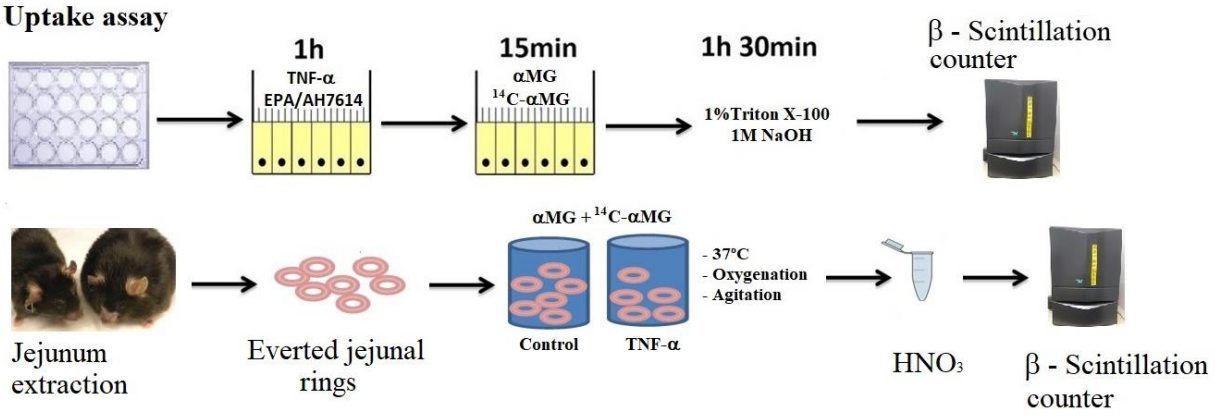
Statistical analysis was performed using the program Stata v12 (Stata, RRID: SCR_012763). Parametric and non-parametric tests (One-way ANOVA, t-student, Kruskal- Wallis test, Median test) followed by the corresponding post-hoc test (Tukey, SNK, Bonferroni) were run depending on the sample size and the normality of the data. Results were expressed as means ± Standard Error of the Mean (SEM), and differences were considered significant at a p value <0.05.

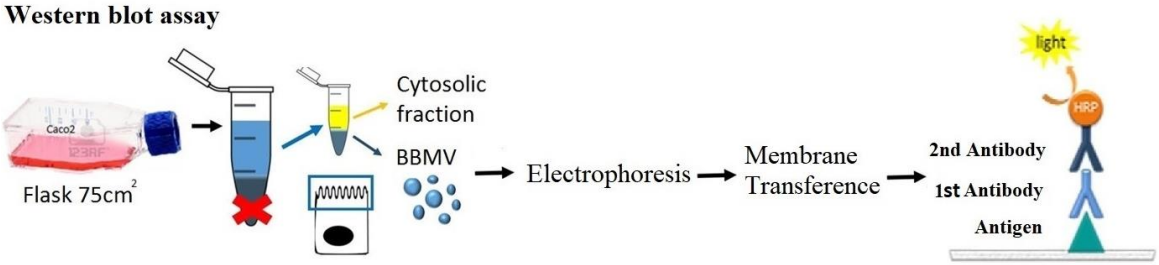
CHAPTER 1

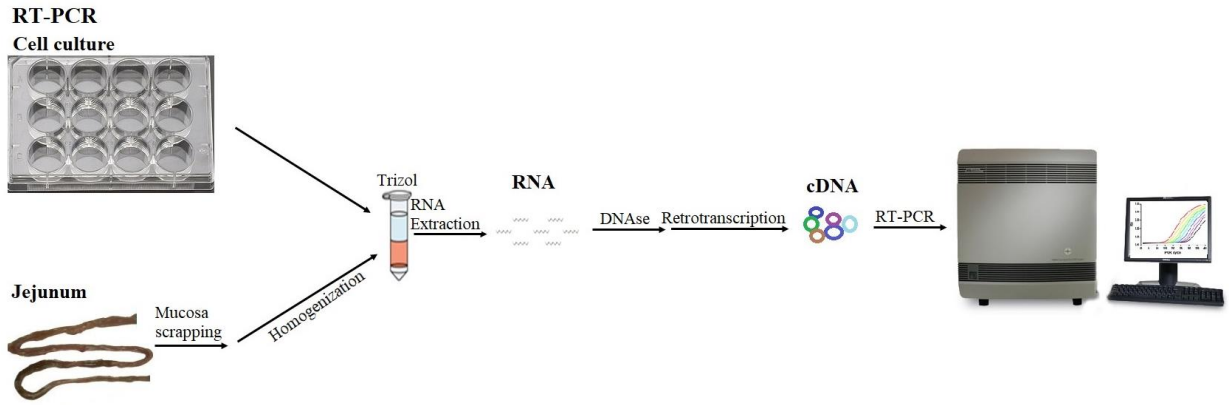

Uptake assay
1h
TNF-α
EPA/AH7614
15min
αMG
^{14}C-αMG
1h 30min
1%Triton X-100
1M NaOH
β - Scintillation counter


Western blot assay
Caco2
Flask 75cm^2
Cytosolic fraction
BBMV
Electrophoresis
Membrane Transference
light
HRP
2nd Antibody
1st Antibody
Antigen

CHAPTER 2

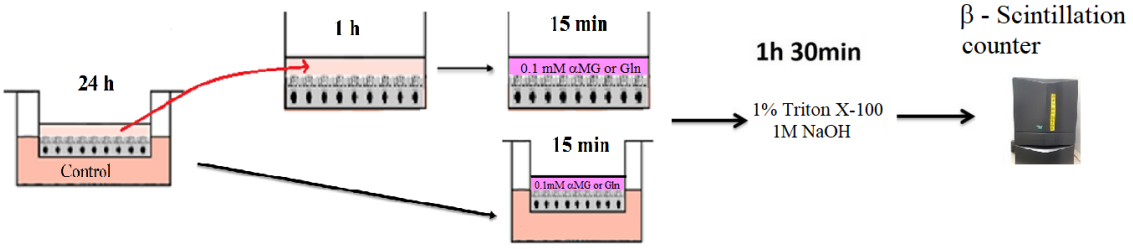

Uptake assay
1h
TNF-α
EPA/AH7614
15min
αMG
14C-αMG
1h 30min
1%Triton X-100
1M NaOH
β - Scintillation counter
αMG + 14C-αMG
- 37°C
- Oxygenation
- Agitation
Jejunum extraction
Everted jejunal rings
Control
TNF-α
HNO3
β - Scintillation counter

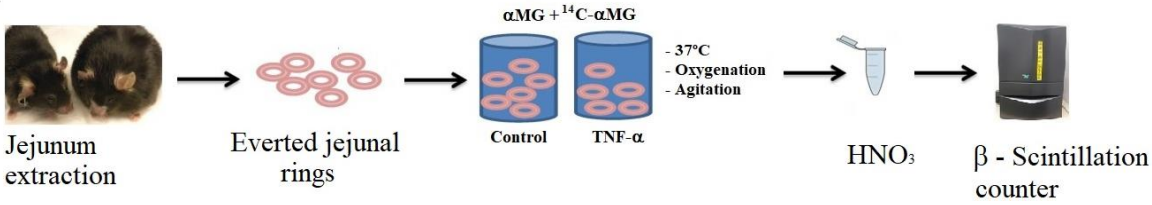

Western blot assay
Caco2
Flask 75cm2
Cytosolic fraction
BBMV
Electrophoresis
Membrane Transference
light
HRP
2nd Antibody
1st Antibody
Antigen


RT-PCR
Cell culture
Jejunum
Mucosa scrapping
Homogenization
Trizol
RNA Extraction
RNA
DNAse
Retrotranscription
cDNA
RT-PCR

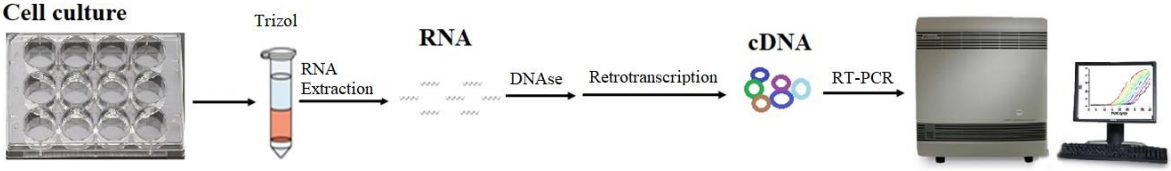
CHAPTER 3

Uptake assay





RT-PCR



References

C

Chanput, W., Mes, J. J. & Wichers, H. J. (2014). THP-1 cell line: An in vitro cell model for immune modulation approach". *International Immunopharmacology* **23** (1): 37–45.

Crane, R. K. & Mandelstam, P. (1960). The active transport of sugars by various preparations of hamster intestine. *Biochimica et Biophysica Acta **45:*** 460–476.

G

Garriga, C., Rovira, N., Moreto, M., Planas, J. (1999). Expression of Na^+-D-glucose cotransporter in brush-border membrane of the chicken intestine. *American Journal of Physiology* **276**: 627–631.

L

Laiglesia, L.M., Lorente-Cebrián, S., Martínez-Fernández, L., Sáinz, N., Prieto-Hontoria, P.L., Burrell, M.A., Rodríguez-Ortigosa, C.M., Martínez, J.A. & Moreno-Aliaga, M.J. (2017). Maresin 1 mitigates liver steatosis in ob/ob and diet-induced obese mice. *International Journal of Obesity*. Doi: 10.1038/ijo.2017.226.

M

Mahraoui, L., Rousset, M., Dussaulx, E., Darmoul, D., Zweibaum, A. & Brot-Laroche, E. (1992). Expression and localization of GLUT-5 in Caco-2 cells, human small intestine, and colon. *American Journal of Physiology* **263** (3 Pt 1): G312–G318.

Martínez-Fernández, L., González-Muniesa, P., Laiglesia, L.M., Sáinz, N., Prieto-Hontoria, P.L., Escoté, X., Odriozola, L., Corrales, F.J., Arbones-Mainar, J.M., Martínez, J.A. & Moreno-Aliaga, M.J. (2017). Maresin 1 improves insulin sensitivity and attenuates adipose tissue inflammation in ob/ob and diet-induced obese mice. *FASEB Journal* **31** (5): 2135–2145.

N

Natoli, M., Leoni, B.D., D'Agnano, I., Zucco, F. & Felsani A. (2012). Good Caco-2 cell culture practices. *Toxicology In Vitro* **26** (8):1243-1246.

Nuttall, F.K. (2015). Body Mass Index. *Nutrition today* **50** (3): 117–128.

P

Perez-Diaz, S., Garcia-Rodriguez, B., Gonzalez-Irazabal, Y., Valero, M., Lagos-Lizan, J. & Arbones-Mainar, J. M. (2017). Knockdown of PTRF ameliorates adipocyte differentiation and functionality of human mesenchymal stem cells. *American Journal of Physiology - Cell Physiology* **312** (1): C83–C91.

S

Shirazi-Beechey, S. P., Davies, a G., Tebbutt, K., Dyer, J., Ellis, A., Taylor, C. J., Fairclough, P. & Beechey, R. B. (1990). Preparation and properties of brush-border membrane vesicles from human small intestine. *Gastroenterology* **98** (3): 676–685.

Z

Zweibaum, A. (1993). Differentiation of human colon cancer cells: a new approach to cancer of the colon. *Annales de Gastroenterologie et d'Hepatologie* **29** (5): 252–257.

IV. RESULTS

CHAPTER 1.

EPA blocks TNF-α-induced inhibition of sugar uptake in Caco-2 cells via GPR120 and AMPK

Rosa Castilla-Madrigal[1,2], Jaione Barrenetxe[1], María J. Moreno-Aliaga[1,2,3,4], María Pilar Lostao[1,2,3]

[1]University of Navarra, Dept. Nutrition, Food Science and Physiology, Irunlarrea 1, 31008 Pamplona, Spain

[2]University of Navarra, Nutrition Research Centre, Irunlarrea 1, 31008 Pamplona, Spain

[3]IdiSNA, Navarra Institute for Health Research, Pamplona, Spain

[4]CIBERobn, Physiopathology of Obesity and Nutrition, Institute of Health Carlos III (ISCIII), Madrid, Spain

J Cell Physiol. 2017

doi: 10.1002/jcp.26115

Impact Factor (2016): 4.080

16/84 in Physiology (Q1)

Abstract

The aim of the present work was to investigate in Caco-2 cells whether eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid, could block the inhibitory effect of tumor necrosis factor-α (TNF-α) on sugar transport, and identify the intracellular signaling pathways involved.

After pre-incubation of the Caco-2 cells with TNF-α and EPA for 1 h, EPA prevented the inhibitory effect of the cytokine on α-methyl-D-glucose (αMG) uptake (15 min) and on SGLT1 expression at the brush border membrane, measured by Western blot. The ERK1/2 inhibitor PD98059 and the AMPK activator AICAR also prevented the inhibitory effect of TNF-α on both αMG uptake and SGLT1 expression. Interestingly, the AMPK inhibitor, Compound C, abolished the ability of EPA to prevent TNF-α-induced reduction of sugar uptake and transporter expression. The GPR120 antagonist, AH7614, also blocked the preventive effect of EPA on TNF-α-induced decrease of αMG uptake and AMPK phosphorylation.

In summary, TNF-α inhibits αMG uptake by decreasing SGLT1 expression in the brush border membrane through the activation of ERK1/2 pathway. EPA prevents the inhibitory effect of TNF-α through the involvement of GPR120 and AMPK activation.

Key words: AMPK, EPA, GPR120, SGLT1, TNF-α.

1. Introduction

The Inflammatory Bowel Disease (IBD) is characterized by chronic inflammation of the gastrointestinal mucosa due to a dysregulation of the immune system (Tabas & Glass, 2013). Genome, diet and intestinal microbiota are implicated in the pathogenesis of IBD (Albenberg *et al.*, 2012). In IBD patients, the gastrointestinal mucosa contains high concentrations of pro-inflammatory molecules such as the tumor necrosis factor α (TNF-α) (Tabas & Glass, 2013). TNF-α is a cytokine produced by activated T-lymphocytes and macrophages that acts in an autocrine and paracrine way by binding to its receptors TNFR1 and TNFR2. This binding induces the activation of the nuclear factor NF-κB which, in turn, stimulates the production of other cytokines, including TNF-α itself (Rossol *et al.*, 2007). It has been found that the levels of TNF-α are a hundred times higher in the intestine and blood of IBD patients than in healthy people (Komatsu *et al.*, 2001).

During intestinal inflammation, the expression and activity of many nutrients and electrolytes transporters can be modified (Bertolo *et al.*, 2002; Sharma *et al*., 2005; Foley *et al*., 2007), which may explain the characteristic malabsorption and diarrhea found in IBD patients. As a consequence, patients may lose weight and suffer from vitamins, micronutrients and protein deficiency (Hébuterne *et al*., 2009). In this sense, we have previously demonstrated in the human intestinal epithelial cell line Caco-2, that TNF-α inhibits sugar uptake by decreasing the expression of the Na^+-glucose cotransporter SGLT1 in the brush border membrane (Barrenetxe *et al*., 2013).

The omega-3 long-chain polyunsaturated fatty acids (n-3 PUFAs) have been proposed to exert beneficial actions in a great variety of human diseases such as asthma, hypertension, myocardial infarction, some cancers and inflammatory diseases such as obesity, atherosclerosis and rheumatoid arthritis (Figueras *et al*., 2011; Hur *et al.,* 2012; Lorente-Cebrián *et al*., 2013, 2015). In patients with IBD, it has been shown that treatment with n-3 PUFAs produces protection against development of illness, improves the intestinal mucosa histology, reduces inflammatory markers (TNF-α) and protects against the effects of oxidative stress (Barbalho *et al.*, 2016). Furthermore, it has been found a strong correlation between the incidence of Crohn's disease and the decrease in n-3 PUFAs in the diet (Shoda *et al.*, 1996; Issa & Saeian, 2011).

The eicosapentaenoic acid (EPA) is an n-3 PUFA found in fish oil. In high-fat-fed rats, it has been observed that EPA blocks the increase of TNF-α production in adipose tissue (Pérez-Matute *et al.*, 2007). In murine cultured adipocytes, EPA directly inhibits the stimulation of lipolysis induced by TNF-α through mechanisms that include inhibition of the pro-inflammatory pathways ERK1/2 and NF-κB, and stimulation of the AMPK pathway (Lorente-Cebrián *et al*., 2009, 2012).

GPR120, also known as free fatty acid receptor 4, is a cell-surface receptor, member of the G protein-coupled receptors (GPCRs) superfamily, which can bind unsaturated medium-to long chain fatty acids (Hirasawa *et al*., 2005; Ichimura *et al.*, 2014). GPR120 is widely expressed in several organs, including intestine, and it is involved in several homeostatic functions such as inflammation, glucose homeostasis and insulin sensitivity (Ichimura *et al.*, 2014). Furthermore, it has been observed that the anti-inflammatory effects of EPA and DHA are mediated by GPR120 acting as a sensor/receptor (Oh *et al.*, 2010).

The goal of the present study was to analyze the ability of EPA to block the inhibitory effect of TNF-α on intestinal sugar transport. Moreover, we aimed to unravel the potential involvement of ERK1/2, AMPK and GPR120 in EPA actions.

2. Material and methods

2.1. Cell culture

Caco-2 cells (RRID: CVCL_Z580) were maintained at 37°C and 5% CO_2 in a humidified atmosphere. The cells were grown in Dulbecco's Modified Eagles medium (DMEM (1X) + GlutaMAX, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% non-essential amino acids (NEAA 100X, LONZA), 1% penicillin (10000 U/ml)–streptomycin (10000 µg/ml) (Gibco) and 1% amphotericin B (250 µg/ml, Gibco). The culture medium was changed every 2 days. When cells reached 80% confluence, confirmed by microscopic observance, they were dissociated with 0.05% trypsin-EDTA (0.25% trypsin 1X, Gibco) and subcultured on 75 cm2 plastic flasks at a density of 25x104 cells/cm2. For the uptake studies, the cells were seeded at a density of 6x104 cells/cm2 in 24-well culture plates. Experiments were performed 15-19 days post seeding.

2.2. Sugar uptake in the presence and absence of TNF-α and EPA

Cells were grown in 24-well culture plates. The cells were pre-incubated for 1 or 24 h in Dulbecco's Modified Eagles Medium without glucose (DMEM, Gibco), supplemented with 1% BSA free fatty acids (Sigma) in control conditions and in the presence of 10 ng/ml TNF-α (PeproTech, Inc.) without or with 10 or 100 µM EPA (Cayman). This EPA concentration range has been reported to be comparable to plasma concentrations of this fatty acid after intake of dietary EPA (Murata et al., 2000; Perez-Matute et al., 2005). After the pre-incubation period, cells were incubated for 15 min with 0.1 mM α-methyl-D-glucose (αMG) and traces of [14C]-α-methyl-glucoside (0.3µCi/ml). After incubation, the reaction was stopped by adding 500 µL of cold Phosphate Buffered Saline with calcium and magnesium (PBS, Sigma Aldrich). Cells were then washed three times with PBS and solubilized by adding 500 µL 1 % Triton X-100 in 1M NaOH for 1 h 30 min at 37°C. After this time, samples were analyzed in the scintillation counter.

2.3. Identification of the intracellular signaling pathways and the implication of GPR120 on EPA effect

Cells were grown in 24-well culture plates. For the intracellular signaling pathways studies, the cells were pre-incubated for 1 h with 10 ng/ml TNF-α in the absence or in the presence of 100 µM EPA plus 1 mM Aicar (AMPK activator), 50 µM PD98059 (ERK inhibitor) or 20 µM Compound C (CC, AMPK inhibitor). To allow the permeability of CC and PD, cells were incubated with each of these compounds for 30 min before starting the pre-incubation period. For the studies about the implication of GPR120 on EPA effect, the cells were pre-incubated for 1 h with 10 ng/ml TNF-α in the absence or in the presence of 100 µM EPA and the GPR120 antagonist AH7614 (Tocris) at 100 µM. After the pre-incubation, cells were incubated for 15 min with 0.1 mM αMG and traces of [14C]-α-methyl-glucoside and treated as described in section 2.2. All experiments were performed in DMEM supplemented with 1% BSA free fatty acids.

2.4. Western Blot Analysis

Cells grown on 75 cm2 plastic flasks were treated as described in section 2.3. After the incubation period, brush border membrane vesicles (BBMV) were isolated using the method of Shirazi-Beechey (1990) with some modifications (Garriga *et al.*, 1999), we also obtained the cytosolic fraction with this technique. All the manipulations were carried out at 4 °C to avoid protein degradation of the samples. The protein content of the vesicles was determined by the standardized method of Bradford (Bio-Rad Protein Assay; Bio-Rad laboratories). Solubilized proteins from BBMV or cytosolic fractions (20 µg) were resolved by electrophoresis on 12% SDS-PAGE mini-Protean TGX gels (Bio-Rad). The resolved proteins were transferred to a PVDF membrane (Hybond P, GE Healthcare) which was then blocked in TBS-Tween 1X buffer (TBS-T 1X) with 10% of milk (Nestle, Sveltesse) for 2 h at room temperature and incubated overnight at 4 °C with the corresponding primary rabbit antibodies used at 1:1000. The primary antibodies were anti SGLT1 (Santa Cruz Biotechnology, Cat# sc-98974, RRID: AB_2191582), anti AMPKα (Cell Signaling Technology, Cat# 2532, RRID: AB_330331), anti Phospho-AMPKα (Cell Signaling Technology, Cat# 2535, RRID: AB_331250), anti ERK1/2(Cell Signaling Technology Cat# 4695, RRID: AB_390779) and anti Phospho-ERK1/2 (Cell Signaling Technology Cat# 4370, RRID: AB_2315112). The β-actin (Santa Cruz Biotechnology Cat# sc-47778, RRID: AB_626632) mouse antibody was also used at 1:1000. After the incubation with the corresponding antibody, the membranes were washed out four times in TBS-T 1X and incubated for 1 h at room temperature with the corresponding peroxidase conjugated secondary antibody, goat anti-rabbit (Santa Cruz Biotechnology, Cat# sc-2004, RRID: AB_631746) and goat anti-mouse (Santa Cruz Biotechnology, Cat# sc-2005, RRID: AB_631736) at 1:10000. The immunoreactive bands were detected by enhanced chemiluminescence (Super Signal West Dura; Thermo Scientific) and quantified by densitometry analysis (Image Studio Lite, RRID: SCR_014211). The results were expressed in percentage of the control value, which was set to 100.

2.5. Statistical analysis

Statistical analysis was performed using the program Stata v12 (Stata, RRID: SCR_012763). Parametric or non-parametric tests (One-way ANOVA, Kruskal- Wallis test, Median test) followed by the corresponding post-hoc test (Tukey, SNK, Bonferroni) were run depending on the sample size and the normality of the data. Results were expressed as means ± Standard Error of the Mean (SEM), and differences were considered significant at a p value <0.05.

3. Results

3.1. Sugar uptake in the presence of TNF-α and EPA

As presented in figure 1, 10 ng/ml TNF-α (1 h) decreased 0.1 mM αMG uptake in Caco-2 cells by ~30%, similar as shown by Barrenetxe *et al.* (2013). Pre-incubation of the cells for 1 h with 100 µM EPA totally blocked TNF-α inhibition of αMG transport. EPA alone did not have any effect on sugar uptake (Fig. 1). A similar preventive effect on TNF-α action was observed after chronic treatment (24 h) with 100 µM EPA, while 10 µM EPA for 1h was not able to significantly counteract the actions of the cytokine on αMG uptake (data not shown).

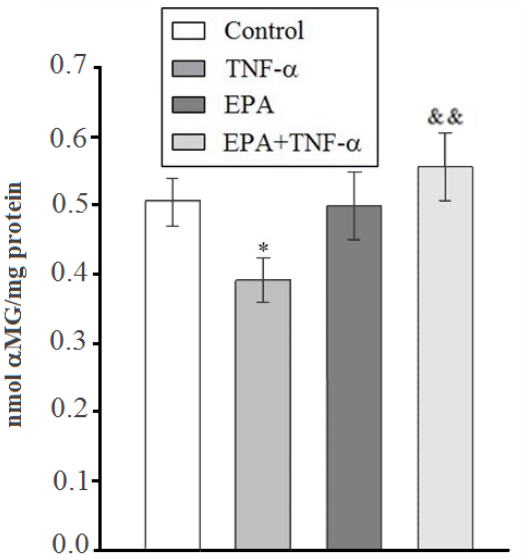


Figure 1. EPA blocks TNF-α inhibition of αMG uptake. Caco-2 cells were pre-incubated for 1h with 10 ng/ml TNF-α in the absence or in the presence of 100 µM EPA before measuring the uptake of 0.1 mM αMG for 15 min. Data (n=12) are represented as mean ± SEM. * p<0.05 vs. control; && p<0.01 vs. TNF-α.

3.2. Implication of the ERK1/2 intracellular pathway in the preventive effect of EPA on TNF-α inhibition of αMG uptake

Since previous studies in adipocytes have shown that EPA acts through the inhibition of the ERK1/2 pathway (Lorente-Cebrián *et al.*, 2012), we first decided to investigate whether ERK1/2 intracellular pathway was involved in the TNF-α inhibition of αMG uptake. As shown in figure 2A, the incubation of the cells with 10 ng/ml TNF-α was accompanied by an increase of ERK1/2 phosphorylation (activation). As expected, the ERK1/2 inhibitor PD98059 (50 μM) alone or together with TNF-α, induced a clear decrease on ERK1/2 phosphorylation. Moreover, the pre-incubation of the cells with PD98059 blocked TNF-α-induced decrease of sugar uptake (Fig. 2B). In agreement with these results, TNF-α reduced the amount of SGLT1 in Caco-2 cells BBMV, but the ERK1/2 inhibitor PD98059 prevented this reduction (Fig. 2C). PD98059 alone did not have any effect on αMG uptake or SGLT1 expression in the apical membrane (Fig. 2B and C). All these results demonstrated that TNF-α decreases αMG uptake by diminishing SGLT1 expression in the apical membrane through ERK1/2 activation.

Next, we studied whether EPA could act through the ERK1/2 pathway to prevent TNF-α-inhibition of sugar uptake. As shown in figure 2D, EPA alone did not alter ERK1/2 phosphorylation with respect to control. On the other hand, the incubation of the cells with 100 μM EPA in the presence of TNF-α did not modify the increase on ERK1/2 phosphorylation levels observed after TNF-α treatment (Fig. 2D). In addition, αMG uptake in the presence of EPA, TNF-α and PD98059 was not altered when compared to control (Fig. 2E), which further suggests that the ERK1/2 pathway is not involved in the EPA blocking effect of TNF-α inhibition of sugar uptake.

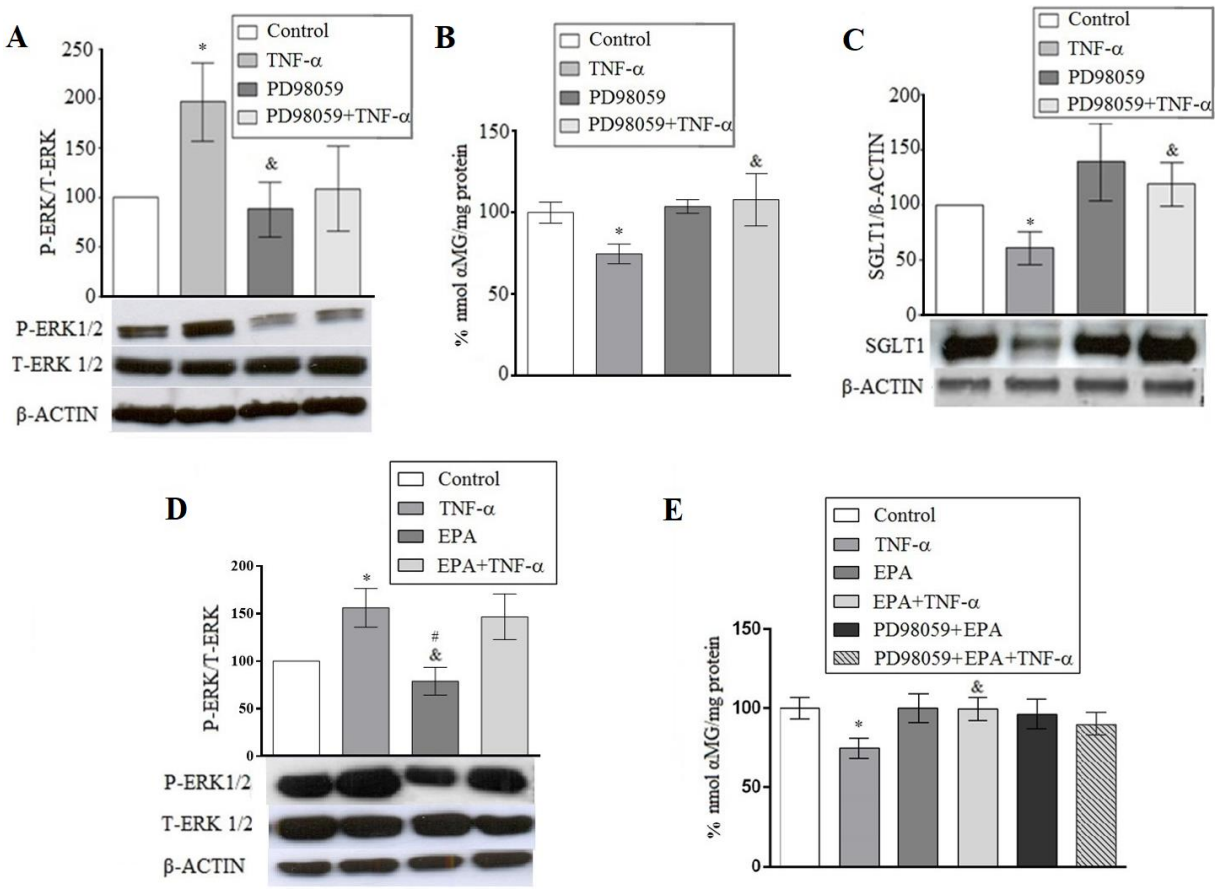


Figure 2. Implication of the ERK1/2 intracellular pathway, using its inhibitor PD98059 (50 µM), in the preventive effect of 100 µM EPA on 10 ng/ml TNF-α inhibition of αMG uptake. Caco-2 cells were pre-incubated for 1h under the different conditions indicated, before measuring the uptake of 0.1 mM αMG for 15 min A. Expression of phospho ERK 1/2 (P-ERK) and total ERK 1/2 (T-ERK) in the presence of TNF-α was measured in the cytosolic fraction of the cells and referred to the expression of β-actin. The mean ratio P-ERK/T-ERK densities (n=4-6) is represented. A representative Western blot image is shown. The molecular weight of both immunoreactive bands was ~45 kDa. B. Effect of PD98059 on TNF-α action on αMG uptake. Data (n=12) are represented as mean ± SEM. C. Effect of PD98059 and TNF-α on SGLT1 expression in BBMV. The ratio SGLT1/β-actin is expressed as % of control (n=3). A representative Western blot image is shown. SGLT1 appears as a ~75 kDa immunoreactive band. D. Expression of phospho ERK 1/2 (P-ERK) and total ERK 1/2 (T-ERK) in the presence of TNF-α and EPA was measured in the cytosolic fraction of the cells and referred to the expression of β-actin. The mean ratio P-ERK/T-ERK densities (n=4-6) is represented. A representative Western blot image is shown. The molecular weight of both immunoreactive bands was ~45 kDa. E. Effect of PD98059 on EPA and TNF-α action on αMG uptake. Data (n=12) are represented as mean ± SEM. *p<0.05 vs. control; & p<0.05 vs. TNF-α; # p<0.05 vs. EPA+TNF-α.

3.3. Implication of AMPK activation in the preventive effect of EPA on TNF-α inhibition of αMG uptake

It is known that EPA activates the AMPK intracellular pathway (Wu *et al*., 2012). Moreover, AMPK is entailed in the EPA blocking effect of lipolysis induced by TNF-α (Lorente-Cebrián *et al.*, 2009, 2012). Therefore, we decided to investigate whether AMPK could be also involved in the preventive effect of EPA on TNF-α inhibition of αMG uptake. Our data showed that the phosphorylation (activation) of AMPK was diminished by the incubation of the cells with 10 ng/ml TNF-α (Fig. 3A). Interestingly, in the presence of 100 μM EPA, TNF-α did not modify AMPK phosphorylation levels compared with the control condition (Fig. 3A). To deepen in the role of AMPK in the effect of EPA, we performed a new set of experiments using the AMPK inhibitor CC (20 μM) and its activator AICAR (1 mM). As expected, the AMPK inhibitor alone reduced the phosphorylation of AMPK and also reversed the ability of EPA to prevent the inhibition of AMPK phosphorylation induced by TNF-α (Fig. 3A). On the other hand, AICAR increased AMPK phosphorylation and this stimulatory effect was maintained in the presence of TNF-α (Fig. 3A).

The uptake studies showed that the treatment of the cells with the AMPK inhibitor CC inhibited αMG uptake in the same magnitude than TNF-α (~40%). In line with figure 3A, EPA was not able to block the inhibitory effect of TNF-α on sugar uptake in the presence of CC. On the other hand, AICAR prevented the decrease of αMG transport induced by TNF-α treatment (Fig. 3B), as occurred with EPA.

Regarding SGLT1 expression in the brush border membrane, as previously shown (Fig. 2C), pre-incubation of the cells with TNF-α decreased the expression of the sugar transporter, whereas in the presence of EPA or AICAR, the inhibition induced by TNF-α on SGLT1 expression was not observed (Fig. 3C). However, CC totally abolished the ability of EPA to prevent the reduction of SGLT1 expression in the apical membrane induced by TNF-α (Fig. 3C).

As expected, EPA diminished the inhibitory effect of CC on AMPK phosphorylation, sugar uptake and SGLT1 expression in the plasma membrane (supplementary figure 1).

All these results demonstrated that the AMPK intracellular pathway is implicated in the insertion of SGLT1 in the plasma membrane and that this pathway is regulated by both, EPA and TNF-α.

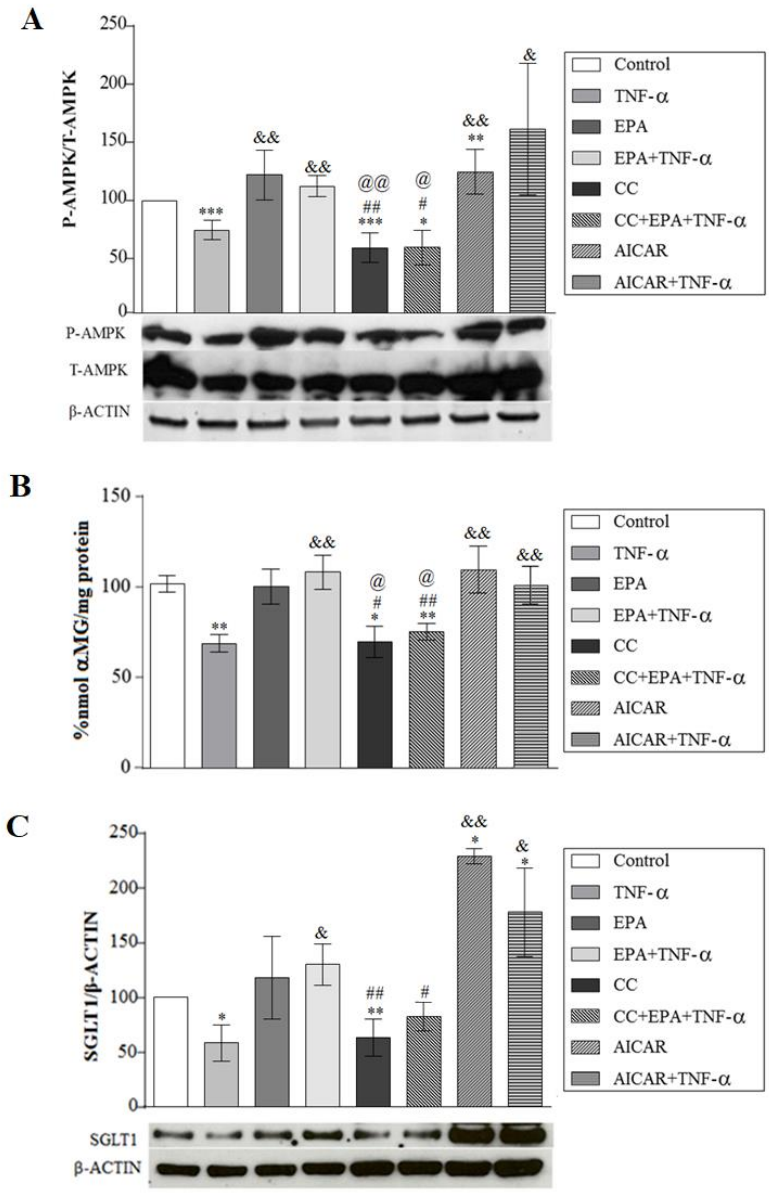
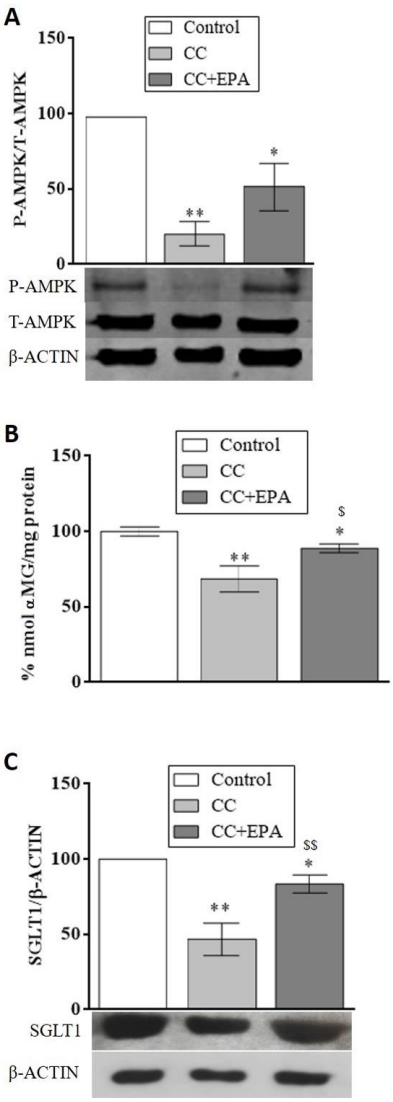


Figure 3. Implication of the AMPK intracellular pathway in the preventive effect of 100 µM EPA on 10 ng/ml TNF-α inhibition of αMG uptake, using its inhibitor Compound C (CC, 20 µM) and its activator AICAR (1mM). Caco-2 cells were pre-incubated for 1h under the different conditions indicated before measuring 0.1 mM αMG uptake for 15 min. A. Effect of TNF-α, EPA and the AMPK regulators CC and Aicar, on AMPK- phosphorylation. Expression of phospho AMPK (P-AMPK) and total AMPK (T-AMPK) was measured in the cytosolic fraction of the cells and referred to the expression of β-actin. The mean ratio P-AMPK/T-AMPK densities (n=4-6) is represented. A representative Western blot image is shown. The molecular weight of both immunoreactive bands was ~62 kDa. B. Effect of TNF-α, EPA and the AMPK regulators, CC and Aicar, on αMG uptake. Data (n=6-8) are represented as mean ± SEM. C. Effect of TNF-α, EPA and the AMPK regulators, CC and Aicar, on SGLT1 expression in BBMV. The intensity ratio SGLT1/β-actin is expressed as % of control (n=3-4). A representative Western blot image is shown. SGLT1 appears as a ~75 kDa immunoreactive band. *p<0.05, **p<0.01, ***p<0.01 vs. control condition; & p<0.05, && p<0.01 vs. TNF-α; @ p<0.05, @@ p<0.01 vs. EPA; #p<0.05, ##p<0.01 vs. EPA+TNF-α.



Supplementary 1. Effect of EPA (100 µM) on Compound C (CC, 20 µM) inhibition of AMPK phosphorylation, sugar uptake and SGLT1 expression. Caco-2 cells were pre-incubated for 1 h under the different conditions indicated before measuring 0.1 mM αMG uptake for 15 min. A. Effect of CC alone and in the presence of EPA on AMPK- phosphorylation. Expression of phospho AMPK (P-AMPK) and total AMPK (T-AMPK) was measured in the cytosolic fraction of the cells and referred to the expression of β-actin. The mean ratio P-AMPK/T-AMPK densities (n=5) is represented. A representative Western blot image is shown. The molecular weight of both immunoreactive bands was ~62 kDa. B. Effect of CC alone and in the presence of EPA on αMG uptake. Data (n=15) are represented as mean ± SEM. C. Effect of CC alone and in the presence of EPA on SGLT1 expression in BBMV. The intensity ratio SGLT1/β-actin is expressed as % of control (n=6). A representative Western blot image is shown. SGLT1 appears as a ~75 kDa immunoreactive band. *p<0.05, **p<0.01 vs. control condition; $ p<0.05, $$ p<0.01 vs. CC- treated cells.

To investigate if ERK1/2 activation is involved in TNF-α effect on AMPK we analyzed the phosphorylation of AMPK in the presence of PD98059 (ERK1/2 inhibitor) alone and with TNF-α. As shown in figure 4, the decrease induced by TNF-α on AMPK phosphorylation was partially prevented by PD98059. In the presence of PD98059 alone, AMPK phosphorylation level was similar than in the control condition (Fig. 4). These results suggest that TNF-α inhibition of AMPK may occur through ERK1/2 activation.

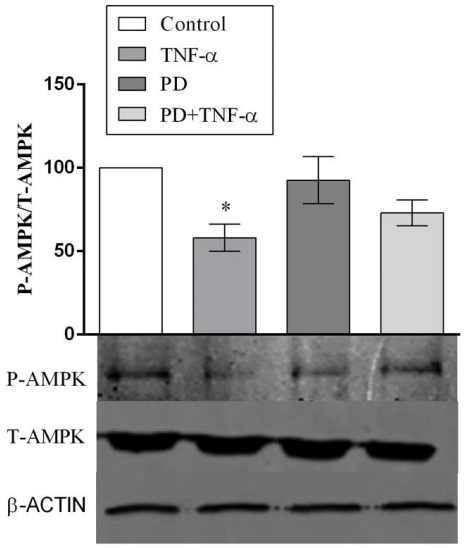


Figure 4. Effect of the ERK1/2 inhibitor PD98059 (50 µM) and TNF-α (10 ng/ml) on AMPK phosphorylation. Expression of phospho-AMPK (P-AMPK) and total AMPK (T-AMPK) was measured in the cytosolic fraction of the cells and referred to the expression of β-actin. The mean ratio P-AMPK/T-AMPK densities (n=5) is represented. A representative Western blot image is shown. The molecular weight of both immunoreactive bands was ~62 kDa. * $p<0.05$ vs. control.

3.4. Implication of GPR120 in EPA effect on TNF-α-induced inhibition of αMG uptake

Finally, we investigated whether EPA action on αMG uptake was mediated through the EPA receptor GPR120. Our data showed that in the presence of the GPR120 antagonist AH7614 (100 µM), EPA was not able to block either TNF-α inhibitory effect on αMG uptake (Fig. 5A) or TNF-α- induced inhibition of AMPK phosphorylation (Fig. 5B). These results suggest the involvement of GPR120 stimulation in the effect of EPA on TNF-α-induced inhibition of sugar uptake.

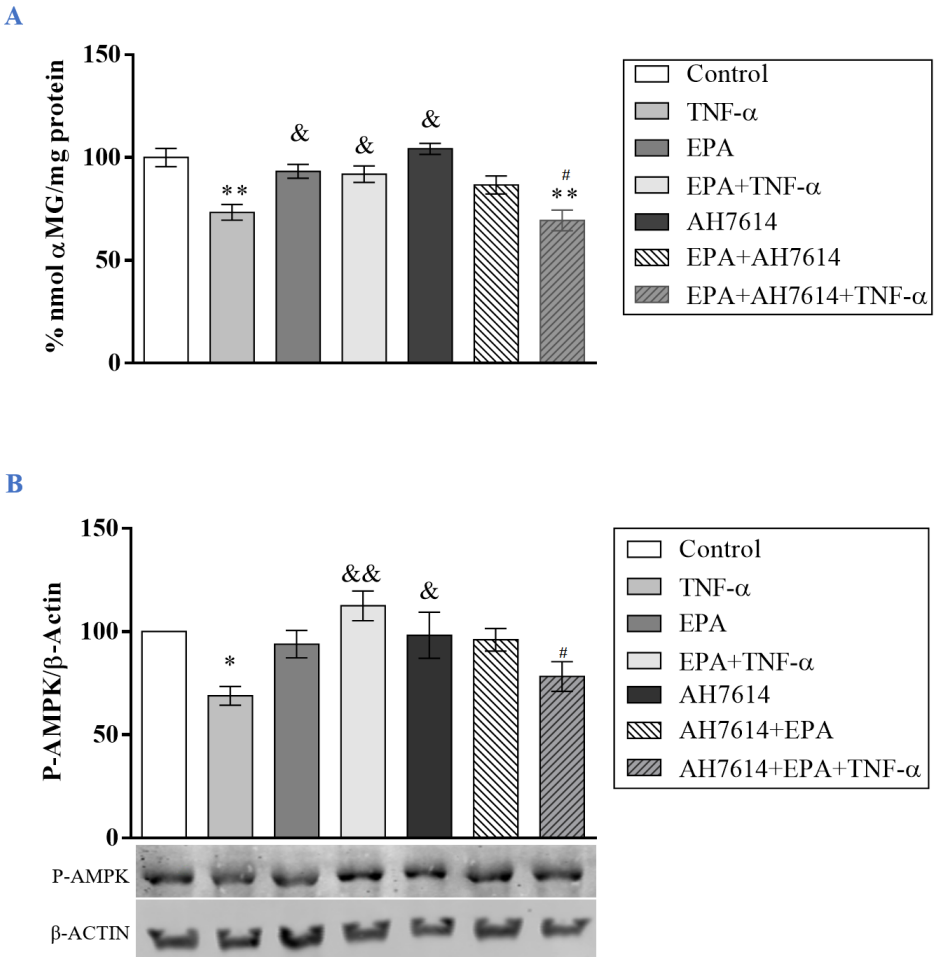


Figure 5. Effect of the ERK1/2 inhibitor PD98059 (50 µM) and TNF-α (10 ng/ml) on AMPK phosphorylation. Expression of phospho-AMPK (P-AMPK) and total AMPK (T-AMPK) was measured in the cytosolic fraction of the cells and referred to the expression of β-actin. The mean ratio P-AMPK/T-AMPK densities (n=5) is represented. A representative Western blot image is shown. The molecular weight of both immunoreactive bands was ~62 kDa. * $p<0.05$ vs. control.

4. Discussion

Our group previously demonstrated in Caco-2 cells that TNF-α, via TNFR1 receptor, decreases galactose and αMG uptake by down-regulating the expression of the Na^+/glucose cotransporter SGLT1 in the apical membrane (Barrenetxe *et al.*, 2013).

In the present work, we show that TNF-α reduces sugar transport in Caco-2 cells through the activation of ERK1/2 that would inhibit the AMPK pathways which, in turn, would induce the recruiting of SGLT1 from the apical membrane into intracellular compartments. Other authors have demonstrated that the activation of intracellular MAPKs-dependent signaling pathways (ERK, P38 and JNK) regulates the expression of rabbit SGLT1 (Castaneda-Sceppa *et al.*, 2010). Also, it has been shown in muscle cells, that endoplasmic reticulum stress activates ERK which, in turn, decreases AMPK activity. The ERK inhibitor U0126, recovered AMPK phosphorylation (Hwang *et al*., 2013). Regarding the role of AMPK in the regulation of intestinal sugar transport, we show that activation of AMPK by AICAR enhances SGLT1 expression in the brush border membrane of Caco-2 cells, as it has been previously observed (Sopjani *et al*., 2010). Moreover, AMPK inhibition by CC reduces the levels of SGLT1 in the brush border membrane, supporting a role of AMPK in the regulation of this sugar transporter. In this context, we have recently shown that Cardiotrophin-1, a cytokine of the IL-6 family, decreases sugar transport by reducing SGLT1 expression in Caco-2 cells apical membrane through the inhibition of AMPK (López-Yoldi *et al*., 2016).

In the last years, the potential beneficial effects of n-3 fatty acids on intestinal inflammation have been widely studied (Belluzzi *et al*., 1996; Almallah *et al*., 1998; Calder, 2008). At the molecular level, EPA and DHA show anti-inflammatory properties in premature rat pups by regulating eicosanoid- and nuclear factor-κB-related metabolite expression (Ohtsuka *et al*., 2011). Here, we demonstrate for the first time, that EPA prevents the inhibitory effect of TNF-α on sugar uptake by activating AMPK. In line with these results, glucose transport in weaning piglets' jejunum is up-regulated when feeding n-3 PUFA during gestation and lactation via activation of AMPK, which increases SGLT1 and GLUT2 expression (Gabler *et al*., 2007). The implication of AMPK on the physiological effect of EPA has already been demonstrated in 3T3-L1 adipocytes, where EPA blunts the lipolytic effect of TNF-α trough the stimulation of

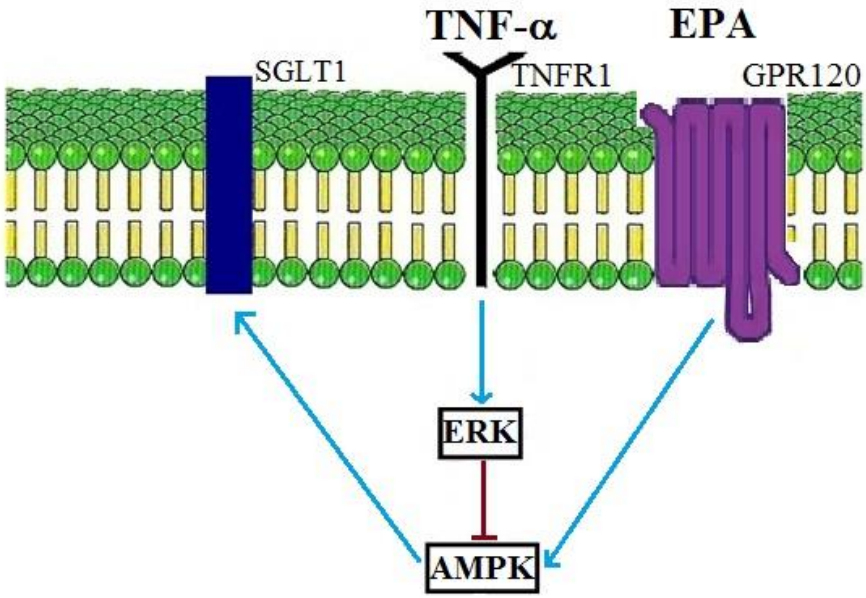
this intracellular pathway (Lorente-Cebrián *et al*., 2012). Also, in diet-induced obese mice, diet containing n-3 PUFAs increases AMPK phosphorylation levels in several tissues (González-Périz *et al*., 2009; Kopecky *et al*., 2009).

It has been proved that GPR120 stimulation by n-3 fatty acids inhibits inflammatory responses mediated by TNF-α (Oh & Lagakos, 2011; Calder, 2015). Moreover, it is widely known that GPR120 usually acts as EPA receptor (Oh & Lagakos, 2011). GPR120 is expressed in Caco-2 cells, where binding of n-3 and n-6 PUFAs induces its activation, which in turn activates Gαq leading to an increase of cytosolic Ca^{2+} and activation of ERK1/2 (Mobraten *et al*., 2013). Also, it has been demonstrated that ligand activation of GPR120 in intestinal epithelial Caco-2 cells exert anti-inflammatory effects via inhibition of NF-κB activation (Anbazhagan *et al*., 2016). All this data supports our current results which strongly suggest that the ability of EPA to reverse the inhibitory effect of TNF-α on αMG uptake and AMPK phosphorylation is mediated by the binding to the receptor GPR120. Other authors have shown that DHA, another marine n-3 PUFAs, stimulates glucose uptake in skeletal muscle through the phosphorylation of AMPK via GPR120 (Kim *et al*., 2015)

4.1. Conclusion

We have demonstrated that TNF-α decreases sugar transport in Caco-2 cells by the activation of ERK1/2 and the inhibition of AMPK pathways. Most importantly, we have shown that EPA prevents TNF-α inhibition of SGLT1 expression in the plasma membrane and sugar uptake by activating the AMPK signaling pathway, probably through GPR120 (Graphical abstract). These results open perspectives to investigate the use of EPA as a beneficial fatty acid for the treatment of nutrients absorption disorders in patients suffering intestinal inflammation.

Graphical abstract



Conflicting interests

The authors declared no conflicts of interest.

Acknowledgements

We thank Asunción Redín for her unconditional technical assistance.

This work was supported by grants from Ministry of Economy and Competitiveness (MINECO) of the Government of Spain (BFU2012-36089), and from Dept. of Health, Navarra Government (67-20165), and CIBER Physiopathology of Obesity and Nutrition (CIBERobn), Carlos III Health Research Institute (CB12/03/30002). RC-M. is supported by a fellowship from Asociación de Amigos de la Universidad de Navarra.

References

A

Albenberg, L. G., Lewis, J. D. and Wu, G. D. (2012). Food and Gut Microbiota in IBD: A Critical Connection. *Current Opinion in Gastroenterology* **28** (4), 314–320.

Almallah, Y. Z., Richardson, S., O'Hanrahan, T., Mowat, N. A. G., Brunt, P. W., Sinclair, T. S., Ewen, S., Heys, S. D. and Eremin, O. (1998). Distal procto-colitis, natural cytotoxicity, and essential fatty acids. *American Journal of Gastroenterology* **93** (5), 804–809.

Anbazhagan, A. N., Priyamvada, S., Gujral, T., Bhattacharyya, S., Alrefai, W. A., Dudeja, P. K. and Borthakur, A. (2016). A Novel Anti-inflammatory Role of GPR120 in Intestinal Epithelial Cells. *American Journal of Physiology - Cell Physiology* **310** (7), C612-621.

B

Barbalho, S.M., de Alvares Goulart, R., Quesada, K., Dib Bechara, M., de Cássio Alves de Carvalho, A. (2016). Inflammatory bowel disease : can omega-3 fatty acids really help?. *Annals Gastroenterology* **29** (1), 37–43.

Barrenetxe, J., Sánchez, O., Barber, A., Gascón, S., Rodríguez-Yoldi, M. J. and Lostao, M. P. (2013). TNFα regulates sugar transporters in the human intestinal epithelial cell line Caco-2. *Cytokin*e **64** (1), 181–187.

Belluzzi, a, Brignola, C., Campieri, M., Pera, A., Boschi, S. and Miglioli, M. (1996). Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease. *The New England journal of medicine* **334** (24), 1557–1560.

Bertolo, C., Barrenetxe, J., Barber, A., Lostao, M. . (2002). Cytokine effect on intestinal galactose absorption. *Journal of Physiology and Biochemistry* **58** (1), 61–62.

C

Calder, P. C. (2008). Polyunsaturated fatty acids, inflammatory processes and inflammatory bowel diseases. *Molecular Nutrition and Food Research* **52** (8) 885–897.

Calder, P. C. (2015). Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. *BBA - Molecular and Cell Biology of Lipids* **1851** (4), 469–484.

Castaneda-Sceppa, C., Subramanian, S. and Castaneda, F. (2010). Protein kinase C mediated intracellular signaling pathways are involved in the regulation of sodium-dependent glucose co-transporter SGLT1 activity. *Journal of Cellular Biochemistry* **109** (6), 1109–1117.

F

Figueras, M., Olivan, M., Busquets, S., López-soriano, F. J. and Argilés, J. M. (2011). Effects of eicosapentaenoic acid (EPA) treatment on insulin sensitivity in an animal model of diabetes: improvement of the inflammatory status. *Obesity*, **19** (2), 362–369.

Foley, K. F., Pantano, C., Ciolino, A. and Mawe, G. M. (2007). IFN-gamma and TNF-αlpha decrease serotonin transporter function and expression in Caco2 cells. *Am Journal of Physiology Gastrointestinal and Liver Physiology* **292** (3), G779-84.

G

Gabler, N. K., Radcliffe, J. S., Spencer, J. D., Webel, D. M. and Spurlock, M. E. (2007). Feeding long-chain n-3 polyunsaturated fatty acids during gestation increases intestinal glucose absorption potentially via the acute activation of AMPK. *Journal of Nutritional Biochemistry* **20** (1), 17–25.

Garriga, C., Rovira, N., Moreto, M., Planas, J. . (1999). Expression of Na^+-D-glucose cotransporter in brush-border membrane of the chicken intestine. *American Journal of Physiology* **276**, 627–631.

González-Périz, A., Horrillo, R., Ferré, N., Gronert, K., Dong, B., Morán-Salvador, E., Titos, E., Martínez-Clemente, M., López-Parra, M., Arroyo, V., Clària, J., Gonza, A., Horrillo, R., Gronert, K., Dong, B., Morán-Salvador, E., Titos, E., Martínez-Clemente, M. and Lo, M. (2009). Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. *FASEB Journal* **23** (6), 1946–57.

H

Hébuterne, X., Filippi, J., Al-Jaouni, R. and Schneider, S. (2009). Nutritional consequences and nutrition therapy in Crohn's disease. *Gastroentérologie clinique et biologique* **33** (S 3), S235-44.

Hirasawa, A., Tsumaya, K., Awaji, T., Katsuma, S., Adachi, T., Yamada, M., Sugimoto, Y., Miyazaki, S. and Tsujimoto, G. (2005). Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nature Medicine* **11** (1), 90–94.

Hur, S. J., Kang, S. H., Jung, H. S., Kim, S. C., Jeon, H. S., Kim, I. H. and Lee, J. D. (2012). Review of natural products actions on cytokines in inflammatory bowel disease. *Nutrition Research* **32** (11), 801–816.

Hwang, S., Jeong, Y., Li, X., Kim, Y. D., Lu, Y., Chang, Y., Lee, I. and Chang, H. W. (2013). Inhibitory cross-talk between the AMPK and ERK pathways mediates endoplasmic reticulum stress-induced insulin resistance in skeletal muscle. *British Journal of Pharmacology* **169**, 69–81.

I

Ichimura, A., Hara, T. and Hirasawa, A. (2014). Regulation of energy homeostasis via GPR120. *Frontiers in Endocrinology* **5**, 1–5. doi: 10.3389/fendo.2014.00111.

Issa, M. and Saeian, K. (2011) 'Diet in inflammatory bowel disease. *Nutrition in clinical practice* **26** (2), 151–4.

K

Kim, N., Lee, J. O., Lee, H. J., Kim, H. I., Kim, J. K., Lee, Y. W., Lee, S. K., Kim, S. J., Park, S. H. and Kim, H. S. (2015). Endogenous ligand for GPR120, docosahexaenoic acid, exerts benign metabolic effects on the skeletal muscles via AMP-activated protein kinase pathway. *Journal of Biological Chemistry* **290** (33), 20438–20447.

Komatsu, M., Kobayashi, D., Saito, K., Furuya, D., Yagihashi, A., Araake, H., Tsuji, N., Sakamaki, S., Niitsu, Y. and Watanabe, N. (2001). Tumor necrosis factor-α in serum of patients with inflammatory bowel disease as measured by a highly sensitive immuno-PCR. *Clinical Chemistry* **47** (7), 1297–1301.

Kopecky, J., Rossmeisl, M., Flachs, P., Kuda, O., Brauner, P., Jilkova, Z., Stankova, B., Tvrzicka, E. and Bryhn, M. (2009). n-3 PUFA: bioavailability and modulation of adipose tissue function. *The Proceedings of the Nutrition Society* **68** (4), 361–9.

L

López-Yoldi, M., Castilla-Madrigal, R., Lostao, M. P., Barber, A., Prieto, J., Martínez, J. A., Bustos, M. and Moreno-Aliaga, M. J. (2016). Cardiotrophin-1 decreases intestinal sugar uptake in mice and in Caco-2 cells. *Acta Physiologica* **217** (3), 217–226.

Lorente-Cebrián, S., Bustos, M., Marti, A., Fernández-Galilea, M., Martinez, J. A. and Moreno-Aliaga, M. J. (2012). Eicosapentaenoic acid inhibits tumour necrosis factor-α-induced lipolysis in murine cultured adipocytes. *Journal of Nutritional Biochemistry* **23** (3), 218–227.

Lorente-Cebrián, S., Bustos, M., Marti, A., Martínez, J. A. and Moreno-Aliaga, M. J. (2009). Eicosapentaenoic acid stimulates AMP-activated protein kinase and increases visfatin secretion in cultured murine adipocytes. *Clinical science* **117** (6), 243–249.

Lorente-Cebrián, S., Costa, A. G. V, Navas-Carretero, S., Zabala, M., Laiglesia, L. M., Martínez, J. A. and Moreno-Aliaga, M. J. (2015). An update on the role of omega-3 fatty acids on inflammatory and degenerative diseases. *Journal of Physiology and Biochemistry* **71** (2), 341–349.

Lorente-Cebrián, S., Costa, A. G. V, Navas-Carretero, S., Zabala, M., Martínez, J. A. and Moreno-Aliaga, M. J. (2013). Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: A review of the evidence. *Journal of Physiology and Biochemistry* **69** (3), 633–651.

M

Mobraten, K., Haug, T. M., Kleiveland, C. R. and Lea, T. (2013). Omega-3 and omega-6 PUFAs induce the same GPR120-mediated signalling events, but with different kinetics and intensity in Caco-2 cells. *Lipids in health and disease* **12** (1), 101.

Murata, M., Kaji, H., Takahashi, Y., Iida, K., Mizuno, I., Okimura, Y., Abe, H. and Chihara, K. (2000). Stimulation by eicosapentaenoic acids of leptin mRNA expression and its secretion in mouse 3T3-L1 adipocytes in vitro. *Biochemical and biophysical research communications* **270** (2), 343–8.

O

Oh, D. Y. and Lagakos, W. S. (2011). The role of G-protein-coupled receptors in mediating the effect of fatty acids on inflammation and insulin sensitivity. *Current Opinion in Clinical Nutrition and Metabolic Care* **14** (4), 322–327.

Oh, D. Y., Talukdar, S., Bae, E. J., Imamura, T., Morinaga, H., Fan, W., Li, P., Lu, W. J., Watkins, S. M. and Olefsky, J. M. (2010). GPR120 Is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-inflammatory and Insulin-Sensitizing Effects. *Cell* **142** (5), 687–698.

Ohtsuka, Y., Okada, K., Yamakawa, Y., Ikuse, T., Baba, Y., Inage, E., Fujii, T., Izumi, H., Oshida, K., Nagata, S., Yamashiro, Y. and Shimizu, T. (2011). Ω-3 Fatty Acids Attenuate Mucosal Inflammation in Premature Rat Pups. *Journal of Pediatric Surgery* **46** (3), 489–495.

P

Perez-Matute, P., Marti, A., Martinez, J. A., Fernandez-Otero, M. P., Stanhope, K. L., Havel, P. J. and Moreno-Aliaga, M. J. (2005). Eicosapentaenoic fatty acid increases leptin secretion from primary cultured rat adipocytes: role of glucose metabolism. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **288** (6), R1682-1688.

Pérez-Matute, P., Pérez-Echarri, N., Martínez, J. A., Marti, A. and Moreno-Aliaga, M. J. (2007). Eicosapentaenoic acid actions on adiposity and insulin resistance in control and high-fat-fed rats: role of apoptosis, adiponectin and tumour necrosis factor-alpha. *The British journal of nutrition* **97** (2), 389–398.

R

Rossol, M., Meusch, U., Pierer, M., Kaltenhäuser, S., Häntzschel, H., Hauschildt, S. and Wagner, U. (2007). Interaction between transmembrane TNF and TNFR1/2 mediates the activation of monocytes by contact with T cells. *Journal of immunology* **179**, 4239–4248.

S

Sharma, N., Laftah, A. H., Brookes, M. J., Cooper, B., Iqbal, T. and Tselepis, C. (2005). A role for tumour necrosis factor alpha in human small bowel iron transport. The *Biochemical journal* **390** (2), 437–46.

Shirazi-Beechey, S. P., Davies, a G., Tebbutt, K., Dyer, J., Ellis, A., Taylor, C. J., Fairclough, P. and Beechey, R. B. (1990) 'Preparation and properties of brush-border membrane vesicles from human small intestine. *Gastroenterology* **98** (3), pp. 676–685.

Shoda, R., Matsueda, K., Yamato, S., Umeda, N. (1996). Epidemiologic analysis of Crohn disease in Japan: increased dietary intake of n-6 polyunsaturated fatty acids and animal protein relates to the increased incidence of Crohn disease in Japan. *American Journal of Clinical Nutrition* **26** (2), 151–154.

Sopjani, M., Bhavsar, S. K., Fraser, S., Kemp, B. E., Föller, M. and Lang, F. (2010) Regulation of Na^+-coupled glucose carrier SGLT1 by AMP-activated protein kinase. *Molecular membrane biology* **27** (2–3), 137–144.

T

Tabas, I. and Glass, C. K. (2013). Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science* **339** (6116), 166–72.

W

Wu, Y., Zhang, C., Dong, Y., Wang, S., Song, P., Viollet, B. and Zou, M. H. (2012). Activation of the AMP-activated protein kinase by eicosapentaenoic acid (EPA, 20:5 n-3) improves endothelial function in vivo. *PLoS ONE* **7** (4), 1–9.

CHAPTER 2.

DHA and its derived lipid mediators, MaR1, RvD1 and RvD2, block TNF-α inhibition of intestinal sugar and glutamine uptake in Caco-2 cells.

Rosa Castilla-Madrigal[1,2], Eva Gil-Iturbe[1,2], Marta López de Calle[1], María J. Moreno-Aliaga[1,2,3,4], María Pilar Lostao[1,2,3]

[1]University of Navarra, Dept. Nutrition, Food Science and Physiology, Irunlarrea 1, 31008 Pamplona, Spain

[2]University of Navarra, Nutrition Research Centre, Irunlarrea 1, 31008 Pamplona, Spain

[3]IdiSNA, Navarra Institute for Health Research, Pamplona, Spain

[4]CIBERobn, Physiopathology of Obesity and Nutrition, Institute of Health Carlos III (ISCIII), Madrid, Spain

Abstract

Tumor necrosis factor alfa (TNF-α) is a pro-inflammatory cytokine which is highly involved in the pathogenesis of intestinal inflammation. Omega-3 polyunsaturated fatty acids (n3-PUFAs) show anti-inflammatory actions on inflammatory-related pathologies including intestinal inflammation. We have previously demonstrated that the omega-3 EPA prevents TNF-α inhibition of sugar uptake in Caco-2 cells through the involvement of GPR120. The goal of the present work was to investigate whether the n3-PUFA DHA and its derived specialized pro-resolving lipid mediators (SPMs) MaR1, RvD1 and RvD2, can block TNF-α inhibition of intestinal sugar and glutamine uptake in Caco-2 cells.

DHA blocked TNF-α-induced inhibition of αMG uptake and SGLT1 expression in the apical membrane, through a pathway which seemed to be independent of GPR120. MaR1, RvD1 and RvD2 showed the same preventive effect. In diet-induced obese (DIO) mice, αMG intestinal transport and SGLT1 expression in the brush border membrane was lower than in lean animals. Oral administration of MaR1 to DIO mice did not reverse those effects. However, MaR1 did reverse the up-regulation of the pro-inflammatory cytokines found in the DIO intestinal mucosa. TNF-α also inhibited glutamine uptake in Caco-2 cells being this inhibition also prevented by EPA, DHA and the DHA derived SPMs. Interestingly, TNF-α increased the expression in the membrane of the glutamine transporter B^0AT1. This increase was partially avoided by the omega-3 fatty acids. These data reveal DHA and its specialized pro-resolving lipid mediators as promising biomolecules to restore intestinal nutrients transport during intestinal inflammation.

1.Introduction

Tumor necrosis factor alfa (TNF-α) is a pro-inflammatory cytokine which is highly involved in the pathogenesis of intestinal inflammation (Tabas & Glass, 2013; Vitale *et al.*, 2017). The blockage of TNF-α has been proved to be effective in the control of intestinal bowel disease (IBD). Therefore, biological drugs such as monoclonal antibodies have been developed to target TNF-α for the treatment of IBD (Argollo *et al*., 2017; Cohen *et al*., 2013). Nevertheless, studies show that around one third of the patients do not improve after the therapy. In other cases, loss of response may occur over time (Argollo *et al*., 2017). Hence, the discovery of other agents/biomolecules to counteract TNF-α deleterious effects is needed for the treatment of intestinal inflammation diseases.

Marine omega-3 polyunsaturated fatty acids (n3-PUFAs) show anti-inflammatory actions on inflammatory-related pathologies such as cardiovascular diseases, atherosclerosis, Alzheimer's disease, asthma, arthritis and colitis (Calder, 2015). Indeed, there is a positive association between high consumption of n-3 PUFAs in the diet and the reduction of ulcerative colitis (UC) risk (Ananthakrishnan *et al*., 2014; Hou *et al.*, 2011). In animal models of chronic intestinal inflammation, n-3 PUFA-rich diet ameliorates inflammation (Hokari *et al.*, 2013; Mbodji *et al*., 2013). Liu *et al.* (2012) reported that fish oil supplementation improved intestinal integrity in LPS-induced intestinal injury, by reducing TNF-α expression and inhibiting TLR4 and NOD2 signaling pathways. Thus, an alternative or complementary treatment for IBD therapy is the supplementation of the diet with n-3 PUFAs (Neuman & Nanau, 2012; Stewart *et al.*, 2011).

Eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are the main n-3 PUFAs in fish oil (Calder, 2013). EPA and DHA serve as substrates for the formation of specialized pro-resolving lipid mediators (SPMs) with potent anti-inflammatory and pro-resolutive properties, namely maresins (MaR), resolvins (Rv), protectins (PD) (Bento *et al.*, 2011; Chatterjee *et al.*, 2014; Serhan, 2007). Different from their precursors, the SPMs exert potent actions at picomolar to nanomolar range *in vivo* (Serhan *et al.*, 2002). Maresin 1 (MaR1) is a macrophage-derived mediator from DHA (Serhan & Chiang, 2009). Administration of MaR1 exerts protective actions in different experimental colitis mice

models by reducing pro-inflammatory cytokines levels (TNF-α) and enhancing the switch of pro-inflammatory M1 macrophages towards the anti-inflammatory M2 phenotype. As a consequence, it reduces body weight loss and colonic tissue damage (Marcon *et al.*, 2013). Serie D Resolvins (resolution-phase interaction products) derived from DHA, such as RvD1 and RvD2, are also endogenous lipid mediators with strong anti-inflammatory and immunomodulatory properties (Serhan & Chiang, 2009). In the ulcerative colitis (UC) mice model, endovenous administration of RvD1 and RvD2 reduced neutrophil infiltration and pro-inflammatory cytokines expression (Bento *et al.*, 2011). After the treatment of mice with dextran sodium sulfate to induce intestinal inflammation, Lee *et al.* (2017) observed increased levels of RvD1 along with decreased levels of its precursor DHA and EPA (precursor of resolvin E), suggesting initiation of mucosal healing by endogenous lipids.

During intestinal inflammation, nutrients and electrolytes malabsorption may occur in relation to alterations on the expression and activity of their intestinal transporters (Bertolo *et al.*, 2002; Foley *et al.*, 2007; Powell, 1990; Sharma *et al.*, 2005). We have previously demonstrated, in the human intestinal epithelial cell line Caco-2, that TNF-α inhibits αMG uptake by decreasing SGLT1 expression in the brush border membrane (Barrenetxe *et al.*, 2013), through the activation of ERK1/2 pathway (Castilla-Madrigal *et al.*, 2017). EPA prevents the inhibitory effect of TNF-α through the involvement of GPR120 and AMPK activation (Castilla-Madrigal *et al.*, 2017).

Here, we set out to investigate the possible blocking effect of DHA and its derived lipid mediators, MaR1, RvD1 and RvD2 on TNF-α inhibition of sugar and glutamine uptake in Caco-2 cells. N-3 PUFAs, maresins and resolvins also show anti-inflammatory actions in adipose tissue during obesity (Martinez-Fernandez *et al.*, 2015; 2017), but their actions on intestinal nutrients uptake and inflammation in obesity are poorly explored. Therefore, we also aim to study the effect of MaR1 on intestinal inflammation and sugar uptake in diet-induced (DIO) mice.

2. Material and methods

2.1. Cell culture

The human intestinal epithelial cell line Caco-2 was maintained at 37°C and 5% CO_2 in a humidified atmosphere. The cells were grown in Dulbecco's Modified Eagles medium (DMEM (1X) + GlutaMAX, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% non-essential amino acids (NEAA 100X, LONZA), 1% penicillin (10000 U/ml)–streptomycin (10,000 µg/ml) (Gibco) and 1% amphotericin B (250 µg/ml, Gibco). The culture medium was changed every 2 days. When cells reached 80% confluence, confirmed by microscopic observance, they were dissociated with 0.05% trypsin-EDTA (0.25% trypsin 1X, Gibco) and subcultured on 75 cm^2 plastic flasks at a density of $25x10^4$ cells/cm^2. For the uptake studies, the cells were seeded at a density of $6x10^4$ cells/cm^2 in 24-well culture plates. Experiments were performed 15-20 days post seeding, when the cells were differentiated into enterocytes.

2.2. Mice model

C57BL/6J male mice were purchased from Harlan Laboratories and fed as previously described (Laiglesia *et al.*, 2017) to obtain the obese phenotype. Diet-induced obesity (DIO) mice were divided into two groups (n= 8), DIO-MaR1 and DIO groups, that received for 10 days a daily oral gavage of MaR1 (50 µg/kg body weight) or the vehicle respectively. The lean (control) group, received vehicle (Laiglesia *et al.*, 2017).

All experimental procedures were performed under protocols approved by the University of Navarra Ethics Committee for the Use of Laboratory Animals, according to the National and Institutional Guidelines for Animal Care and Use (Protocols 029-12 and 047-15).

2.3. Uptake assays

2.3.1. Uptake assays in Caco-2 cells

Caco-2 cells were grown in 24-well culture plates and pre-incubated for 1 h in Dulbecco's Modified Eagles Medium without glucose (DMEM, Gibco), for αMG uptake studies, or in Krebs modified buffer (5.4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 137 mM NaCl, 0.3 mM KH_2PO_4, 10 mM glucose and 10 mM HEPES/Tris, pH 7.5), for glutamine uptake assays. Both media were supplemented with 1% BSA free fatty acids (Sigma). The pre-incubations were performed in control conditions and in the presence of 10 ng/ml TNF-α (PeproTech, Inc.) without and with 100 µM EPA (Cayman), 100 µM DHA (Cayman), 100 nM RvD1 (Cayman), 100 nM RvD2 (Cayman) or 100 nM MaR1 (Cayman). For the studies of the implication of the free fatty acid receptor 4, GPR120, on DHA effect, cells were pre-incubated with 10 ng/ml TNF-α in the presence of 100 µM DHA plus the GPR120 antagonist AH7614 (Tocris) at 100 µM. Uptake assays were performed by incubating the cells for 15 min with 0.1 mM α-methyl-D-glucose (αMG; Sigma) and traces of [^{14}C]-α-methyl-glucoside (0.08 µCi/ml; ARC 0131) or with 0.1 mM L-Glutamine (Gln; Sigma) and traces of [^{14}C]-L-Glutamine (0.1 µCi/ml; ARC 0196). The reaction was stopped by adding 500 µL of cold Phosphate Buffered Saline with calcium and magnesium (PBS, Sigma Aldrich). Cells were washed three times with PBS and solubilized by adding 500 µL 1% Triton X-100 in 1M NaOH for 1 h 30 min at 37°C. Samples (100 µL) were taken to measure radioactivity by liquid scintillation counter. Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay).

2.3.2. Uptake assays in everted intestinal rings

Uptake of αMG was measured in everted jejunal rings from lean (control), DIO and DIO-MaR1 mice to determine the effects of diet-induced obesity and the administration of MaR1 on sugar uptake. Four animals from each experimental group were used. After the sacrifice, a portion of jejunum was removed, everted and cut in small rings. Rings were incubated in Krebs-Ringer-Tris (KRT) solution with 1 mM αMG and traces of [^{14}C]-αMG (0.0025 µCi mL^{-1}). The incubation was performed at 37 °C for 15 min, under continuous shaking and gassed with O_2. Then, rings were washed in ice cold KRT solution, and incubated for 24 h in a

solution containing 0.1 M HNO_3 to denature the proteins and allow the exit of the cellular radioactivity, which was finally determined by liquid scintillation counting (Ducroc *et al.*, 2010).

All uptake results are expressed as nmol mg^{-1} of protein. Data are presented as % compared to controls which are normalized at 100 %.

2.4. Proteins isolation and Western blot analysis

2.4.1. Brush border membrane vesicle extraction

Caco-2 cells grown on 75 cm^2 plastic flasks were incubated under the different experimental conditions as described in section 2.3.1. After the incubation period, brush border membrane vesicles (BBMV) were isolated using the method of Shirazi-Beechey *et al.* (1990) with some modifications (Garriga *et al.*, 1999). BBMV were also obtained from jejunal mucosa of lean, DIO and DIO-MaR1 mice following the same procedure.

2.4.2. Protein surface biotinylation

Caco-2 cells grown on 12-well plates were treated under the different experimental conditions as described in section 2.3.1. Then, the cells were washed with PBS with 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ and incubated for 1 h under continuous shaking with sulfo-NHS-SS-biotin (1.5 mg/ml; 100 µL/well; Thermo Scientific) in biotinylation buffer (20 mM Hepes, 150 mM NaCl and 2 mM $CaCl_2$; pH 8.5). Next, cells were washed with glycine buffer (15 min; 100 mM Glycine in PBS) and PBS with 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$. Then, they were lysed in lysis buffer (8 mM NaH_2PO_4, 42 mM Na_2HPO_4, 1 % SDS, 100 mM NaCl, 0,1 % NP4O, 1 mM NaF, 10 mM sodium orthovanadate, 2 mM PMSF, 10 mM EDTA and 1% protease inhibitor cocktail, Sigma), homogenized with sonicator, and centrifuged at 13.000 rpm for 5 min. Supernatant was mixed with StreptAvidin-agarose beads (100 µL/ 150 µg protein; Thermo Scientific) and incubated overnight to pull down biotinylated antigens. Next, samples were centrifuged at 13,000 rpm to allow pelleting of the beads containing the membrane surface protein bound to them. Beads were washed with 100 µL lysis buffer and centrifuged for 5 min at 13,000 rpm. This procedure was repeated three times. Biotinylated fractions were boiled in gel loading buffer containing 100 µM DTT to separate the beads from the proteins.

All the manipulations were carried out at 4°C to avoid protein degradation of the samples. The protein content of the samples was determined by the standardized method of Bradford (Bio-Rad Protein Assay; Bio-Rad laboratories).

2.4.3. Western blot analysis

Solubilized proteins (30-40 µg) were resolved by electrophoresis on 12% SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane (Hybond P, GE Healthcare) which was then blocked in TBS-Tween 1X buffer (TBS-T 1X) with 10% of milk (Sveltesse, Nestle) for 1 h at room temperature, and incubated overnight at 4°C with the corresponding primary rabbit antibodies, used at 1:1000. The primary antibodies were: anti SGLT1 (Santa Cruz Biotechnology, Cat# sc-98974, RRID: AB_2191582) and anti-B^0AT1 (Abcam, Cat# ab180516). The β-actin mouse antibody (Santa Cruz Biotechnology Cat# sc-47778, RRID: AB_626632) was also used at 1:1000. After the incubation with the corresponding primary antibody, the membranes were washed out four times in TBS-T 1X and incubated for 1 h at room temperature with the corresponding peroxidase conjugated secondary antibody, goat anti-rabbit (Santa Cruz Biotechnology, Cat# sc-2004, RRID: AB_631746) and goat anti-mouse (Santa Cruz Biotechnology, Cat# sc-2005, RRID: AB_631736) at 1:10000. The immunoreactive bands were detected by enhanced chemiluminescence (Super Signal West Dura; Thermo Scientific) and quantified by densitometry analysis (Image Studio Lite, RRID: SCR_014211).

The results are expressed in percentage of the control value, which was set to 100.

2.5. Gene expression by Real-Time PCR

Total RNA was isolated from mice jejunal mucosa using TRIzol® reagent (Invitrogen, CA, USA) according to manufacturer's procedures. RNA-concentrations and quality were measured using Nanodrop Spectrophotometer ND1000 (Thermo Scientific, DE, USA). RNA was reverse transcribed to cDNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen). Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Monocyte chemoattractant protein 1 (MCP1/ CCL2) and TNF-α mRNA levels were determined using predesigned Taqman® Assays-on-Demand and Taqman Universal Master Mix (Applied

Biosystems, CA, USA). Amplification and detection of specific products were performed in the ABI PRISM 7900HT Fast Sequence Detection System (Applied Biosystems).

The levels of mRNA were normalized to 18S as housekeeping gene obtained from Applied Biosystems. Samples were analyzed in duplicate. Ct values were generated by the ABI PRISM 7900HT (Applied Biosystems). Finally, the relative expression of the genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.6. Statistical analysis

Statistical analysis was performed using the program Stata v12 (Stata, RRID: SCR_012763). Parametric or non-parametric tests (Kruskal-Wallis test, Student's t test and Mann Whitney's U test) were run depending on the sample size and the normality of the data. Results were expressed as means ± Standard Error of the Mean (SEM), and differences were considered significant at a $p<0.05$.

3. Results

3.1. Effect of DHA on the inhibition of αMG uptake by TNF-α in Caco-2 cells

Alpha methyl-glucose (αMG) is a glucose analog, specific substrate for the Na^+/glucose cotransporter (SGLT) family (Bormans *et al.*, 2003).

We first investigated whether DHA was able to block TNFα-induced inhibition of αMG in Caco-2 cells. As we previously reported, 10 ng/ml TNF-α decreased 0.1 mM αMG uptake by ~30% (Barrenetxe *et al*., 2013). Pre-incubation of the cells for 1 h with 100 μM DHA totally blocked TNF-α inhibition of αMG transport. DHA alone did not have any effect on sugar uptake (Fig. 1A). These effects were accompanied by a reduction of SGLT1 expression in BBMV in TNF-α treated cells (Barrenetxe *et al.*, 2013) that was prevented by DHA (Fig.1 B). Then, we investigated whether DHA action on αMG uptake was mediated through the DHA receptor GPR120. Our data showed that, in the presence of the GPR120 antagonist AH7614 (100 μM), DHA was also able to prevent TNF-α inhibitory effect on αMG uptake (Fig. 1C). This suggested that GPR120 stimulation was not involved in the blocking effect of DHA on TNF-α actions.

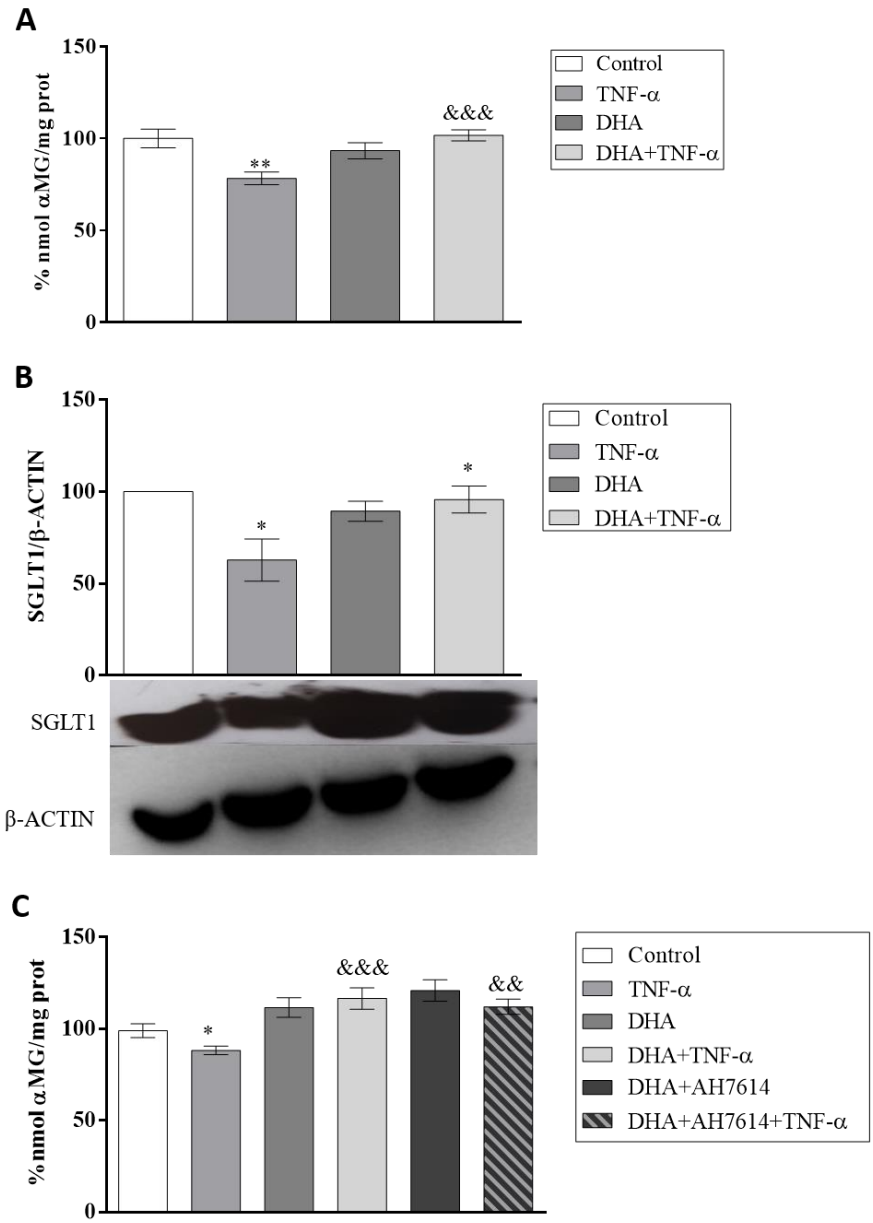


Figure 1. Effects of DHA on the inhibition of αMG uptake by TNF-α in Caco-2 cells. A. Caco-2 cells were pre-incubated for 1 h with 10 ng/mL TNF-α in the absence and in the presence of 100 µM DHA before measuring the uptake of 0.1 mM αMG for 15 min. Data (n= 16) are represented as mean ± SEM. **B.** SGLT1 expression in BBMV from Caco-2 cells treated as in **A**. The ratio SGLT1/β-Actin is expressed as % of control (n= 4). A representative Western blot image is shown. SGLT1 appears as a ~75 kDa immunoreactive band. **C.** Caco-2 cells were pre-incubated for 1 h with 10 ng/ml TNF-α in the absence and in the presence of 100 µM of the GPR120 antagonist AH7614 and 100 µM DHA, before measuring the uptake of 0.1 mM αMG for 15 min. Data (n= 16) are represented as mean ± SEM. * p<0.05, ** p<0.01 *vs.* control; $^{\&\&}$ p<0.01, $^{\&\&\&}$ p<0.001 *vs.* TNF-α.

3.2. Effect of MaR1, RvD1 and RvD2 on the inhibition of αMG uptake by TNF-α in Caco-2 cells

Next, we investigated whether the DHA lipid mediators MaR1, RvD1 and RvD2 showed the same blocking effect than its precursor on TNF-α inhibition of αMG uptake. As shown in Figures 2A, 2B and 2C, the three SPMs were able to block TNF-α effect by preventing the decrease of SGLT1 expression in the BBMV induced by the cytokine (Fig. 2D, 2E and 2F, respectively).

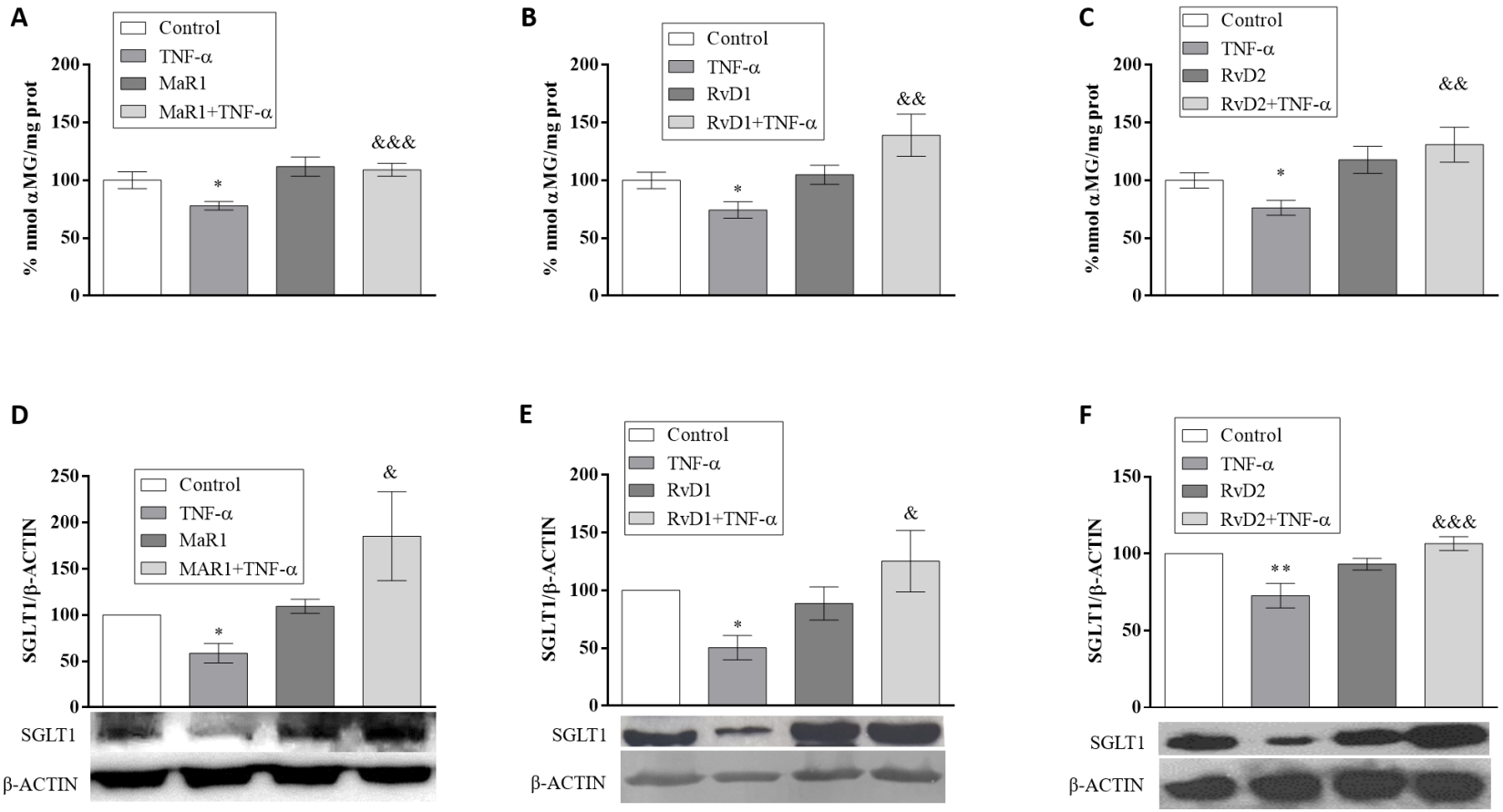


Figure 2. Effect of MaR1, RvD1 and RvD2 on the inhibition of αMG uptake by TNF-α in Caco-2 cells. A-C: Caco-2 cells were pre-incubated for 1 h with 10 ng/mL TNF-α in the absence and in the presence of 100 nM MaR1 (**A,** n=18), 100 nM RvD1 (**B,** n=10) or 100 nM RvD2 (**C,** n=10) before measuring the uptake of 0.1 mM αMG for 15 min. Data are represented as mean ± SEM. **D-F:** SGLT1 expression in BBMV from Caco-2 cells treated as previously described with 100 nM MaR1 (**D,** n=5), 100 nM RvD1 (**E,** n=4) or 100 nM RvD2 (**F,** n=5). The ratio SGLT1/β-actin is expressed as % of control. A representative Western blot image is shown. SGLT1 appears as a ~75 kDa immunoreactive band. * $p<0.05$, ** $p<0.01$ *vs.* control; $^{\&}$ $p<0.05$, $^{\&\&}$ $p<0.01$, $^{\&\&\&}$ $p<0.001$ *vs.* TNF-α.

3.3. Effect of the administration of MaR1 on αMG intestinal uptake in DIO mice

Obesity is known as a low-grade chronic inflammatory disease (Gonçalves *et al.*, 2015; Max *et al.*, 2013) in which there is an increase in the secretion of pro-inflammatory cytokines (Maurizi *et al.*, 2017; Mazur-Bialy *et al.,* 2017). In obesity, visceral adipose tissue surrounding the intestine grows, triggering intestinal inflammation (Kredel & Siegmund, 2014). To get closer to the in *vivo* model, we decided to study αMG intestinal transport in diet-induced obese (DIO) mice and DIO mice treated with MaR1 (DIO-MaR1) by oral gavage, and compare it with the transport in lean animals.

Uptake of 0.1 mM αMG by intestinal rings of DIO mice was decreased (~30%) when compared with the uptake of lean mice. However, MaR1 did not significantly reverse this reduction (Fig. 3A). In line with these results, SGLT1 expression in BBMV was reduced in the DIO mice. In DIO-MaR1 mice, the transporter expression showed an intermediate value between the expression observed in lean and DIO mice (Fig. 3B).

We also determined the gene expression of some pro-inflammatory proteins in the jejunal mucosa from the three experimental groups. The expression of *Tnf-α, Il-1β* and *Il-6* genes was increased in the DIO mice compared to the lean animals. The values returned to the control levels after treatment with MaR1. Macrophage chemotactic protein (MCP1) expression was not modified in any of the DIO groups compared to the control mice (Fig. 3C).

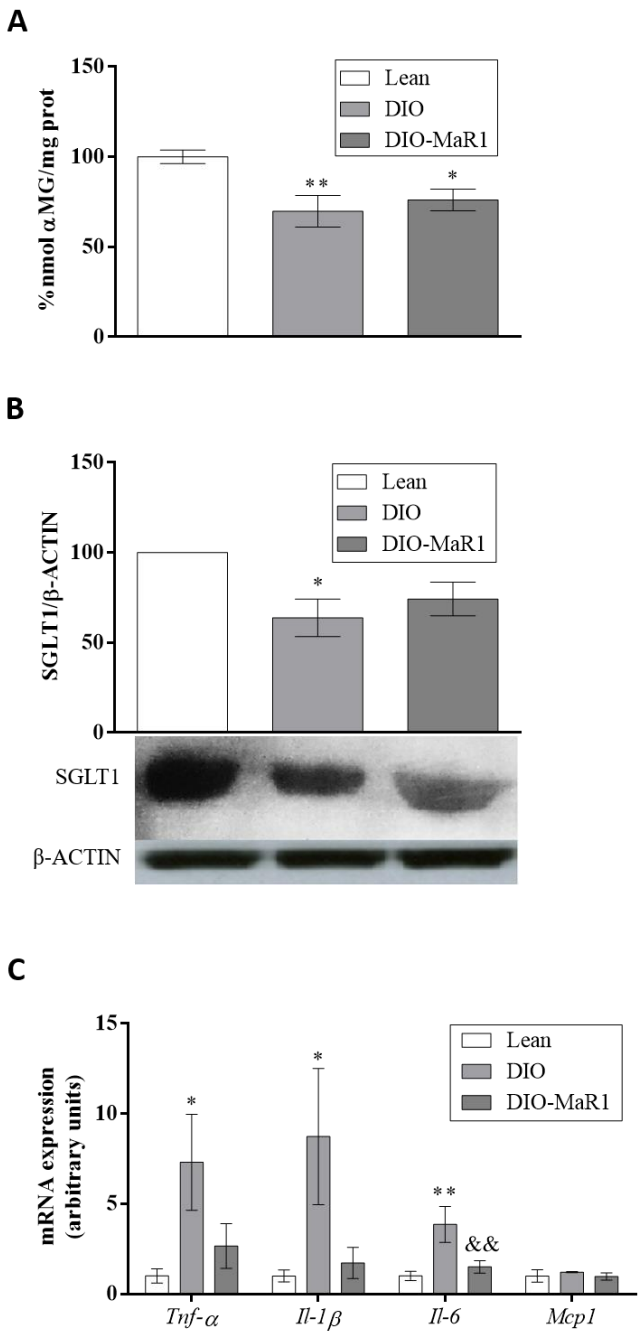


Figure 3. Effect of administration of MaR-1 (oral gavage) on αMG intestinal uptake in DIO mice. A. Uptake of 1 mM αMG (15 min) in intestinal everted jejunal rings from lean (control), DIO and DIO-MaR1 mice. Data (n= 15) are represented as mean ± SEM. **B**. SGLT1 expression in BBMV from lean (control), DIO and DIO-MaR1 mice jejunum. The ratio SGLT1/β-Actin is expressed as % of control (n= 3-4). A representative Western blot image is shown. SGLT1 appears as a ~75 kDa immunoreactive band. **C.** mRNA expression levels of the pro-inflammatory cytokines *Tnf-α, Il-1ß, Il-6* and the monocyte chemotactic protein-1 *Mcp1* in jejunal mucosa from lean, DIO and DIO-MaR1 mice. Data (n= 5-7; mean ± SEM) are expressed as fold change ($2^{-\Delta\Delta CT}$) relative to control group set as 1. * p<0.05, ** p<0.01 *vs.* control; $^{\&\&}$ p<0.01 *vs.* DIO.

3.4. Effects of TNF-α and EPA or DHA on glutamine uptake in Caco-2 cells

Glutamine is an essential amino acid for the maintenance of the gut barrier function and the intestinal cell proliferation and differentiation (Chun *et al.*, 1997; Reeds & Burrin, 2001; Windmueller, 1978). The main Gln transporter in the small intestine is the Na^+-dependent neutral amino acid transporter B^0AT1 (Bröer *et al*., 2004; Pochini *et al.*, 2014). We decided to investigate whether TNF-α could also inhibit Gln uptake and B^0AT1 expression in the brush border membrane in Caco-2 cells and, if that was the case, whether n-3 PUFAs (EPA and DHA) could block these TNF-α effects.

As observed in Figures 4A and 4B, 10 ng/ml TNF-α significantly inhibited Gln uptake by ~20%. This inhibition was blocked by both EPA and DHA. EPA alone but not DHA increased Gln uptake. Interestingly, brush border membrane expression of B^0AT1 was significantly increased by TNF-α (Fig. 4C and 4D). This increase was partially blocked by EPA and DHA. EPA and DHA alone slightly increased B^0AT1 recruitment into the apical membrane (Fig. 4C and 4D).

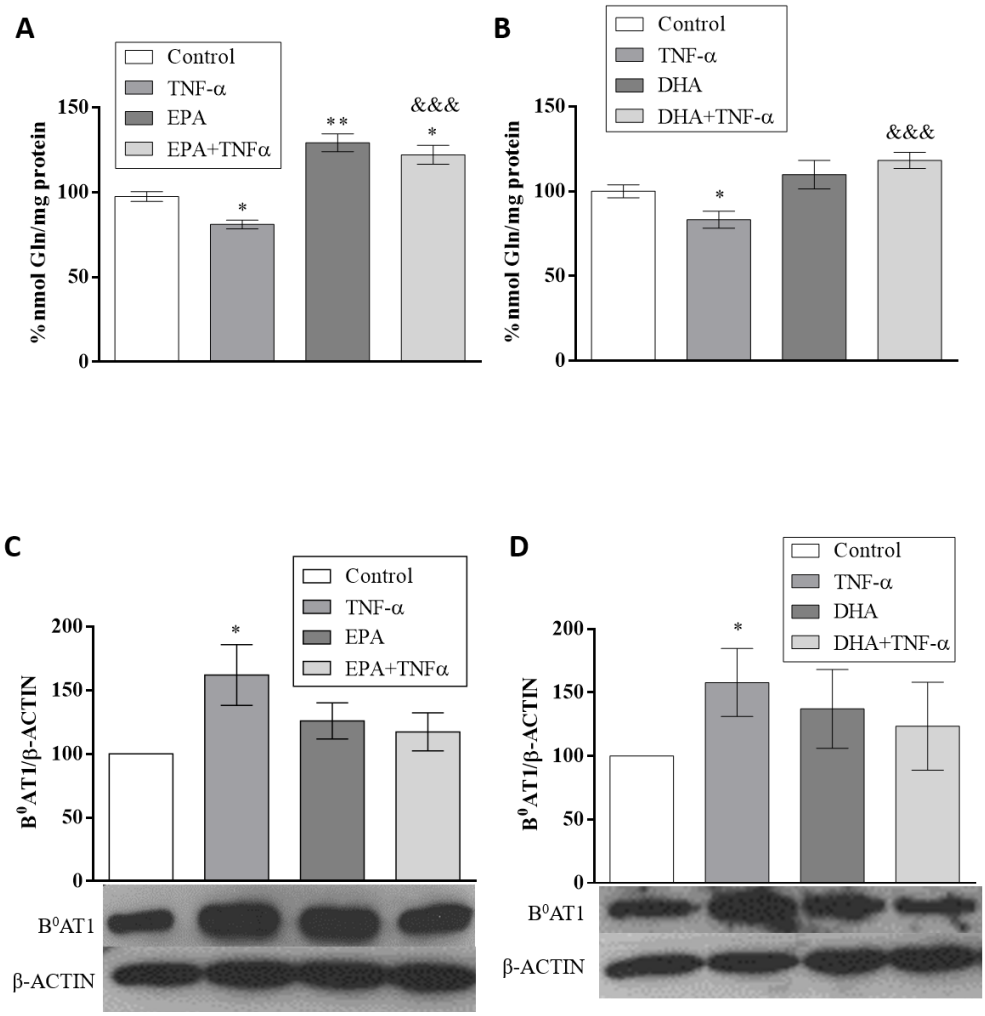


Figure 4. Glutamine uptake in the presence of TNF-α and EPA or DHA. A-B: Caco-2 cells were pre-incubated for 1 h with 10 ng/mL TNF-α in the absence and in the presence of 100 µM EPA (**A**) or 100 µM DHA (**B**) before measuring the uptake of 0.1 mM Gln for 15 min. Data (n= 18) are represented as mean ± SEM. **C-D:** B^0AT1 expression in brush border biotinylated proteins from Caco-2 cells treated as in the uptakes assays. The ratio B^0AT1/β-Actin is expressed as % of control (n= 4). A representative Western blot image is shown. B^0AT1 appears as a ~75 kDa immunoreactive band. * $p<0.05$, ** $p<0.01$ *vs.* control; $^{\&\&\&}$ $p<0.001$ *vs.* TNF-α.

3.5. Effects of MaR1, RvD1 and RvD2 on glutamine uptake in the presence of TNF-α in Caco-2 cells

Finally, we investigated if the DHA lipid mediators MaR1, RvD1 and RvD2 showed the same blocking effect than its precursor on TNF-α inhibition of Gln uptake. As shown in Figures 5A, 5B and 5C, the three SPMs were able to block TNF-α effect.

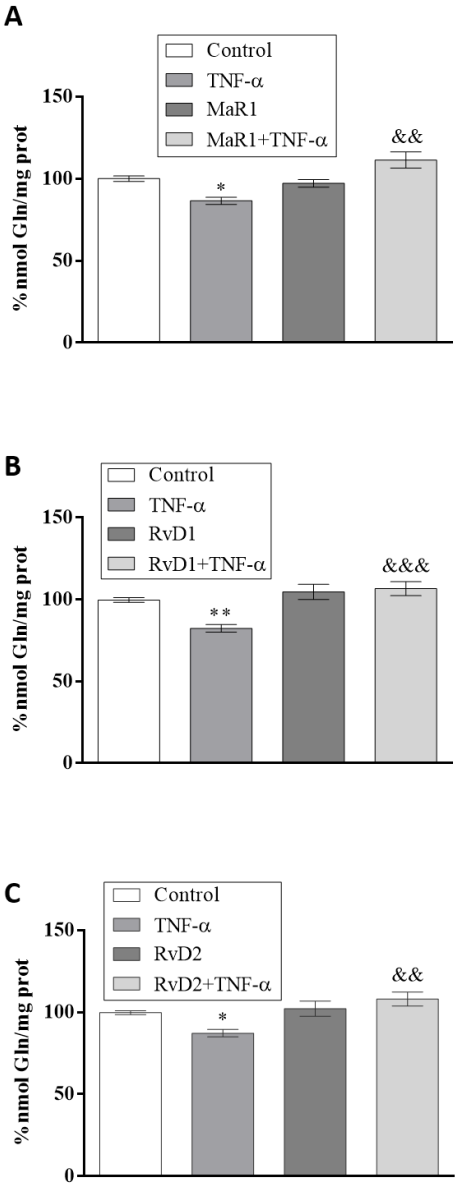


Figure 5. Glutamine uptake in the presence of TNF-α and MaR1, RvD1 or RvD2. Caco-2 cells were pre-incubated for 1 h with 10 ng/mL TNF-α in the absence and in the presence of 100 nM MaR1 (**A**),100 nM RvD1 (**B**) or 100 nM RvD2 (**C**) before measuring the uptake of 0.1 mM Gln for 15 min. Data (n= 18) are represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs.* control; $^{\&\&}$ $p<0.01$, $^{\&\&\&}$ $p<0.001$ *vs.* TNF-α.

4. Discussion

Some previous studies from our group and others have suggested the ability of n-3 PUFAs to regulate intestinal inflammation and sugar uptake (SGLT1) *in vitro* and *in vivo* (Gabler *et al.* , 2007; Castilla-Madrigal *et al.*, 2017; Cabré *et a*l., 2012; Zhao *et al.*, 2015). In the present study, we have demonstrated that DHA, as previously reported for EPA (Castilla-Madrigal *et al.*, 2017), blocks TNFα-inhibition of sugar uptake by preventing the recruitment of SLGT1 from the apical membrane into intracellular compartments. Moreover, we observed that contrary to EPA (Castilla-Madrigal *et al.*, 2017), DHA action did not seem to implicate the omega-3 fatty acid receptor GPR120 (Oh *et al.*, 2010) activation. In this context, several previous works have shown different outcomes concerning the involvement of GPR120 in the actions of DHA. Thus, it has been described in Caco-2 cells that DHA decreases NF-κB activation, included in TNF-α signaling pathway, by binding to GPR120 (Anbazhagan *et al.*, 2016). Accordingly, in a mice colitis model (IL-10 KO mice), DHA partially reduces inflammation through the inhibition of the NF-κB signaling pathway by activating GPR120 (Zhao *et al.,* 2017). Arantes *et al*. (2016) observed that skin wound healing was improved by topical DHA, which induced TGF-β1 production in the keratinocytes through GPR120. However, TGF-β1 synthesis by fibroblast was independent of GPR120, since the receptor is not expressed in these cells. Interestingly, the protective effects of n-3 PUFAs against insulin-resistance and inflammation in obesity was also observed in GPR120 KO mice, suggesting that GPR120 signaling is not required for these effects (Pærregaard *et al*., 2016). EPA and DHA might act through three possible alternative mechanisms: 1) Binding and activation of GPR120; 2) Interfering with early membrane events involved in other receptors activation; 3) Diffusing through the membrane and activation of PPAR-γ (Calder, 2015). In the present work, DHA seems to exert its effect by a mechanism different from the binding to GPR120. Nevertheless, further studies are needed to confirm this.

It has been suggested that n-3 PUFAs-derived SPMs are able to counteract inflammation (Spite *et al.*, 2014). We found that MaR1, RvD1 and RvD2, as their precursor DHA, block TNF-α-induced inhibition of αMG uptake and SGLT1 expression

in the apical membrane of Caco-2 cells, but with a concentration a thousand times lower.

Cytokines secreted by adipose tissue are closely associated with intestinal inflammation (Teixeira *et al.*, 2011). In DIO mice, Martínez-Fernández *et al*. (2017) found that the intraperitoneal administration of MaR1 ameliorated obesity-induced insulin resistance and up-regulated adiponectin and GLUT4 genes. It also reduced adipose tissue inflammation, revealed by the decrease of pro-inflammatory M1 macrophage phenotype and MCP-1, TNF-α, and IL-1β gene expression in adipose tissue. In our DIO model, we did not find reversion of the decrease on intestinal sugar uptake or SGLT1 expression in the DIO mice by MaR1, but we did observe reversion on the increase of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 gene expression in the intestine. In line with these results, intraperitoneal administration of MaR1 in mice with spontaneous colitis attenuated histological colitis and diminished the concentration of TNF-α, IFN-γ, IL-6 and IL-17, which improved iron-deficient anemia (Wang *et al.*, 2016). Likewise, in mice with induced experimental colitis, intraperitoneal administration of MaR1 reduced TNF-α levels and enhanced macrophages M2 phenotype, reverting colon damage (Marcon *et al.*, 2013). Also, in vascular endothelial and smooth muscle cells, MaR1 diminished TNF-α inflammatory pathway (Chatterjee *et al.*, 2014). Similarly, intravenous administration of RvD1 and RvD2 to a mice model of colitis reduced pro-inflammatory cytokines secretion (Bento *et al.*, 2011). Interestingly, Lee *et al*. (2017) found in intestinal mucosa of colitis model mice, increased levels of RvD1, together with decreased levels of its precursor DHA, suggesting the initiation of healing by endogenous lipids. Remarkably, Caco-2 cells exposed to LPS can also produce MaR1 (Le Faouder *et al.*, 2013). In our work, the fact that MaR1 was able to block TNF-α-induced decrease on sugar uptake in Caco-2 cells, but not in the mice model, may suggest that longer treatment with MaR1 would be required to achieve the recovery of sugar transport in the DIO mice model.

Glutamine is the main energy source for intestine and is necessary to maintain the gut integrity (Pochini *et al.*, 2014). Intravenous administration of Gln to protein malnourished mice, previously injected with LPS, reduced synthesis and circulating levels of TNF-α (Santos *et al*., 2016).

Here, we show that TNF-α inhibits Gln uptake in Caco-2 cells, contrary to the findings of Souba *et al.* (1992). As occurs for sugar, EPA (Castilla-Madrigal *et al.*, 2017), DHA and its derived lipid mediators MaR1, RvD1 and RvD2 prevented TNF-α-induced inhibition of Gln uptake. However, while decrease of sugar uptake by the cytokine was due to SGLT1 expression reduction in the apical membrane (Barrenetxe *et al.*, 2013), TNF-α inhibitory effect of Gln transport was accompanied by an increase on the Na^+-dependent glutamine transporter B^0AT1 expression in membrane. This increase was partially blunted by EPA and DHA. In a rabbit model of chronic enteritis, glutamine uptake by the intestine was decreased due to B^0AT1 expression reduction in the plasma membrane (Arthur *et al.*, 2012). However, in the same animal model, the activity of ATB^0 (a Na^+-dependent neutral amino-acid cotransporter) and PepT1 (a H^+-dipeptide transporter) was diminished due to a reduction on their substrate affinity, without modification on their expression in the membrane (Sundaram *et al.*, 2006; Talukder *et al.*, 2008). In rat colon and Caco-2 cells, TNF-α down-regulates the Na^+/K^+ ATPase (Markossian & Kreydiyyeh, 2005). These data suggest that in Caco-2 cells, TNF-α would indirectly decrease B^0AT1 activity by reducing the Na^+ gradient across the membrane. In the case of the reduction of sugar uptake by TNF-α, the internalization of SGLT1 does not exclude the implication of the Na^+ gradient in the decrease of its activity. TNF-α could also alter B^0AT1 function by inducing its phosphorylation (Böhmer *et al.*, 2010; Pochini *et al*., 2014), that would alter its turnover rate or affinity, as it happens in other transporters (Vayro & Silverman, 1999). The decrease of B^0AT1 activity, in turn, would be a signal for the insertion of more molecules of B^0AT1 into the membrane.

In conclusion, DHA and its lipid mediators MaR1, RvD1 and RvD2 block the TNF-α-induced inhibition of αMG and Gln uptake. Thus, n-3 PUFAs and their derived pro-resolving lipid mediators are presented as promising biomolecules to restore intestinal nutrients transport during intestinal inflammatory processes.

References

A

Ananthakrishnan, A. N., Khalili, H., Konijeti, G. G., Higuchi, L. M., de Silva, P., Fuchs, C. S., Willett, W.C., Richter, J.M. & Chan, A. T. (2014). Long-term intake of dietary fat and risk of ulcerative colitis and Crohn's disease. *Gut* ***63*** (5), 776–784.

Anbazhagan, A. N., Priyamvada, S., Gujral, T., Bhattacharyya, S., Alrefai, W. A., Dudeja, P. K. & Borthakur, A. (2016). A Novel Anti-inflammatory Role of GPR120 in Intestinal Epithelial Cells. *American Journal of Physiology - Cell Physiology* **310** (7), C612-21, ajpcell.00123.2015.

Arantes, E.L., Dragano, N., Ramalho, A., Vitorino, D., de-Souza, G.F., Lima, M.H.M., Velloso, L.A. & Araújo, E. P. (2016). Topical Docosahexaenoic Acid (DHA) Accelerates Skin Wound Healing in Rats and Activates GPR120. *Biological Research For Nursing* ***18*** (4), 411–419.

Argollo, M., Fiorino, G., Hindryck, P., Peyrin-Biroulet, L. & Danese, S. (2017). Novel therapeutic targets for inflammatory bowel disease. *Journal of Autoimmunity*. https://doi.org/10.1016/j.jaut.2017.07.004

Arthur, S., Saha, P., Sundaram, S., Kekuda, R. & Sundaram, U. (2012). Regulation of sodium-glutamine cotransport in villus and crypt cells by glucocorticoids during chronic enteritis. *Inflammatory Bowel Diseases* ***18*** (11), 2149–2157.

B

Barrenetxe, J., Sánchez, O., Barber, A., Gascón, S., Rodríguez-Yoldi, M.J. & Lostao, M.P. (2013). TNFα regulates sugar transporters in the human intestinal epithelial cell line Caco-2. *Cytokine* ***64*** (1), 181–187.

Bento, A. F., Claudino, R. F., Dutra, R. C., Marcon, R. & Calixto, J. B. (2011). Omega-3 Fatty Acid-Derived Mediators 17(R)-Hydroxy Docosahexaenoic Acid, Aspirin-Triggered Resolvin D1 and Resolvin D2 Prevent Experimental Colitis in Mice. *The Journal of Immunology* ***187*** (4), 1957–1969.

Bertolo, C., Barrenetxe, J., Barber, A., Lostao, M.P. (2002). Cytokine effect on intestinal galactose absorption. *Journal of Physiology and Biochemistry* ***58*** (1), 61–62.

Böhmer, C., Sopjani, M., Klaus, F., Lindner, R., Laufer, J., Jeyaraj, S., Lang, F. & Palmada, M. (2010). The serum and glucocorticoid inducible kinases SGK1-3 stimulate the neutral amino acid transporter SLC6A19. *Cellular Physiology and Biochemistry* ***25*** (6), 723–732.

Bormans, G.M., Van Oosterwyck, G., De Groot, T.J., Veyhl, M., Mortelmans, L., Verbruggen, A.M. & Koepsell, H. (2003). Synthesis and biologic evaluation of (11)c-methyl-d-glucoside, a tracer of the sodium-dependent glucose transporters. *Journal of Nuclear Medicine : Official Publication, Society of Nuclear Medicine* ***44*** (7), 1075–1081.

Bröer, A., Klingel, K., Kowalczuk, S., Rasko, J. E. J., Cavanaugh, J. & Bröer, S. (2004). Molecular cloning of mouse amino acid transport system B0, a neutral amino acid transporter related to Hartnup disorder. *Journal of Biological Chemistry* ***279*** (23), 24467–24476.

C

Cabré, E., Mañosa, M. & Gassull, M. A. (2012). Omega-3 fatty acids and inflammatory bowel diseases - a systematic review. *The British Journal of Nutrition* ***107*** (S2), S240-252.

Calder, P. C. (2013). Omega-3 polyunsaturated fatty acids and inflammatory processes: Nutrition or pharmacology? *British Journal of Clinical Pharmacology* ***75*** (3), 645–662.

Calder, P. C. (2015). Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. *BBA - Molecular and Cell Biology of Lipids* ***1851*** (4), 469–484.

Castilla-Madrigal, R., Barrenetxe, J., Moreno-Aliaga, M.J. & Lostao, M.P. (2017). EPA blocks TNF-α-induced inhibition of sugar uptake in Caco-2 cells via GPR120 and AMPK. *Journal of Cell Physiol.*https://doi.org/10.1002/jcp.26115

Chatterjee, A., Sharma, A., Chen, M., Toy, R., Mottola, G. & Conte, M. S. (2014). The pro-resolving lipid mediator maresin 1 (MaR1) attenuates inflammatory signaling pathways in vascular smooth muscle and endothelial cells. *PLoS ONE* ***9*** (11). https://doi.org/10.1371/journal.pone.0113480

Chun, H., Sasaki, M., Fujiyama, Y. & Bamba, T. (1997). Effect of enteral glutamine on intestinal permeability and bacterial translocation after abdominal radiation injury in rats. *Journal of Gastroenterology* ***32*** **(**2), 189–195.

Cohen, A.B., Lee, D., Long, M.D., Kappelman, M.D., Martin, C.F., Sandler, R.S., & Lewis, J.D. (2013). Dietary patterns and self-reported associations of diet with symptoms of inflammatory bowel disease. *Digestive Diseases and Sciences* ***58*** (5), 1322–1328.

D

Ducroc, R., Sakar, Y., Fanjul, C., Barber, A., Bado, A. & Lostao, M. P. (2010). Luminal leptin inhibits L-glutamine transport in rat small intestine: involvement of ASCT2 and B^0AT1. *American Journal of Physiology Gastrointestinal and Liver Physiology* ***299*** (1), G179-185.

F

Foley, K. F., Pantano, C., Ciolino, A. & Mawe, G. M. (2007). IFN-gamma and TNF-α lpha decrease serotonin transporter function and expression in Caco2 cells. *American Journal of Physiology Gastrointestinal and Liver Physiology* ***292*** (3), G779-84.

G

Gabler, N. K., Radcliffe, J. S., Spencer, J. D., Webel, D. M. & Spurlock, M. E. (2007). Feeding long-chain n-3 polyunsaturated fatty acids during gestation increases intestinal glucose absorption potentially via the acute activation of AMPK. *Journal of Nutritional Biochemistry* ***20*** (1), 17–25.

Garriga, C., Rovira, N., Moreto, M. & Planas, J. (1999). Expression of Na^+-D-glucose cotransporter in brush-border membrane of the chicken intestine. *American Journal of Physiology* ***276***, 627–631.

Gonçalves, P., Magro, F., & Martel, F. (2015). Metabolic Inflammation in Inflammatory Bowel Disease: Crosstalk Between Adipose Tissue and Bowel. *Inflammatory Bowel Diseases* ***21***(2), 453–467. https://doi.org/10.1097/MIB.0000000000000209

H

Hokari, R., Matsunaga, H. & Miura, S. (2013). Effect of dietary fat on intestinal inflammatory diseases. *Journal of Gastroenterology and Hepatology* ***28*** (S4), 33–36.

Hou, J. K., Abraham, B. & El-Serag, H. (2011). Dietary Intake and Risk of Developing Inflammatory Bowel Disease: A Systematic Review of the Literature. *The American Journal of Gastroenterology* ***106*** (4), 563–573.

K

Kredel, L. I. & Siegmund, B. (2014). Adipose-tissue and intestinal inflammation - visceral obesity and creeping fat. *Frontiers in Immunology* ***5***, 1–12.

L

Laiglesia, L.M., Lorente-Cebrián, S., Martínez-Fernández, L., Sáinz, N., Prieto-Hontoria, P.L., Burrell, M.A., Rodríguez-Ortigosa, C.M., Martínez, J.A. & Moreno-Aliaga, M.J. (2017). Maresin 1 mitigates liver steatosis in ob/ob and diet-induced obese mice. *International Journal of Obesity*. Doi: 10.1038/ijo.2017.226

Le Faouder, P., Baillif, V., Spreadbury, I., Motta, J. P., Rousset, P., Chêne, G., Guigné, C., Tercé, F., Vanner, S., Vergnolle, N., Bertrand-Michel, J., Dubourdeau, M. & Cenac, N. (2013). LC-MS/MS method for rapid and concomitant quantification of pro-inflammatory and pro-resolving polyunsaturated fatty acid metabolites. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* ***932***, 123–133.

Lee, Y., Choo, J., Kim, S. J., Heo, G., Pothoulakis, C., Kim, Y. H. & Im, E. (2017). Analysis of endogenous lipids during intestinal wound healing. *PLoS ONE* ***12*** (8), 1–23.

Liu, Y., Chen, F., Odle, J., Lin, X., Jacobi, S. K., Zhu, H., Wu, Z. & Hou, Y. (2012). Fish Oil Enhances Intestinal Integrity and Inhibits TLR4 and NOD2 Signaling Pathways in Weaned Pigs after LPS Challenge. *Journal of Nutrition* ***142*** (11), 2017–2024.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real- Time Quantitative PCR and the 2 (-Delta Delta C(T)) Method. *METHODS* ***25***, 402–408.

M

Marcon, R., Bento, A. F., Dutra, R. C., Bicca, M. a, Leite, D. F. P. & Calixto, J. B. (2013). Maresin 1, a proresolving lipid mediator derived from omega-3 polyunsaturated Fatty acids, exerts protective actions in murine models of colitis. *Journal of Immunology* ***191*** (8), 4288–98.

Markossian, S. & Kreydiyyeh, S. I. (2005). TNF-α down-regulates the Na^+-K^+ ATPase and the Na^+-K^+-2Cl-cotransporter in the rat colon via PGE2. *Cytokine* ***30*** (6), 319–327.

Martínez-Fernández, L., González-Muniesa, P., Laiglesia, L.M., Sáinz, N., Prieto-Hontoria, P.L., Escoté, X., Odriozola, L., Corrales, F.J., Arbones-Mainar, J.M., Martínez, J.A. & Moreno-Aliaga, M.J. (2017). Maresin 1 improves insulin sensitivity and attenuates adipose tissue inflammation in ob/ob and diet-induced obese mice. *FASEB Journal* ***31*** (5), 2135–2145.

Martinez-Fernandez, L., Laiglesia, L.M., Huerta, A.E., Martinez, J.A. & Moreno-Aliaga, M.J. (2015). Omega-3 fatty acids and adipose tissue function in obesity and metabolic syndrome. *Prostaglandins & Other Lipid Mediators* ***121***, 1–18.

Maurizi, G., Della Guardia, L., Maurizi, A. & Poloni, A. (2017). Adipocytes properties and crosstalk with immune system in obesity-related inflammation. *Journal of Cellular Physiology*.

Max, J. B., Stidham, R., Su, G. L. & Waljee, A. K. (2013). Obesity and IBD: Are We Tipping the Scales Toward an Epidemic? *Gastroenterology* ***14*** (2), 478–479.

Mazur-Bialy A.I., Bilski J., Pochec E., Brzozowski T. (2017). New insight into the direct anti-inflammatory activity of a myokine irisin against proinflammatory activation of adipocytes. Implication for exercise in obesity. *Journal of Physiology and Pharmacology* ***68*** (2), 243–251.

Mbodji, K., Charpentier, C., Guérin, C., Querec, C., Bole-Feysot, C., Aziz, M., Savoye, G., Déchelotte, P. & Marion-Letellier, R. (2013). Adjunct therapy of n-3 fatty acids to 5-ASA ameliorates inflammatory score and decreases NF-κB in rats with TNBS-induced colitis. *Journal of Nutritional Biochemistry* ***24*** (4), 700–705.

N

Neuman, M. G. & Nanau, R. M. (2012). Inflammatory bowel disease: Role of diet, microbiota, life style. *Translational Research*.

O

Oh, D. Y., Talukdar, S., Bae, E. J., Imamura, T., Morinaga, H., Fan, W., Li, P., Lu, W. J., Watkins, S. M. and Olefsky, J. M. (2010). GPR120 Is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-inflammatory and Insulin-Sensitizing Effects. *Cell* **142** (5), 687–698.

P

Pærregaard, S.I., Agerholm, M., Serup, A.K., Ma, T., Kiens, B., Madsen, L., Kristiansen, K. & Jensen, B. A. H. (2016). FFAR4 (GPR120) Signaling Is Not Required for Anti-Inflammatory and Insulin-Sensitizing Effects of Omega-3 Fatty Acids. *Mediators of Inflammation*, *2016*. https://doi.org/10.1155/2016/1536047

Pochini, L., Scalise, M., Galluccio, M. & Indiveri, C. (2014). Membrane transporters for the special amino acid glutamine: structure/function relationships and relevance to human health. *Frontiers in Chemistry* ***2*****,** 1–23.

Powell, D.W. (1990). Immunophysiology of intestinal electrolyte transport. In *Handbook of Physiology. The Gastrointestinal System. Intestinal Absorption and Secretion.* (pp. 591–564).

R

Reeds, P.J. & Burrin, D.G. (2001). Glutamine and the bowel. *The Journal of Nutrition* ***131*** (9 Suppl), 2505S–2508S; discussion 2523S–2524S.

S

Santos, A.C.A., Correia, C.A., de Oliveira, D.C., Nogueira-Pedro, A., Borelli, P. & Fock, R. A. (2016). Intravenous Glutamine Administration Modulates TNF-α /IL-10 Ratio and Attenuates NF-κB Phosphorylation in a Protein Malnutrition Model. *Inflammation* ***39*** (6), 1883–1891.

Serhan, C.N. (2007). Resolution Phase of Inflammation: Novel Endogenous Anti-Inflammatory and Proresolving Lipid Mediators and Pathways. *Annual Review of Immunology* ***25*** (1), 101–137.

Serhan, C.N. & Chiang, N. (2009). Endogenous pro-resolving and anti-inflammatory lipid mediators: a new pharmacologic genus. *British Journal of Pharmacology* ***153*** (S1), S200–S215.

Serhan, C.N., Hong, S., Gronert, K., Colgan, S.P., Devchand, P.R., Mirick, G. & Moussignac, R.L. (2002). Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *The Journal of Experimental Medicine* ***196*** (8), 1025–1037.

Sharma, N., Laftah, A.H., Brookes, M.J., Cooper, B., Iqbal, T. & Tselepis, C. (2005). A role for tumour necrosis factor alpha in human small bowel iron transport. *The Biochemical Journal* ***390***, 437–46.

Shirazi-Beechey, S. P., Davies, a G., Tebbutt, K., Dyer, J., Ellis, A., Taylor, C. J., Fairclough, P. and Beechey, R. B. (1990) 'Preparation and properties of brush-border membrane vesicles from human small intestine. *Gastroenterology* **98** (3), pp. 676–685.

Souba, W.W., Pan, M. & Stevens, B.R. (1992). Kinetics of the sodium-dependent glutamine transporter in human intestinal cell confluent monolayers. *Biochemical and Biophysical Research Communications* ***188*** (2), 746–753. https://doi.org/10.1016/0006-291X(92)91119-B

Spite, M., Clària, J., & Serhan, C.N. (2014). Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. *Cell Metabolism* **19** (1):21-36.

Stewart, M., Day, A.S. & Otley, A. (2011). Physician Attitudes and Practices of Enteral Nutrition as Primary Treatment of Paediatric Crohn Disease in North America. *Journal of Pediatric Gastroenterology and Nutrition* ***52*** **(**1), 38–42.

Sundaram, U., Wisel, S. & Coon, S. (2006). Neutral Na-amino acid cotransport is differentially regulated by glucocorticoids in the normal and chronically inflamed rabbit small intestine. *AJP: Gastrointestinal and Liver Physiology* ***292*** (2), G467–G474.

T

Tabas, I. & Glass, C.K. (2013). Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science* ***339*** (6116), 166–72.

Talukder, J.R., Kekuda, R., Saha, P., Arthur, S. & Sundaram, U. (2008). Identification and characterization of rabbit small intestinal villus cell brush border membrane Na-glutamine cotransporter. *AJP: Gastrointestinal and Liver Physiology* ***295*** (1), G7–G15.

Teixeira, L.G., Leonel, A.J., Aguilar, E.C., Batista, N.V, Alves, A.C., Coimbra, C.C., Ferreira, A.V., de Faria, A.M., Cara, D.C. & Alvarez Leite, J.I. (2011). The combination of high-fat diet-induced obesity and chronic ulcerative colitis reciprocally exacerbates adipose tissue and colon inflammation. *Lipids in Health and Disease* ***10*** (1), 204.

V

Vayro, S. & Silverman, M. (1999). PKC regulates turnover rate of rabbit intestinal Na^+-glucose transporter expressed in COS-7 cells. *The American Physiological Society* **276**, 1053–1060.

Vitale, S., Strisciuglio, C., Pisapia, L., Miele, E., Barba, P., Vitale, A., Cenni, S., Bassi, V., Maglio, M., Del Pozzo, G., Troncone, R., Staiano, A. & Gianfrani, C. (2017). Cytokine production profile in intestinal mucosa of paediatric inflammatory bowel disease. *PLoS ONE* ***12*** (8), 1–20.

W

Wang, H., Shi, P., Huang, C. & Liu, Q. (2016). Maresin 1 ameliorates iron-deficient anemia in IL-10-/- mice with spontaneous colitis by the inhibition of hepcidin expression though the IL-6/STAT3 pathway. *American Journal of Translational Research* ***8*** (6), 2758–2766.

Windmueller, H & Spaeth, E. (1978). Identification of ketone bodies and glutamine as the major respiratory fuels in vivo for postabsorptive rat small intestine. *The journal of Biological Chemistry* ***253*** (1), 69–76.

Z

Zhao, J., Shi, P., Sun, Y., Sun, J., Dong, J.N., Wang, H.G., Zuo, L.G., Gong, J.F., Li, Y., Gu, L.L., Li, N., Li, J.S. & Zhu, W.M. (2015). DHA protects against experimental colitis in IL-10-deficient mice associated with the modulation of intestinal epithelial barrier function. *British Journal of Nutrition* **114** (11), 1–8.

Zhao, J., Wang, H., Shi, P., Wang, W. & Sun, Y. (2017). GPR120, a potential therapeutic target for experimental colitis in IL-10 deficient mice. *Oncotarget* ***8*** (5), 8397–8405.

CHAPTER 3.

The pro-inflammatory cytokine TNF-α reduces intestinal sugar transport acting from the basolateral membrane in relation with obesity

Rosa Castilla-Madrigal[1,2], Eva Gil-Iturbe[1,2], Neira Sáinz[2], María J. Moreno-Aliaga[1,2,3,4], María Pilar Lostao[1,2,3]

[1]*University of Navarra, Dept. Nutrition, Food Science and Physiology, Irunlarrea 1, 31008 Pamplona, Spain*

[2]*University of Navarra, Nutrition Research Centre, Irunlarrea 1, 31008 Pamplona, Spain*

[3]*IdiSNA, Navarra Institute for Health Research, Pamplona, Spain*

[4]*CIBERobn, Physiopathology of Obesity and Nutrition, Institute of Health Carlos III (ISCIII), Madrid, Spain*

Abstract

We have previously demonstrated in the human intestinal epithelial cell line Caco-2, that TNF-α inhibits sugar uptake, acting from the apical membrane, by decreasing the expression of the Na^+-glucose cotransporter SGLT1 in the brush border membrane. The goal of this study was to investigate the hypothesis that in obesity, TNF-α from abdominal adipose tissue (adipocytes and macrophages) would decrease sugar transport acting from the basolateral membrane of the enterocytes.

Basal TNF-α (10 ng/mL, 24 h incubation) decreased α-methyl-D-glucose (αMG) and glutamine uptake (15 min). The apical medium derived from these Caco-2 cells and placed apically for 1 h in another set of cells, also reduced sugar and glutamine transport. RT-PCR analysis demonstrated up-regulation of *TNF-α, IL-1β* and *MCP1* gene expression in Caco-2 cells expose to basal TNF-α. Similarly, αMG uptake was inhibited after Caco-2 cells were incubated for 24 h with medium from subcutaneous or visceral overweight human mesenchymal stem cells derived adipocytes (hMSC-DA) placed in the basal compartment. The apical medium collected from those Caco-2 cells, placed in the upper side of other set of cells, also induced reduction of sugar uptake. None of these effects were observed with medium from morbidly obese subcutaneous or visceral hMSC-DA. RT-PCR analysis demonstrated up-regulation of *TNF-α, IL-1β, IL-6* and *MCP1* in overweight visceral hMSC-DA but not in morbid visceral hMSC-DA. Basal presence for 24 h of medium derived from LPS-activated macrophages and non-activated macrophages also decreased αMG uptake. Diet induced obesity (DIO) produced an increase in the visceral adipose tissue surrounding the intestine in mice. In this physiological condition, there was a decrease on αMG uptake in jejunal everted rings which was not further decreased in the presence of TNF-α.

These results suggest that basolateral TNF-α, which can be produced by macrophages and adipocytes during obesity, would be able to activate pro-inflammatory cytokines expression in the small intestine and diminish intestinal sugar uptake.

1. Introduction

Tumor necrosis factor α (TNF-α) is a pro-inflammatory cytokine involved in the regulation of many important cellular processes such as proliferation, differentiation, growth and immune response (Hayashi *et al.*, 2013). This cytokine is also implicated in numerous diseases with an inflammatory component, as inflammatory bowel disease (IBD) (Argollo *et al*., 2017) and obesity (Peluso & Palmery, 2016).

IBD is characterized by chronic inflammation of the gastrointestinal mucosa due to a dysregulation of the immune system (Tabas & Glass, 2013). Genome, diet and intestinal microbiota are implicated in the pathogenesis of IBD (Albenberg *et al.*, 2012). Cytokines play a crucial role in this pathogenesis of IBD as they orchestrate many aspects of intestinal inflammation. A disturbed balance between pro-inflammatory and immunoregulatory cytokines has been reported in IBD (Soufli *et al.*, 2016).

Obesity is a low grade chronic inflammatory state where inflammation results in secondary diseases in the long run, and impacts the progression of other illnesses (Rocha & Folco, 2011). Obesity is associated with a worst diagnosis and development of IBD (Gonçalves *et al.*, 2015; Max *et al.*, 2013). Patients with IBD exhibit ectopic adipose tissue extending from the mesenteric depot along the intestinal surface. Dysfunctional inflamed mesenteric adipose tissue in obesity could also contribute to the chronic intestinal inflammation in IBD (Sideri *et al.*, 2015). Fat accumulation can also be locally restricted (Kredel & Siegmund, 2014). In normal to mildly overweight healthy women, it was found a positive association between visceral adiposity and intestinal permeability (Gummesson *et al.*, 2011). A progressive increase in visceral adiposity is also observed with aging (Huffman & Barzilai, 2009; Lumeng *et al.*, 2011).

Macrophages are classified as pro-inflammatory M1 macrophages and immuno-modulatory M2 macrophages (Mantovani *et al*., 2004). Directly underneath the epithelium of the intestine there are tissue-resident intestinal M2 macrophages (Mahida *et al.*, 1989, Davies *et al.*, 2013; Mantovani *et al.*, 2013). During the triggering of intestinal inflammation different mediators stimulate the switch of M2 resident macrophages into M1, and also induce M1 macrophages infiltration. These M1 macrophages, in turn, secrete pro-inflammatory cytokines such as TNF-α, IL-6, IL-8, IL-

23, IL-1β, and IFNγ as well as the chemokine MCP1 (Kamada *et al*., 2008; Schenk *et al.*, 2007; Zareie *et al.*, 2001), which maintain the inflammatory state. In obesity, there is recruitment and activation of macrophages (Nishimura *et al.*, 2009) that can impact the intestinal function (Neuman, 2007).

We have previously demonstrated in the human intestinal epithelial cell line Caco-2, that TNF-α inhibits sugar uptake, acting from the apical membrane, by decreasing the expression of the Na^+-glucose cotransporter SGLT1 in the brush border membrane (Barrenetxe *et al*., 2013).

The goal of this study was to investigate the hypothesis that in obesity, TNF-α from abdominal adipose tissue (adipocytes and macrophages) would decrease sugar transport acting from the basolateral membrane of the enterocytes. For this purpose, Caco-2 cells were exposed to secretions from human mesenchymal stem cells derived adipocytes (hMSC-DA) of lean, overweight and morbidly obese individuals, and from LPS-activated and non-activated macrophages.

2. Material and methods

2.1. Cells culture

The human intestinal epithelial cell line Caco-2 was maintained at 37°C and 5% CO_2 in a humidified atmosphere. The cells were grown in Dulbecco's Modified Eagles medium (DMEM (1X) + GlutaMAX, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% non-essential amino acids (NEAA 100X, LONZA), 1% penicillin (10,000 U/ml)–streptomycin (10,000 µg/ml) (Gibco) and 1% amphotericin B (250 µg/ml, Gibco). The culture medium was changed every 2 days. For the uptake studies, the cells were seeded at a density of $2x10^5$ cells/cm^2 in 12-well culture plates or 12-well insert plates (Transwel TM Costar). Experiments were performed 15-20 days post seeding, when the cells were differentiated into enterocytes.

Human mesenchymal stem cells (hMSC) were kindly provided by Dr. Arbones-Mainar (Perez-Diaz *et al.*, 2017). They were obtained from subcutaneous abdominal and visceral adipose tissue from three subjects with different body mass index (BMI): lean (24 kg/m^2), overweight (27 kg/m^2) and morbidly obese (44.1 kg/m^2), undergoing elective laparoscopic surgery. None of the patients presented chronic diseases derived

from metabolic syndrome. All donors signed the written consent, and the study was approved by the local Institutional Review Board (CEIC-A) and the Ethics Committee of the University of Navarra. Human mesenchymal stem cells were isolated as previously described (Perez-Diaz *et al*. 2017) and grown in low-glucose DMEM (Lonza) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin (10,000 U/ml)–streptomycin (10,000 µg/ml) (Gibco) at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were seeded at a density of $2x10^5$ cells/cm^2 in 12-well culture plates. After confluence, the cells were differentiated into adipocytes by adding an adipogenic cocktail (500 µM IBMX, 1.67 µM insulin, 1 µM dexamethasone, 1 µM rosiglitazone, and 10% FBS high-glucose DMEM) for 3 days. Then, the medium was refreshed with new adipogenic cocktail and the cells incubated for three more days with 10% FBS high-glucose medium until cells displayed typical features of mature white adipocytes (hMSC-derived adipocytes, hMSC-DA).

Transformed human mononuclear cell line (THP-1) was maintained at 37°C under a humidified atmosphere of 5% CO_2, and cultured with RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin (10000 U/ml)–streptomycin (10000 µg/ml) (Gibco). The cells were differentiated to macrophage-like cells (inactivated macrophages), by treatment for 2 days with 200 nM phorbol myristate acetate (PMA) in 12-well plates at a density of $2x10^5$ cells/cm^2. The inactivated macrophages were activated by 4 h treatment with LPS.

2.2. Experimental design for uptake studies

Uptakes in Caco-2 cells were performed by incubating the cells for 15 min with apical 0.1 mM α-methyl-D-glucose (αMG; Sigma) and traces of [^{14}C]-α-methyl-glucoside (0.08 µCi/ml; ARC 0131) or with 0.1 mM L-Glutamine (Gln; Sigma) and traces of [^{14}C]-L-Glutamine (0.1 µCi/ml; ARC 0196). Then, the reaction was stopped by adding 500 µL of cold Phosphate Buffered Saline with calcium and magnesium (PBS, Sigma Aldrich). Cells were washed three times with PBS and solubilized by adding 500 µL 1% Triton X-100 in 1M NaOH for 1 h 30 min at 37°C. Samples (100 µL) were taken to measure radioactivity by liquid scintillation counter. Protein concentration was

determined by the Bradford method (Bio-Rad Protein Assay). Alpha-methyl-D-glucose is specific substrate of the Na^+-glucose cotransporter SGLT1.

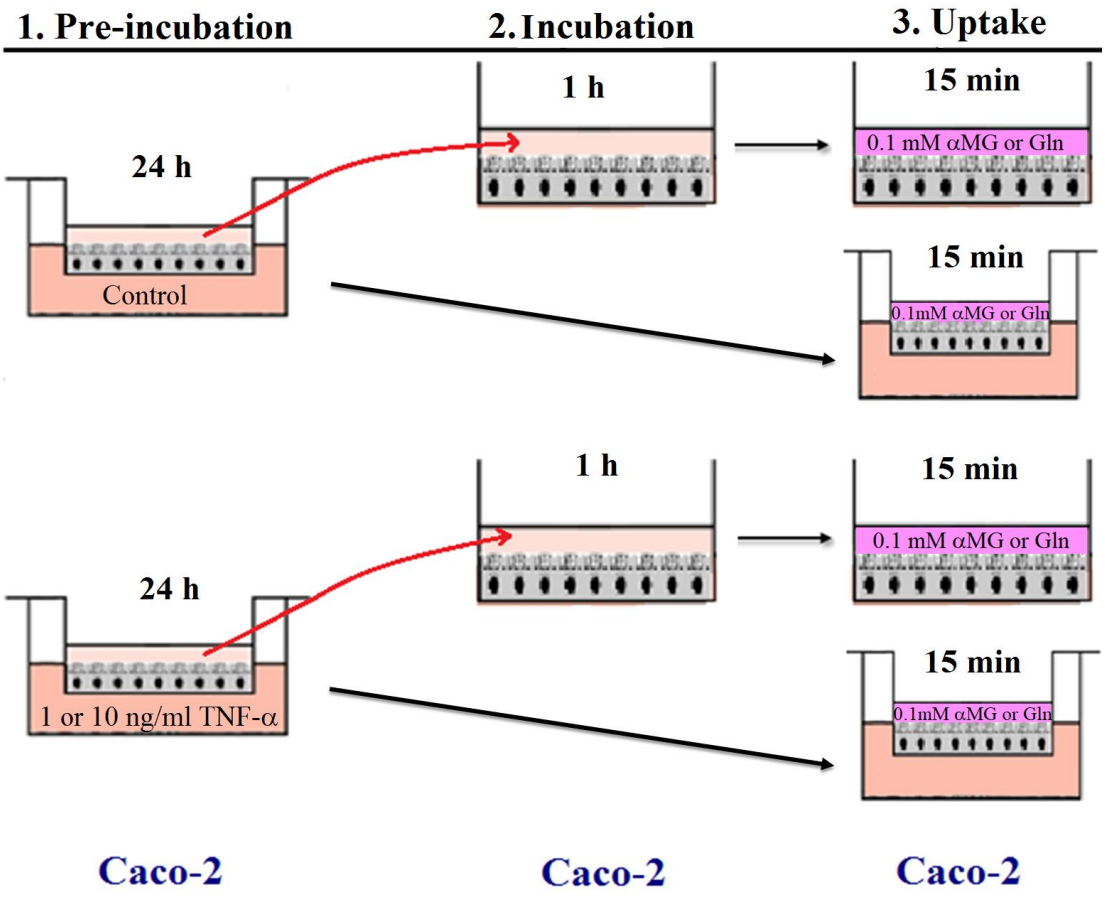
Results of uptake experiments were expressed as nmol mg^{-1} of protein. All data are presented as % compared to controls which were normalized at 100 %

2.2.1. Experimental design for the uptake assays after basal exposure of Caco-2 cells to TNF-α

Scheme 1 depicts the experimental design which is described below.

Caco-2 cells were grown in 12-well insert plates and pre-incubated for 24 h with Dulbecco's Modified Eagles Medium without glucose (DMEM, Gibco) for αMG uptake studies, or with Krebs modified buffer (5.4 mM KCl, 2.8 mM CaCl2, 1 mM MgSO4, 0.3 mM NaH2PO4, 137 mM NaCl, 0.3 mM KH2PO4, 10 mM glucose and 10 mM HEPES/Tris, pH 7.5), for Gln uptake assays. Both media were supplemented with 1% non-essential amino acids (NEAA 100X, LONZA) and placed in the apical compartment. The pre-incubations were performed in the absence (control) and in the presence of 10 ng/ml TNF-α (PeproTech, Inc.), placed in the basal compartment and diluted in the same DMEM or Krebs media without NEAA. A pre-incubation of 24 h was performed to allow secretion by the cells. Next, the apical media were collected and placed for 1 h in the apical side of Caco-2 cells grown on plates while αMG and Gln uptake (15 min) was measured in the inserts. After the 1 h incubation, the apical media were removed and the uptake of αMG and Gln was measured as well.

Scheme 1. Experimental design for the uptake assays after basal exposure of Caco-2 cells to TNF-α.



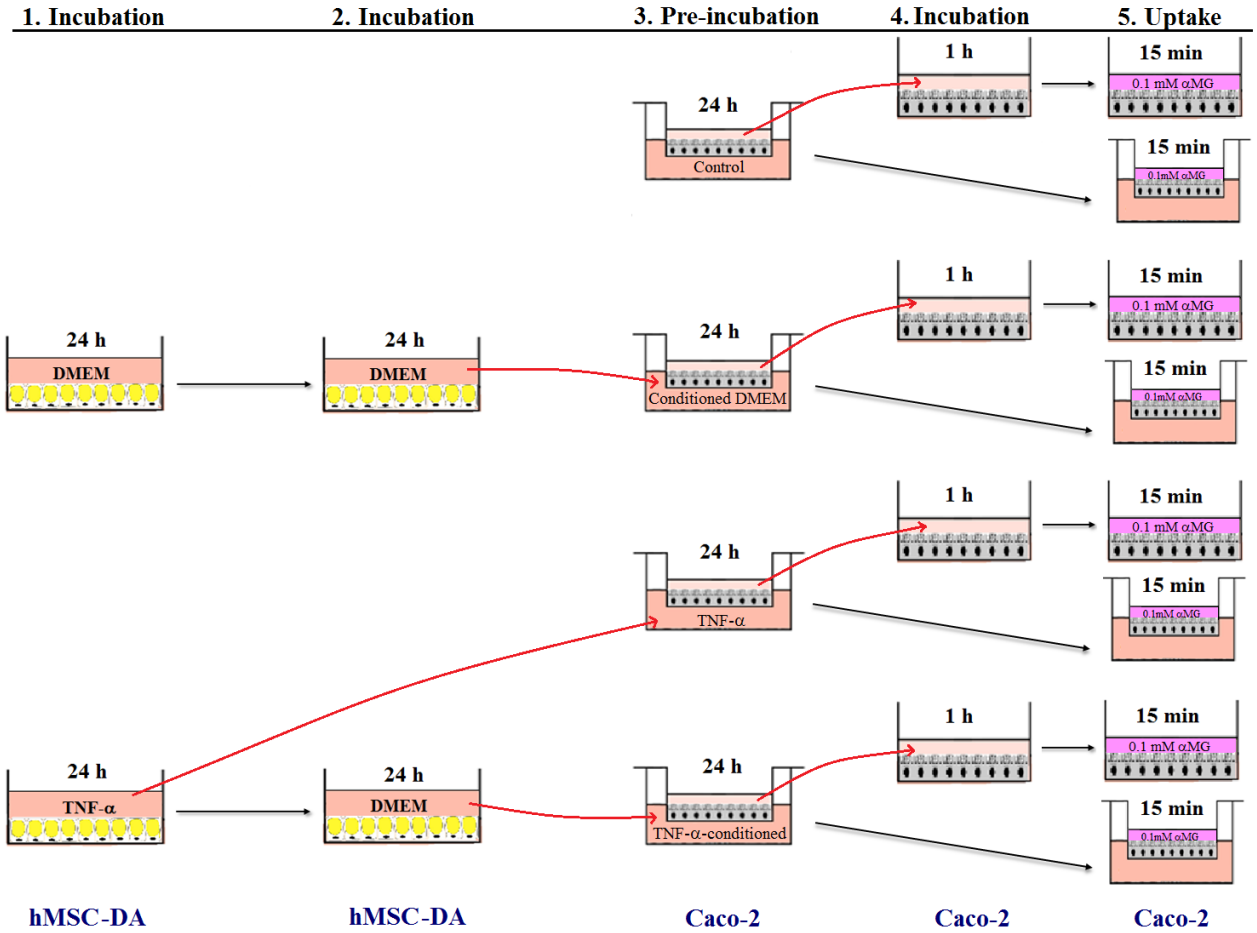
2.2.2. Experimental design for the uptake assays after basal exposure of Caco-2 cells to human mesenchymal stem cell derived adipocytes (hMSC-DA) media

Scheme 2 depicts the experimental design which is described below.

Subcutaneous and visceral hMSC-DA from lean, overweight and morbidly obese donors were incubated with high-glucose DMEM in the absence or presence of 10 ng/ml TNF-α for 24 h. After that time, the media were removed and new fresh DMEM was added to the cells for another 24 h to allow secretion. The collected medium from the hMSC-DA cells treated with TNF-α was kept for the following pre-incubation of Caco-2 cells.

After the 24 h incubation, the media were collected and placed in the basal compartment of Caco-2 cells grown on filters. Those media were named: Conditioned DMEM, TNF-α-conditioned medium and TNF-α medium (collected medium from the first incubation of the hMSC-DA). Fresh DMEM was placed in the basal compartment of another insert as control. Then, Caco-2 cells were pre-incubated for 24 h with the different media to allow secretion. After that, the apical medium from each experimental condition was collected and placed for 1 h in the apical side of Caco-2 cells grown on plates, while αMG uptake (15 min) was measured in the Caco-2 cells grown on filters. After the 1 h incubation, the apical media were removed and uptake of αMG was measured as well.

Scheme 2. Experimental design for the uptake assays after basal exposure of Caco-2 cells to human mesenchymal stem cell derived adipocytes (hMSC-DA) media.

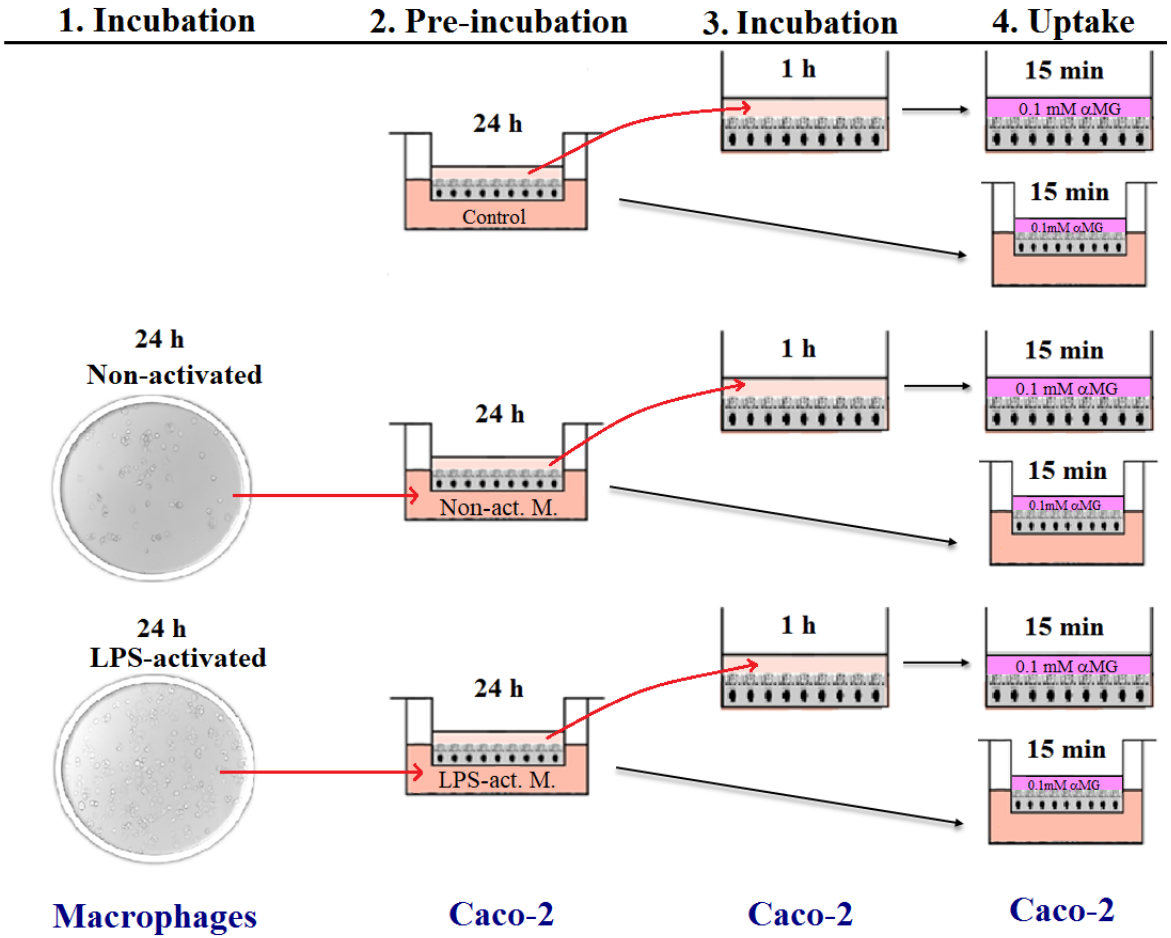


2.2.3. Experimental design for the uptake assays after basal exposure of Caco-2 cells to human macrophages media

Scheme 3 depicts the experimental design which is described below.

LPS-activated and non-activated macrophages were incubated for 24 h with RPMI. Then, each media was placed for 24 h in the basal compartment of Caco-2 cells grown on filters. Fresh RPMI was used as control. Next, the apical media from each experimental condition was collected and placed in the apical side of Caco-2 cells grown on plates for 1 h, while αMG uptake (15 min) was measured in the filters. After the 1 h incubation, the apical media were removed and uptake of αMG was measured as well.

Scheme 3. Experimental design for the uptake assays after basal exposure of Caco-2 cells to human macrophages media



2.3. Uptake assays in everted intestinal rings from diet-induced obese mice

C57BL/6J male mice were purchased from Harlan Laboratories and fed as previously described to obtain the obese phenotype (Laiglesia *et al*., 2017).

All experimental procedures were performed under protocols approved by the University of Navarra Ethics Committee for the use of laboratory animals, according to the National and Institutional Guidelines for Animal Care and Use (Protocols 029-12 and 047-15).

The effects of diet induced obesity (DIO) on the uptake of αMG, was determined in everted jejunal rings obtained as previously described (Ducroc *et al*., 2010). Four animals from lean (control) and DIO experimental groups were used. After the sacrifice, a portion of jejunum was removed, everted and cut in small rings. Rings were incubated in Krebs-Ringer-Tris (KRT) solution with 1 mM αMG and traces of [^{14}C]-αMG (0.0025 μCi mL^{-1}), in the absence or presence of 10 ng/ml TNF-α. The incubation was performed at 37 °C for 15 min, under continuous shaking, and gassed with O_2.

After the incubation period, rings were washed in ice cold KRT solution and then, incubated for 24 h in a solution containing 0.1 M HNO_3 to denature the proteins and allow the exit of the cellular radioactivity, which was finally determined by liquid scintillation counting (Ducroc *et al.*, 2010).

2.5. Gene expression by Real-Time PCR

Total RNA was isolated from the cell lines and mice jejunal mucosa using TRIzol® reagent (Invitrogen, CA, USA) according to manufacturer's procedures. RNA-concentrations and quality were measured using Nanodrop Spectrophotometer ND1000 (Thermo Scientific, DE, USA). RNA was reverse transcribed to cDNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen). Interleukin-1β (*Il-1β*), Interleukin-6 *(Il-6*), Monocyte chemoattractant protein 1(*Mcp1/ Ccl2*) and *Tnf-α* mRNA levels were determined using predesigned Taqman® Assays-on-Demand and Taqman Universal Master Mix (Applied Biosystems, CA, USA). Amplification and detection of specific products were performed in the ABI PRISM 7900HT Fast Sequence Detection System (Applied Biosystems).

The levels of mRNA were normalized to 18S as housekeeping gene. Samples were analyzed in duplicate. Ct values were generated by the ABI PRISM 7900HT (Applied Biosystems). Finally, the relative expression of the genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.6. Statistical analysis

Statistical analysis was performed using the program Stata v12 (Stata, RRID: SCR_012763). Parametric and non-parametric tests (One-way ANOVA, t-student, Kruskal- Wallis test, Median test) followed by the corresponding post-hoc test (Tukey, SNK, Bonferroni), were run depending on the sample size and the normality of the data. Results were expressed as means ± Standard Error of the Mean (SEM), and differences were considered significant at a p value <0.05.

3. Results

3.1. Effect of basal TNF-α on αMG and glutamine uptake and pro-inflammatory protein genes expression in Caco-2 cells

In order to investigate whether basal TNF-α could inhibit sugar uptake, Caco-2 cells grown on inserts were pre-incubated with 10 ng/ml TNF-α present in the basal compartment for 24 h. Next, the apical medium was collected and placed for 1 h in the upper side of Caco-2 cells grown on plates to check if it could affect sugar uptake. Then, uptake of 0.1 mM αMG was measure for 15 min in both, the cells exposed to basal TNF-α and the cells incubated with the apical medium. Similar experiments were performed with Gln to verify whether another nutrient uptake could be altered.

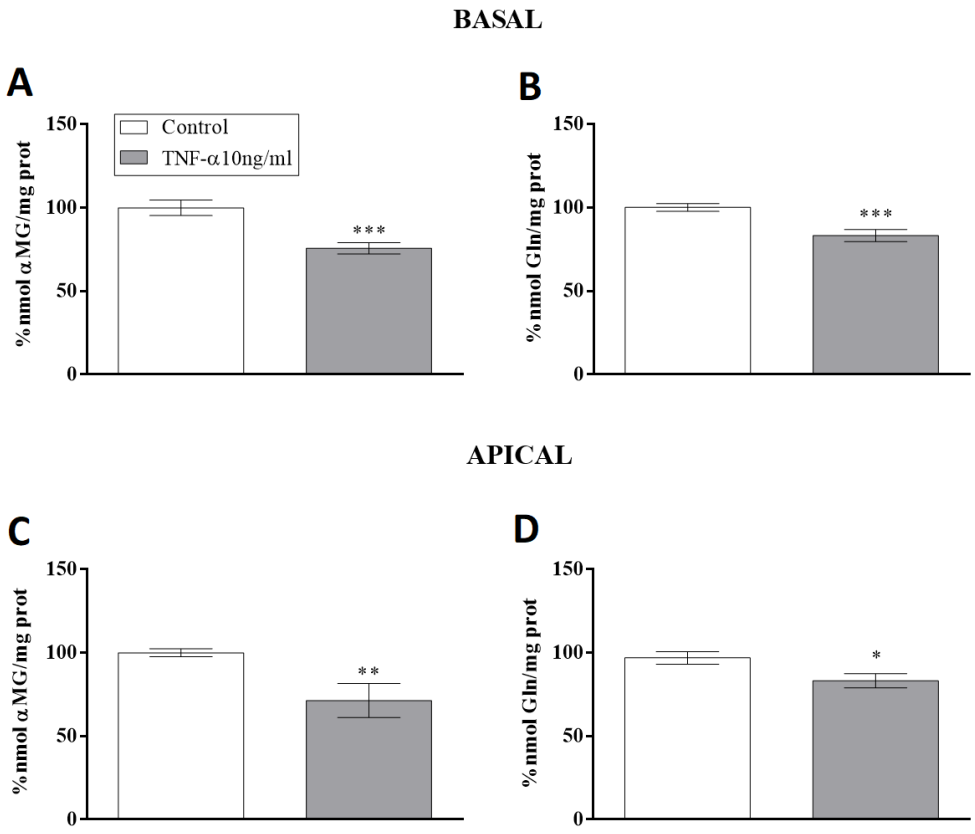


Figure 1. Effect of basal TNF-α on sugar (αMG) and glutamine (Gln) uptake in Caco-2 cells. A-B: Caco-2 cells were pre-incubated for 24 h with 10 ng/ml TNF-α placed in the basal side, before 15 min uptake of 0.1 mM αMG **(A)** and 0.1 mM Gln **(B). C-D:** Caco-2 cells were pre-incubated for 1 h with apical medium from Caco-2 cells exposed to basal TNF-α for 24 h, before 15 min uptake of 0.1 mM αMG **(C)** and 0.1 mM Gln **(D)**. Data (n=12-18) are represented as mean ± SEM.*p<0.05, **p<0.01, ***p<0.001 *vs* control group.

As shown in figure 1A and B, basal TNF-α treatment significantly decreased (by 20-30%) αMG and Gln uptake. Interestingly, the apical medium derived from the Caco-2 cells exposed basally to TNF-α also significantly reduced the nutrients uptake (Fig.1C and D). These data suggested the presence of TNF-α in the apical medium of the cells pre-incubated with the basal cytokine. In order to check if TNF-α was able to up-regulate its own secretion by the Caco-2 cells, we analyzed *TNF-α* gene expression by RT-PCR. We also studied the expression of *IL-1β*, another pro-inflammatory cytokine with local and systemic effects, which also decreases intestinal sugar transport (Bertolo *et al.*, 2002), and the expression of *MCP1* (monocyte chemotactic protein-1).

Figure 2 shows significant increases on the gene expression of *TNF-α*, *IL-1β* and *MCP1*, three pro-inflammatory proteins.

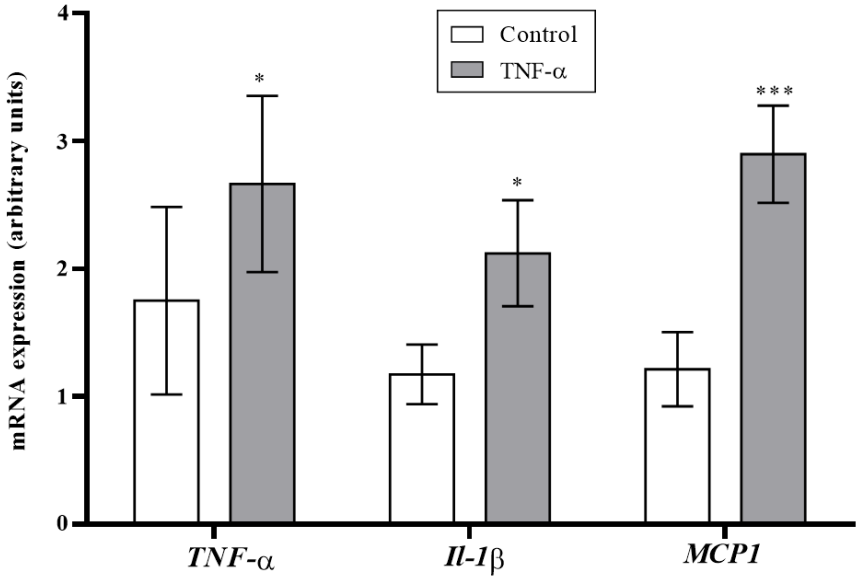


Figure 2. Effects of basal TNF-α treatment on *TNF-α*, *IL-1β* and *MCP1* gene expression in Caco-2 cells. Caco-2 cells grown in 12 well-plate inserts were incubated for 24 h with 10 ng/ml of TNF-α present in the basal compartment before RNA extraction and RT-PCR. Data (n=11; mean ± SEM) are expressed as fold change ($2^{-\Delta\Delta CT}$) relative to control group considered as 1. *p<0.05, ***p<0.001 *vs.* control group.

The data demonstrate that basal TNF-α treatment inhibits αMG and glutamine uptake in Caco-2 cells and stimulates its own expression, together with that of *IL-1β* and *MCP1*.

3.2. Effect of basal exposure of Caco-2 cells to hMSC-DA secretions on αMG uptake

In pathophysiological conditions, TNF-α that access to the basolateral membrane of the enterocytes may come from the blood, due to systemic inflammation, such as sepsis or obesity, or from inflamed intestine (inflammatory bowel diseases) and surrounding tissues (visceral fat in obese individuals). Under inflammatory stimulus, enterocytes can also contribute to the secretion of TNF-α (Jung *et al*., 1995).

Since obesity is a low grade chronic inflammatory disease, accompanied by higher secretion of TNF-α from adipose tissue (Tzanavari *et al.*, 2010), we decided to investigate whether the exposure of Caco-2 cells to the medium coming from mature

adipocytes inhibited sugar uptake. The adipocytes derived from human mesenchymal stem cells (hMSC-DA) of subcutaneous and visceral fat from lean, overweight and morbidly obese donors.

The detailed experimental procedures are described in the Material and Methods (section 2.2.2)

αMG uptake was significantly inhibited in Caco-2 cells exposed in the basal side to conditioned-DMEM from overweight subcutaneous adipocytes compared to control (Fig. 3A). However, non-significant changes were observed in Caco-2 cells incubated with the TNF-α medium or TNF-α-conditioned medium from overweight hMSC-DA (Fig. 3 B and C). In the assays using the apical media collected from the former Caco-2 cells, that was apically placed for 1 h in another Caco-2 cells grown on plates, αMG uptake was also significantly inhibited by conditioned-DMEM and TNF-α medium from overweight hMSC-DA (Fig. 3D, E). Conditioned media coming from subcutaneous adipocytes of a morbidly obese subject did not produce any effect on sugar uptake.

Visceral adipose tissue differs in cellular composition and molecular properties from subcutaneous adipose tissue due to enhanced secretion of pro-inflammatory factors (Caesar *et al*., 2010). For this reason, we performed similar studies using visceral hMSC-DA from overweight and morbidly obese patients.

Interestingly, as observed with subcutaneous hMSC-DA, αMG uptake was significantly inhibited, compared to control, by conditioned-DMEM and TNF-α medium from overweight hMSC-DA, both in the basal (Fig. 4 A, B) and apical assays (Fig. 4 D and E).

These results suggest that the hMSC-DA from overweight individuals would be the most active secretor adipocytes of pro-inflammatory cytokines, both in basal condition and after stimulation with TNF-α. The TNF-α medium may contain traces of this cytokine (see scheme 2) together with TNF-α secreted by overweight adipocytes, since the medium obtained from lean or morbidly obese hMSC-DA did not inhibit sugar uptake.

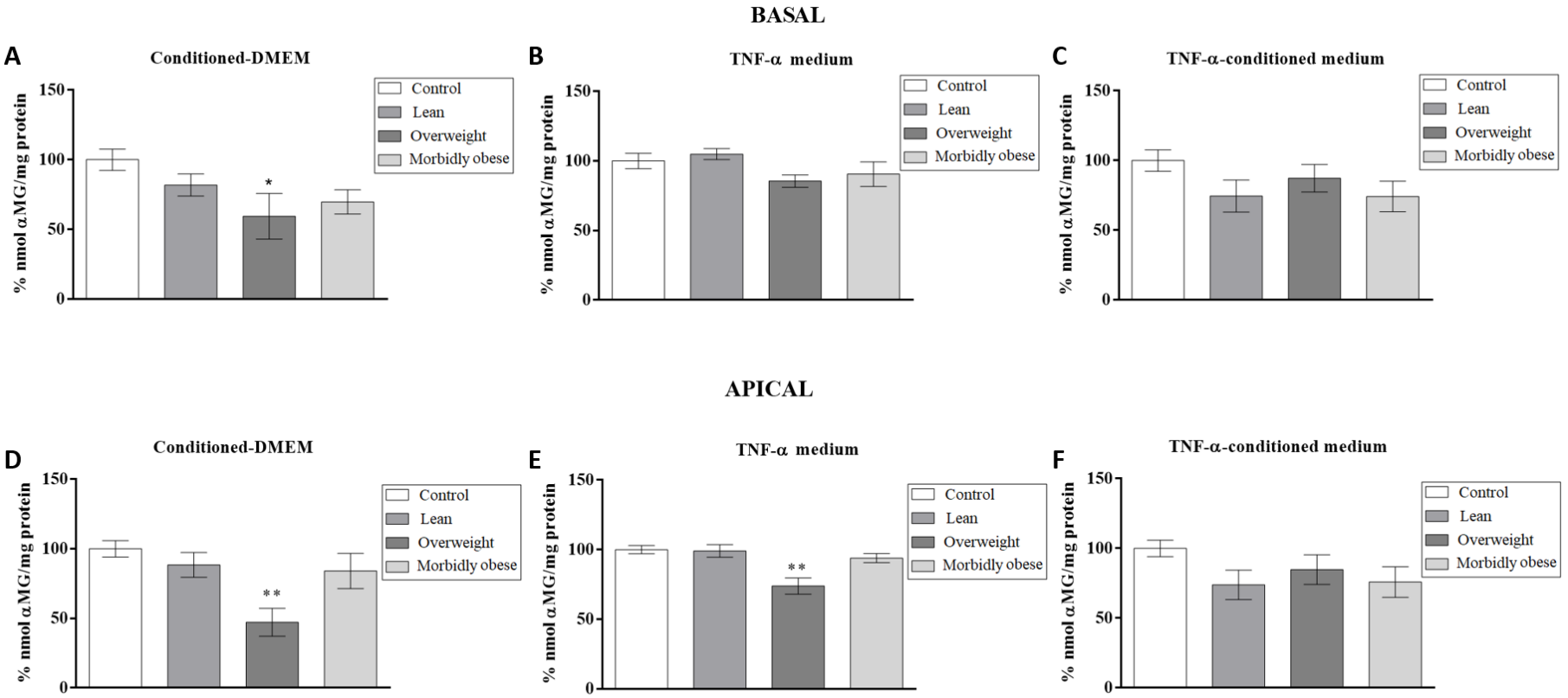


Figure 3. Effect of basal exposure of Caco-2 cells to subcutaneous hMSC-DA media on αMG uptake. A-C: Caco-2 cells grown on inserts were pre-incubated, in the basal compartment for 24 h, with the media obtained from subcutaneous hMSC-DA that have been incubated during 24 h with and without TNF-α. Then, the upper medium was collected and uptake of 0.1 mM αMG was measured for 15 min. **D-F:** The mentioned collected medium was placed on the apical side of Caco-2 cells grown on plates for 1h. Then, the medium was removed and uptake of 0.1 mM αMG was measured for 15 min. Conditioned-DMEM, medium coming from hMSC-DA 24 h incubated solely with DMEM; TNF-α medium, medium coming from hMSC-DA incubated with TNF-α; TNF-α-conditioned medium, medium coming from hMSC-DA after 24 h removal of TNF-α. Data (n=6-12) are represented as mean ± SEM. *p<0.05, **p<0.01 *vs.* control.

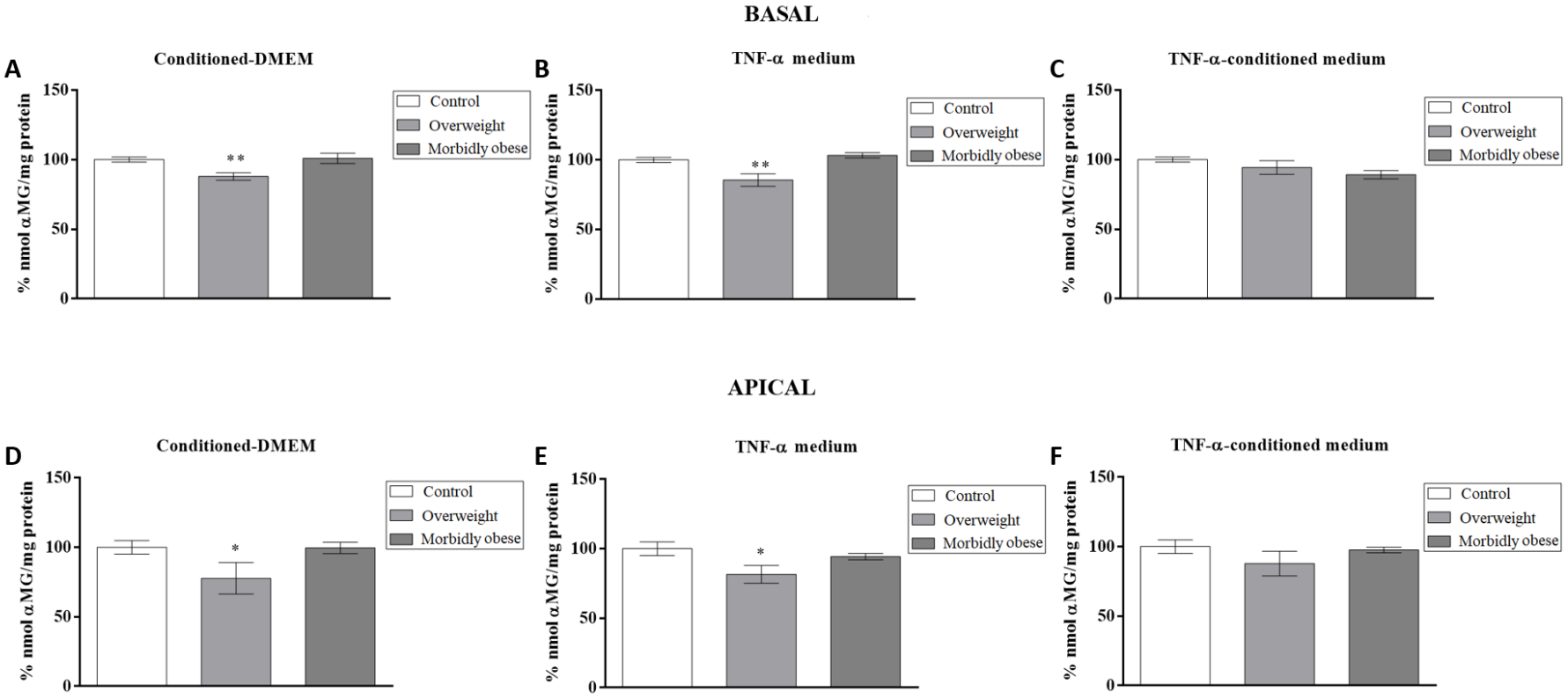


Figure 4. Effect of basal exposure of Caco-2 cells to visceral hMSC-DA media on αMG uptake. A-C: Caco-2 cells grown on inserts were pre-incubated, in the basal compartment for 24 h, with the media obtained from visceral hMSC-DA that have been incubated during 24 h with and without TNF-α. Then, the upper medium was collected and uptake of 0.1 mM αMG was measured for 15 min. **D-F:** The mentioned collected medium was placed on the apical side of Caco-2 cells grown on plates for 1h. Then, the medium was removed and uptake of 0.1 mM αMG was measured for 15 min. Conditioned-DMEM, medium coming from hMSC-DA 24 h incubated solely with DMEM; TNF-α medium, medium coming from hMSC-DA incubated with TNF-α; TNF-α-conditioned medium, medium coming from hMSC-DA after 24 h removal of TNF-α. Data (n=6) are represented as mean ± SEM. *p<0.05, **p<0.01 *vs.* control.

3.3. Expression of pro-inflammatory genes in hMSC-DA.

Human MSC-DA from the overweight subject seemed to be the most active secretor cells of pro-inflammatory cytokines. To check this hypothesis, we set out to investigate the gene expression of the pro-inflammatory cytokines *TNF-α, IL-1β* and *IL-6*, and the chemotactic protein *MCP1*, in visceral hMSC-DA from overweight and also from morbidly obese individuals. For that, we used the hMSC-DA employed for the uptake experiments (section 3.3 of Results). Briefly, matured adipocytes derived from hMSC where incubated for 24 h with DMEM or 10 ng/ml TNF-α. Next, the media were collected, and the cells were incubated for another 24 h with fresh DMEM. Then, media were recovered again for the Caco-2 uptake studies (section 3.3) and hMSC-DA used for mRNA isolation and RT-PCR. The cells were named conditioned DMEM and TNF-α- conditioned medium respectively.

Figure 5 A shows higher expression of *TNF-α, IL-1β, IL-6* and *MCP1* in overweight visceral hMSC-DA compared to adipocytes from a morbidly obese subject in basal conditions (incubation with DMEM, conditioned-DMEM). In the cells incubated first with TNF-α and afterwards with DMEM, only the *IL-6* expression was significantly higher in the overweight adipocytes (Fig. 5 B). Moreover, the gene expression levels of the pro-inflammatory proteins were higher in overweight hMSC-DA from conditioned DMEM than in the overweight cells from TNF-α-conditioned medium.

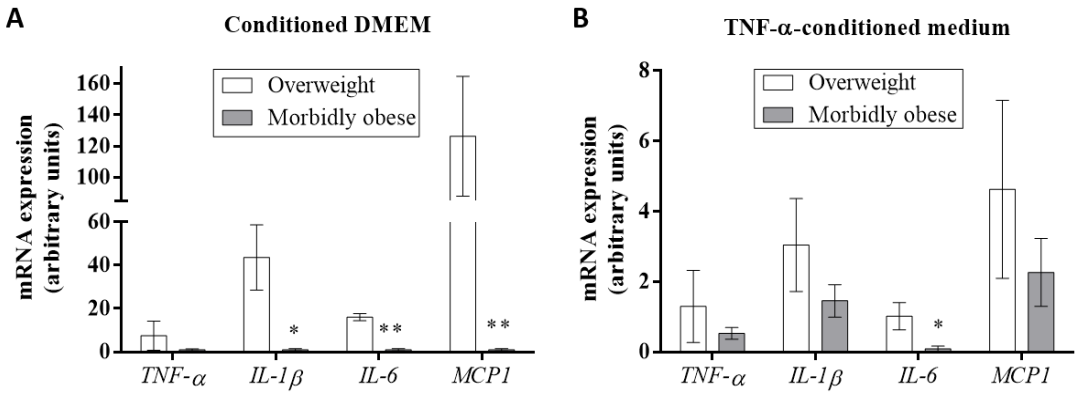


Figure 5. *TNF-α, IL-1β, IL-6* and *MCP1* gene expression in visceral hMSC-DA from overweight and morbidly obese individuals. Visceral matured adipocytes derived from hMSC where incubated for 24 h with DMEM or 10 ng/ml TNF-α. Next, the media were collected, and the cells were incubated for another 24 h with fresh DMEM. Then, media were recovered for the Caco-2 uptake studies and hMSC-DA used for mRNA isolation and RT-PCR. The cells were named conditioned DMEM and TNF-α-conditioned medium respectively. Data (n=5-6; mean ± SEM) are expressed as fold change ($2^{-\Delta\Delta CT}$) relative to morbidly group considered as 1. *p <0.05, ***p <0.001 *vs.* overweight group.

Taken together, these results suggest that mature adipocytes derived from hMSC of overweight subjects, in basal conditions, may secrete pro-inflammatory cytokines which would produce inhibition of sugar uptake in Caco-2 cells.

3.4. Effect of basal exposure of Caco-2 cells to macrophages secretions on αMG uptake and genes expression

Macrophages are directly implicated in the development of inflammation (Johnson *et al.*, 2012). Among their actions, they secrete pro-inflammatory cytokines including TNF-α (Kamada *et al.*, 2008; Schenk *et al*., 2007; Zareie *et al*., 2001). Deposits of M2 macrophages are found underneath the enterocytes (Mahida *et al.*, 1989). During inflammation, M2 macrophages switch into the pro-inflammatory M1 macrophages. In obese subjects, it has been observed increase of M1 macrophages (Peluso & Palmery, 2016; Weisberg *et al.*, 2003).

We decided to investigate whether secretions from LPS-activated and non-activated macrophages could inhibit sugar uptake in Caco-2 cells. Before that, we analyzed the expression of anti-inflammatory and pro-inflammatory genes in both types of macrophages. As observed in figure 6, the pro-inflammatory genes *TNF-α, IL-1β* and *IL-6* were increased in the LPS-activated macrophages compared to the non-

activated ones. However, the gene expression of *MCP1* was significantly decreased in the LPS-activated macrophages. As expected, the anti-inflammatory cytokine *IL-10 was* significantly decreased in the LPS-activated macrophages compared to the non-activated.

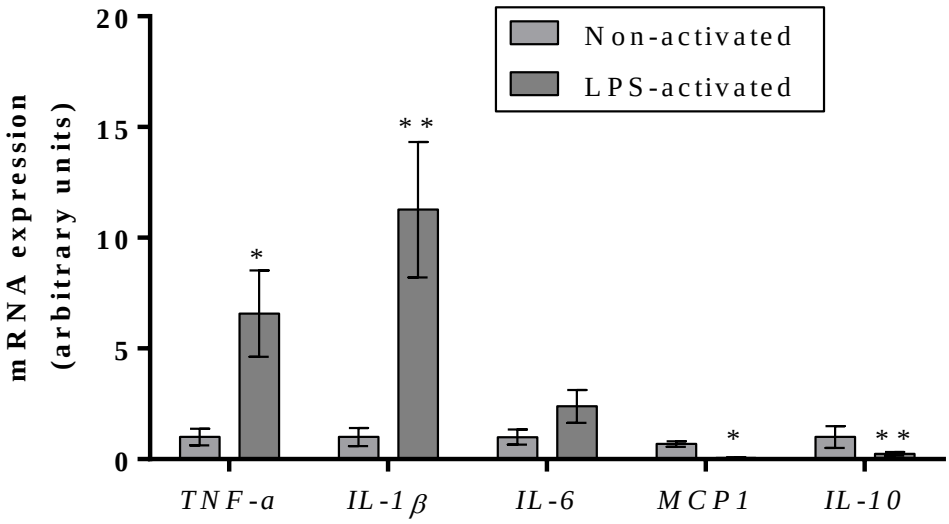


Figure 6. *TNF-α, IL-1β, IL-6, IL-10* and *MCP1* gene expression in LPS-activated and non-activated macrophages. Data (n=6; mean ± SEM) are expressed as fold change ($2^{-\Delta\Delta CT}$) relative to non-activated macrophages group considered as 1. *p<0.05, **p<0.01 *vs.* non-activated macrophages.

As it is shown in figure 7 A, the presence in the basal side (24 h) of the media secreted by both LPS-activated and non-activated macrophages, significantly reduced αMG uptake, being the inhibition higher in the Caco-2 cells incubated with medium from the LPS-activated macrophages (35% for LPS-activated macrophages *vs.* 20% for the non-activated macrophages). On the other hand, the apical medium collected from those Caco-2 cells and placed for 1 h in the apical compartment of another Caco-2 set of cells had no effect on αMG uptake (Fig. 7B).

Increase on *TNF-α, IL-1β, IL-6* and *MCP1* expression was found in Caco-2 cells basally exposed to LPS-activated macrophages secretion, being significant for *IL-1β* and *IL-6* compared to control. In Caco-2 cells basally exposed to non-activated macrophages secretion, a non-significant increased on *IL-1β, IL-6* and *MCP1* was observed compared to control (Fig. 7C).

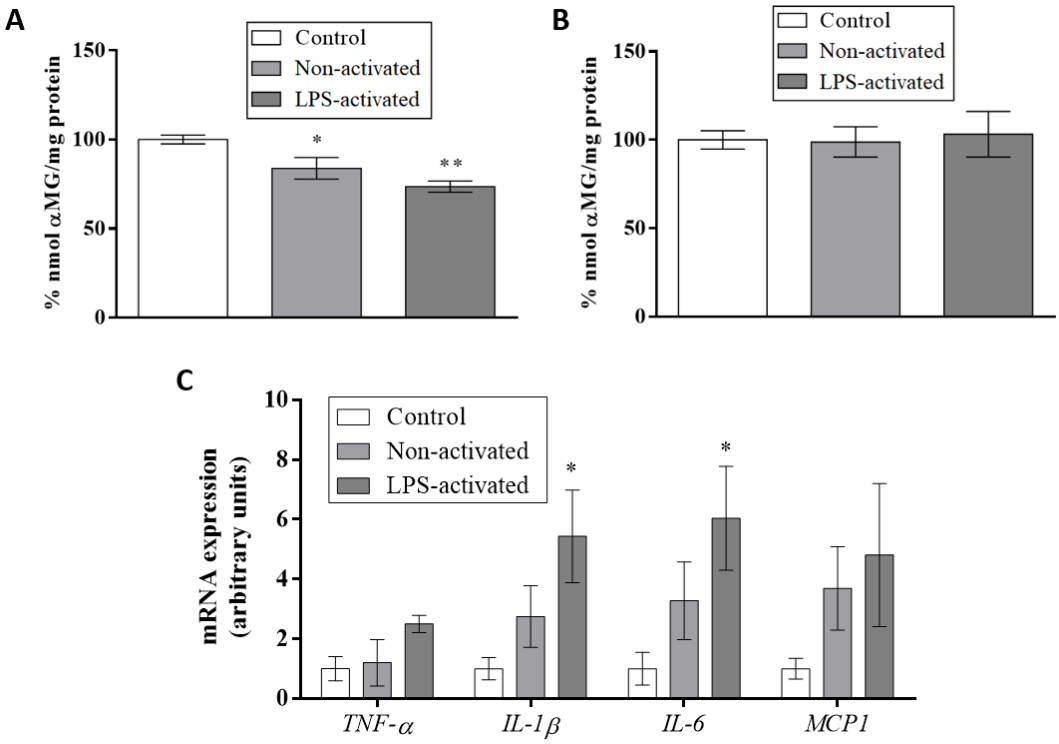


Figure 7. Effect of basal exposure of Caco-2 cells to macrophages media on αMG uptake and Caco-2 cells pro-inflammatory gene expression. A. Caco-2 cells were grown on filters and pre-incubated for 24 h with medium derived from LPS-activated and non-activated macrophages placed in the basal compartment. Then, the upper medium was collected and uptake of 0.1 mM αMG was measured for 15 min. **B.** The mentioned collected medium was placed on the apical side of Caco-2 cells grown on plates for 1h. Then, the medium was removed and uptake of 0.1 mM αMG was measured for 15 min. Data (n=8) are represented as mean ± SEM. **C.** *TNF-α, IL-1ß, IL-6* and *MCP1* gene expression in Caco-2 cells grown on inserts and pre-incubated for 24 h with medium derived from LPS-activated and non-activated macrophages placed in the basal compartment. Data (n=4; mean ± SEM) are expressed as fold change ($2^{-\Delta\Delta CT}$) relative to control group considered as 1. *p<0.05, **p<0.01 *vs.* control.

3.5. Effect of diet induced obesity on intestinal αMG uptake

To get closer to the *in vivo* model, we set out to investigate whether intestinal sugar uptake was altered in diet-induced obese mice, a physiological condition in which the secretion of pro-inflammatory cytokines by adipose tissue is increased (Makki *et al.*, 2013).

Figure 8A shows, at the moment of the sacrifice, that DIO mice presented more adipose tissue around the intestine than the control mice.

In the lean animals, uptake of 0.1 mM αMG in intestinal everted rings was significantly decreased by the presence of 10 ng/mL TNF-α, as we previously reported

(Bertolo *et al*., 2002). Sugar uptake in the DIO animals was also decreased by the same magnitude (~40%) when compared to lean animals uptake (Fig. 8B) as shown in chapter 2. Interestingly in the DIO animals, the presence of TNF-α maintained the same inhibition level of αMG uptake, indicating that the cytokine did not cause additional effect on αMG uptake.

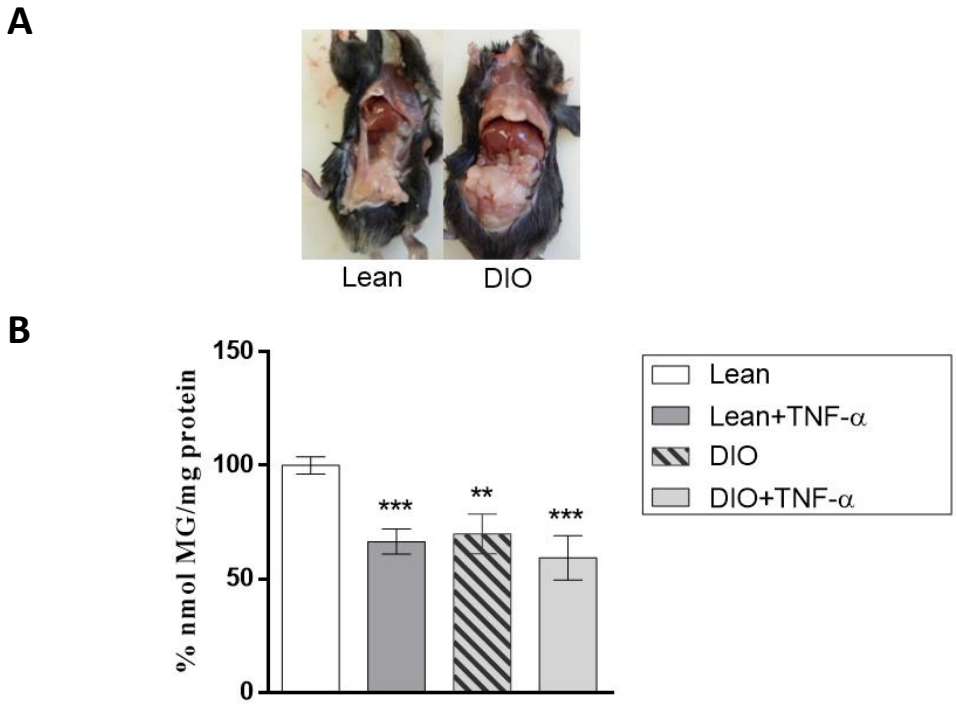


Figure 8. Effect of diet induced obesity (DIO) on intestinal αMG uptake. A. Visceral adipose tissue surrounding the intestine in lean and DIO mice. **B.** Uptake of 1 mM αMG (15 min) in jejunal everted rings from lean (control) and DIO mice in the absence and presence of 10 ng/ml TNF-α. Data (n=15) are represented as mean ± SEM. **p<0.01, ***p<0.01 *vs.* control.

4. Discussion

In the present work, we have demonstrated in Caco-2 cells that αMG uptake is decreased by basolateral exposure to TNF-α, to secretions from human visceral and subcutaneous obese adipocytes and to secretions from macrophages. Intestinal αMG uptake is also diminished in diet-induced obese (DIO) mice, but TNF-α does not increase this inhibition. These functional data are accompanied by increase of TNF-α and other pro-inflammatory genes expression in the Caco-2 cells and in human visceral obese adipocytes.

We have previously demonstrated that TNF-α decreases galactose and αMG uptake in Caco-2 cells via apical TNFR1, by down-regulating the expression of the Na^+/glucose cotransporter SGLT1 in the brush border membrane (Barrenetxe *et al.*, 2013). Likewise, apical TNF-α decreases glutamine uptake (chapter 2). Here, we show that TNF-α, acting from the basolateral membrane, is also able to inhibit αMG and glutamine uptake. In fact, TNFR1 is expressed in the basolateral membrane of Caco-2 cells where it responds to TNF-α (Sonnier *et al.*, 2010).

We also have demonstrated that basal TNF-α stimulates in the Caco-2 cells its own gene expression, together with the expression of *IL-1β* and *MCP1*. Similarly, basal TNF-α induces apical IL-8 secretion (Sonnier *et al.*, 2010). On the other hand, apical TNFα and IL-1 stimulation of human colon epithelial cells up-regulates gene expression and apical secretion of TNF-α, IL-1, IL8 and MCP1 (Jung *et al.*, 1995; Wang *et al.*, 2010).

We have found that apical medium from Caco-2 cells activated with basal TNF-α and placed in the apical side of other set of cells, also decreases sugar and Gln uptake. We can anticipate that this medium will contain pro-inflammatory cytokines, secreted by the Caco-2 cells, able to inhibit nutrients transport as TNF-α (Barrenetxe et al., 2013; Foley *et al.*, 2007; Johnson *et al.*, 2004) and IL-1β (Viñuales *et al.*, 2013). Whether basal TNF-α signaling is directly contributing to the regulation of the nutrients transporters, would need further investigation.

Obesity is known as a low-grade chronic inflammatory disease (Gonçalves *et al.*, 2015; Max *et al.*, 2013) in which there is an increase on the secretion of pro-inflammatory cytokines (TNF-α, IL-1, IL-6 among others) by adipocytes (Maurizi *et al.*, 2017; Mazur-Bialy *et al.*, 2017) and macrophages (Kredel & Siegmund, 2014; Trayhurn & Wood, 2004).

In overweight adipocytes derived from human mesenchymal stem cells (hMSC-DA) of visceral origin, we found up-regulation of the pro-inflammatory cytokines *TNF-α, IL-1 β, IL-6*. The medium coming from these cells and also from overweight hMSC-DA of subcutaneous origin, acting from the basolateral membrane, produces a decrease on αMG uptake in Caco-2 cells. Moreover, the apical medium of these exposed Caco-2 cells is also able to decrease sugar uptake in other set of intestinal cells. This would suggest that the overweight hMSC-DA would secrete pro-inflammatory cytokines to the medium. In turn, the medium would activate cytokines secretion by Caco-2 cells

that would inhibit sugar uptake. *In vivo*, this would mean that in obesity, visceral adipose tissue surrounding the intestine would decrease intestinal nutrients transport through the secretion and diffusion of TNF-α and other pro-inflammatory cytokines. Subcutaneous fat depots would contribute to the alteration of nutrients absorption through systemic TNF-α.

Interestingly, medium from morbidly obese hMSC-DA did not modify sugar uptake in Caco-2 cells. Solá *et al*. (2009) have shown that morbidly obese patients show an extreme chronic inflammatory state, due to an increase in pro-inflammatory cytokine secretion. In these subjects, moderate weight loss did not ameliorate this pro-inflammatory state. We hypothesize that in this chronic inflammatory condition, infiltrated macrophages and T-lymphocytes (Kredel & Siegmund, 2014) would maintain the inflammatory state, and adipocytes would acquire inflammatory resistance to the secretion of pro-inflammatory cytokines. In fact, we demonstrate that expression of *TNF-α, IL-1β* and *IL-6* and *Mcp1* genes is downregulated in morbidly obese hMSC-DA compared to overweight hMSC-DA from visceral origin.

Surprisingly, the medium coming from TNF-α stimulated hMSC-DA did not alter αMG uptake in none of the experimental conditions. These results would suggest that the exposure of the adipocytes to TNF-α could induce resistance to the secretion of pro-inflammatory cytokines, at least in our experimental model. In line with these results, we found very low expression of *TNF-α, IL-1β, IL-6* and *MCP1* in these TNF-α stimulated hMSC-DA in comparison to the non-stimulated cells.

The growth of the adipose tissue in obesity triggers the recruitment and activation (M2 shift into pro-inflammatory M1 phenotype) of macrophages (Nishimura *et al*., 2009; Odegaard & Chawla, 2013). These macrophages produce pro-inflammatory cytokines that are involved in the generation of low grade chronic inflammation, being TNF-α one of the main mediators (Hamdy *et al.*, 2006; Peluso & Palmery, 2016; Weisberg *et al.*, 2003). Macrophages in the lamina propria of the small intestine can be stimulated by pro-inflammatory cytokines coming from the surrounding adipose tissue and, in turn, secrete TNF-α, IL-1β, IL-6, IL-12 (Peluso & Palmery, 2016) which enhances local inflammation (Neuman, 2007) altering intestinal function.

We have found that secretions from LPS-activated and non-activated macrophages inhibit sugar uptake in Caco-2 cells, acting from the basolateral membrane. This inhibition is higher in the Caco-2 exposed to LPS-activated medium which is in agreement with the higher expression of *TNF*-α and *IL*-1β in LPS-activated macrophages. Other authors have also found expression of *IL-1β, IL-6, IL-8* and *TNF-α* in non-activated macrophages. Moreover, co-culture of Caco-2 cells with those macrophages for 48 h, triggered Caco-2 cells damage through TNF-α binding to TNFR1 (Satsu *et al.*, 2006). Tanoue *et al.* (2008) observed that LPS-stimulated-RAW264.7 cells (murine macrophages), in co-culture with Caco-2 cells, secreted TNF-α which induced *Il-8* mRNA expression in the Caco-2 cells. Our Caco-2 cells exposed to LPS-activated and non-activated macrophages secretions did not significantly increase *TNF-α* expression. These data could explain the lack of effect of the apical medium of these cells on sugar uptake in another set of Caco-2 cells.

We have found that diet induced obesity in mice showed a development of the visceral adipose tissue surrounding the intestine. This was accompanied by up-regulation of the pro-inflammatory cytokines *Tnf-α, Il-1β, Il-6* genes expression in the jejunum and decrease on αMG uptake by intestinal rings and SGLT1 expression in the apical membrane of the enterocytes (chapter 2). The sugar uptake reduction was of the same magnitude than that observed in the lean animals intestinal rings in the presence of TNF-α. Interestingly, in DIO mice there was not further decrease of sugar uptake by addition of TNF-α, which would suggest resistance to the cytokine in the obese animals

Our earlier data support these results and confirm our hypothesis. Thus, obesity would induce the growth of visceral adipose tissue next to the intestine. TNF-α coming from adipocytes would bind to the basolateral receptor TNFR1 (Sonnier *et al*., 2010) and stimulate TNF-α apical secretion by the enterocytes which, in turn, would inhibit sugar transport by binding to apical TNFR1 (Barrenetxe *et al.*, 2013). Secretion from activated macrophages would also inhibit sugar uptake by directly signaling from the basolateral membrane.

References

A

Albenberg, L.G., Lewis, J.D. & Wu, G.D. (2012). Food and Gut Microbiota in IBD: A Critical Connection. *Curr Opin Gastroenterol* ***28*** (4), 314–320.

Argollo, M., Fiorino, G., Hindryck, P., Peyrin-Biroulet, L. & Danese, S. (2017). Novel therapeutic targets for inflammatory bowel disease. *Journal of Autoimmunity*. https://doi.org/10.1016/j.jaut.2017.07.004

B

Barrenetxe, J., Sánchez, O., Barber, A., Gascón, S., Rodríguez-Yoldi, M.J. & Lostao, M.P. (2013). TNFα regulates sugar transporters in the human intestinal epithelial cell line Caco-2. *Cytokine* ***64*** (1), 181–187.

Bertolo, C., Barrenetxe, J., Barber, A. & Lostao, M.P. (2002). Cytokine effect on intestinal galactose absorption. *Journal of Physiology and Biochemistry* ***58*** (1), 61–62.

C

Caesar, R., Manieri, M., Kelder, T., Boekschoten, M., Evelo, C., Müller, M., Kooistra, T., Cinti, S., Kleemann, R. & Drevon, C. A. (2010). A combined transcriptomics and lipidomics analysis of subcutaneous, epididymal and mesenteric adipose tissue reveals marked functional differences. *PLoS ONE* ***5*** (7): e11525.

D

Davies, L.C., Jenkins, S.J., Allen, J.E. & Taylor, P.R. (2013). Tissue-resident macrophages. *Nature Immunology* ***14*** (10), 986–995.

Ducroc, R., Sakar, Y., Fanjul, C., Barber, A., Bado, A. & Lostao, M.P. (2010). Luminal leptin inhibits L-glutamine transport in rat small intestine: involvement of ASCT2 and B^0AT1. *American Journal of Physiology Gastrointestinal and Liver Physiology* ***299*** (1), G179-85.

F

Foley, K.F., Pantano, C., Ciolino, A. & Mawe, G.M. (2007). IFN-gamma and TNF-αlpha decrease serotonin transporter function and expression in Caco2 cells. *American Journal of Physiology Gastrointestinal and Liver Physiology* ***292*** (3), G779-84.

G

Gonçalves, P., Magro, F. & Martel, F. (2015). Metabolic Inflammation in Inflammatory Bowel Disease: Crosstalk Between Adipose Tissue and Bowel. *Inflammatory Bowel Diseases* ***21*** (2), 453–467.

Gummesson, A., Carlsson, L.M.S., Storlien, L.H., Bäckhed, F., Lundin, P., Löfgren, L., Stenlöf, K., Lam, Y.Y., Fagerberg, B. & Carlsson, B. (2011). Intestinal Permeability Is Associated With Visceral Adiposity in Healthy Women. *Obesity* ***19*** (11), 2280–2282.

H

Hamdy, O., Porramatikul, S. & Al-Ozairi, E. (2006). Metabolic obesity: the paradox between visceral and subcutaneous fat. *Current Diabetes Reviews* ***2*** (4), 367–373.

Hayashi, K., Piras, V., Tabata, S., Tomita, M. & Selvarajoo, K. (2013). A systems biology approach to suppress TNF-induced proinflammatory gene expressions. *Cell Communication and Signaling* ***11*** (1), 84.

Huffman, D. M. & Barzilai, N. (2009). Role of visceral adipose tissue in aging. *Biochimica et Biophysica Acta - General Subjects* **1790** (10): 1117–1123.

J

Johnson, A.R., Milner, J.J. & Makowski, L. (2012). The inflammation highway: metabolism accelerates inflammatory traffic in obesity. *Immunology Review* ***249***(1), 218–238.

Johnson, D., Bayele, H., Johnston, K., Tennant, J., Srai, S.K. & Sharp, P. (2004). Tumour necrosis factor alpha regulates iron transport and transporter expression in human intestinal epithelial cells. *FEBS Letters* ***573*** (1–3), 195–201.

Jung, H.C., Eckmann, L., Yang, S.K., Panja, A., Fierer, J., Morzycka-Wroblewska, E. & Kagnoff, M.F. (1995). A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *The Journal of Clinical Investigation* ***95*** (1), 55–65.

K

Kamada, N., Hisamatsu, T., Okamoto, S., Chinen, H., Kobayashi, T., Sato, T., Sakuraba, A., Kitazume, M.T., Sugita, A., Koganei, K., Akagawa, K.S. & Hibi, T. (2008). Unique CD14+ intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-γ axis. *The Journal of Clinical Investigation* ***118*** (6), 2269–2280.

Kredel, L.I. & Siegmund, B. (2014). Adipose-tissue and intestinal inflammation - visceral obesity and creeping fat. *Frontiers in Immunology* ***5***, 1–12.

L

Laiglesia, L.M., Lorente-Cebrián, S., Martínez-Fernández, L., Sáinz, N., Prieto-Hontoria, P.L., Burrell, M.A., Rodríguez-Ortigosa, C.M., Martínez, J.A. & Moreno-Aliaga, M.J. (2017). Maresin 1 mitigates liver steatosis in ob/ob and diet-induced obese mice. *International Journal of Obesity*. Doi: 10.1038/ijo.2017.226

Livak, K.J. & Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real- Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. *METHODS* ***25*** *(4)*, 402–408.

Lumeng, C.N., Liu, J., Geletka, L., Delaney, C., Delproposto, J., Desai, A., Oatmen, K., Martinez-Santibanez, G., Julius, A., Garg, S. & Yung, R. L. (2011). Aging is associated with an increase in T cells and inflammatory macrophages in visceral adipose tissue. *Journal of Immunology* ***187*** (12), 6208–6216.

M

Mahida, Y.R., Patel, S., Gionchetti, P., Vaux, D. & Jewell, D.P. (1989). Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. *Gut* ***30*** (6), 826–834.

Makki, K., Froguel, P. & Wolowczuk, I. (2013). Adipose Tissue in Obesity-Related Inflammation and Insulin Resistance: Cells, Cytokines, and Chemokines. *ISRN Inflammation* ***2013***, 1–12.

Mantovani, A., Biswas, S. K., Galdiero, M. R., Sica, A. & Locati, M. (2013). Macrophage plasticity and polarization in tissue repair and remodelling. *Journal of Pathology* ***229*** (2), 176–185.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology* **25** (12):677-686.

Maurizi, G., Della Guardia, L., Maurizi, A. & Poloni, A. (2017). Adipocytes properties and crosstalk with immune system in obesity-related inflammation. *Journal of Cellular Physiology*. https://doi.org/10.1002/jcp.25855

Max, J.B., Stidham, R., Su, G.L. & Waljee, A.K. (2013). Obesity and IBD: Are We Tipping the Scales Toward an Epidemic? *Gastroenterology* **145** (2), 478–479.

Mazur-Bialy A.I., Bilski J., Pochec E., Brzozowski T. (2017). New insight into the direct anti-inflammatory activity of a myokine irisin against proinflammatory activation of adipocytes. Implication for exercise in obesity. *Journal of Physiology and Pharmacology* ***68*** (2), 243–251.

N

Neuman, M.G. (2007). Immune dysfunction in inflammatory bowel disease. *Translational Research* ***149*** (4), 173–186.

Nishimura, S., Manabe, I., Nagasaki, M., Eto, K., Yamashita, H., Ohsugi, M., Otsu, M., Hara, K., Ueki, K., Sugiura, S., Yoshimura, K., Kadowaki, T. & Nagai, R. (2009). CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nature Medicine* **15** (8), 914–920.

O

Odegaard, J. & Chawla, A. (2013). Pleiotropic actions of insulin resistance and inflammation in metabolic homeostasis. *Science* **339** (6116), 172–177.

P

Peluso, I. & Palmery, M. (2016). The relationship between body weight and inflammation: Lesson from anti-TNF-α antibody therapy. *Human Immunology* **77** (1), 47–53.

Perez-Diaz, S., Garcia-Rodriguez, B., Gonzalez-Irazabal, Y., Valero, M., Lagos-Lizan, J. & Arbones-Mainar, J.M. (2017). Knockdown of PTRF ameliorates adipocyte differentiation and functionality of human mesenchymal stem cells. *American Journal of Physiology - Cell Physiology* ***312*** (1), C83–C91.

R

Rocha, V. Z. & Folco, E. J. (2011). Inflammatory Concepts of Obesity. *International Journal of Inflammation* ***2011***, 1–14.

S

Satsu, H., Ishimoto, Y., Nakano, T., Mochizuki, T., Iwanaga, T. & Shimizu, M. (2006). Induction by activated macrophage-like THP-1 cells of apoptotic and necrotic cell death in intestinal epithelial Caco-2 monolayers via tumor necrosis factor-alpha. *Experimental Cell Research* ***312*** (19), 3909–3919.

Schenk, M., Bouchon, A., Seibold, F., & Mueller, C. (2007). TREM-1–expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. *The Journal of Clinical Investigation* ***117*** (10), 3097–3106.

Sideri, A., Stavrakis, D., Bowe, C., Shih, D. Q., Fleshner, P., Arsenescu, V., Arsenescu, R., Turner, J.R., Pothoulakis, C. & Karagiannides, I. (2015). Effects of obesity on severity of colitis and cytokine expression in mouse mesenteric fat. Potential role of adiponectin receptor 1. *American Journal of Physiology. Gastrointestinal and Liver Physiology* ***308*** (7), G591-604.

Solá, E., Jover, A., López-Ruiz, A., Jarabo, M., Vayá, A., Morillas, C., Gómez-Balaguer, M. & Hernández-Mijares, A. (2009). Parameters of inflammation in morbid obesity: Lack of effect of moderate weight loss. *Obesity Surgery* ***19*** (5), 571–576.

Sonnier, D.I., Bailey, S.R., Schuster, R.M., Lentsch, A.B. & Pritts, T.A. (2010). TNF-α Induces Vectorial Secretion of IL-8 in Caco-2 Cells. *Journal of Gastrointestinal Surgery* ***14*** (10), 1592–1599.

Soufli, I., Toumi, R., Rafa, H. & Touil-Boukoffa, C. (2016). Overview of cytokines and nitric oxide involvement in immuno-pathogenesis of inflammatory bowel diseases. *World Journal of Gastrointestinal Pharmacology and Therapeutics* ***7*** (3), 353.

T

Tabas, I. & Glass, C.K. (2013). Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science* ***339*** (6116), 166–172.

Tanoue, T., Nishitani, Y., Kanazawa, K., Hashimoto, T. & Mizuno, M. (2008). In vitro model to estimate gut inflammation using co-cultured Caco-2 and RAW264.7 cells. *Biochemical and Biophysical Research Communications* ***374*** (3), 565–569.

Trayhurn, P. & Wood, I.S. (2004). Adipokines: inflammation and the pleiotropic role of white adipose tissue. *British Journal of Nutrition* ***92*** (3), 347.

Tzanavari, T., Giannogonas, P. & Karalis, K.P. (2010). TNF-alpha and obesity. *Current Directions in Autoimmunity* ***11,*** *145-56*.

V

Viñuales, C., Gascón, S., Barranquero, C., Osada, J. & Rodríguez-Yoldi, M.J. (2013). Interleukin-1 beta reduces galactose transport in intestinal epithelial cells in a NF-κB and protein kinase C-dependent manner. *Veterinary Immunology and Immunopathology* ***155*** (3), 171–181.

W

Wang, B., Li, J., Chen, J., Huang, Q., Li, N. & Li, J. (2010). Effect of live Lactobacillus plantarum L2 on TNF-α-induced MCP-1 production in Caco-2 cells. *International Journal of Food Microbiology* ***142*** (1–2), 237–241.

Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L. & Ferrante, A.W. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigation* ***112*** (12), 1796–1808.

Z

Zareie, M., Singh, P.K., Irvine, E.J., Sherman, P.M., Mckay, D.M., & Perdue, M.H. (2001). Monocyte / Macrophage Activation by Normal Bacteria and Bacterial Products Implications for Altered Epithelial Function in Crohn ' s Disease. *American Journal of Pathology* ***158*** (3), 1101–1109.

V. GENERAL DISCUSSION

The results obtained in the present work have been discussed in detail in each chapter; here, a general discussion is presented.

The gastrointestinal mucosa is continuously exposed to external potential antigenic substances. As a consequence, the immune system is activated and inflammatory reaction may occur. If this reaction is not properly controlled, chronic inflammation can develop, with high production of pro-inflammatory cytokines as TNF-α (Tabas & Glass, 2013; Vitale *et al.*, 2017). Under inflammatory stimulus, enterocytes also contribute to the secretion of TNF-α (Jung *et al.*, 1995). TNF-α can bind to its receptors, TNFR1 and TNFR2, located in the apical and basolateral membrane of the enterocytes, in an autocrine and paracrine way (Sonnier *et al.*, 2010).

During intestinal inflammation, nutrients and electrolytes malabsorption may occur in relation to alterations on the expression and activity of their intestinal transporters (Powel, 1990; Bertolo *et al.*, 2002; Foley *et al.*, 2007; Sharma *et al*., 2005). We have previously demonstrated, in the human intestinal epithelial cell line Caco-2, that TNF-α, acting from the apical membrane, inhibits αMG uptake by decreasing the Na^+/glucose cotransporter SGLT1 expression in the brush border membrane (Barrenetxe *et al*., 2013).

Marine omega-3 polyunsaturated fatty acids (n-3 PUFAs) show anti-inflammatory actions on inflammatory-related pathologies, including intestinal inflammation and obesity (Calder, 2015; Martínez-Fernández *et al.*, 2015). EPA and DHA are the main n-3 PUFAs in fish oil (Calder, 2013). N-3 PUFAs serve as substrates for the formation of specialized pro-resolving lipid mediators (SPMs) with potent anti-inflammatory and pro-resolving properties, namely maresins (MaR), resolvins (Rv) and protectins (PD) (Bento *et al*., 2011; Chatterjee *et al.*, 2014; Serhan, 2007). To our knowledge, the possible beneficial actions of the n-3 PUFAs and SPMs on nutrients intestinal transport affected during intestinal inflammation, remains unknown.

In the present work, we have demonstrated that apical TNF-α decreases sugar uptake and SGLT1 expression in the brush border membrane of Caco-2 cells, by the activation of ERK 1/2 and the inhibition of AMPK pathways. EPA, DHA, MaR1, RvD1 and RvD2 block apical TNF-α-induced inhibition of sugar uptake and SGLT1 expression in the brush border membrane. EPA prevents these TNF-α effects by activating the AMPK signaling pathway, probably through GPR120. Interestingly, DHA acts through a

mechanism different from the binding to GPR120. Apical TNF-α also decreases glutamine uptake in Caco-2 cells, but increases the expression in the brush border membrane of the Na^+-dependent glutamine transporter B^0AT1, suggesting an alteration on the activity of the transporter. EPA, DHA and DHA derived SPMs blunt TNF-α-induced inhibition of glutamine transport. EPA and DHA also partially block the B^0AT1 increased expression in the membrane. Altogether, our data suggest that EPA, DHA and DHA derived SPMs are presented as promising biomolecules to restore intestinal nutrients transport during intestinal inflammation processes.

Obesity is a low grade chronic inflammatory state, where inflammation results in secondary diseases in the long run, and impacts the progression of other illnesses (Rocha & Folco, 2011). In obesity, there is an increase of the secretion of pro-inflammatory cytokines by adipocytes (Mazur-Bialy *et al.*, 2017; Maurizi *et al*., 2017) and macrophages (Trayhurn & Wood, 2004; Kredel & Siegmund, 2014). TNF-α production in obesity may access the basolateral membrane of the enterocytes coming from the blood or diffusing from the visceral adipose tissue surrounding the intestine or from infiltrated macrophages.

Here, we show that TNF-α and the secretions from overweight adipocytes (hMSC-DA) and LPS-activated and non-activated macrophages, acting from the basolateral membrane of Caco-2 cells, decrease sugar uptake. In Caco-2 cells under basolateral TNF-α stimulation and in overweight hMSC-DA, there is up-regulation of *TNF-α, IL-1β* and *MCP1* gene expression.

To approach our *in vitro* results to the *in vivo* model, we used diet induced obese (DIO) mice. DIO mice presented a clear increase in visceral adipose tissue surrounding the intestine. In these animals, intestinal sugar transport is decreased in comparison to lean animals. This effect is accompanied by a reduction on SGLT1 expression in the brush border membrane of the enterocytes, and by an increase on *Tnf-α, Il-1β* and *Il-6* expression in the jejunal mucosa. Oral administration of MaR1 reverses the increase of the pro-inflammatory cytokines genes expression, but not the decrease on sugar uptake or SGLT1 amount in the brush border membrane.

Thus, it can be suggested that in obesity, TNF-α coming from adipocytes of the visceral fat next to the intestine would bind to the basolateral TNF-α receptor TNFR1 (Sonnier *et al*., 2010) and stimulate TNF-α apical secretion by the enterocytes which, in

turn, would inhibit sugar transport by binding to the apical TNFR1 (Barrenetxe *et al*., 2013). Secretion from infiltrated macrophages would also inhibit sugar uptake by signaling from the basolateral membrane.

In conclusion, the results of this work support that TNF-α secreted by the small intestine, macrophages and adipocytes during intestinal inflammation and obesity can decrease sugar and glutamine absorption and that the omega-3 EPA, DHA and the DHA derived pro-resolving lipid mediators are able to block this effect.

References

B

Barrenetxe, J., Sánchez, O., Barber, A., Gascón, S., Rodríguez-Yoldi, M.J. & Lostao, M.P. (2013). TNFα regulates sugar transporters in the human intestinal epithelial cell line Caco-2. *Cytokine* ***64*** (1), 181–187.

Bento, A.F., Claudino, R.F., Dutra, R.C., Marcon, R. & Calixto, J.B. (2011). Omega-3 Fatty Acid-Derived Mediators 17(R)-Hydroxy Docosahexaenoic Acid, Aspirin-Triggered Resolvin D1 and Resolvin D2 Prevent Experimental Colitis in Mice. *The Journal of Immunology* ***187*** (4), 1957–1969.

Bertolo, C., Barrenetxe, J., Barber, A. & Lostao, M.P. (2002). Cytokine effect on intestinal galactose absorption. *Journal of Physiology and Biochemistry* ***58*** (1), 61–62.

C

Calder, P.C. (2013). Omega-3 polyunsaturated fatty acids and inflammatory processes: Nutrition or pharmacology? *British Journal of Clinical Pharmacology* ***75*** (3), 645–662.

Calder, P.C. (2015). Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance . *BBA - Molecular and Cell Biology of Lipids* ***1851*** (4), 469–484.

Chatterjee, A., Sharma, A., Chen, M., Toy, R., Mottola, G. & Conte, M.S. (2014). The pro-resolving lipid mediator maresin 1 (MaR1) attenuates inflammatory signaling pathways in vascular smooth muscle and endothelial cells. *PLoS ONE* ***9*** (11).

F

Foley, K.F., Pantano, C., Ciolino, A. & Mawe, G.M. (2007). IFN-gamma and TNF-αlpha decrease serotonin transporter function and expression in Caco2 cells. *American Journal of Physiology Gastrointestinal and Liver Physiology* ***292*** (3), G779-84.

J

Jung, H.C., Eckmann, L., Yang, S.K., Panja, A., Fierer, J., Morzycka-Wroblewska, E. & Kagnoff, M.F. (1995). A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *The Journal of Clinical Investigation* ***95*** (1), 55–65

K

Kredel, L.I. & Siegmund, B. (2014). Adipose-tissue and intestinal inflammation - visceral obesity and creeping fat. *Frontiers in Immunology **5***, 1–12.

M

Martinez-Fernandez, L., Laiglesia, L.M., Huerta, A.E., Martinez, J.A., & Moreno-Aliaga, M.J. (2015). Omega-3 fatty acids and adipose tissue function in obesity and metabolic syndrome. *Prostaglandins & Other Lipid Mediators **121***, 1–18.

Maurizi, G., Della Guardia, L., Maurizi, A. & Poloni, A. (2017). Adipocytes properties and crosstalk with immune system in obesity-related inflammation. *Journal of Cellular Physiology*. https://doi.org/10.1002/jcp.25855

Mazur-Bialy A.I., Bilski J., Pochec E., Brzozowski T. (2017). New insight into the direct anti-inflammatory activity of a myokine irisin against proinflammatory activation of adipocytes. Implication for exercise in obesity. *Journal of Physiology and Pharmacology **68*** (2), 243–251.

P

Powell, D.W. (1990). Immunophysiology of intestinal electrolyte transport. In *Handbook of Physiology. The Gastrointestinal System. Intestinal Absorption and Secretion.* (pp. 591–564). Bethesda, MD: American Physiological Society

R

Rocha, V.Z. & Folco, E.J. (2011). Inflammatory Concepts of Obesity. *International Journal of Inflammation **2011***, 1–14.

S

Serhan, C.N. (2007). Resolution Phase of Inflammation: Novel Endogenous Anti-Inflammatory and Proresolving Lipid Mediators and Pathways. *Annual Review of Immunology **25*** (1), 101–137.

Sharma, N., Laftah, A.H., Brookes, M.J., Cooper, B., Iqbal, T. & Tselepis, C. (2005). A role for tumour necrosis factor alpha in human small bowel iron transport. *The Biochemical Journal **390***, 437–46.

Sonnier, D.I., Bailey, S.R., Schuster, R.M., Lentsch, A.B. & Pritts, T.A. (2010). TNF-α Induces Vectorial Secretion of IL-8 in Caco-2 Cells. *Journal of Gastrointestinal Surgery **14*** (10), 1592–1599.

T

Tabas, I. & Glass, C.K. (2013). Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science* ***339*** (6116), 166–72.

Trayhurn, P., & Wood, I.S. (2004). Adipokines: inflammation and the pleiotropic role of white adipose tissue. *British Journal of Nutrition* ***92*** (3), 347.

V

Vitale, S., Strisciuglio, C., Pisapia, L., Miele, E., Barba, P., Vitale, A., Cenni, S., Bassi, V., Maglio, M., Del Pozzo, G., Troncone, R., Staiano, A. & Gianfrani, C. (2017). Cytokine production profile in intestinal mucosa of paediatric inflammatory bowel disease. *PLoS ONE* ***12*** (8), 1–20.

VI. CONCLUSIONS

1. Apical TNF-α decreases sugar uptake and SGLT1 expression in the brush border membrane of Caco-2 cells by the activation of ERK 1/2 and the inhibition of AMPK pathways. EPA prevents these TNF-α effects by activating the AMPK signaling pathway, probably through GPR120.

2. DHA and its derived specialized pro-resolving lipid mediators (SPMs) MaR1, RvD1 and RvD2 also block apical TNF-α-induced inhibition of sugar uptake and SGLT1 expression in the brush border Caco-2 cells. DHA may exert its effect through a mechanism different from GPR120 binding.

3. Apical TNF-α decreases glutamine uptake in Caco-2 cells but increases B^0AT1 expression in the brush border membrane. EPA, DHA and DHA derived SPMs blunt TNF-α-induced inhibition of glutamine transport. EPA and DHA also partially block the increased expression of B^0AT1.

4. Basolateral TNF-α is able to inhibit sugar and glutamine uptake and stimulate *TNF-α IL-1β* and *MCP1* gene expression in Caco-2 cells.

5. Media from subcutaneous and visceral overweight hMSC-DA decrease sugar uptake in Caco-2 cells acting from the basolateral membrane. In turn, apical media from these Caco-2 cells also reduce sugar uptake. In visceral overweight hMSC-DA there is gene up-regulation of the pro-inflammatory cytokines *TNF-α, IL-1β* and *IL-6*. None of these effects are observed with media from morbidly obese hMSC-DA.

6. Media from LPS-activated and non-activated macrophages decrease sugar uptake in Caco-2 cells acting from the basolateral membrane. TNF-α expression was not significantly increase in these Caco-2 cells. Apical media from these Caco-2 cells do not alter sugar uptake.

7. Diet-induced obese (DIO) mice show a higher content of visceral adipose tissue surrounding the intestine. This is accompanied by up-regulation of *Tnf-α, Il-1β, Il-6* gene expression in the jejunal mucosa. Also, there is a decrease on intestinal sugar uptake and SGLT1 expression in the bush border membrane of DIO mice. Oral administration of MaR1 to DIO mice reverses the increase in the expression of the pro-inflammatory cytokines, but not the decrease on sugar uptake or SGLT1 expression observed in DIO mice.

GENERAL CONCLUSION

In conclusion, the results of this work suggest that TNF-α secreted by the small intestine, macrophages and adipocytes during intestinal inflammation and obesity can decrease sugar and glutamine absorption and that the omega-3 EPA, DHA and the DHA derived pro-resolving lipid mediators (MaR1, RvD1 and RvD2) are able to block this effect.

1. El TNF-α apical disminuye la captación de azúcar y la expresión de SGLT1 en la membrana de borde en cepillo de las células Caco-2, por activación de la vía intracelular de ERK 1/2 e inhibición de AMPK. EPA previene los efectos de TNF-α por activación de la vía de AMPK, probablemente mediante la unión a su receptor GPR120.

2. DHA y sus mediadores lipídicos pro-resolutivos (SPMs) MaR1, RvD1 and RvD2 también pueden bloquear la inhibición de la captación de azúcar inducida por el TNF-α apical y de la expresión de SGLT1 en la membrana en cepillo de células Caco-2. El DHA no parece ejercer sus efectos a través de la unión con GPR120.

3. El TNF-α apical disminuye la captación de glutamina en células Caco-2, pero aumenta la expresión de B^0AT1 en la membrana de borde en cepillo. EPA, DHA y SPMs derivados de DHA bloquean el efecto inhibitorio de TNF-α en la captación de glutamina. EPA y DHA también bloquean parcialmente el aumento en la expresión de B^0AT1.

4. El TNF-α basolateral es capaz de inhibir la captación de azúcar y glutamina y estimular la expresión de los genes de *TNF-α, IL-1β* y *MCP1* en células Caco-2.

5. Medios procedentes de hMSC-DA, con origen subcutáneo y visceral de un paciente con sobrepeso, disminuyen la captación de azúcar en células Caco-2 actuando desde la membrana basolateral. A su vez, los medios apicales de estas células Caco-2 también reducen la captación de azúcar. En las hMSC-DA viscerales de un paciente con sobrepeso hay un aumento de la expresión de genes de las citoquinas pro-inflamatorias *TNF-α, IL-1β* e *IL-6*. Ninguno de estos efectos se produce con hMSC-DA procedentes de un paciente obeso mórbido.

6. Medios procedentes de macrófagos activados con LPS y macrófagos sin activar disminuyen la captación de azúcar en células Caco-2 actuando desde su membrana basolateral. La de TNF-α expresión no estaba significativamente aumentada en estas células Caco-2. Los medios apicales obtenidos de estas células Caco-2 no alteran la captación de azúcar.

7. Ratones con obesidad inducida por la dieta (DIO) muestran un aumento de la grasa visceral que recubre el intestino. Esto está acompañado por un aumento en la expresión de los genes de *Tnf-α, Il-1β, Il-6* en la mucosa del yeyuno. Además, estos ratones DIO presentan una disminución de la captación de azúcar en el intestino y de la expresión de SGLT1 en la membrana del borde en cepillo de los enterocitos. La administración oral de MaR1 a los ratones DIO revierte el aumento en la expresión de citoquinas pro-inflamatorias, pero no la disminución en la captación de azúcar y la expresión de SGLT1.

Conclusión general

En resumen, los resultados de este trabajo sugieren la hipótesis de que el TNF-α secretado por el intestino delgado, macrófagos o adipocitos durante el proceso de inflamación intestinal u obesidad, pueden disminuir la absorción de azúcar y glutamina, y que los omega-3, EPA y DHA, así como los derivados lipídicos pro-resolutivos del DHA (MaR1, RvD1 y RvD2), son capaces de bloquear estos efectos adversos.

VII. THESIS SUMMARY

Tumor necrosis factor alfa (TNF-α) is a pro-inflammatory cytokine that acts in an autocrine and paracrine way by binding to its receptors TNFR1 and TNFR2. This cytokine is implicated in numerous diseases with an inflammatory component, as inflammatory bowel disease (IBD). Obesity is a low grade chronic inflammatory state that could contribute to the chronic intestinal inflammation in IBD. Also, high amounts of TNF-α have been found during obesity. During the triggering of intestinal inflammation different mediators induce the switch of tissue-resident M2 macrophages into pro-inflammatory M1 macrophages, and M1 macrophages infiltration. In turn, M1 macrophages secrete pro-inflammatory cytokines including TNF-α. Obesity would impact the intestinal function through the recruitment and activation of macrophages.

Omega-3 polyunsaturated fatty acids (n3-PUFAs) and its derived lipid mediators show anti-inflammatory actions on inflammatory-related pathologies, including intestinal inflammation.

In enterocytes, glucose is actively transported by the Na^+/glucose cotransporter SGLT1. Glutamine, an essential amino acid for the maintenance of the gut barrier function and the intestinal cell proliferation, is mainly transported by the Na^+-dependent glutamine transporter B^0AT1. We have previously demonstrated, in the human intestinal epithelial cell line Caco-2, that TNF-α inhibits αMG uptake by decreasing SGLT1 expression in the brush border membrane.

The goal of this study was to investigate the double hypothesis: 1) That n-3 PUFAs and DHA derived specialized pro-resolving lipid mediators (MaR1, RvD1 and RvD2) can counteract TNF-α-induced inhibition of nutrients intestinal transport, acting from the apical membrane of the enterocytes. 2) That basolateral TNF-α, in relation with obesity, can inhibit nutrients transport and induce its own expression in the enterocytes.

This research demonstrates that, after pre-incubation of the Caco-2 cells with apical TNF-α and EPA, EPA prevented the inhibitory effect of the cytokine on α-methyl-D-glucose (αMG) uptake and on SGLT1 expression at the brush border membrane, measured by Western blot. The ERK1/2 inhibitor PD98059 and the AMPK activator AICAR also prevented the inhibitory effect of TNF-α on both αMG uptake and SGLT1 expression. Interestingly, the AMPK inhibitor, Compound C, abolished the ability of

EPA to prevent TNF-α-induced reduction of sugar uptake and transporter expression. The GPR120 antagonist, AH7614, also blocked the preventive effect of EPA on TNF-α-induced a decrease of αMG uptake and AMPK phosphorylation.

Moreover, DHA blocked apical TNF-α-induced inhibition of αMG uptake and SGLT1 expression in the apical membrane, through a pathway which seemed to be independent of GPR120. MaR1, RvD1 and RvD2 showed the same preventive effect on TNF-α action, but acting at concentration 1,000 times lower. TNF-α also inhibited glutamine (Gln) uptake in Caco-2 cells being this inhibition also blunted by EPA, DHA and the DHA-SPMs. Interestingly, TNF-α increased the expression in the membrane of the glutamine transporter B^0AT1. This increase was partially avoided by the omega-3 fatty acids. These data present DHA and its specialized pro-resolving lipid mediators as promising biomolecules to restore intestinal nutrients transport during intestinal inflammation.

On the other hand, TNF-α and the secretions from overweight adipocytes (hMSC-DA) and macrophages (inactive and active), acting from the basolateral membrane of Caco-2 cells, decrease sugar uptake. In Caco-2 cell under basolateral TNF-α stimulation and in overweight hMSC-DA, there is up-regulation of *TNF-α, IL-1β* and *MCP1* gene expression.

To approach our *in vitro* results to the *in vivo* model, we used diet induced obese (DIO) mice. In DIO mice, αMG intestinal transport and SGLT1 expression in the brush border membrane was lower than in lean animals. This effect was accompanied by an increase on *Tnf-α, Il-1β* and *Il-6* gene expression in the jejunal mucosa. Oral administration of MaR1 reversed the up-regulation of the pro-inflammatory cytokines found in the DIO intestinal mucosa, but not the decrease on sugar uptake or SGLT1 amount in the brush border membrane.

In conclusion, the results of this work support that TNF-α secreted by the small intestine, macrophages and adipocytes during intestinal inflammation and obesity can decrease sugar and glutamine absorption. They also support that the omega-3 EPA, DHA and the DHA derived pro-resolving lipid mediators are able to block TNF-α effect This suggest that EPA, DHA and DHA-SPMs could be beneficial biomolecules to restore intestinal nutrients transport in patients suffering intestinal inflammation.