Sónia Margarida Neto Rosa Pereira

Protective Role of Anthocyanins on Intestinal Inflammation in Comparison with 5-Aminosalicylic Acid:
In Vitro and *In Vivo* Approaches

Agosto de 2017

· U C ·

UNIVERSIDADE DE COIMBRA

Sónia Margarida Neto Rosa Pereira

Protective Role of Anthocyanins on Intestinal Inflammation in Comparison with 5-Aminosalicylic Acid: *In Vitro* and *In Vivo* Approaches

Efeito Protetor de Antocianinas na Inflamação Intestinal em Comparação com o Ácido 5-Aminosalicílico: Abordagens *In Vitro* e *In Vivo*

Tese de Doutoramento em Ciências Farmacêuticas, ramo de Bioquímica, apresentada à Faculdade de Farmácia da Universidade de Coimbra para obtenção do grau de Doutor

Orientadores: Professora Doutora Leonor Almeida e Professora Doutora Teresa Dinis

Coimbra, 2017

Dissertation presented to the Faculty of Pharmacy, University of Coimbra, in fulfilment of the requirements for obtaining a Doctoral degree in Pharmaceutical Sciences, branch of Biochemistry.

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra, para prestação de provas de Doutoramento em Ciências Farmacêuticas, no ramo de Bioquímica.

"It's the possibility of having a dream come true

that makes life interesting."

Paulo Coelho, *The Alchemist*

Agradecimentos

Gostaria de expressar os meus sinceros agradecimentos a todos aqueles que contribuíram, de forma direta ou indireta, para o desenvolvimento do trabalho presente nesta dissertação.

Às minhas orientadoras, Doutora Leonor Almeida e Doutora Teresa Dinis, por me possibilitarem dar os primeiros passos no mundo da ciência. Agradeço a permanente disponibilidade para ensinar, para discutir ideias e ultrapassar as dificuldades inerentes ao trabalho laboratorial, culminando no auxílio na elaboração do texto presente nesta dissertação. Acima de tudo, gostaria de agradecer a sua amizade e carinho, assim como o apoio que me facultaram durante estes anos, contribuindo, de forma inequívoca, para o meu crescimento pessoal e profissional.

Ao laboratório do Doutor Victor Freitas, pela ajuda fundamental para a caracterização da fração de antocianinas de mirtilo utilizado no presente trabalho experimental. À Doutora Isabel Figueiredo, pelo auxílio no trabalho executado com animais. À Cassilda Pereira e à Cátia Marques, pela ajuda (e muita paciência) na determinação de óxido nítrico, que consta na última parte do trabalho experimental aqui apresentado.

Queria deixar um agradecimento muito especial à Joana Paixão e à Carla Nunes, pelo apoio fundamental, quer no início do trabalho de laboratório, quer ao longo destes anos. Expresso aqui a minha maior gratidão por toda a sua disponibilidade para ajudar e ensinar. Obrigada pela amizade e companheirismo.

Também queria agradecer profundamente à Rita (Francisca), pela ajuda no trabalho desenvolvido *in vivo*. Partilhámos momentos de trabalho, por vezes extenuantes, e ensinou-me as bases de trabalho com animais de laboratório. Com a sua simpatia maravilhosa, de forma alguma poderia deixar de mencionar a amizade que se construiu.

A todos aqueles que partilharam comigo o espaço de laboratório, com os quais partilhei muitos bons momentos. Com o risco de me esquecer de alguém, não posso deixar de mencionar a Diana, a Mariana, a Sara, a Clarissa e o Fábio. Agradeço igualmente à Maria João e à Aninhas, por todo o apoio prestado.

Aos meus amigos, aqueles que estão sempre no sítio quer sejam precisos ou não, por todo o apoio e motivação ao longo destes anos: à Filipa Contente, amiga, confidente e pedra basilar de apoio incondicional, capaz de me fazer rir quando mais preciso, e muito mais (coelhos, vacas voadoras, ta-ta-ra-ta!); à Isabel Onofre, agradeço toda a tua ajuda e disponibilidade, minha grande companheira de planos maléficos; à Vânia Boal, amiga de longa data; à Filipa Matos, um exemplo de força; à Rita Costa, afilhada do meu coração, sempre com uma história mirabolante para contar; ao Nuno Fonseca, compincha de "gaming". Apesar de mais recentes, não posso deixar de mencionar a Joana Vindeirinho e os seus (agora também meus) amigos de Leiria, Jacinta, Mário, Mariana, Hélio e a Vânia; o Rui Benfeitas, guitarrista/gamer/cenas. Perdoem-me os que faltam na lista (não posso escrever três páginas de agradecimentos), mas vocês sabem quem são, obrigada por todos os momentos partilhados, que me ajudaram a chegar aqui.

À minha família, especialmente aos meus pais e irmão, por acreditarem em mim e me apoiarem durante este percurso, nem sempre fácil. Obrigada por me ensinarem a ser a pessoa que hoje sou e a lutar para alcançar os meus objetivos.

Um agradecimento muito especial ao Pedro, meu companheiro de vida e, acima de tudo, amigo. Não há palavras que descrevam todo o apoio dado ao longo destes anos. Companheiro de bons e menos bons momentos, sempre pronto a ajudar e a colocar-me um sorriso no rosto. Obrigada por acreditares em mim, me compreenderes e ajudares a alcançar os meus objetivos.

À "Panaceia", amiga de mundos fantásticos, capaz de aliviar o stress diário e me fazer sonhar.

Table of Contents

CHAPTER 2: CYANIDIN-3-GLUCOSIDE IMPROVES THE ANTI-INFLAMMATORY ACTIVITY OF 5-AMINOSALICYLIC ACID IN A LIPOPOLYSACCHARIDE-ACTIVATED RAW 264.7 MACROPHAGE CELL LINE THROUGH INHIBITION OF AP-1 AND MAPK SIGNALLING PATHWAYS 67

Abbreviations

5-ASA	5-Aminosalicylic acid
ALP	Alkaline phosphatase
AMP	Antimicrobial peptides
AP-1	Activator protein 1
APCs	Antigen-presenting cells
ARE	Antioxidant response element
ARF	Anthocyanin-rich fraction
ASCA	Anti-*Saccharomyces cerevisiaea* antibody
ATG16L1	Autophagy related 16-like1
CAT	Catalase
CD	Crohn's disease
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
CTAB	Hexadecyltrimethylammonium bromide
Cy	Cyanidin
Cy3glc	Cyanidin-3-glucoside
Cys	Cysteine
CySS	Cystine
DAN	2,3-Diaminonaphthalene
DCs	Dendritic cells
DNBS	Dinitrobenzene sulfonic acid
Dp	Delphinidin
Dp3glc	Delphinidin-3-glucoside
DSS	Dextran sulphate sodium
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GAE	Gallic acid equivalents
GI	Gastrointestinal tract
GPX	Glutathione peroxidase

Abbreviations

GSH	Glutathione
GSSG	Glutathione disulphide
GWAS	genome-wide association studies
IκB-α	Inhibitor κB alpha
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule-1
IECs	Intestinal epithelial cells
IFN-γ	Interferon-gamma
IKK	Inhibitor of κB kinases
IL	Interleukin
IL23R	Interleukin 23 receptor
iNOS	Inducible nitric oxide synthase
IRGM	Immunity-related GTPase family M protein
ISC	Intestinal stem cells
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPKs	mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein-1
MPO	Myeloperoxidase
Mv	Malvidin
Mv3glc	Malvidin-3-glucoside
NF-κB	Nuclear factor κB
NFAT	Nuclear factor of activated T cells
NK cells	Natural killer cells
nNOS	Neuronal nitric oxide synthase
NOD	Nucleotide-binding oligomerization domain
NOX	NADPH oxidase
Nrf2	Nuclear factor E2-related factor
ORAC	Oxygen radical absorbance capacity
PAMPs	Pathogen-associated molecular patterns
pANCA	Perinuclear anti-neutrophil antibody
Pg	Pelargonidin
Pg3glc	Pelargonidin-3-glucoside
PGE$_2$	Prostaglandin E$_2$

Pn	Peonidin
PPAR	Peroxisome proliferator-activated receptor
PRRs	Pattern-recognition receptors
Pt	Petunidin
Rac1	Ras-related C3 botulinum toxin substrate 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNPs	Single nucleotide polymorphisms
SOD	Superoxide dismutase
STAT1	Signal transducer and activator of transcription 1
TAK1	Transforming growth factor β-activated kinase 1
Th cells	T helper cells
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
Treg cells	T regulatory cells
Trx	Thioredoxin
TrxSS	Thioredoxin disulfide
UC	Ulcerative colitis
UPR	Unfolded protein response
XO	Xanthine oxidase

Resumo

Resumo

A doença inflamatória intestinal (DII) abrange um conjunto de doenças inflamatórias crónicas recidivantes do trato gastrointestinal, cuja incidência tem aumentado visivelmente a nível mundial. Infelizmente, a etiologia desta doença ainda não está completamente esclarecida e, como tal, ainda não tem cura. Neste sentido, tem havido um interesse crescente em alternativas farmacológicas, especialmente de origem natural. As antocianinas, uma das subclasses de polifenóis abundantes na dieta Mediterrânica, são moléculas promissoras no contexto da DII, tendo em consideração as suas conhecidas propriedades anti-inflamatórias e antioxidantes.

Assim, o trabalho apresentado nesta tese explora a possibilidade de utilizar antocianinas da dieta no tratamento da DII, quer *per si* ou em combinação com fármacos convencionais. Para tal, avaliou-se a atividade de uma antocianina típica, a cianidina-3-glucósido (Cy3glc), em comparação e em associação com o ácido 5-aminosalicílico (5-ASA), em vias de sinalização celular relevantes no contexto da DII, utilizando um modelo *in vitro* de macrófagos. Adicionalmente, foi explorada a atividade anti-inflamatória e antioxidante de um extrato enriquecido em antocianinas preparado a partir de mirtilos (*Vaccinium corymbosum* L.) da Região Centro de Portugal, em comparação com o 5-ASA, num modelo *in vivo* de colite induzida por TNBS em rato. De forma a melhor explorar os mecanismos de ação do extrato e a interação entre macrófagos e células intestinais, foi ainda utilizado um modelo *in vitro* de inflamação intestinal constituído por uma co-coltura de macrófagos (RAW 264.7) e de células epiteliais intestinais (Caco-2).

Os resultados obtidos demonstram que a Cy3glc isolada tem capacidade para inibir a secreção de vários mediadores pró-inflamatórios ($^{\bullet}$NO, PGE_2 e TNF-α) e a produção de espécies reactivas de oxigénio (ROS) numa extensão muito superior ao 5-ASA em macrófagos. A diminuição da secreção de $^{\bullet}$NO e PGE_2 está relacionada com a inibição da expressão, respectivamente, de iNOS e COX-2 mas sem interferir com a

Resumo

ativação do NF-κB. Alternativamente, esta antocianina interfere na via de sinalização da AP-1, reduzindo a fosforilação de c-Jun no núcleo e inibindo a ativação das MAPKs p38 e JNK. Além disso, a utilização combinada de Cy3glc e 5-ASA chega a sugerir um efeito sinérgico.

Quanto ao extrato obtido a partir de mirtilos demonstrou conter um elevado teor e variedade de antocianinas, e ser de facto capaz de reverter vários parâmetros de inflamação intestinal *in vivo*. A sua administração melhorou o aumento de peso corporal dos ratos tratados e reduziu as lesões no colon, o seu encurtamento e o espessamento da parede causados pela instilação local de TNBS. Tal foi acompanhado por uma marcada redução da MPO ativa e da ALP no tecido do colon, biomarcadores de infiltração leucocitária e de inflamação ativa. Diminuiu igualmente mediadores inflamatórios relevantes, como a iNOS e a COX-2, melhorando simultaneamente as defesas antioxidantes endógenas, nomeadamente a proporção de glutatião reduzido *versus* oxidado (GSH/GSSG) e a atividade da glutatião peroxidase (GPX). No geral, os efeitos benéficos observados foram muito superiores aos do 5-ASA, apesar da dose molar de antocianinas ser muito inferior.

Resultados *in vitro* em células epiteliais intestinais e macrófagos ativados, num sistema de co-cultura, permitiu confirmar a maior eficácia do extrato relativamente ao 5-ASA. A incubação das células epiteliais com o extrato (compartimento apical) reduziu a produção de mediadores pró-inflamatórios por ambos os tipos celulares (compartimento apical e basolateral), diminuindo a secreção de IL-6 pelas células epiteliais apicais, e de ˙NO e IL-1β pelos macrófagos basolaterais. A formação de ROS intracelulares foi igualmente reduzida em ambos os tipos celulares. Estes resultados sugerem ainda a capacidade das antocianinas atravessarem a barreira intestinal.

Em conclusão, os resultados apresentados destacam o potencial da utilização de antocianinas, quer isoladas ou em combinação, no controlo da inflamação intestinal. A atividade destas moléculas é complexa, interferindo em vários mecanismos e vias de

sinalização celulares estreitamente relacionadas com a patogénese da DII. Desta forma, fica reforçada a hipótese de utilização de antocianinas alimentares como uma abordagem terapêutica natural na DII.

Abstract

Abstract

Inflammatory bowel disease (IBD) encompasses a set of chronic and relapsing inflammatory conditions of the gastrointestinal tract, whose incidence has markedly increased worldwide. Unfortunately, the etiology of this disease has not yet been fully elucidated and, as such, has yet no cure. In this sense, there has been an increasing interest in pharmacological alternatives, especially of natural origin. Anthocyanins, one of the subclasses of polyphenols abundant in the Mediterranean diet, are promising molecules in the context of IBD, considering their acknowledged anti-inflammatory and antioxidant properties.

Thus, the work presented in this thesis explores the possibility of using dietary anthocyanins in the treatment of IBD, either *per se* or in combination with conventional drugs. For this, the activity of a typical anthocyanin, the cyanidin-3-glucoside (Cy3glc), was evaluated in comparison and in association with 5-aminosalicylic acid (5-ASA), in cellular pathways of relevance in the context of IBD, by using an *in vitro* model of macrophages. In addition, the anti-inflammatory and antioxidant activity of a fraction of anthocyanins prepared from blueberries (*Vaccinium corymbosum* L.) from the center region of Portugal was evaluated in comparison with 5-ASA, in an *in vivo* model of colitis induced by TNBS in rats. To better explore the mechanisms of action of the prepared fraction, and the interaction between macrophage and intestinal cells, it was also used an *in vitro* model of intestinal inflammation, consisting of a co-culture of macrophages (RAW 264.7) and intestinal epithelial cells (Caco-2).

The results show that the isolated Cy3glc can inhibit the secretion of various proinflammatory mediators ($^{\bullet}$NO, PGE_2 and TNF-α) and reactive oxygen species (ROS) production to a much greater extent than 5-ASA in macrophages. The decreased secretions of $^{\bullet}$NO and PGE_2 are related to the inhibition of iNOS and COX-2 expressions, respectively, but without interfering with NF-κB activation. Instead, this anthocyanin

Abstract

interferes with AP-1 signalling pathway, by reducing the phosphorylated of c-Jun in the nucleus, and inhibiting the activation of p38 and JNK MAPKs. Moreover, the combined use of Cy3glc with 5-ASA even suggests a synergistic effect.

Concerning the blueberry fraction, it showed a high content and variety of anthocyanins, and it was in fact capable of reversing several parameters of intestinal inflammation *in vivo*. Its administration improved the body weight gain of treated rats and reduced colon lesions, its shortening, and wall thickening caused by local instillation of TNBS. This was accompanied by a marked reduction of active MPO and ALP in the colon tissue, a leukocyte infiltration indicator and an active inflammation biomarker respectively. The fraction also decreased relevant inflammatory mediators, such as iNOS and COX-2, while improving endogenous antioxidant defenses, namely the ratio of reduced *versus* oxidized glutathione (GSH/GSSG) and gluthatione peroxidade (GPX) activity. In general, the beneficial effects observed were much higher than those of 5-ASA, despite the much lower molar dose of anthocyanins.

In vitro results from intestinal epithelial cells and activated macrophages in a co-cultured system allowed to confirm the higher efficiency of this fraction in relation to 5-ASA. Incubation of the epithelial cells (apical compartment) with the fraction reduced the production of proinflammatory mediators by both cell types (apical and basolateral compartment), decreasing IL-6 secretion by apical epithelial cells, and ˙NO and IL-1β by basolateral macrophages. Intracellular ROS formation was also reduced in both cell types. These results also suggest the ability of anthocyanins to cross the intestinal barrier.

In conclusion, the results presented highlight the potential of using anthocyanins, as isolated or in combination, for the control of intestinal inflammation. The activity of these molecules is complex, interfering with several mechanisms and signalling pathways closely related to the pathogenesis of IBD. Thus, the hypothesis of using dietary anthocyanins as a natural therapeutic approach in IBD is reinforced.

XX

Chapter 1

General Introduction

General Introduction

1. Inflammatory Bowel Disease

The gastrointestinal tract (GI) is a central organ in nutrient and water uptake, while providing defence against the constant threat of several microorganisms and chemical substances from the environment. Thus, it is under permanent antigenic pressure [1]. In fact, the intestinal epithelium is the largest barrier to the outer-body environment (32 m^2 per individual), even exceeding that of the skin [2]. Therefore, the intestinal barrier plays a very active role in the control of the host microbial community and in the prevention of unnecessary immune stimulation. This equilibrium is achieved by the dynamic interplay of several mechanisms (**Fig 1**), such as: i) production of a mucous layer by Goblets cells; ii) secretion of antimicrobial peptides (AMPs) by Paneth cells; iii) maintenance of intercellular tight junction complexes by intestinal epithelial cells; and (iv) presence of pathogen-associated molecular pattern (PAMP) recognition systems. However, in some individuals this intestinal homeostasis can become altered and lead to abnormal inflammation responses that may progress to chronic GI disorders such as inflammatory bowel disease (IBD) [3,4]. The two most common forms of this broad category of IBD are Crhon's disease (CD) and ulcerative colitis (UC). Both are marked by episodes of relapse and remission, with uncontrolled response of the intestinal immune system against the normal enteric microflora, resulting in a digestive tract prone to infection and with diminished ability to food processing and water absorption. Consequently, patients suffer from fatigue, abdominal pain, diarrhea, weight loss and tissue damage along the GI, which greatly impairs their quality of life [5].
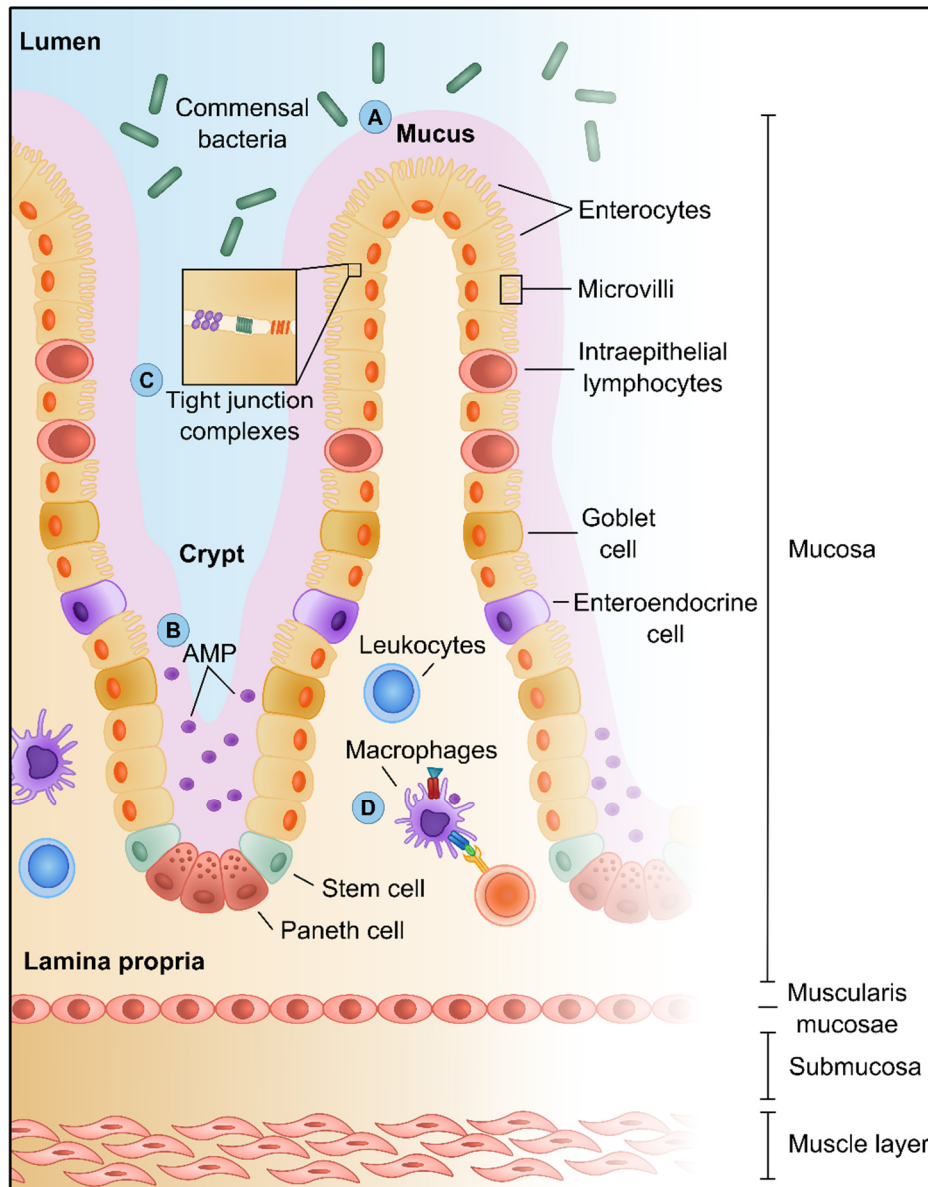
Figure 1: Intestinal homeostasis – defence mechanisms of the gut. To maintain a healthy gut, the host-microbe interaction mechanisms must be finely tuned, simultaneously defending mucosal tissues from pathogens while maintaining immune tolerance against commensal bacteria. The intestinal epithelial barrier depends on several mechanisms: production of a mucous layer (**A**), secretion of AMPs (**B**), maintenance of cellular tight junction complexes (**C**) and PAMP recognition systems (**D**). Rupture of this homeostasis may allow bacteria to invade lamina propria and mucosa, thus triggering an immune response by macrophages, dendritic cells and neutrophils, which may lead to chronic intestinal inflammation.

1.1. Epidemiology of inflammatory bowel disease

Historically, the first time a condition resembling CD was reported, described as a transmural inflammation of the small and large intestines, was in 1913 by Kennedy Dalziel. Afterwards in 1932, more data was published by Dr Burrill Crohn, Dr Leon Ginzburg, and Dr Gordon Oppenheimer reporting a "regional or terminal ileitis", later known as CD. The first description of UC came much earlier, dating from Hippocrates, and it was thought to be related with chronic diarrhea and bloody stools. This disease was then named "bloody flux" in 1600s by Thomas Sydenham, and only acknowledged as UC in 1859 by Samuel Wilks [6]. Even though IBD was once considered a rare disorder, in the last decades its incidence and prevalence rates have increased considerably all over the world, in both adult and pediatric populations [7].

1.1.1. IBD as an emerging global disease

The incidence and prevalence of IBD have been clearly rising worldwide (**Figs 2** and **3**) and it has been increasingly considered an emerging global disease. The highest number of identified cases per year were reported in Australia for CD (29 cases per 100,000 person-years), and in the Northern Europe for UC (24 cases per 100,000 person-years) [7]. But the overall prevalence of IBD achieves the highest values in Europe (322 CD and 505 UC patients per 100,000 persons) and in North America (319 CD and 249 UC patients per 100,000). Interestingly, time-trend analysis shows that while incidence rates plateaued after 1970s in Western countries, they continued to increase in areas with previously low UC incidence such as Eastern Europe, Asia and Africa. These trends seem to be related with the country industrialization development, which is associated with changes in sanitation, pollution exposure, diet, lifestyle behaviours, medication and microbiota alterations [8].
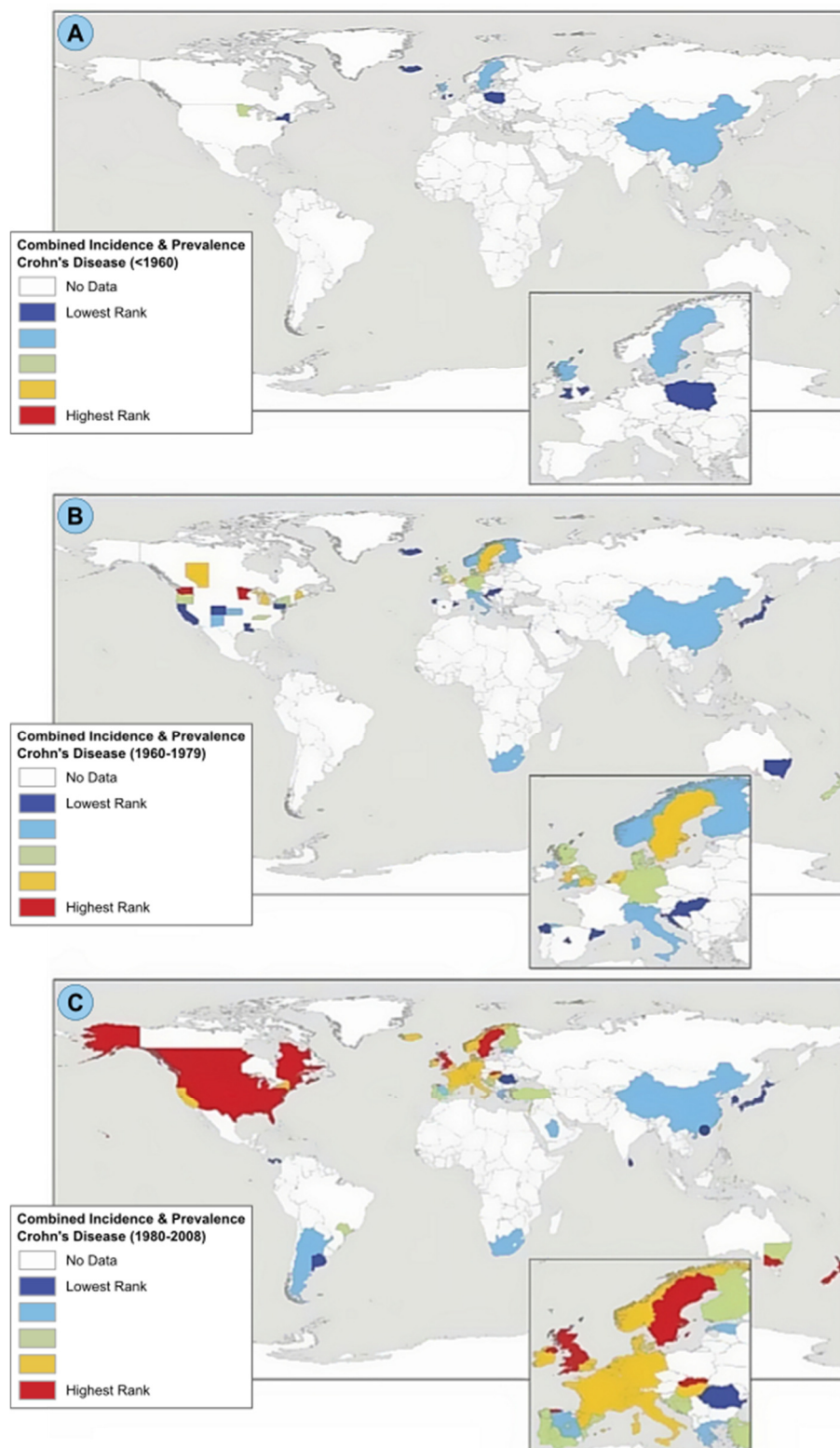
Figure 2: Combined incidence and prevalence of CD worldwide. Data reported before 1960 **(A)**, from 1960 to 1979 **(B)**, and after 1980 **(C)**. The occurrence of the disease reported from each country is represented from low (dark and light blue), intermediate (green) and high (yellow and red). *Image from M'Koma AE* [8].
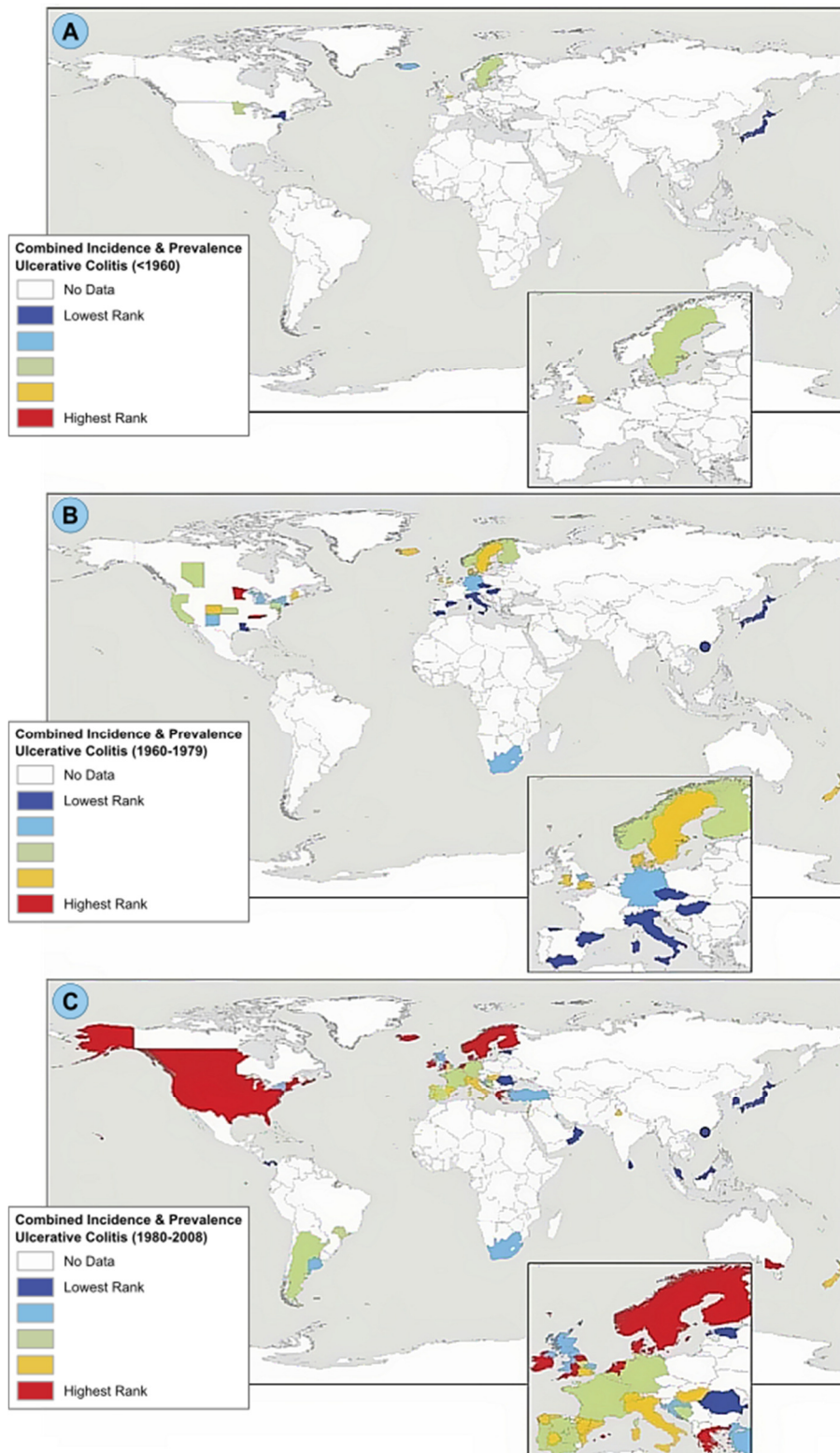
Figure 3: Combined incidence and prevalence of UC worldwide. Data reported before 1960 **(A)**, from 1960 to 1979 **(B)**, and after 1980 **(C)**. The occurrence of the disease reported from each country is represented from low (dark and light blue), intermediate (green) and high (yellow and red). *Image from M'Koma AE* [8].

1.1.2. Personal and social impact of the disease

The current trends observed in epidemiological studies in combination with the chronic nature of these diseases result in significant personal and social repercussions. The most relevant ones include the reduced quality of life of patients and the financial burden related to medical care. Not to mention that IBD can be very incapacitating due to painful flair-ups or development of complications. Moreover, additional concerns have arisen due to an increasing incidence of IBD at younger age. These patients are more prone to develop colorectal cancer related to the duration of the disease [9].

On the perspective of the healthcare systems, IBD is ranked in the top of five most expensive GI disorders, especially in the US where it was estimated a total annual financial burden of about \$14.6 to \$31.6 billion in 2014. This scenario may worsen with the introduction of a new group of biologic pharmaceutical treatments. The newly approved tumor necrosis factor (TNF) inhibitors, such as infliximab and adalimumab, although more effectives are costlier. Therefore, the overall scenario demands attention for this group of diseases in order to address the major needs of the patients, either in terms of proper diagnosis and disease management [9].

1.2. Understanding IBD: Crohn's disease and ulcerative colitis

Even though CD and UC belong to the same broader group of IBD and share several clinical features, it is of the utmost importance to distinguish them to adequately treat and monitor each patient. There is however a small percentage of patients that remain difficult to classify. The major differences between CD and UC are the affected regions of the GI and the depth of these lesions. While CD can affect any part of the GI, from the mouth to the anus, and extend through the entire depth of the gut tissue, UC is restricted to the colon and only affects the large bowel mucosa and submucosa [10].

1.2.1. Clinical diagnosis of Crohn's disease and ulcerative colitis

Currently, there is no single test to diagnose IBD or distinguish its subtypes with certainty. Therefore, the clinical diagnosis relies on the combination of symptoms, endoscopy, radiology and histological criteria, which requires the collaboration of a multidisciplinary team of specialists, such as gastroenterologists, pathologists and radiologists. Together, they perform multiple imaging and laboratory tests and seek the possible causes for the disease onset [11].

A. *The first awareness: symptoms*

Although symptoms may sometimes overlap in terms of CD and UC, they are usually the first indication of the disease. In CD, symptoms may vary greatly, from abdominal pain with non-bloody diarrhoea to more severe intestinal manifestations, depending on the extension and depth of the lesioned area(s). Abdominal pain, chronic diarrhoea, weight loss and fatigue are the most common symptoms. In patients with colonic involvement, it may occur rectal bleeding or bloody diarrhoea. Still, more aggressive manifestations may result in fistulas formation, strictures, and abscesses [12].

The presence of blood in the stool and diarrhoea are more common in UC. These patients may also report urgency, incontinence, fatigue, increased frequency of bowel movements, mucus discharge, nocturnal defecations, lower abdominal pain, and tenesmus (sensation of incomplete evacuation), which is correlated to the major involvement of the rectum. The most common complications include toxic megacolon, massive haemorrhage or colon perforation [13].

B. *Crossing the data towards a diagnosis*

Imaging tools, such as endoscopies and X-rays, allow to observe the localization and extent of the inflamed tissues, and permit the identification of complications (e.g. narrowing and blockage regions of the intestine, colorectal neoplasia). The endoscopic technique remains the gold standard for IBD diagnosis, and more recently it gained a relevant role in monitoring the disease activity, in terms of mucosal healing [12-14].

The collection and analysis of biopsies from patients are crucial to identify the subtype of IBD through their characteristic cellular features [11]. In CD patients, biopsies present mild-to-severe inflammation and alterations near skip lesion regions (areas of inflamed tissue separated by healthy ones), which usually extend below the surface layer of the intestinal wall. The disease hallmark is the marked infiltration of immune cells, usually forming characteristic granulomas (**Fig 4A**) [12]. The tissue alterations in UC are limited to the top mucosal cell layer, presenting profound modifications of the intestinal crypts structure (**Fig 4B**), with infiltrated immune and plasma cells [13].

Serological testing for IBD can give a small contribution to the diagnosis, nonetheless it provides supporting information and allows to monitor the disease progression and the efficacy of the treatments. Nonspecific markers of the disease involve the count of red blood cells, fecal occult blood, increased acute phase proteins (particularly C-reactive protein) and electrolytes concentrations [12,13]. More specific tests take advantage of antibody and biomarkers assays, such as anti-*Saccharomyces cerevisiaea* antibodies (ASCA) for CD and perinuclear anti-neutrophil antibody (pANCA) for UC. However, due to the low sensitivity of these tests, they are not generally used to distinguish between CD and UC [15].
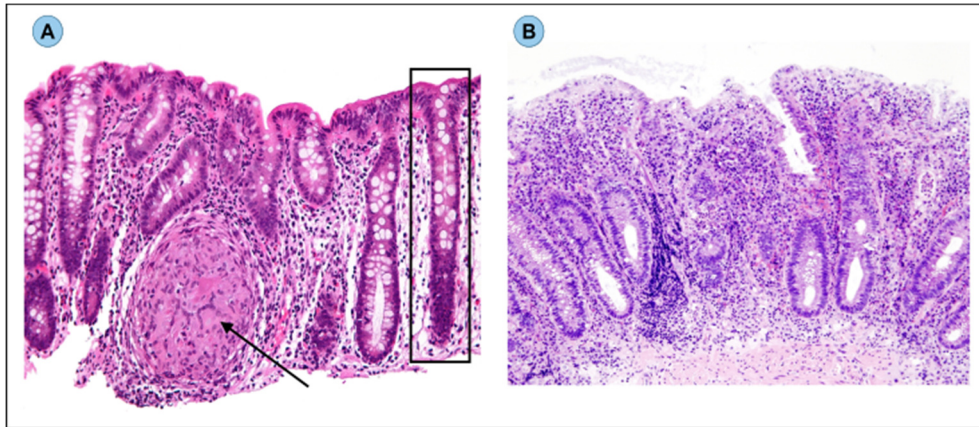
Figure 4: Representative biopsy slides of CD and UC patients. Histopathological images of inflamed colon tissues from a CD **(A)** and a UC **(B)** patients, with hematoxylin (violet) and eosin (red) stain. Dark purple dots correspond to immune cells. In **(A)** the arrow points to a characteristic granuloma and the square highlights a crypt; **(B)** shows profound alterations in the crypts structure of the intestinal surface layer. *Images from Wikimedia commons.*

1.2.2. The clinical course of a long-life disorder

The correct identification of the disease subset allows a better prediction of its natural progression. This knowledge is crucial to select the most appropriate intervention at each step of the disease, from treatment to the prevention of relapses. Moreover, in about 80% of the cases, IBD are diagnosed during the second or third decade of life and accompanies the patients through the rest of their lives. While half of the patients may show a slightly progressive disease with few relapses, hospitalizations and complications, the other half may experience a more aggressive progression, with complications that may even require surgery. In this scenario, although life expectancy of IBD patients is usually similar to that of unaffected individuals, those complications decrease significantly the quality of life and may increase the mortality risk [10,16].

Therefore, to achieve an effective delay of the disease progression, an appropriate intervention must be carried out at the right time, towards a specific therapeutic endpoint (**Fig 5**) [10].
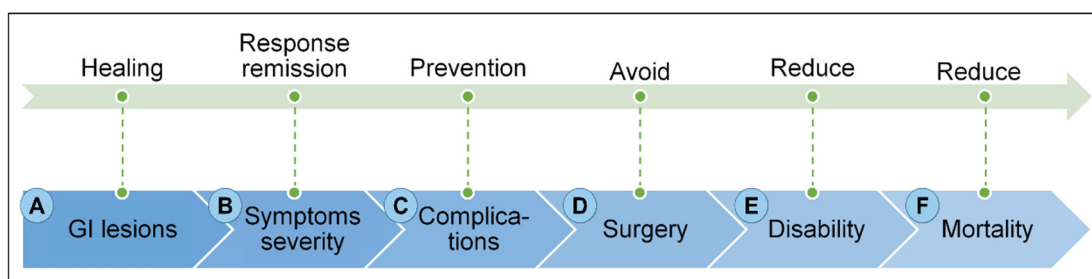
Figure 5: Step of the natural course of the disease. The management of IBD should aim to **(A)** heal GI lesions and **(B)** induce remission of the symptoms, thus **(C)** preventing complications and **(D)** the need for surgery. At later stages, it should reduce both **(E)** disability and **(F)** mortality.

1.3. The pathogenesis of inflammatory bowel disease

Although the pathogenesis of IBD is still not completely understood, it is certainly a multifactorial disease that results from the combination of personal genetic susceptibility, environment, intestinal microbiota and immune system. According to the most accepted hypothesis, an uncontrolled immune-mediated inflammatory response may occur towards an environmental trigger and/or alteration in the host intestinal flora, in genetically susceptible individuals, resulting in the disease onset [17,18]. In this scenario, the intestinal homeostasis is broken, the tolerance to commensal flora is altered, and the epithelial barrier is compromised. Innate immune defences are triggered, leading to an uncontrolled production of pro-inflammatory cytokines and mediators, that further aggravates the inflammatory response by recruiting additional immune cells to the tissue (**Fig 6**) [19].
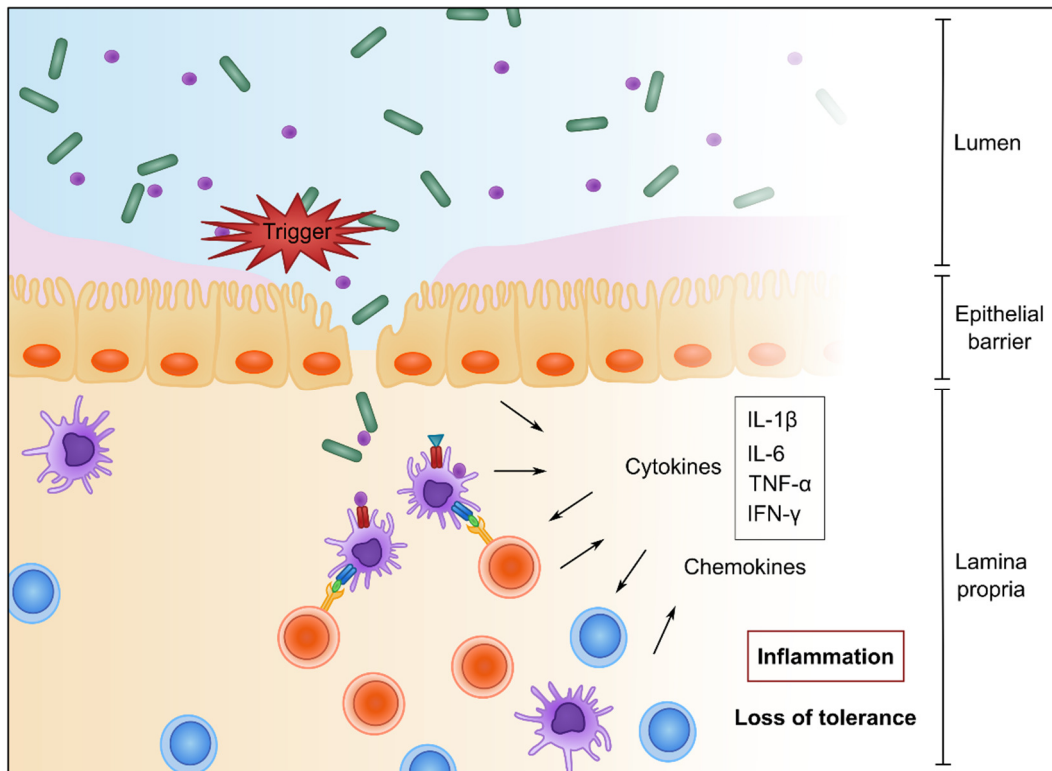
Figure 6: Initiation of gut inflammation in IBD. In susceptible individuals, the epithelial barrier may be disrupted, allowing the otherwise inoffensive bacteria to invade the lamina propria and mucosa. The interaction with macrophages, dendritic cells and neutrophils elicits the production of pro-inflammatory cytokines and chemokines that further recruit immune cells to the region. When persistent, this may trigger the development of IBD.

1.3.1. Acknowledged risk factors for disease onset

A. Disease susceptibility related to genetic background

In the last decade, huge advances have been made in the field of IBD genetics, shedding light on the pathogenesis of this disease. The new genotyping and sequencing technologies allowed to perform genome-wide association studies (GWAS) and identify new single nucleotide polymorphisms (SNPs) [20]. From recent GWAS involving European individuals and East Asian, Indian or Iranian descents, it was possible to identify 163 potential candidate susceptibility loci for IBD, from which 30 loci were unique to CD and

23 to UC. The study of specific populations however, demonstrated some heterogeneity among them [21].

The first identified genetic association was with CD susceptibility and the replicated genetic mutations in the nucleotide-binding oligomerization domain 2 (*NOD2*). This was followed by immunity-related GTPase family M protein (*IRGM*) and autophagy related 16-like 1 (*ATG16L1*) gene mutations. Defects in *NOD2* can affect the host's capacity to localize and eliminate bacteria, resulting in aberrant inflammatory responses in the constant presence of antigens [22]. Like *NOD2*, the *IRGM* and *ATG16L1* genes have relevant roles in autophagy. Moreover, mutations in interleukin 23 receptor (*IL23R*) gene, also identified in other autoimmune diseases, have been associated with CD, affecting T-cell signalling. This indicates the importance of innate immunity, autophagy and phagocytosis on IBD pathogenesis [23].

While this may be true for the European people, the major variants in *NOD2* and *ATG16L1* are not present in individuals from East Asian ancestry. Instead, in CD genome studies with this people, it has been identified genetic susceptibility to this disease with *ATG16L2* variants [21]. On the other hand, in the modern Ashkenazi Jewish population, who has shown a higher disease prevalence, several new IBD susceptible genes (*RPL7*, *CPAMD8*, *PRG2*, *PRG3* and *HEATR3*) have been identified, nevertheless these loci individually account only for a small percentage of the expected heritability in IBD [20].

In most cases, IBD results from the contribution of various genes. Yet, in rare cases of very-early-onset IBD (before two years old), monogenic defects alter intestinal immune homeostasis by disrupting the epithelial barrier and reducing clearance of bacteria by phagocytes. In other cases, the disease has been associated to induced hyperinflammation and autoinflammation, or disrupted T- and B-cell selection and activation. Mutations in *IL10*, *IL10RA*, or *IL10RB* genes lead to IL-10 pathway defects that can result in the hyperactivation of the immune response [17,24].

B. *The crucial role of the environment: epigenetics*

Studies with Swedish-Danish twins have previously described a relatively high monozygotic concordance rate in IBD [25]. After re-running those studies with a better methodology, Halfvarson found that these rates were overestimated [26]. Moreover, evidence supports that the complex interaction of multiple genetic mutations and environmental factors is crucial for the disease onset. IBD epigenetics is therefore an emerging concept to explain differences in disease expression in monozygotic twins [27].

Recent population based studies support the strong contribution of environment in IBD incidence. Children of immigrants, from low incidence countries to Canada, tended to approach the IBD incidence rates of long-term inhabitants in this country [28], and the opposite effect was reported in Faroese immigrants to Denmark, showing decreased incidence over time and over generations. This indicates that IBD incidence is strongly modified by the environment and lifestyle in the new country, mainly in terms of UC incidence [29].

Several environmental factors have already been identified as modifiers of the disease incidence. Smoking habits (only for CD), high fat and refined sugar consumption, antibiotic usage during childhood, and low levels of vitamin D have been correlated with higher incidence of IBD. In contrast, breastfeeding, consumption of tea or coffee, and Mediterranean diet have been acknowledged to reduce the risk of the disease. The diet seems in fact a key element, and France is a good example. In its Northern region, with higher incidence of CD, it is common a diet rich in beer, butter, eggs and potatoes, whereas the Southern part usually follows a Mediterranean diet, consuming many vegetables, fruits, fish, olive oil and wine [30], which has been associated to a lower risk of disease.

C. _The intestinal microbiota dysbiosis found in IBD patients_

Under physiological conditions, the gut microbiota beneficially contributes to the digestion of some substrates, educates the host immune system and controls the growth of harmful microorganisms. However, in IBD patients, a profound alteration occurs in their intestinal microbiota profile, which has been extensively implicated in the disease pathogenesis [31]. Previous GWAS studies support this correlation, since several loci related with aberrant immune responses toward the intestinal microbiota are found in IBD patients [23]. Also, environment factors such as diet, smoking habits or drug treatments, may also alter the resident flora and its functional properties [30]. On the other hand, modifications induced by short-term treatment with antibiotics, pro- and prebiotics seem to elicit remission in IBD patients [32].

Among the broad pattern of dysbiosis found in IBD, it was described a reduction in biodiversity, mainly in terms of _Firmicutes phylum_, and an increase in the large bacterial class of _Gammaproteobacteria_. The reduced biodiversity is also found within the same patient when comparing inflamed regions versus non-inflamed ones in CD [33]. Lack of some bacteria species may allow the colonization by pathogens and impair the development of tolerogenic mechanisms of the host. As an example, _Bacteroides_, _Clostridium_, _Bifidobacterium_, _Lactobacillus_ and _Faecalibacterium_ seem to induce expansion of regulatory T cells, downregulate inflammatory cytokines or stimulate the anti-inflammatory cytokine IL-10. In contrast, the increased _Enterobacteriaceae_, especially adherent-invasive _Escherichia coli_ strains, may pass the colonic mucus layer, invade epithelial cells and replicate in macrophage cells forming granulomas. _Fusobacterium_ species, also a group of adherent and invasive bacteria, have been found increased in colonic mucosa of UC patients and seem to influence IBD pathology and colorectal cancer development [34].

D. *The dysregulation of the immune response*

In what concerns the immune response, it is crucial to maintain mechanisms of tolerance to suppress inappropriate responses towards non-pathogens in the gut lumen (commensal flora and dietary antigens), while retaining the ability to defend against pathogens. In IBD patients however, several of the most recent identified susceptibility genes are related with the host immunity, including defects in autophagy, barrier defence and in T-cell differentiation signalling [35]. This fact supports the idea that an excessive immune response towards a normal microbiota may occur due to the genetic susceptibility in some individuals. Though, some doubts remain about whether this is caused by the previous described intestinal dysbiosis [36].

In healthy individuals, the innate immunity is critical for an early immune response to localize and eradicate invading pathogens. It involves the mucosal epithelial barrier and cells such as neutrophils, macrophages, natural killer (NK) cells, innate lymphoid cells and mast cells. Complementing these first-line mechanisms, the adaptive immunity has the ability of specificity and memory, being mainly mediated by T and B cells. Macrophages and dendritic cells (DCs) mediate the two types of immunity, by acting as antigen-presenting cells (APCs) to T cells, and secreting cytokines [37]. Although CD and UC may have distinct pro-inflammatory profiles, both present significant alterations of the balanced state of the immune system. In these disorders, antigens that may permeate the epithelial barrier promote the development of a classic adaptive immune response, thus initiating and propagating gut inflammation. This occurs by the activation of T helper lymphocytes (Th1, Th2, Th17 and Th22 cells) and suppression of T regulatory (Treg) cells. Locally, it also occurs an extensive production of cytokines and chemokines, as found during BD flares [38].

1.3.2. Disruption of mucosal barrier integrity

All the previous described susceptibility factors may have impact on the intestinal epithelium function. This barrier confronts environmental and microbial factors, interacts with the immune cells and cytokines produced, and deals with inherent genetic factors (**Fig 7**) [39]. Since the gut mucosa is constantly challenged by a wide range of foreign antigens and environmental microorganisms, it requires a precise regulation of the intestinal barrier to maintain the mucosal immune homeostasis and prevent the onset of uncontrolled inflammation. The disruption of this barrier results in an enhanced permeability, which is a key factor in IBD pathogenesis [38].
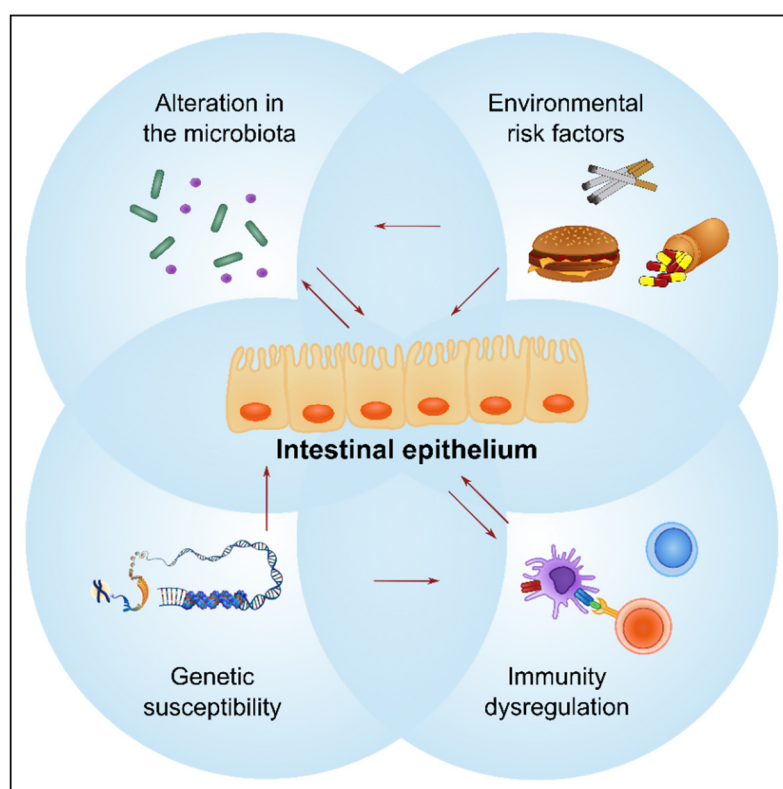
Figure 7: Central role of the intestinal epithelium in IBD pathogenesis. The intestinal epithelium directly interacts with all acknowledged factors in IBD pathogenesis: microbiota and environmental factors, immune responses and genetic susceptibility.

A healthy intestinal barrier relies on different elements that include the integrity of the intestinal epithelial cells (IECs), a robust immune response and the production of a mucus layer. The IECs are the principal structural elements of the epithelial barrier, showing a high ability to respond and adapt to the surrounding environment and stimuli. IECs determine the selective absorption of nutrients and control undesired antigens entry. Their permeability is controlled by the presence of tight junctions and adherent junctions that maintain the physical structure between cells, sealing the intercellular spaces. In addition to the physical barrier, IECs contribute to the immune response towards pathogens. They express innate immune receptors that recognize pathogens, and produce anti-microbial molecules, cytokines and chemokines, linking both innate and adaptive immune responses. The production of a mucous layer further enhances the membrane protection, either preventing the cells from the contact with pathogens, or by the presence of glycoproteins, trefoil peptides, IgA and AMPs, acting as a biochemical barrier. Therefore, several defects in these mechanisms have been associated with IBD pathogenesis [40].

Genetic studies have identified defects related to epithelial regeneration, control of crypt cell proliferation and IECs differentiation, which may destabilize the mucosal layer structure. Also, cell junction destabilization due to inappropriate localization or molecular structure of tight junction proteins, may alter the intestinal permeability in IBD patients. Defects in the recognition of antigens may result in an overactive mucosal immune response, eliciting cellular inflammatory pathways activation. The mucous layer produced by Goblet cells has been frequently compromised in IBD patients, either by inherent alterations or resulting from an increased number of mucolytic bacteria. The AMPs production by Paneth cells has also been found dysregulated in some patients. Moreover, since these cells have high synthetic and secretory activities, they are prone to high levels of endoplasmic reticulum (ER) stress, that may be aggravated in individuals with impaired defence mechanisms against oxidative stress [39].

It is interesting to note that, in recent years, the "mucosal healing" has gained high relevance as a key prognostic parameter in the management of IBD patients, instead of the traditional clinical remission. In fact, the repair of the mucosa has been associated with better long-term outcomes, low risk of surgical treatment and seems to be a good predictor of the long-term prognosis [41,42].

1.4. The intestinal inflammatory response in IBD

The development of inflammation is part of the innate immune response and it is crucial for the protection against harmful stimuli such as pathogens, injury, and metabolic stress. Nevertheless, it may be involved in several pathologies, such as cardiovascular diseases, malignancies and autoimmune diseases. The inflammatory response is characterized by pain, redness, heat, swelling and in extreme cases even by loss of function. Currently it is known that inflammation is mediated by several mechanisms. One of the most relevant innate immune mechanisms is based on innate immune receptor molecules, the pattern-recognition receptors (PRRs), that recognize PAMPs from several pathogens. Among these receptors, the nucleotide-binding oligomerization domain (NOD)-like receptors and Toll-like receptors (TLRs) recognise PAMPs from microorganisms or associated with tissue injury, triggering inflammatory signalling pathways. The following complex processes are usually mediated by phosphorylation cascades that trigger inflammatory genes transcription, thus eliciting the production of large amounts of inflammatory mediators that propagate the inflammatory response (**Fig 8**). Macrophages, DCs, and other sentinel cells are crucial to monitor these mechanisms and to re-establish the initial tissue homeostasis [43].
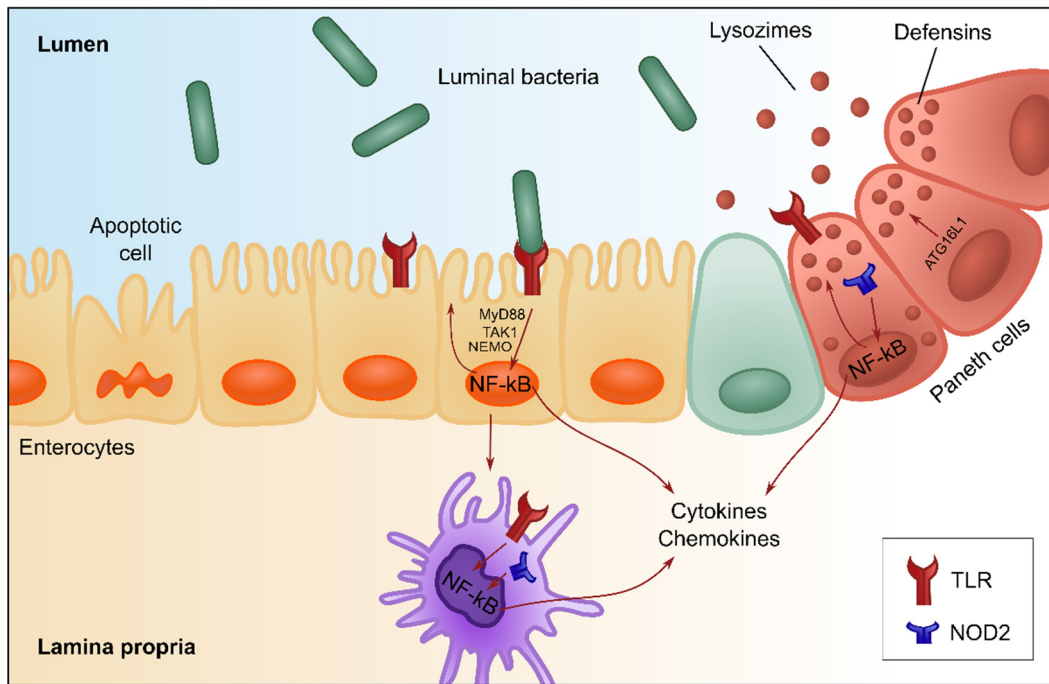
Figure 8: Initial steps of the inflammatory response. IECs and immune cells express PRRs such as NOD-like receptors and TLRs, that recognize PAMPs. Upon activation, they initiate signalling cascades that result in the activation of pro-inflammatory genes transcription, such as those induced by NF-κB. This elicits the production of pro-inflammatory cytokines and chemokines.

1.4.1. The role of intestinal resident versus inflammation-elicited macrophages

Monocyte-derived mononuclear phagocytes, particularly macrophages, respond to immunogenic challenges aiming to re-establish tissue homeostasis. Resident macrophages, one of the macrophage subsets found in the lamina propria close to the epithelial cell layer, support functions to maintain the epithelial barrier structure and integrity. In the face of an immunogenic challenge, they quickly respond by performing phagocytosis and producing chemokines to recruit effector cells from the blood, in a controlled manner. To maintain an hyporeactive environment towards commensal bacteria and preserve the barrier homeostasis, intestinal resident macrophages constitutively produce the anti-inflammatory cytokine IL-10, low levels of TNF-α, and are anergic to TLR activation. Impaired IL-10 signalling leads to overproduction of inflammatory cytokines by

macrophages in response to bacterial stimulation, being closely related with severe gastrointestinal inflammation and colitis development [44,45].

The other macrophage subset, the inflammation-elicited (inflammatory) macrophages, derives from circulating monocytes and infiltrates the tissue following intestinal damage or infection. These cells produce larger amounts of pro-inflammatory factors, such as TNF-α, prostaglandin E$_2$ (PGE$_2$), IL-1β, IL-6, reactive oxygen species (ROS) and nitric oxide ($^\bullet$NO), via inducible nitric oxide synthase (iNOS). In fact, these cells seem to accumulate in the inflamed gut of either IBD patients or animal models in response to elevated levels of chemokines. The abnormal production of pro-inflammatory mediators can be highly deleterious, disrupting normal cell function and propagating local inflammation. Also, macrophages can interact with effector T cells, thus contributing to the disease progression. Once the inflammatory trigger is removed or becomes tolerated, if the homeostasis is not recovered, chronic inflammation may develop [44,45].

1.4.2. The chronic inflammation status and risk of carcinogenesis

The episodes of inflammation during flares, in auto-immune diseases, usually result from genetic dysregulation of the suppressive components of the inflammatory response [43]. Loss of tolerance mechanisms towards the normal intestinal microflora lead to chronic intestinal inflammation. In IBD, this status is characterized by infiltration of lymphocytes, intestinal tissue injury and failed attempts of repair. In this state, the epithelial barrier integrity is compromised, resulting in abnormal mucosal permeability, overexpression of pro-inflammatory cytokines/chemokines, overactivation of TLR signalling and lack of immunosuppression mechanisms [46,47]. The perpetuation of this imbalance may be correlated with an increased risk of mutagenesis. In fact, patients with IBD have been shown to have an increased risk for the development of colorectal cancer (CRC), primarily due to chronic inflammation. Concerning UC, the associated CRC risk

seems to increase significantly with the disease longevity (2% after 10 years, 8% after 20 years, and 18% after 30 years of the disease). Although the underlying carcinogenic mechanisms may be similar to other sporadic colorectal carcinomas, in IBD patients this process seems to be faster and/or multifocal. For that contributes a combination of factors, namely the IBD-related oxidative stress (ROS and RNS), immune responses (TNF-α, IL-1, IL-6, etc), inflammatory-related genes (COX-2 and iNOS) and microbiota (*Fusobacterium* and *Enterococcus* populations, among others). In addition, the long-term immunomodulators and biologic treatments can effectively promote carcinogenesis [48].

1.4.3. The activation of inflammatory signalling pathways via NOD and TLR

As part of the innate immune defences, the NOD-like receptors, NOD1 and NOD2, recognise either PAMPs from invading bacteria or stress signals within the cells. While the NOD1 receptors are ubiquitously expressed, the NOD2 is particularly abundant in macrophages, monocytes, Paneth cells and DCs. Their activation results in the downstream activation of signalling kinases and transcription factors that induce pro-inflammatory genes transcription. In these signalling cascades, the binding and activation of the transforming growth factor β-activated kinase 1 (TAK1) is a key event, since it can activate both nuclear factor κB (NF-κB) and mitogen-activating protein kinases (MAPKs)/activator protein-1 (AP-1) signalling pathways (**Fig 9**). As previously mentioned, genetic defects in these receptors have been implicated in IBD pathogenesis. Actually, biopsies form UC patients expressed higher levels of NOD1, while polymorphisms in NOD2 gene have been highly correlated with CD [49,50].

The TLR signalling is another mechanism to recognise infection via PRRs and to activate the immune response, whose dysregulation has also been implicated in several autoimmune diseases. These receptors can identify a wide range of molecules, from viral genetic material to bacterial-derived lipoproteins, lipopolysaccharide (LPS) or flagellin.
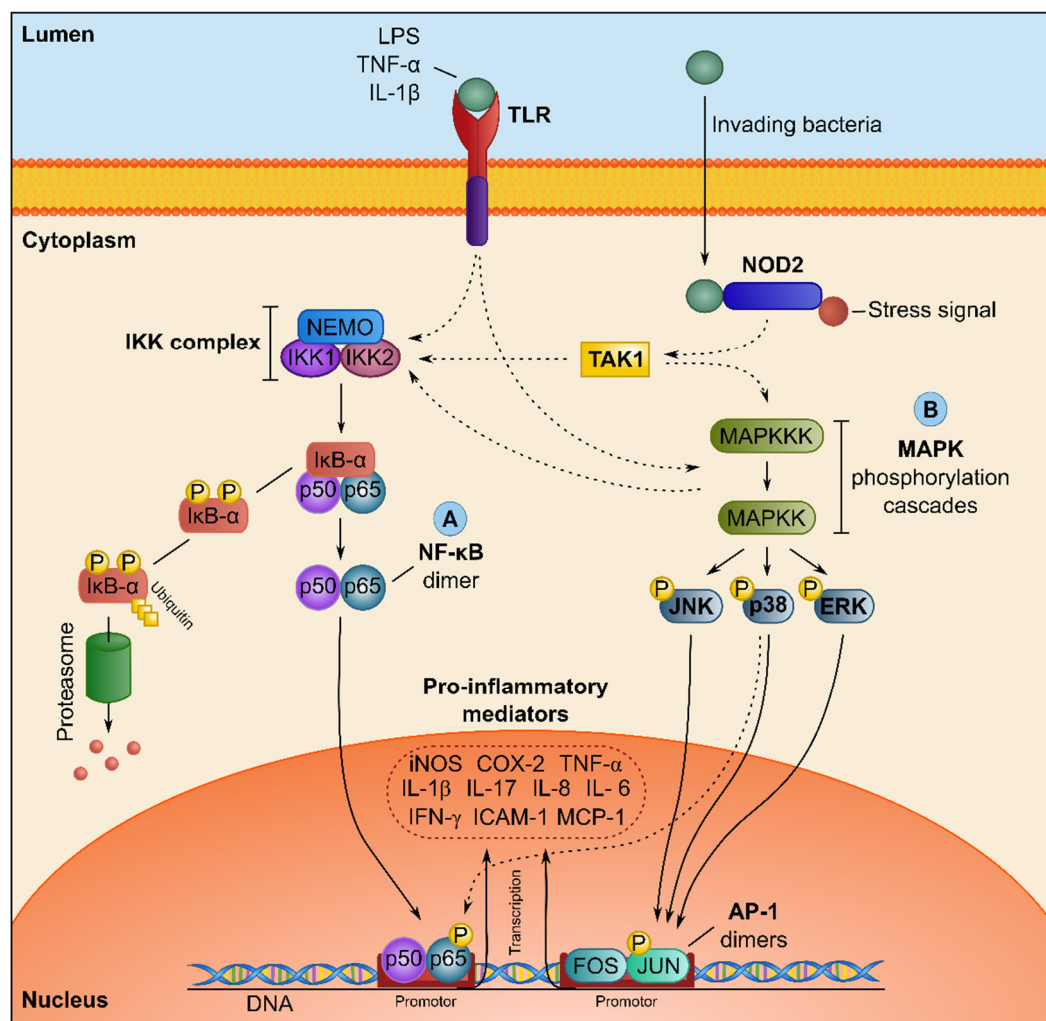
Figure 9: Signalling pathways activated by TLR and NOD2 - NF-κB and MAPKs/AP-1. Upon activation of TLR and NOD2 due to either extra or intracellular stimuli, various signalling pathways are triggered, including the NF-κB **(A)** and MAPKs cascades **(B)**, both of which may be activated by TAK1. In the classical activation, the IKK complex mediates the phosphorylation of IκB-α, thereby initiating its proteasomal degradation. The released NF-κB dimer can then translocate to the nucleus, where it mediates the transcription of target genes. Activation of the MAPK phosphorylation cascades results in JNK, p38 and ERK MAPKs activation, that in turn phosphorylates substrate proteins, such as AP-1 transcription factor. The p38 MAPK can also modulate p65 subunit of NF-κB, thus enhancing its signalling. Their coordinated action, modulate the expression of target genes involved in inflammation.

Both immune and non-immune cells express these receptors, namely DCs and epithelial cells. Like the NOD-like receptors, activation of the TLR signalling components may result in the NF-κB and MAPKs signalling pathways induction (**Fig 9**). In the GI, due to its complex homeostasis with the microbiota, the TLRs have specific signalling

mechanisms to prevent persistent inflammation. As an example, under healthy conditions, the TLR5 seems to have a protective effect, since there is no cell activation when exposed to flagellin [51], while spontaneous colitis occurs in TLR5 deficient mice models [52]. Therefore, defects in the TLRs regulation may contribute to the development of IBD chronic inflammation. Furthermore, the role of TLRs and NOD2 on inflammatory signalling pathways can't be considered individually. Both seem to act synergistically on the secretion of TNF-α, IL-1β and IL-8 in human peripheral blood mononuclear cells. This mechanism of signalling amplification seems to be relevant to the recruitment and activation of neutrophils. When impaired, as occurs in NOD2-related CD patients, it may result in failure of pathogen clearance, abnormal immune responses and dysregulated cytokine production [50,53,54].

A. *NF-κB signalling pathway*

The NF-κB signalling pathway is therefore one of the most relevant in terms of inflammation, being able to induce the expression of many immediate early genes. To date, five proteins have been identified in mammals from the NF-κB family: p65 (RelA), cRel, RelB, p50 and p52. In the absence of stimulus, cells contain an inactive form of this dimeric protein, formed most commonly by p65 with p50 in the cytoplasm, where it is bound to the inhibitor of κB alpha (IκB-α). In the classic canonical pathway (**Fig 9A**), upon stimulation by endogenous inducers (e.g. TNF-α and IL-1β) or exogenous stimuli (e.g. lipopolysaccharides), ubiquitylation/phosphorylation cascades may trigger the recruitment of inhibitor of κB kinases (IKK). Activated components via NOD-like receptors (e.g. TAK1) and/or TLRs are described to recruit IKK complex (the catalytic IKK1 and IKK2, and the regulatory NEMO) for activation. This event, mainly due to the IKK2 activity, allows the rapid phosphorylation of IκB-α that is then degraded by ubiquitination in the proteasome. The inhibitor dissociation from the NF-κB dimer (p65- and cRel-containing

dimers), renders it free to translocate to the nucleus, where it binds with high affinity to regulator κB elements and promote the transcription of target genes. Such genes include inflammatory and immunoregulatory genes, cell cycle regulating genes, anti-apoptotic genes and genes that encode negative regulators of NF-κB. An alternative IKK1-dependent pathway may also occur but it is poorly understood yet in terms of its role in intestinal inflammation [55,56].

This transcription factor has been associated with the pathophysiology of several chronic inflammatory diseases, including IBD. Some of the first studies have demonstrated a higher content of p65 in the nucleus of cells from IBD patients' biopsies, and a higher activation of NF-κB in both IECs and macrophages [57-59]. The relevance of this signalling pathway in IBD was also explored in animal models of colitis (e.g. TNBS- and DSS-induced colitis and IL-10-deficient mice), where its inhibition reduced the intestinal inflammation. Inhibition of NF-κB activity has been demonstrated to reduce pro-inflammatory mediators such as iNOS, intercellular adhesion molecule-1 (ICAM-1), monocyte chemotactic protein-1 (MCP-1), cyclooxygenase-2 (COX-2), interferon-gamma (IFN-γ), TNF-α, IL-1β, IL-6 and IL-17 [60-63]. Moreover, NF-κB signalling dysregulation has been correlated with CRC progression [64]. The pathological mechanism by which NF-κB is implicated in IBD is somewhat dependent of its cell-specific functions. As an example, NF-κB activation in mucosal macrophages results in a higher secretion of TNF-α, IL-1 and IL-6, while in IECs it seems to exert some anti-inflammatory effect [56].

A. *MAPK/AP-1 signalling pathway*

As described for the NF-κB signalling pathway, extracellular and intracellular stimulus may activate specific receptors (TLR or NOD-like receptors) that trigger complex MAPK signalling cascades [49,50]. These are involved in a wide range of cellular

processes, including cell growth, proliferation, differentiation, migration, inflammation and survival. They respond to growth factors, cytokines and hormones, in addition to several cellular stresses, from which their title derived as stress-activated MAPKs. The above mentioned TAK1 kinase can activate the three major MAPK family members: c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK). Activated MAPKs translocate to the nucleus, where they phosphorylate numerous transcription factors, thus regulating the expression of downstream target genes. The duration and magnitude of MAPK activation are crucial for the gene expression patterns and cells cycle decisions. Since this pathway regulates several pivotal cell functions, its dysregulation is implicated in the pathogenesis of diseases such as IBD, being found with increased expression in IBD patients. Inflammatory cytokines (e.g. TNF-α and IL-1β) can activate the MAPKs cascades, including by this way the expression of additional pro-inflammatory cytokines (e.g. IFN-γ, TNF-α, IL-1β and IL-8). Also, they participate in the controlled renewal of the intestinal epithelium, thus being of major relevance to maintain its proper barrier function [65,66].

The AP-1 transcription factor is one of the MAPKs downstream targets that promote the expression of pro-inflammatory mediators. This transcription factor is a homo- or heterodimeric protein that contains one of the Jun (c-Jun, JunB, and JunD) or Fos family of proteins (c-Fos, FosB, Fra1, and Fra2). The JNK MAPK can directly activate c-Jun by phosphorylation, thus inducing its transcriptional activity. This results in the expression of pro-inflammatory mediators such as iNOS and COX-2, and extracellular matrix proteins. Partly due to the perpetuation of the inflammatory status, AP-1 has also been correlated with tumor promotion and progression, as occurs in CRC [67,68]. The MAPKs cascades can also regulate NF-κB signalling. As an example, the p38 MAPK can induce the phosphorylation of p65 subunit of NF-κB, thus enhancing the expression of its target genes [69,70].

1.4.4. The oxidative status of the GI in IBD

Another key aspect of the GI is the ability to maintain its homeostasis under the constant contact with luminal oxidants and high susceptibility to oxidative damage. It is well known that intense oxidative stress-related tissue damage is crucial to the onset and development of chronic gut inflammation. Even though it is not yet clear if intestinal oxidative stress is cause or consequence of the inflammatory stress, it has been proposed as a mechanism underlying the pathophysiology of IBD [71,72].

Although both reactive oxygen species (ROS) and reactive nitrogen species (RNS) play physiological roles, such as mitogenic signalling and host defences against invading pathogens, when their production exceeds the cellular antioxidant mechanisms capacity, they may cause oxidative/nitrosative damage to cellular constituents [73]. In addition to the direct damage to intestinal cells, the oxidative stress may trigger inflammatory signalling pathways, such as the previous described NF-κB and MAPKs cascades, thus perpetuating the disease. Therefore, the oxidative and nitrosative stress may occur due to an overproduction of ROS and RNS respectively, or by insufficient antioxidant mechanisms that fail to maintain them at physiological levels [71,72]. This was observed in both animal models and in patients with either UC or CD, where it has been found an increased formation of ROS/RNS and decreased levels of endogenous antioxidants [74,75]. Moreover, the higher levels of reactive species could be correlated with the disease severity and progression in most cases. The use of exogenous antioxidant compounds seems to prevent or retard the disease, usually associated with a decrease in both inflammatory and oxidative stress biomarkers in the colonic tissue and peripheral blood [76].

A. *The major reactive oxygen and nitrogen species formed in the GI*

The main ROS are formed by unstable forms of oxygen: superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) (**Table 1** and **Fig 10**). The $O_2^{\bullet-}$ is constantly being generated in the mitochondrial electron transport chain, in complex I and II (**A**). It is also produced by the enzymatic reaction catalysed by xanthine oxidase (XO) (**B**) and, in several cells and tissues, the NADPH oxidases (NOX) also catalyse its production (**C**) as a mechanism of defence against pathogens [77]. In the healthy GI, the superoxide dismutase (SOD) converts $O_2^{\bullet-}$ to H_2O_2 (**D**) which is subsequently converted to water, through a reaction catalysed by catalase (CAT) (**E**) and/or by glutathione peroxidase (GPX) (**F**). Under stress conditions, the H_2O_2 is somehow problematic since it can easily cross cellular membranes and react with $O_2^{\bullet-}$, forming the highly reactive $HO^{\bullet}$, as described by the Haber-Weiss reaction (**G**). This reaction is very slow and is catalysed by the ferrous ion (Fe^{2+}) or other transient metals, which drive a reaction cycle where $O_2^{\bullet-}$ reduces the oxidized metal generated by the Fenton reaction (**H**), ensuring the formation of $HO^{\bullet}$ (Haber-Weiss net reaction) [71,78].

As already mentioned, low levels of ROS are crucial for several cell mechanisms of signalling and defence, nonetheless their excessive production is implicated in IBD initiation and progression. In UC patients for example, a strong neutrophil infiltration of the inflamed intestinal lamina propria occurs, and higher levels of myeloperoxidase (MPO) are found. This is particularly relevant since these immune cells produce H_2O_2 that is used by MPO yielding hypochlorite ion (OCl^-), a species with strong oxidizing activity [77]. Also, increasing concentration of $O_2^{\bullet-}$ from neutrophil NOX enzymes can generate $HO^{\bullet}$ and perhydroxyl ($HOO^{\bullet}$) radicals [79]. The first is known to inactivate mitochondrial enzyme pyruvate dehydrogenase, to depolymerise GI mucin, and to inflict mitochondrial RNA and DNA damages. $HOO^{\bullet}$ initiates fatty acid peroxidation, disturbing the permeability of biomembranes, modifying lipoproteins to pro-inflammatory forms and generating

potentially toxic and mutagenic products. Such species have been associated with mutagenesis in chronic intestinal inflammation [71,78].

Table 1: Enzymatic reactions involved with ROS and RNS in the GI

Enzyme	Reaction	Site of action	
Complex I and III/ubiquinone of the mitochondrial transport chain	*Complex I (NADH dehydrogenase):* $O_2 + NADH \rightarrow O_2^{\bullet-} + NAD^+$ *Complex III (cytochrome bc$_1$):* $O_2 \rightarrow O_2^{\bullet-}$	Mitochondria	A
Xanthine oxidase	$Xanthine + O_2 + NADPH \rightarrow O_2^{\bullet-} + H_2O_2 + NADP^+ + uric\ acid$	Plasma and cytoplasm of epithelial cells	B
NADPH oxidase	$2O_2 + NADPH \rightarrow 2O_2^{\bullet-} + NADP^+ + H^+$	Cell membrane	C
Superoxide dismutase	$2H^+ + 2O_2^{\bullet-} \rightarrow O_2 + H_2O_2$	Cytoplasm, mitochondria and small amount in the nucleus; Plasma	D
Catalase	$2H_2O_2 \rightarrow O_2 + H_2O$	Cytoplasm and peroxisomes of epithelium and lamina propria; Leukocytes	E
Glutathione peroxidase	$H_2O_2 + 2GSH \rightarrow GSSG + 2\ H_2O$	Peroxisomes of colon lymphatic tissue, luminal epithelium, and secreted by IECs	F
——	$H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + HO^- + HO^{\bullet}$ (*Haber-Weiss reaction*)	Plasma and cytoplasm of cells	G
——	$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO^{\bullet}$ (*Fenton reaction*)	Plasma and cytoplasm of cells	H
Nitric oxide synthase	$L\text{-}arginine + O_2 \rightarrow L\text{-}citrulline + {}^{\bullet}NO$	Cell membrane of endothelial cells	I
——	${}^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^-$	Cytoplasm of inflammatory and epithelial cells	J
Glutathione reductase	$GSSG + NADPH \rightarrow GSH + NADP^+$	Peroxisomes of colon lymphatic tissue, luminal epithelium, and secreted by IECs	K
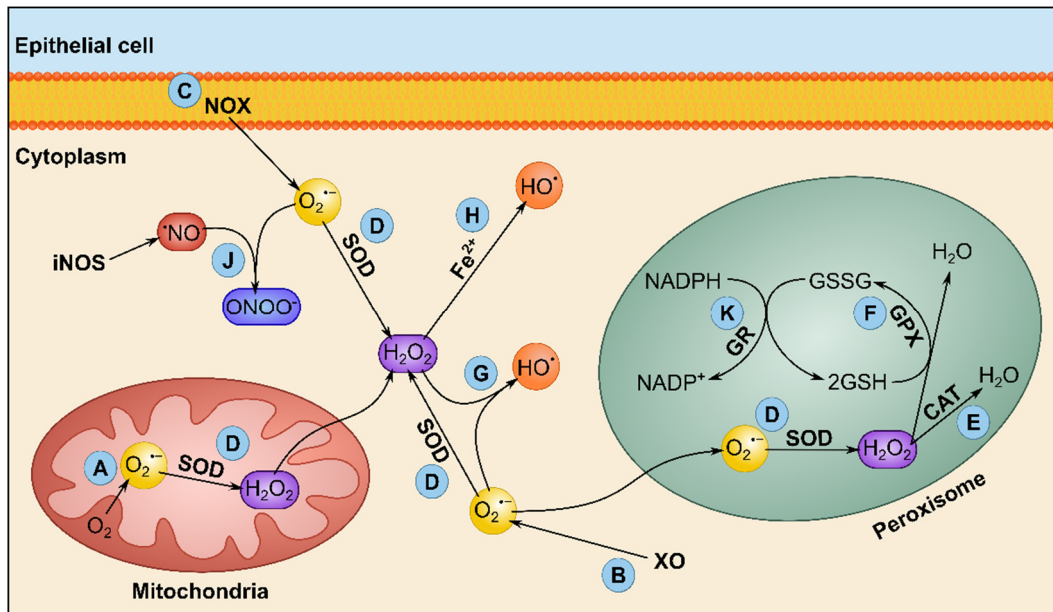
Figure 10: ROS and RNS formation within an IEC. The major ROS and RNS originate from various sources within the cell (**A**, **B** and **C**). When their production exceeds the endogenous antioxidant mechanisms capacity (**D**, **E**, **F** and **K**), they react with each other originating high levels of deleterious reactive species (**G**, **H** and **J**). The letters correspond to **Table 1** reactions.

RNS encompass NO and its derived metabolites, being the most common ˙NO, nitrogen dioxide (NO_2), and peroxynitrite ($ONOO^-$). ˙NO is produced by nitric oxide synthases (**Table 1I**) and participates in several physiological functions, including neurotransmission, vasodilation and immunomodulation. To date, three isoforms of NOS have been identified: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III). Both nNOS and eNOS isoforms are constitutively present in neuronal and endothelial cells respectively, while iNOS is an induced isoform and mainly regulated at the transcriptional level. The latter is only detected in inflamed tissues and is responsible for RNS overproduction by activated macrophages, leukocytes and epithelial cells in the intestinal mucosa. In these cells, the ˙NO may also react with $O_2^{˙-}$ thus forming $ONOO^-$ (**Table 1J** and **Fig 10J**), a species much more reactive than the previous two, causing generalized oxidative/nitrosative damage, including DNA fragmentation and lipid degradation [71,77,78]. Therefore, activation of iNOS and COX-2

in UC, leading to the consequent production of $^\bullet$NO and PGE_2 respectively, contributes to the damage of intestinal mucous membrane [80].

B. *The GI antioxidant mechanisms*

To deal with ROS and RNS production, several antioxidant mechanisms are in place to sustain healthy levels of these radicals. These involve enzymes such as CAT, SOD or GPX, and radical scavengers like reduced glutathione (GSH). Although during inflammatory conditions CAT is highly expressed to deal with H_2O_2 conversion to water, the GPX enzyme has higher affinity to H_2O_2. Its activity depends on GSH as a proton donor, which in turn is oxidised to glutathione disulfide (GSSG) in the presence of NADPH (**Table 1F** and **Fig 10F**). The GSH pool is afterwards replenished by de novo synthesis, and by regeneration from GSSG mediated by GSH reductase (**Table 1K** and **Fig 10K**) [71,77,78].

In IBD patients, the mucosal inflammatory severity has also been correlated with an elevated ratio of the oxidized glutathione versus its reduced form (GSSG/GSH), reflecting disturbances in the redox metabolism. This has been associated with hyperplasia, mucosal inflammation and clinical symptoms of colitis. It is noteworthy that the promoter region of γ-glutamylcysteine synthetase, an enzyme involved in GSH synthesis, contains a ROS-sensitive AP-1 binding site and an antioxidant response element (ARE). Therefore, when these regions are activated, the GSH production increases to counter the oxidative challenge [71].

Besides the endogenous antioxidants, dietary antioxidants like vitamins C and E, carotenoids and polyphenols, are another important antioxidant source which may help coping with the oxidative stress [81]. Although some controversy about their biodisponibility, epidemiological studies suggest that long term consumption of diets rich in antioxidants like polyphenols, could bring benefits in preventing some diseases [82].

C. *The redox signalling*

The controlled production of ROS undertakes an important role as physiological signalling molecules. The most described targets are the catalytic cysteine residues of tyrosine kinases and MAPKs phosphatases. This results in the activation of not only the previously described AP-1 and NF-κB transcription factors (**Fig 11B**), but also the nuclear factor-erythroid-2-related factor 2 (Nfr2) pathway (**Fig 11A**), which is a key regulator of cellular antioxidant and detoxifying defences, being responsible for the elimination of xenobiotics and oxidants [77]. It is particularly interesting that controlled levels of H_2O_2 trigger the oxidation of oxidant-sensitive proteins in a reversible way. Usually this requires mechanisms involving thioredoxin (Trx) or GSH, and results in an on/off regulation like that of phosphorylation/dephosphorylation cascades. As a result, it allows a graded perception of the intracellular ROS and the control of signalling pathways [79]. Concerning Nrf2, it is bound to its inhibitor Keap1 in the cytoplasm, under reducing conditions. In response to pro-oxidant or electrophilic stress, Keap1 is oxidized in its cysteine domains, allowing the Nrf2 translocation to the nucleus and dimerization with small Maf proteins. This results in its binding to antioxidant response elements (ARE) sequence and activation of an extensive gene network, promoting the expression of antioxidant enzymes (e.g. SOD, GPX, and Trx) and xenobiotic detoxification enzymes (**Fig 11A**). Thus, deficiencies in Nrf2 factor have been associated with gastrointestinal inflammation and colorectal carcinogenesis [79,83].
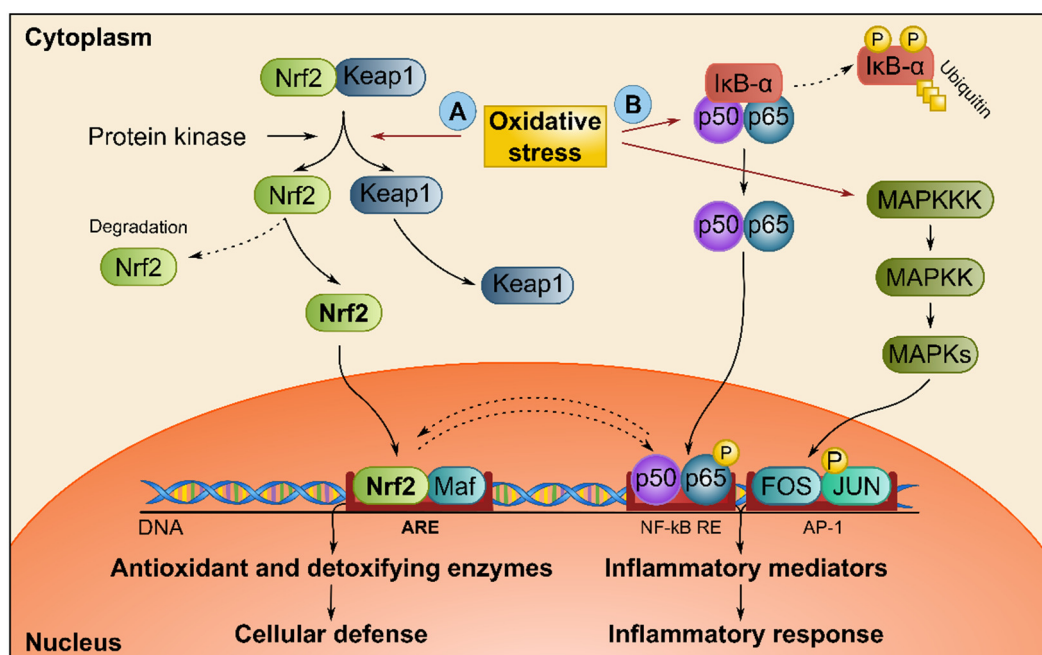
Figure 11: Activation of Nrf2, NF-κB and AP-1 signalling due to oxidative stress. Oxidative stress can cause the dissociation of Keap1 from Nrf2 (**A**), allowing this factor to translocate to the nucleus. Here, it forms a dimer with small Maf proteins and bind to ARE, promoting the transcription of antioxidant and detoxifying enzymes. Simultaneously, the inflammatory response is also triggered by NF-κB and AP-1 signalling activation (**B**). The Nrf2 and NF-κB pathways influence one another.

In the intestinal epithelium, the redox signalling is also responsible for regulating cell cycle decisions, maintaining mucosal integrity. Changes in the intracellular redox potential of GSH (GSH/GSSG) orient decisions from cell proliferation (−260 to −240 mV), through differentiation (−220 to −200 mV), and finally till apoptosis (−170 to −150 mV). The GSH/GSSG couple, together with the cysteine/cystine (Cys/CySS) and the thioredoxin/thioredoxin disulfide (Trx/TrxSS) couples, are believed to function as distinct control nodes that regulate metabolic processes and enzymatic reactions. This role is also supported by their shift between active and inactive sulphur forms within each subcellular compartment (mitochondria, nucleus, endoplasmic reticulum, and cytosol). Again, the disruption of these unique control nodes may result in mucosal oxidative stress, contributing to the development of intestinal inflammation and cancer [84,85].

1.5. The current medical therapy approaches for IBD

Understanding the disease is crucial to effectively modify IBD progression. Objective evaluation of biomarkers of inflammation have been correlated with better long-term outcomes. In recent years, targeting mucosal healing rather than ameliorating symptoms, also seems to achieve better results. The symptoms may be misleading, since they may be present when the mucosal inflammation is not substantially active, or conversely, patients may have active inflammation without reporting symptoms. Thus, it is fundamental to select an effective intervention, at the right time, for a specific therapeutic endpoint [86].

1.5.1. Drug therapies

Currently, the drug medical options to treat IBD include antibiotics, aminosalicylates, corticosteroids, immunosuppressants and biologic therapies (**Fig 12**). Each medication category is useful to control symptoms, treat mild-to-moderate episodes of CD and UC, prevent relapses, and maintain remission. In addition, nutritional and hygienic measures must also be taken into consideration. For each case, physicians must decide the most appropriate therapy, evaluating its efficacy and safety [87,88]. The main aim of treatment should be to achieve a profound remission, characterized by the clinical state, together with mucosal healing and cessation of steroid administration [89,90].
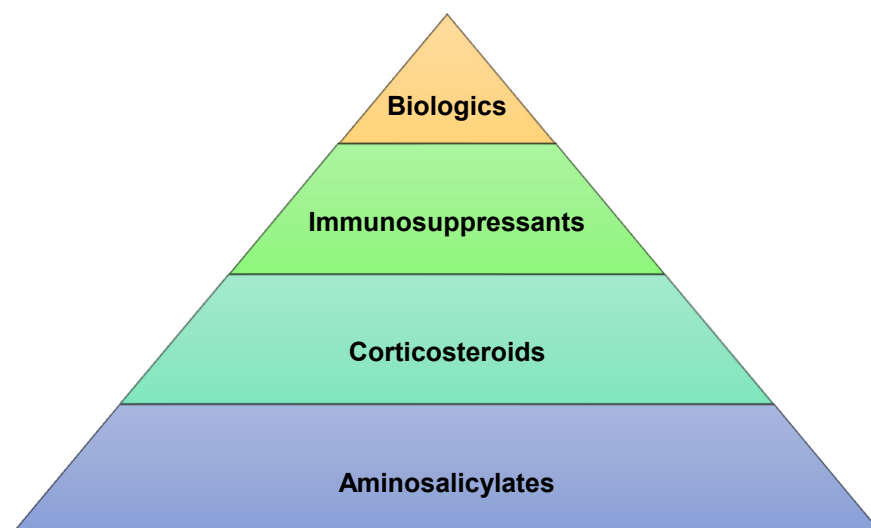
Figure 12: The classical therapeutic pyramid for IBD. In a step-up approach, the use of aminosalicylates (e.g. 5-ASA) continues to be in the basis of treatment. Corticosteroids are regarded as a second step, followed by immunosuppressants and lastly by biologics, such as anti-TNF antibodies. In patients with higher risks of complications (e.g. younger onset of the disease), it is preferable a step-down approach to treatment. (Adapted from [91]).

A. *Aminosalicylates*

Aminosalicylates are one of the most used drug classes to treat mild-to-moderate episodes of CD and UC (**Fig 12**), encompassing mesalazine or 5-aminosalicylic acid (5-ASA) and its pro-drug form sulfasalazine. 5-ASA seems to act locally on colonic mucosa, by reducing inflammation through several mechanisms, which include the inhibition of either lipid mediators release, inflammatory cells, or cytokines and ROS formation. These actions seem to be mediated by the activation of γ-form peroxisome proliferator-activated receptors (PPAR-γ), known to be able to trans-repress several inflammatory effector genes, including NF-κB target genes. Therefore, this drug down-regulates inflammatory cytokine production, p65 dephosphorylation, accompanied by NF-κB decreased transcriptional activity, reducing by this way the synthesis of prostaglandins and leukotrienes. This action is highly relevant in IBD since PPAR-γ is highly expressed in the colonic epithelial and macrophage cells. Additionally, 5-ASA also

exhibits potent antioxidant activity as a free radicals' scavenger [87,92,93], suggesting that this action mechanism may contribute to its intestinal anti-inflammatory activity.

Although the recognized efficacy, 5-ASA is not devoid of side effects. They occur in 10-45% of patients, being the most common headache, nausea, epigastric pain, diarrhoea, and oligospermia in men. Also, all aminosalicylates have been associated with nephrotoxicity, in part related to the drug dosage. This way, renal function must be under surveillance to stop the treatment if renal function deteriorates [88,94].

B. *Corticosteroids*

Corticosteroids have a potent anti-inflammatory effect and are usually used to treat moderate to severe relapses of both CD and UC. They inhibit T cell activation and pro-inflammatory cytokines, by binding to the intracellular glucocorticoid receptor. Upon activation of this receptor, it translocates into the nucleus where it binds to the glucocorticoid responsive elements, preventing the binding of AP-1 and NF-κB to these promoter regions and inducing the trans-activation of their inhibitor proteins [87,95].

Since the glucocorticoid receptors are expressed in most cells, the choice of these drugs should be carefully evaluated taking into consideration the benefits versus side effects, which may range from weight gain and acne to more serious effects, such as impaired glucose tolerance, bone loss, cataracts and venous thromboembolism. Therefore, their use must be restricted to a short interval and it is recommended the early initiation of corticosteroid-sparing medications, like immunosuppressants or biologics [96,97].

C. *Immunosuppressants*

Some of the immunosuppressants used in IBD include thiopurines, methotrexate and calcineurin inhibitors [98]. The immunomodulatory properties of thiopurines are due to their capacity to control T cell apoptosis via Ras-related C3 botulinum toxin substrate 1 (Rac1). Activation of this gene is responsible for MAPK, NF-κB and mitochondrial pathway of apoptosis. Modulation of Rac1 by thiopurines converts the stimulatory signalling into an apoptotic one [87,99]. Regarding methotrexate, most of the its metabolites have inhibitory effects on folate-dependent enzymes, thus interfering with the folic acid metabolic pathway. Although its mechanism of action is not yet completely clear, in low-dose therapy it seems to be related with inhibition of lymphocyte proliferation through DNA synthesis inhibition, down-regulation of proliferation cytokines (e.g. IL-2) and upregulation of anti-inflammatory ones (e.g. IL-10) [87,100]. Calcineurin inhibitors, such as ciclosporin, act by binding to immunophilins that in turn bind to calcineurin. The formation of this complex with calcineurin prevents the activation of this phosphatase, thus inhibiting nuclear factor of activated T cells (NFAT). This inhibits T cells and antigen presenting cells activation, supresses inflammatory cytokines and interferes with NF-κB signalling [87,101].

Major adverse effects occur up to 20% with thiopurines, 27-49% with methotrexate, and up to 17% with calcineurin inhibitors treatment. They may cause allergic reactions, severe leucopenia, increased risk of infection, hepatotoxicity, and renal impairment. Thus, patients treated with these drugs require close monitoring, with frequent blood tests for full blood count, liver and renal function [88].

D. *Biologics*

Although biologics appear at the top of the step-up approach in IBD treatment, their introduction may be recommended earlier in patients with poor prognosis and refractory

to the conventional drugs. The most used biologic agents are monoclonal antibodies against TNF-α, namely infliximab and adalimumab. They bind with high affinity to this pro-inflammatory cytokine, thus interfering with NF-κB activation and cytokine production. These drugs control TNF-induced excessive inflammation and tissue injury, and some evidences also suggest a down-regulation of inflammatory cells via apoptotic mechanisms [87,102]. Infliximab therapy demonstrated high efficacy in patients with CD and UC that were refractory to conventional treatments (CD: 81% response rate with 5 mg.kg^{-1}, for 4 weeks; UC: 9.4% response rate with 5 mg.kg^{-1}, for 8 weeks). Unfortunately, they render the users more susceptible to infections from intracellular (tuberculosis) or opportunistic pathogens, autoimmunity, and other side-effects. Therefore, the use of biologics must be carefully evaluated and monitored [88,103]. The high costs of biologic drugs also limit their use, but the scenario might change with the introduction of biosimilars to the market after patents expiration [102].

E. *Antibiotics*

The use of antibiotics is limited, being mainly relevant to control symptoms or treat secondary complications of IBD, including abscesses and pathogenic bacterial over-growth. Evidence suggests their utility in some situations by reducing the concentration and altering the composition of bacteria in the gut, therefore decreasing the disease activity and inducing remission. There is limited data supporting the efficacy of antibiotic treatment in UC, but a modest effect in CD has been reported. Usually physicians prescribe metronidazole, ciprofloxacin or a combination of both to treat complications of CD. Metronidazole, a synthetic nitroimidazole antibiotic and antiprotozoal drug, elicits the reduction of inflammatory markers in the blood. Ciprofloxacin is a broad-spectrum antibiotic and seems more effective than metronidazole in some scenarios. Yet, their

prolonged use may cause serious adverse effects that limit their use, and contribute to antibiotic resistance [88,104].

1.5.2. Nutrition

As mentioned above, one of the risk factors for IBD onset is diet, thus diet management should be considered during treatment. Certain types of foods may directly alter the gut microbiome and its produced metabolites, act as antigens that trigger an immune response or impair the defensive mucous layer. Animal models have allowed the identification of several nutrients that help control IBD, namely: omega-3 polyunsaturated fatty acids, amino acids such as glutamine, arginine, histidine and threonine, vitamin D, plant-derived polysaccharides, poorly digestible fibers, curcumin, green tea, fermented grains and polyphenols. In IBD patients, the Mediterranean diet seems to improve clinical parameters of the disease and the diversity of microbiome [105,106]. Even though there are limited data about which diets should be recommended, it seems reasonable to adopt a well-balanced low-fat and -sugar diet, such as the Mediterranean diet, and avoid commercially prepared foods and other self-identified triggers. Higher consumption of fruits and vegetables have also been associated with a lower risk of IBD. Further studies on how diet may interfere with medication or how it may be used to improve drug's efficacy are still lacking and should be addressed [105].

1.5.3. Surgery

Although disease modulation aims to avoid surgery, many IBD patients require surgical interventions at some point after diagnosis. It is important to note that surgery is not curative but evidence suggests good long-term remission, and should be considered when the other medical options have failed. In UC, the benefits of surgery seem more

controversial, depending on the disease location and number of segments affected. Although the decision for surgery is not easy, it must not be delayed since it may progress to more severe complications The procedures must minimize the operative trauma, and favour the postoperative recovery [88,107]. The introduction of biologics was expected to reduce the need for surgery, but recent data revealed otherwise. Around 30-40% of CD and 20-30% of UC patients still require surgery at some point during their lifetime. Additionally, there are some concerns about their effects on postoperative complications [108].

1.6. Experimental models of IBD to find new treatments

Taking into account that the exact etiology of IBD is still not fully understood, several experimental animal models have been developed in the last decades. They try to mimic clinical and histological features of the disease, aiming to discover a viable IBD treatment in humans. Most of these models are based on chemical induction of the disease, immune cell transfer or gene targeting. This way, they allow to study defects in epithelial integrity or permeability, and alterations in the innate or adaptive immune systems. Unfortunately, there is no ideal model, since none can reproduce simultaneously the complicated interactions of genetic, immunological, environmental, and physiologic factors of the IBD pathophysiology. For this reason, the most appropriate model must be chosen to address each question [103].

1.6.1. *In vitro models*

Several *in vitro* models have been used to study IBD molecular mechanisms and to test therapeutic agents. Both human and animal cells lines have been used to represent the different intestinal cell types, from IECs to macrophages. IEC lines have been

commonly used, such as Caco-2 or HT-29 from human origin, and IEC-6 or IEC-18 from rodents [110-113]. To achieve an inflammatory profile, these cells have been usually stimulated with one or more cytokines, namely TNF-α, IL-1β, IFN-γ or LPS [110,113]. To reach another level of complexity, the cross-talk between IECs and immune or microbial cells has also been explored in co-culture systems, which allow the growth of IECs in a porous membrane, achieving a polarized monolayer, thus mimicking the epithelial barrier. In the chamber bellow the membrane, immune cells may be cultured, such as human THP-1 or RAW 264.7 macrophages [114,115]. Bacteria, like *E. coli*, may also be co-cultured with IECs, by adding them to the top compartment [116].
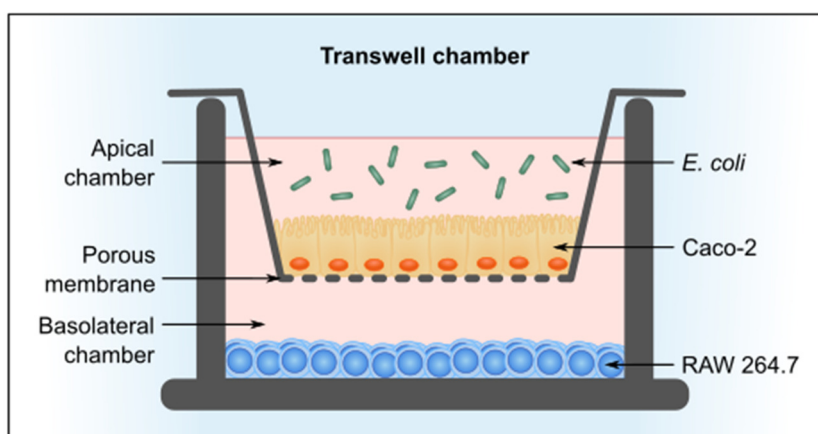
Figure 13: Schematic representation of an *in vitro* co-culture model of IBD. Taking advantage of a transwell chamber it is possible to mimic the intestinal lumen with cultured bacteria (apical chamber), the epithelial barrier (seeded on the porous membrane), and the immune cells environment of lamina propria (basolateral chamber). *E. coli*, Caco-2 and RAW 264.7 are examples of cells that can be used in this system.

1.6.2. In vivo models

Most of the *in vivo* studies have been carried out in rodent models, where acute and/or chronic intestinal inflammation are chemically induced. They rely on: i) rectal administration of trinitrobenzene sulfonic acid (TNBS), dinitrobenzene sulfonic acid

(DNBS) or acetic acid; ii) oral administration of dextran sodium sulphate (DSS); or iii) intraperitoneal injection of LPS. Transgenic animals that develop colitis spontaneously have also been used, like IL-10 knock-out mice. It is important to note that these models vary in terms of their immune and pathological characteristics, which may account for different treatment outcomes depending on the model. For instance, DSS elicits colitis primarily by disruption of the epithelial layer, while IL-10 knockout mice develop colonic inflammation in a more progressive and subtle fashion due to an immunological imbalance [109].

A. *Chemically-induced colitis*

When evaluating the safety and effectiveness of new therapeutic drugs, the chemically induced models of IBD seem to be preferable. They resemble clinical aspect of the human disease, while being cost-effective and reproducible [109]. Among these, the TNBS-induced colitis model has been used since 1989, and continues to be highly relevant to test the anti-inflammatory and/or anti-oxidant action of chemical and natural active compounds in the preclinical phase of their development. The rectal administration of TNBS requires the ethanol action to break the intestinal mucosal barrier. Then, TNBS acts as a hapten which when coupled with colonic autologous or microbiota proteins, with high molecular weight, renders them immunogenic to the host immune system. A single administration of TNBS with ethanol results in an excessive cell mediated immune response, with a dense infiltration of lymphocytes/macrophages and secretion of several pro-inflammatory cytokines. This leads to the development of transmural inflammation that closely resembles the histopathological lesions present in CD patients. Macroscopic and microscopic damage score are therefore useful to determine the severity of these lesions. At a clinical level, the animals present symptoms of severe acute colitis, namely bloody diarrhea and serious weight loss. Thus, this model is useful to study crucial aspects of GI

inflammation, namely cytokine secretion patterns, mechanisms of tolerance, cell adhesion and immunotherapy [117].

The DSS-induced colitis can also reproduce an acute phase of colitis, characterized by bloody diarrhea, ulcerations and infiltration with granulocytes. In contrast with TNBS, oral administration of DSS directly affects the integrity of the mucosal barrier by exerting a toxic effect on IECs. Changing the duration of its administration may reproduce either acute, chronic or relapsing features of colitis [118-120]. The inflammation-related CRC can also be achieved with co-administration of an initial single dose of azoxymethane, a genotoxic colon carcinogen. Therefore, this model has been reported to be useful to study the intestinal epithelial barrier and innate immune mechanisms of colitis [109].

B. *Genetically-induced colitis*

The genetically-induced models usually involve the inactivation or deletion of an existing gene, and its replacement with an artificial piece of DNA. This knock-out strategy may involve a single organ instead of the whole animal body. As an example, IL-10 knock-out mice develop spontaneous transmural pancolitis by 2-4 months of age, that resembles human CD. This is mainly due to the lack of the suppressive effect of IL-10 to maintain physiological inflammatory conditions. The A20-deficent mice also develop spontaneous inflammation related with an impaired inhibition of TNF-induced NF-κB activity. Other models instead, target the epithelial barrier, as in mice models expressing a dominant negative mutant of N-cadherin, a cell adhesion molecule in intestinal epithelial cells, which develop defects in the epithelial barrier function. Therefore, the inflammatory reaction occurs near the porous sites of the epithelial barrier, due to the abnormal contact with mucosal microflora [109,121].

2. Dietary polyphenols and their benefits in the management of IBD

The use of medication to treat or reduce the symptoms of IBD continues to be the first-line medical approach, followed by surgery when the first fails. Although pharmacotherapy may achieve good outcomes in many patients, many others experience adverse effects that may limit their use or affect the medication adherence by the patients. Additionally, in some cases the disease is not fully controlled, requiring invasive surgical interventions. Therefore, in the past few years, with the consciousness about how diet can affect the disease, dietary supplements have deserved increasing interest to achieve extra benefits, and the use of natural products and dietary components for the prevention of IBD have gained in popularity. Around half of patients with IBD seek dietary advice, and among them around 76% use supplements and vitamins [122,123].

In this context, several dietary compounds have been investigated as potential alternatives for the management of IBD. Polyphenols are a large group of compounds widely distributed in vegetables and fruits, with recognised health promoting activities. They seem to afford protection against several chronic and neurodegenerative diseases, including GI diseases [124-127]. Although their biological activities are diverse, polyphenols possess recognized antioxidant and anti-inflammatory properties. It has been demonstrated that they can act by several mechanisms, as direct antioxidants or by promoting antioxidant signalling pathways, and by reducing pro-inflammatory ones (**Fig 14**) [128,129]. Several *in vivo* studies have demonstrated their efficacy in experimental models of IBD, either as pure polyphenols or plant extracts, by reducing colitis severity in terms of morphological, histological and biochemical parameters [130,131]. Overall, this makes them promising candidates for the management of IBD.
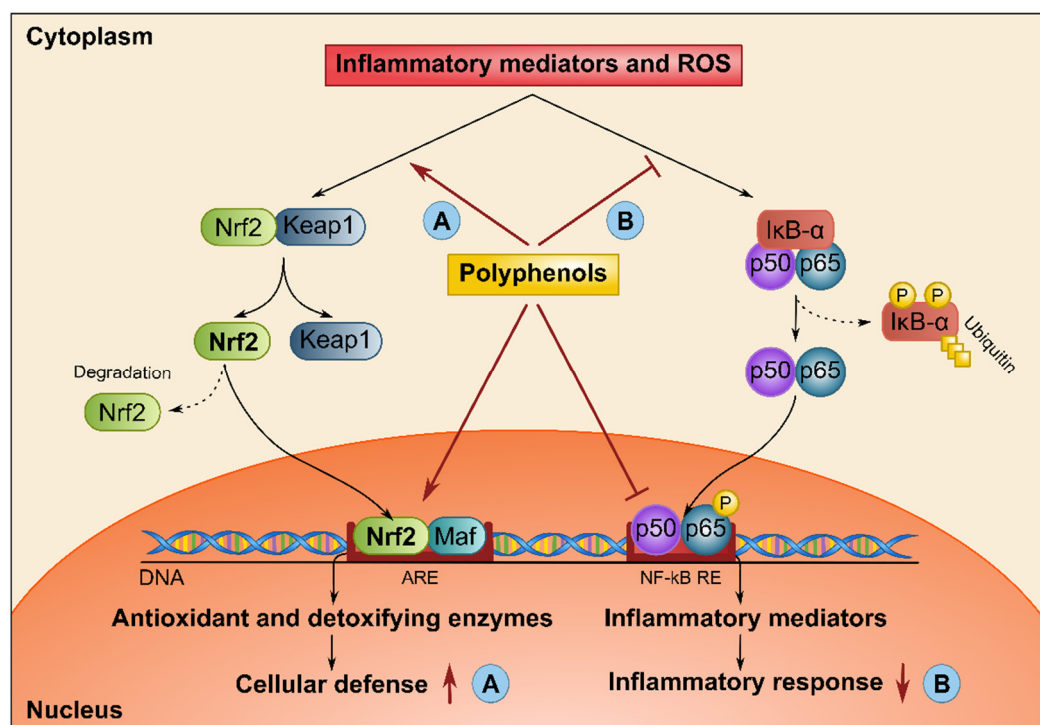
Figure 14: Polyphenols modulate antioxidant and inflammatory signalling pathways. In addition to directly scavenging free radicals, polyphenols seem to attenuate inflammation by up-regulating antioxidant genes transcription (**A**) and down-regulating inflammatory ones (**B**).

2.1. Anthocyanins as a promising subclass of polyphenols

There are many subclasses of polyphenols commonly found in the Mediterranean diet. Among them, anthocyanins are a large group of dietary flavonoids with a relatively high consumption, which have been reported to possess relevant health-promoting benefits. In addition to their well-known antioxidant activity, a wide variety of other activities have been ascribed to these compounds, namely cytoprotective, antimicrobial and antitumour activities. Even though, data about their therapeutic potential for chronic and degenerative disorders are still scarce [132].

2.1.1. Major anthocyanins and their sources

Nowadays, 702 anthocyanins have already been identified, derived from naturally occurring anthocyanidins [133]. From these, 6 are commonly found in pants: pelargonidin (Pg), cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt) and malvidin (Mv). Anthocyanins diversity arises from the binding to several glycosidic moieties (glucose, galactose, xylose, arabinose, fructose and rhamnose), and different patterns of hydroxyl and methoxy groups on B ring of the anthocyanidin structure (**Fig 15**) [132].

Anthocyanidin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3
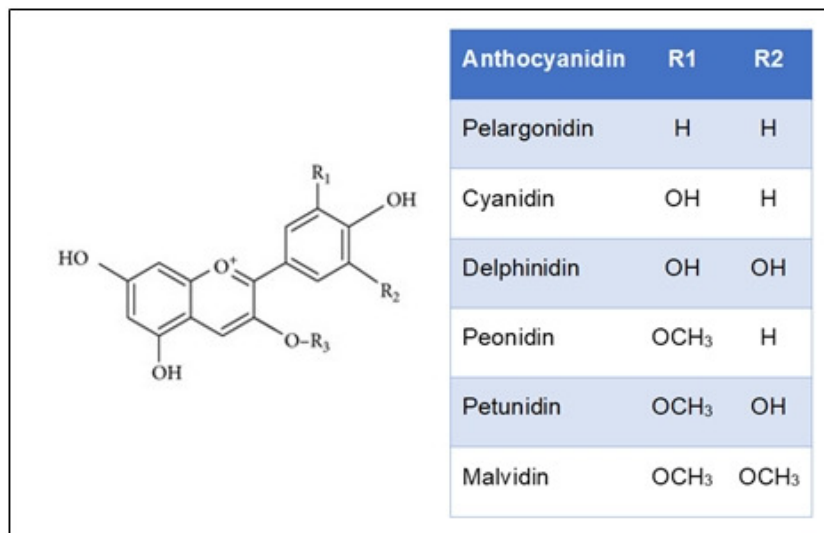
Figure 15: General chemical structures of dietary anthocyanins. R1 and R2 = patterns of hydroxyl and methoxy groups; R3 = glycosidic moiety.

Since these compounds are widely distributed in our diet, their daily intake may be quite high depending on the eating habits. In a study targeting ten western European countries, it was found that the daily anthocyanin intake could reach 64.9 mg/day and 44.1 mg/day in Italy, for men and women respectively. Northern countries however, showed the lowest levels of anthocyanins consumption. These differences may be correlated with the Mediterranean diet, which includes fruits, vegetables and red wine, with high anthocyanin contents. In fact, high sources of anthocyanins are red fruits such as

blueberries, blackberries, raspberries, strawberries, red grapes, and saskatoon berries, as well some vegetables like red cabbage [134].

2.1.2. Anthocyanins health benefits

Several experimental studies support the correlation between a high consumption of anthocyanins and lower incidence of chronic diseases. A great diversity of beneficial health effects have been assigned to these dietary compounds, such as antidiabetic, anti-obesity, neuroprotective, cardiovascular protection, anti-inflammatory and antitumour effects [132]. The underlying molecular mechanisms by which the anthocyanins exert these effects are diverse. Besides their potent antioxidant capacity, including direct radical scavenging ability, they may interfere with several signalling pathways, such as MAPKs and NF-κB, and cellular processes involved in cell cycle decisions, autophagy and biochemical metabolism [135].

Regarding the antioxidant properties, cyanidin-3-glucoside (Cy3glc) has been reported to possess the highest activity, when evaluated by the oxygen radical absorbance capacity (ORAC) assay [136]. When studied *in vitro*, this anthocyanin has been demonstrated to afford protection against endothelial dysfunction and vascular failure induced by peroxynitrite radical [137]. Besides its action as scavenger of peroxynitrite radicals, it was further reported Cy3glc ability to regulate the enzymatic production of •NO via iNOS, and to induce cellular protective mechanisms such as the mediator stress protein heme oxygenase-1 (HO-1) [138]. Previous work from our group further explored the vascular protective effects of dietary anthocyanins, in the context of cardiovascular diseases prevention. Three of the most common anthocyanins, Cy3glc, delphinidin-3-glucoside (Dp3glc) and pelargonidin-3-glucoside (Pg3glc), exhibited cytoprotective effects on endothelial cells, by scavenging peroxynitrite, as aforementioned for Cy3glc, and by disrupting the mitochondrial apoptotic pathway [139]. Later, malvidin-3-glucoside (Mv3glc)

was also shown similar effects, countering ROS formation, peroxynitrite-induced mitochondrial apoptotic signaling pathway and NF-κB activation [140,141].

The anti-inflammatory properties of anthocyanins have drawn particular interest since inflammation has been associated with the pathogenesis of several diseases, such as cancer [142]. Therefore, fruit-derived anthocyanins have been reported to inhibit the expression of COX-2, the NF-κB activation and the secretion of several interleukins, either *in vitro* or *in vivo* [143-145].

2.1.3. Anthocyanins for the management of IBD

Some controversy may arise in terms of the *in vivo* benefits of anthocyanins due to their lower bioavailability when compared with other polyphenols [146]. Nonetheless, it was shown that high percentages of anthocyanins (some more than 70%) can reach the colon after consumption of anthocyanin-rich foods like blueberries. Furthermore, anthocyanins such as cy3glc and pg3glc could be absorbed by the GI wall in their intact form. Not to mention that their metabolites can also contribute to the ascribed health benefits of anthocyanins [147]. Thus, in contrast with the low reached plasma concentrations, the GI is the compartment where dietary anthocyanins achieve their highest concentrations, with consequent potential therapeutic benefits against IBD. Therefore, some studies have explored the effect of some species of anthocyanin-rich fruits, namely strawberries, grapes and some varieties of berries, on IBD animal models and some patients [148-151]. Overall, and despite the variety in anthocyanin structures and mixtures, they seem to possess relevant protective and therapeutic effects on the management of IBD. Some of the described mechanisms include mitigation of the oxidative stress, scavenging of free radicals, cytoprotective functions, inflammatory cytokines and mediators countering, and down-regulation of pro-inflammatory pathways.

Not to mention that anthocyanins and their metabolites interact with GI microbiota, being able to modulate its composition [148].

Recent data from our group also show the beneficial effect of Cy3glc in the context of IBD, by using an *in vitro* model of human HT-29 intestinal cells. In these studies, Cy3glc demonstrated high efficacy in countering inflammation when compared to a conventional treatment (5-ASA), by suppressing cytokine production and STAT1 activation, while inducing endogenous antioxidant mechanisms [152,153].

2.1.4. Anthocyanins in IBD: the missing pieces

Evidences from several studies have demonstrated preventive and therapeutic functions of anthocyanins that are highly relevant in the context of IBD [148]. Their role as antioxidant and anti-inflammatory agents may contribute to treating flares or delay relapses, thus improving the patient's quality of life. Moreover, as part of our diet, they may be a safer strategy without the adverse effects ascribed to current drug therapy. However, so that anthocyanins can be considered as part of any therapeutic regimen for IBD, many questions still need clarification.

Considering the huge variety of anthocyanins, modification of the basic chemical structures may account for different effects, action mechanisms, potency or cell absorption. Therefore, further studies need to address their metabolism, bioavailability, efficacy, and action mechanisms, especially in the context of intestinal inflammation.

Another consideration is the fact that anthocyanin-rich food and isolated anthocyanin-rich fractions activity do not necessarily reflect that of isolated single anthocyanins. Thus, anthocyanins interactions should be analysed to identify beneficial synergisms and/or avoid antagonisms that may hinder anthocyanins activity. This may be highly relevant when developing a nutraceutical formulation, for example.

At least, a concern that has been disregarded is the possible interactions between anthocyanins and conventional drug therapies. Since most IBD patients are medicated, this should also be explored.

3. Objectives

The continuous effort to understand the pathophysiology of IBD have led to important breakthroughs in IBD management. Unfortunately, there is still no cure available and patients undergo life-long therapies, with inherent adverse effects that may be quite severe. Therefore, the need to develop more effective and safer therapies for IBD, together with preventative strategies, continues to persist. Dietary polyphenols, particularly anthocyanins, have been envisaged as promising natural alternatives due to their high abundance in fruits and vegetables and to recognized anti-inflammatory activity against chronic inflammatory disorders. In the context of IBD, although some studies have demonstrated their anti-inflammatory and antioxidant activities, the underlying action mechanisms and efficacy still need clarification.

Thus, the main goal of this work was to evaluate the efficacy of typical anthocyanins commonly found in the Mediterranean diet to ameliorate the inflammation underlying IBD, in comparison with 5-ASA, a well-known anti-inflammatory drug commonly used in this disease, by using representative models of IBD.

Therefore, to achieve our main goal, the following tasks were performed with specific objectives:

1. A first line of work aimed to assess the role of a typical single anthocyanin, Cy3glc, in inflammatory pathways of importance for management of IBD, in comparison and in association with 5-ASA, by using a macrophage cell model. Cy3glc was chosen because it is one of the most common dietary anthocyanins found to reach the colon in its intact form after anthocyanin-rich food consumption. Therefore, following a previous work from our group showing the anti-inflammatory response by Cy3glc in human intestinal cells (HT29) [152] and taking into consideration that macrophages play a key role in intestinal inflammation onset and progression, we explored its anti-inflammatory efficacy in a model of LPS-stimulated RAW 264.7 cells, as compared with 5-ASA, in terms of capacity to

counter $^\bullet$NO, PGE$_2$, TNF-α and ROS production, and iNOS and COX-2 expressions. The action mechanisms were tentatively clarified by measuring its ability to modulate crucial signalling pathways closely implicated in IBD, namely NF-κB and MAPKs/AP-1 signalling (**Chapter 2**).

2. Secondly, we aimed to investigate the *in vivo* anti-inflammatory action of an anthocyanin-rich fraction (ARF) of Portuguese blueberries (*Vaccinium corymbosum* L.), as a natural anthocyanin mixture, in a TNBS-induced colitis rat model, in comparison with 5-ASA. Thus, after colitis induction, the animals were treated with either ARF or 5-ASA for 8 days. After the sacrifices, the animals were evaluated in terms of clinically relevant parameters that closely resemble the human pathology, namely macroscopic colon damage, leukocyte infiltration (MPO activity and expression), ALP activity, antioxidant defences status (GSH/GSSG ratio and GPX activity) and expression of pro-inflammatory enzymes (COX-2 and iNOS) in the colonic tissue (**Chapter 3**).

3. To further get a better knowledge on the action mechanisms by which the prepared ARF exerts its effects and to evaluate the impact of anthocyanins on the interaction between macrophages and intestinal epithelial cells, we used an *in vitro* model of gut inflammation, a co-cultured model of intestinal Caco-2 and macrophage RAW 264.7 cells. In a transwell system, the apical compartment with a differentiated and polarized monolayer of Caco-2 cells mimics the epithelial barrier, while the basolateral compartment, containing RAW 264.7 macrophage cells, initiates the inflammatory response by LPS stimulation. This model allowed to study the ARF capacity to counter pro-inflammatory cytokines (IL-6, IL-1β), $^\bullet$NO and ROS produced by both cell types, when incubated only in the apical compartment, representative of the intestinal lumen. The ARF efficacy was again compared with that of 5-ASA (**Chapter 4**).

4. References

1. Greenwood-Van Meerveld B, Johnson AC, Grundy D (2017) Gastrointestinal physiology and function. *Handb Exp Pharmacol* **239**: 1-16.

2. Helander HF, Fändriks L (2014) Surface area of the digestive tract – revisited. *Scand J Gastroenterol* **49**: 681-689.

3. Ohland CL, Jobin C (2015) Microbial activities and intestinal homeostasis: a delicate balance between health and disease. *Cell Mol Gastroenterol Hepatol* **1**: 28-40.

4. König J, Wells J, Cani PD, Garcia-Rodenas CL, MacDonald T, et al. (2016) Human intestinal barrier function in health and disease. *Clin Transl Gastroenterol* **7**: e196.

5. Wallace KL, Zheng LB, Kanazawa Y, Shih DQ (2014) Immunopathology of inflammatory bowel disease. *World J Gastroenterol* **20**: 6-21.

6. Malik TA (2015) Inflammatory bowel disease: historical perspective, epidemiology, and risk factors. *Surg Clin North Am* **95**: 1105-1122.

7. Harlan WR, Meyer A, Fisher J (2016) Inflammatory bowel disease: epidemiology, evaluation, treatment, and health maintenance. *N C Med J* **77**: 198-201.

8. M'Koma AE (2013) Inflammatory bowel disease: an expanding global health problem. *Clin Med Insights Gastroenterol* **6**: 33-47.

9. Mehta F (2016) Report: economic implications of inflammatory bowel disease and its management. *Am J Manag Care* **22**: s51-60.

10. Latella G, Papi C (2012) Crucial steps in the natural history of inflammatory bowel disease. *World J Gastroenterol* **18**: 3790-3799.

11. Magro F, Langner C, Driessen A, Ensari A, Geboes K, et al. (2013) European consensus on the histopathology of inflammatory bowel disease. *J Crohns Colitis* **7**: 827-851.

12. Torres J, Mehandru S, Colombel JF, Peyrin-Biroulet L (2017) Crohn's disease. *Lancet* **389**: 1741-1755.

13. Ungaro R, Mehandru S, Allen PB, Peyrin-Biroulet L, Colombel JF (2017) Ulcerative colitis. *Lancet* **389**: 1756-1770.

14. Knieling F, Waldner MJ (2016) Light and sound - emerging imaging techniques for inflammatory bowel disease. *World J Gastroenterol* **22**: 5642-5654.

15. Kochhar G, Lashner B (2017) Utility of biomarkers in the management of inflammatory bowel disease. *Curr Treat Options Gastroenterol* **15**: 105-115.

16. Cosnes J, Gower-Rousseau C, Seksik P, Cortot A (2011) Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* **140**: 1785-1794.

17. Loddo I, Romano C (2015) Inflammatory bowel disease: genetics, epigenetics, and pathogenesis. *Front Immunol* **6**: 551.

18. Mayer L (2010) Evolving paradigms in the pathogenesis of IBD. *J Gastroenterol* **45**: 9-16.

19. Zaki MH, Lamkanfi M, Kanneganti T-D (2011) The Nlrp3 inflammasome: contributions to intestinal homeostasis. *Trends Immunol* **32**: 171-179.

20. Liu TC, Stappenbeck TS (2016) Genetics and pathogenesis of inflammatory bowel disease. *Annu Rev Pathol* **11**: 127-148.

21. McGovern DPB, Kugathasan S, Cho JH (2015) Genetics of inflammatory bowel diseases. *Gastroenterology* **149**: 1163-1176.

22. Cooney R, Baker J, Brain O, Danis B, Pichulik T, et al. (2010) NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* **16**: 90-97.

23. Uniken Venema WT, Voskuil MD, Dijkstra G, Weersma RK, Festen EA (2017) The genetic background of inflammatory bowel disease: from correlation to causality. *J Pathol* **241**: 146-158.

24. Pigneur B, Escher J, Elawad M, Lima R, Buderus S, et al. (2013) Phenotypic characterization of very early-onset IBD due to mutations in the IL10, IL10 receptor alpha or beta gene: a survey of the GENIUS Working Group. *Inflamm Bowel Dis* **19**: 2820-2828.

25. Halfvarson J, Jess T, Bodin L, Jarnerot G, Munkholm P, et al. (2007) Longitudinal concordance for clinical characteristics in a Swedish-Danish twin population with inflammatory bowel disease. *Inflamm Bowel Dis* **13**: 1536-1544.

26. Halfvarson J (2011) Genetics in twins with Crohn's disease: less pronounced than previously believed? *Inflamm Bowel Dis* **17**: 6-12.

27. Ventham NT, Kennedy NA, Nimmo ER, Satsangi J (2013) Beyond gene discovery in inflammatory bowel disease: the emerging role of epigenetics. *Gastroenterology* **145**: 293-308.

28. Benchimol EI, Mack DR, Guttmann A, Nguyen GC, To T, et al. (2015) Inflammatory bowel disease in immigrants to Canada and their children: a population-based cohort study. *Am J Gastroenterol* **110**: 553-563.

29. Hammer T, Lophaven SN, Nielsen KR, von Euler-Chelpin M, Weihe P, et al. (2017) Inflammatory bowel diseases in Faroese-born Danish residents and their offspring: further evidence of the dominant role of environmental factors in IBD development. *Aliment Pharmacol Ther* **45**: 1107-1114.

30. Kaplan GG, Ng SC (2017) Understanding and preventing the global increase of inflammatory bowel disease. *Gastroenterology* **152**: 313-321.

31. Takaishi H, Matsuki T, Nakazawa A, Takada T, Kado S, et al. (2008) Imbalance in intestinal microflora constitution could be involved in the pathogenesis of inflammatory bowel disease. *Int J Med Microbiol* **298**: 463-472.

32. Sartor RB (2004) Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* **126**: 1620-1633.

33. Sepehri S, Kotlowski R, Bernstein CN, Krause DO (2007) Microbial diversity of inflamed and noninflamed gut biopsy tissues in inflammatory bowel disease. *Inflamm Bowel Dis* **13**: 675-683.

34. Kostic AD, Xavier RJ, Gevers D (2014) The microbiome in inflammatory bowel disease: current status and the future ahead. *Gastroenterology* **146**: 1489-1499.

35. de Lange KM, Barrett JC (2015) Understanding inflammatory bowel disease via immunogenetics. *J Autoimmun* **64**: 91-100.

36. Babickova J, Gardlik R (2015) Pathological and therapeutic interactions between bacteriophages, microbes and the host in inflammatory bowel disease. *World J Gastroenterol* **21**: 11321-11330.

37. Huang Y, Chen Z (2016) Inflammatory bowel disease related innate immunity and adaptive immunity. *Am J Transl Res* **8**: 2490-2497.

38. Kmiec Z, Cyman M, Slebioda TJ (2017) Cells of the innate and adaptive immunity and their interactions in inflammatory bowel disease. *Adv Med Sci* **62**: 1-16.

39. Khor B, Gardet A, Xavier RJ (2011) Genetics and pathogenesis of inflammatory bowel disease. *Nature* **474**: 307-317.

40. Pastorelli L, De Salvo C, Mercado JR, Vecchi M, Pizarro TT (2013) Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics. *Front Immunol* **4**: 280.

41. Shah SC, Colombel JF, Sands BE, Narula N (2016) Mucosal healing is associated with improved long-term outcomes of patients with ulcerative colitis: a systematic review and meta-analysis. *Clin Gastroenterol Hepatol* **14**: 1245-1255.

42. Neurath MF, Travis SPL (2012) Mucosal healing in inflammatory bowel diseases: a systematic review. *Gut* **61**: 1619-1635.

43. Antonelli M, Kushner I (2017) It's time to redefine inflammation. *FASEB J* **31**: 1787-1791.

44. Grainger JR, Konkel JE, Zangerle-Murray T, Shaw TN (2017) Macrophages in gastrointestinal homeostasis and inflammation. *Pflugers Arch* **469**: 527-539.

45. Ohashi W, Hattori K, Hattori Y (2015) Control of macrophage dynamics as a potential therapeutic approach for clinical disorders involving chronic inflammation. *J Pharmacol Exp Ther* **354**: 240-250.

46. Sakai Y, Kobayashi M (2015) Lymphocyte 'homing' and chronic inflammation. *Pathol Int* **65**: 344-354.

47. Uluckan O, Wagner EF (2017) Chronic systemic inflammation originating from epithelial tissues. *FEBS J* **284**: 505-516.

48. Axelrad JE, Lichtiger S, Yajnik V (2016) Inflammatory bowel disease and cancer: the role of inflammation, immunosuppression, and cancer treatment. *World J Gastroenterol* **22**: 4794-4801.

49. Feerick CL, McKernan DP (2017) Understanding the regulation of pattern recognition receptors in inflammatory diseases - a 'Nod' in the right direction. *Immunology* **150**: 237-247.

50. Haller D (2006) Intestinal epithelial cell signalling and host-derived negative regulators under chronic inflammation: to be or not to be activated determines the balance towards commensal bacteria. *Neurogastroenterol Motil* **18**: 184-199.

51. Rhee SH, Im E, Riegler M, Kokkotou E, O'Brien M, et al. (2005) Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. *Proc Natl Acad Sci U S A* **102**: 13610-13615.

52. Vijay-Kumar M, Sanders CJ, Taylor RT, Kumar A, Aitken JD, et al. (2007) Deletion of TLR5 results in spontaneous colitis in mice. *J Clin Invest* **117**: 3909-3921.

53. Drexler SK, Foxwell BM (2010) The role of Toll-like receptors in chronic inflammation. *Int J Biochem Cell Biol* **42**: 506-518.

54. van Heel DA, Ghosh S, Butler M, Hunt K, Foxwell BMJ, et al. (2005) Synergistic enhancement of Toll-like receptor responses by NOD1 activation. *Eur J Immunol* **35**: 2471-2476.

55. Wullaert A (2010) Role of NF-kappaB activation in intestinal immune homeostasis. *Int J Med Microbiol* **300**: 49-56.

56. Atreya I, Atreya R, Neurath MF (2008) NF-kappaB in inflammatory bowel disease. *J Intern Med* **263**: 591-596.

57. Schreiber S, Nikolaus S, Hampe J (1998) Activation of nuclear factor κB in inflammatory bowel disease. *Gut* **42**: 477-484.

58. Rogler G, Brand K, Vogl D, Page S, Hofmeister R, et al. (1998) Nuclear factor κB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology* **115**: 357-369.

59. Ellis RD, Goodlad JR, Limb GA, Powell JJ, Thompson RPH, et al. (1998) Activation of nuclear factor kappa B in Crohn's disease. *Inflamm Res* **47**: 440-445.

60. Sugimoto K, Hanai H, Tozawa K, Aoshi T, Uchijima M, et al. (2002) Curcumin prevents and ameliorates trinitrobenzene sulfonic acid-induced colitis in mice. *Gastroenterology* **123**: 1912-1922.

61. Davé SH, Tilstra JS, Matsuoka K, Li F, Karrasch T, et al. (2007) Amelioration of chronic murine colitis by peptide-mediated transduction of the IκB kinase inhibitor NEMO binding domain peptide. *J Immunol* **179**: 7852-7859.

62. Shibata W, Maeda S, Hikiba Y, Yanai A, Ohmae T, et al. (2007) Cutting edge: the IκB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks inflammatory injury in murine colitis. *J Immunol* **179**: 2681-2685.

63. Dou W, Zhang J, Ren G, Ding L, Sun A, et al. (2014) Mangiferin attenuates the symptoms of dextran sulfate sodium-induced colitis in mice via NF-kappaB and MAPK signaling inactivation. *Int Immunopharmacol* **23**: 170-178.

64. Wang S, Liu Z, Wang L, Zhang X (2009) NF-kappaB signaling pathway, inflammation and colorectal cancer. *Cell Mol Immunol* **6**: 327-334.

65. Coskun M, Olsen J, Seidelin JB, Nielsen OH (2011) MAP kinases in inflammatory bowel disease. *Clin Chim Acta* **412**: 513-520.

66. Broom OJ, Widjaya B, Troelsen J, Olsen J, Nielsen OH (2009) Mitogen activated protein kinases: a role in inflammatory bowel disease? *Clin Exp Immunol* **158**: 272-280.

67. Matthews CP, Colburn NH, Young MR (2007) AP-1 a target for cancer prevention. *Curr Cancer Drug Targets* **7**: 317-324.

68. Vaiopoulos AG, Papachroni KK, Papavassiliou AG (2010) Colon carcinogenesis: learning from NF-kappaB and AP-1. *Int J Biochem Cell Biol* **42**: 1061-1065.

69. Carter AB, Knudtson KL, Monick MM, Hunninghake GW (1999) The p38 mitogen-activated protein kinase is required for NF-kappaB-dependent gene expression. The role of TATA-binding protein (TBP). *J Biol Chem* **274**: 30858-30863.

70. Hoffmann A, Baltimore D (2006) Circuitry of nuclear factor kappaB signaling. *Immunol Rev* **210**: 171-186.

71. Piechota-Polanczyk A, Fichna J (2014) Review article: the role of oxidative stress in pathogenesis and treatment of inflammatory bowel diseases. *Naunyn Schmiedebergs Arch Pharmacol* **387**: 605-620.

72. Balmus IM, Ciobica A, Trifan A, Stanciu C (2016) The implications of oxidative stress and antioxidant therapies in inflammatory bowel disease: clinical aspects and animal models. *Saudi J Gastroenterol* **22**: 3-17.

73. Ferguson LR (2010) Chronic inflammation and mutagenesis. *Mutat Res* **690**: 3-11.

74. Koutroubakis IE, Malliaraki N, Dimoulios PD, Karmiris K, Castanas E, et al. (2004) Decreased total and corrected antioxidant capacity in patients with inflammatory bowel disease. *Dig Dis Sci* **49**: 1433-1437.

75. D'Odorico A, Bortolan S, Cardin R, D'Inca' R, Martines D, Ferronato A, Sturniolo GC (2001) Reduced plasma antioxidant concentrations and increased oxidative DNA damage in inflammatory bowel disease. *Scand J Gastroenterol* **36**: 1289-1294.

76. Zhu H, Li YR (2012) Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. *Exp Biol Med (Maywood)* **237**: 474-480.

77. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE (2014) Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev* **94**: 329-354.

78. Pereira C, Gracio D, Teixeira JP, Magro F (2015) Oxidative stress and DNA damage: implications in inflammatory bowel disease. *Inflamm Bowel Dis* **21**: 2403-2417.

79. Lambeth JD, Neish AS (2014) Nox enzymes and new thinking on reactive oxygen: a double-edged sword revisited. *Annu Rev Pathol* **9**: 119-145.

80. Sklyarov AY, Panasyuk NB, Fomenko IS (2011) Role of nitric oxide-synthase and cyclooxygenase/lipooxygenase systems in development of experimental ulcerative colitis. *J Physiol Pharmacol* **62**: 65-73.

81. Bouayed J, Bohn T (2010) Exogenous antioxidants--double-edged swords in cellular redox state: health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxid Med Cell Longev* **3**: 228-237.

82. Li AN, Li S, Zhang YJ, Xu XR, Chen YM, et al. (2014) Resources and biological activities of natural polyphenols. *Nutrients* **6**: 6020-6047.

83. Li W, Khor TO, Xu C, Shen G, Jeong WS, et al. (2008) Activation of Nrf2-antioxidant signaling attenuates NFkappaB-inflammatory response and elicits apoptosis. *Biochem Pharmacol* **76**: 1485-1489.

84. Perez S, Talens-Visconti R, Rius-Perez S, Finamor I, Sastre J (2017) Redox signaling in the gastrointestinal tract. *Free Radic Biol Med* **104**: 75-103.

85. Circu ML, Aw TY (2012) Intestinal redox biology and oxidative stress. *Semin Cell Dev Biol* **23**: 729-737.

86. Sands BE (2015) Biomarkers of inflammation in inflammatory bowel disease. *Gastroenterology* **149**: 1275-1285.

87. Quetglas EG, Mujagic Z, Wigge S, Keszthelyi D, Wachten S, et al. (2015) Update on pathogenesis and predictors of response of therapeutic strategies used in inflammatory bowel disease. *World J Gastroenterol* **21**: 12519-12543.

88. Mowat C, Cole A, Windsor A, Ahmad T, Arnott I, et al. (2011) Guidelines for the management of inflammatory bowel disease in adults. *Gut* **60**: 571-607.

89. Caprilli R, Latella G, Frieri G (2012) Treatment of inflammatory bowel diseases: to heal the wound or to heal the sick? *J Crohns Colitis* **6**: 621-625.

90. Pineton de Chambrun G, Peyrin-Biroulet L, Lemann M, Colombel JF (2010) Clinical implications of mucosal healing for the management of IBD. *Nat Rev Gastroenterol Hepatol* **7**: 15-29.

91. Rogler G (2015) Where are we heading to in pharmacological IBD therapy? *Pharmacol Res* **100**: 220-227.

92. Sonu I, Lin MV, Blonski W, Lichtenstein GR (2010) Clinical pharmacology of 5-ASA compounds in inflammatory bowel disease. *Gastroenterol Clin North Am* **39**: 559-599.

93. Dinis TC, Maderia VM, Almeida LM (1994) Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys* **315**: 161-169.

94. Heap GA, So K, Weedon M, Edney N, Bewshea C, et al. (2016) Clinical features and HLA association of 5-aminosalicylate (5-ASA)-induced nephrotoxicity in inflammatory bowel disease. *J Crohns Colitis* **10**: 149-158.

95. Katz JA (2004) Treatment of inflammatory bowel disease with corticosteroids. *Gastroenterol Clin North Am* **33**: 171-189.

96. Waljee AK, Wiitala WL, Govani S, Stidham R, Saini S, et al. (2016) Corticosteroid use and complications in a US inflammatory bowel disease cohort. *PLoS One* **11**: e0158017.

97. Targownik LE, Nugent Z, Singh H, Bernstein CN (2014) Prevalence of and outcomes associated with corticosteroid prescription in inflammatory bowel disease. *Inflamm Bowel Dis* **20**: 622-630.

98. Renna S, Cottone M, Orlando A (2014) Optimization of the treatment with immunosuppressants and biologics in inflammatory bowel disease. *World J Gastroenterol* **20**: 9675-9690.

99. Axelrad JE, Roy A, Lawlor G, Korelitz B, Lichtiger S (2016) Thiopurines and inflammatory bowel disease: current evidence and a historical perspective. *World J Gastroenterol* **22**: 10103-10117.

100. Herfarth HH, Kappelman MD, Long MD, Isaacs KL (2016) Use of methotrexate in the treatment of inflammatory bowel diseases. *Inflamm Bowel Dis* **22**: 224-233.

101. Naganuma M, Fujii T, Watanabe M (2011) The use of traditional and newer calcineurin inhibitors in inflammatory bowel disease. *J Gastroenterol* **46**: 129-137.

102. Chan HC, Ng SC (2017) Emerging biologics in inflammatory bowel disease. *J Gastroenterol* **52**: 141-150.

103. Sousa P, Allez M (2015) Complications of biologics in inflammatory bowel disease. *Curr Opin Gastroenterol* **31**: 296-302.

104. Nitzan O, Elias M, Peretz A, Saliba W (2016) Role of antibiotics for treatment of inflammatory bowel disease. *World J Gastroenterol* **22**: 1078-1087.

105. Shivashankar R, Lewis JD (2017) The role of diet in inflammatory bowel disease. *Curr Gastroenterol Rep* **19**: 22.

106. Rapozo DC, Bernardazzi C, de Souza HS (2017) Diet and microbiota in inflammatory bowel disease: the gut in disharmony. *World J Gastroenterol* **23**: 2124-2140.

107. Neumann PA, Rijcken E (2016) Minimally invasive surgery for inflammatory bowel disease: review of current developments and future perspectives. *World J Gastrointest Pharmacol Ther* **7**: 217-226.

108. Ferrari L, Krane MK, Fichera A (2016) Inflammatory bowel disease surgery in the biologic era. *World J Gastrointest Surg* **8**: 363-370.

109. Wirtz S, Neurath MF (2007) Mouse models of inflammatory bowel disease. *Adv Drug Deliv Rev* **59**: 1073-1083.

110. Lim KJ, Lee SJ, Kim S, Lee SY, Lee MS, et al. (2017) Comparable immune function inhibition by the infliximab biosimilar CT-P13: implications for treatment of inflammatory bowel disease. *J Crohns Colitis* **11**: 593-602.

111. Ostvik AE, Granlund A, Gustafsson BI, Torp SH, Espevik T, et al. (2014) Mucosal toll-like receptor 3-dependent synthesis of complement factor B and systemic complement activation in inflammatory bowel disease. *Inflamm Bowel Dis* **20**: 995-1003.

112. Hughes KR, Sablitzky F, Mahida YR (2011) Expression profiling of Wnt family of genes in normal and inflammatory bowel disease primary human intestinal myofibroblasts and normal human colonic crypt epithelial cells. *Inflamm Bowel Dis* **17**: 213-220.

113. Bein A, Zilbershtein A, Golosovsky M, Davidov D, Schwartz B (2017) LPS induces hyper-permeability of intestinal epithelial cells. *J Cell Physiol* **232**: 381-390.

114. Al-Ghadban S, Kaissi S, Homaidan FR, Naim HY, El-Sabban ME (2016) Cross-talk between intestinal epithelial cells and immune cells in inflammatory bowel disease. *Sci Rep* **6**: 29783.

115. Kim KM, Kim YS, Lim JY, Min SJ, Ko HC, et al. (2015) Intestinal anti-inflammatory activity of *Sasa quelpaertensis* leaf extract by suppressing lipopolysaccharide-stimulated inflammatory mediators in intestinal epithelial Caco-2 cells co-cultured with RAW 264.7 macrophage cells. *Nutr Res Pract* **9**: 3-10.

116. Banskota S, Regmi SC, Gautam J, Gurung P, Lee YJ, et al. (2017) Serotonin disturbs colon epithelial tolerance of commensal *E. coli* by increasing NOX2-derived superoxide. *Free Radic Biol Med* **106**: 196-207.

117. Antoniou E, Margonis GA, Angelou A, Pikouli A, Argiri P, et al. (2016) The TNBS-induced colitis animal model: an overview. *Ann Med Surg (Lond)* **11**: 9-15.

118. Munyaka PM, Rabbi MF, Khafipour E, Ghia JE (2016) Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbial dysbiosis in mice. *J Basic Microbiol* **56**: 986-998.

119. Ghia JE, Blennerhassett P, El-Sharkawy RT, Collins SM (2007) The protective effect of the vagus nerve in a murine model of chronic relapsing colitis. *Am J Physiol Gastrointest Liver Physiol* **293**: G711-718.

120. Zhang CL, Zhang S, He WX, Lu JL, Xu YJ, et al. (2017) Baicalin may alleviate inflammatory infiltration in dextran sodium sulfate-induced chronic ulcerative colitis via inhibiting IL-33 expression. *Life Sci* **186**: 125-132.

121. Goyal N, Rana A, Ahlawat A, Bijjem KR, Kumar P (2014) Animal models of inflammatory bowel disease: a review. *Inflammopharmacology* **22**: 219-233.

122. Holt DQ, Strauss BJ, Moore GT (2017) Patients with inflammatory bowel disease and their treating clinicians have different views regarding diet. *J Hum Nutr Diet* **30**: 66-72.

123. Haskey N, Gibson DL (2017) An examination of diet for the maintenance of remission in inflammatory bowel disease. *Nutrients* **9**: 259

124. Zhang PY (2015) Polyphenols in health and disease. *Cell Biochem Biophys* **73**: 649-664.

125. de Pascual-Teresa S (2014) Molecular mechanisms involved in the cardiovascular and neuroprotective effects of anthocyanins. *Arch Biochem Biophys* **559**: 68-74.

126. Wang S, Moustaid-Moussa N, Chen L, Mo H, Shastri A, et al. (2014) Novel insights of dietary polyphenols and obesity. *J Nutr Biochem* **25**: 1-18.

127. Lakey-Beitia J, Berrocal R, Rao KS, Durant AA (2015) Polyphenols as therapeutic molecules in Alzheimer's disease through modulating amyloid pathways. *Mol Neurobiol* **51**: 466-479.

128. Farzaei MH, Rahimi R, Abdollahi M (2015) The role of dietary polyphenols in the management of inflammatory bowel disease. *Curr Pharm Biotechnol* **16**: 196-210.

129. Bonaccio M, Pounis G, Cerletti C, Donati MB, Iacoviello L, et al. (2017) Mediterranean diet, dietary polyphenols and low grade inflammation: results from the MOLI-SANI study. *Br J Clin Pharmacol* **83**: 107-113.

130. Romier B, Schneider YJ, Larondelle Y, During A (2009) Dietary polyphenols can modulate the intestinal inflammatory response. *Nutr Rev* **67**: 363-378.

131. Martin DA, Bolling BW (2015) A review of the efficacy of dietary polyphenols in experimental models of inflammatory bowel diseases. *Food Funct* **6**: 1773-1786.

132. Smeriglio A, Barreca D, Bellocco E, Trombetta D (2016) Chemistry, pharmacology and health benefits of anthocyanins. *Phytother Res* **30**: 1265-1286.

133. Santos-Buelga C, Mateus N, De Freitas V (2014) Anthocyanins. Plant pigments and beyond. *J Agric Food Chem* **62**: 6879-6884.

134. Zamora-Ros R, Knaze V, Lujan-Barroso L, Slimani N, Romieu I, et al. (2011) Estimation of the intake of anthocyanidins and their food sources in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *Br J Nutr* **106**: 1090-1099.

135. Li D, Wang P, Luo Y, Zhao M, Chen F (2017) Health benefits of anthocyanins and molecular mechanisms: update from recent decade. *Crit Rev Food Sci Nutr* **57**: 1729-1741.

136. Wang H, Cao G, Prior RL (1997) Oxygen radical absorbing capacity of anthocyanins. *J Agric Food Chem* **45**: 304-309.

137. Serraino I, Dugo L, Dugo P, Mondello L, Mazzon E, et al. (2003) Protective effects of cyanidin-3-O-glucoside from blackberry extract against peroxynitrite-induced endothelial dysfunction and vascular failure. *Life Sci* **73**: 1097-1114.

138. Sorrenti V, Mazza F, Campisi A, Di Giacomo C, Acquaviva R, et al. (2007) Heme oxygenase induction by cyanidin-3-O-beta-glucoside in cultured human endothelial cells. *Mol Nutr Food Res* **51**: 580-586.

139. Paixão J, Dinis TC, Almeida LM (2011) Dietary anthocyanins protect endothelial cells against peroxynitrite-induced mitochondrial apoptosis pathway and Bax nuclear translocation: an in vitro approach. *Apoptosis* **16**: 976-989.

140. Paixão J, Dinis TC, Almeida LM (2012) Protective role of malvidin-3-glucoside on peroxynitrite-induced damage in endothelial cells by counteracting reactive species formation and apoptotic mitochondrial pathway. *Oxid Med Cell Longev* 2012: 428538.

141. Paixão J, Dinis TC, Almeida LM (2012) Malvidin-3-glucoside protects endothelial cells up-regulating endothelial NO synthase and inhibiting peroxynitrite-induced NF-kB activation. *Chem Biol Interact* **199**: 192-200.

142. Coussens LM, Werb Z (2002) Inflammation and cancer. *Nature* **420**: 860-867.

143. Seeram NP, Momin RA, Nair MG, Bourquin LD (2001) Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. *Phytomedicine* **8**: 362-369.

144. Afaq F, Saleem M, Krueger CG, Reed JD, Mukhtar H (2005) Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF-kappaB pathways and inhibits skin tumorigenesis in CD-1 mice. *Int J Cancer* **113**: 423-433.

145. Garcia-Alonso M, Minihane AM, Rimbach G, Rivas-Gonzalo JC, de Pascual-Teresa S (2009) Red wine anthocyanins are rapidly absorbed in humans and affect monocyte chemoattractant protein 1 levels and antioxidant capacity of plasma. *J Nutr Biochem* **20**: 521-529.

146. Fang J (2014) Some anthocyanins could be efficiently absorbed across the gastrointestinal mucosa: extensive presystemic metabolism reduces apparent bioavailability. *J Agric Food Chem* **62**: 3904-3911.

147. Fang J (2014) Bioavailability of anthocyanins. *Drug Metab Rev* **46**: 508-520.

148. Sodagari HR, Farzaei MH, Bahramsoltani R, Abdolghaffari AH, Mahmoudi M, et al. (2015) Dietary anthocyanins as a complementary medicinal approach for

management of inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol* **9**: 807-820.

149. Kanodia L, Borgohain M, Das S (2011) Effect of fruit extract of *Fragaria vesca* L. on experimentally induced inflammatory bowel disease in albino rats. *Indian J Pharmacol* **43**: 18-21.

150. Osman N, Adawi D, Ahrne S, Jeppsson B, Molin G (2008) Probiotics and blueberry attenuate the severity of dextran sulfate sodium (DSS)-induced colitis. *Dig Dis Sci* **53**: 2464-2473.

151. Biasi F, Deiana M, Guina T, Gamba P, Leonarduzzi G, et al. (2014) Wine consumption and intestinal redox homeostasis. *Redox Biol* **2**: 795-802.

152. Serra D, Paixão J, Nunes C, Dinis TC, Almeida LM (2013) Cyanidin-3-glucoside suppresses cytokine-induced inflammatory response in human intestinal cells: comparison with 5-aminosalicylic acid. *PLoS One* **8**: e73001.

153. Serra D, Almeida LM, Dinis TC (2016) Anti-inflammatory protection afforded by cyanidin-3-glucoside and resveratrol in human intestinal cells via Nrf2 and PPAR-gamma: comparison with 5-aminosalicylic acid. *Chem Biol Interact* **260**: 102-109.

Chapter 2

Cyanidin-3-glucoside improves the anti-inflammatory activity of 5-aminosalicylic acid in a lipopolysaccharide-activated RAW 264.7 macrophage cell line through inhibition of AP-1 and MAPK signalling pathways
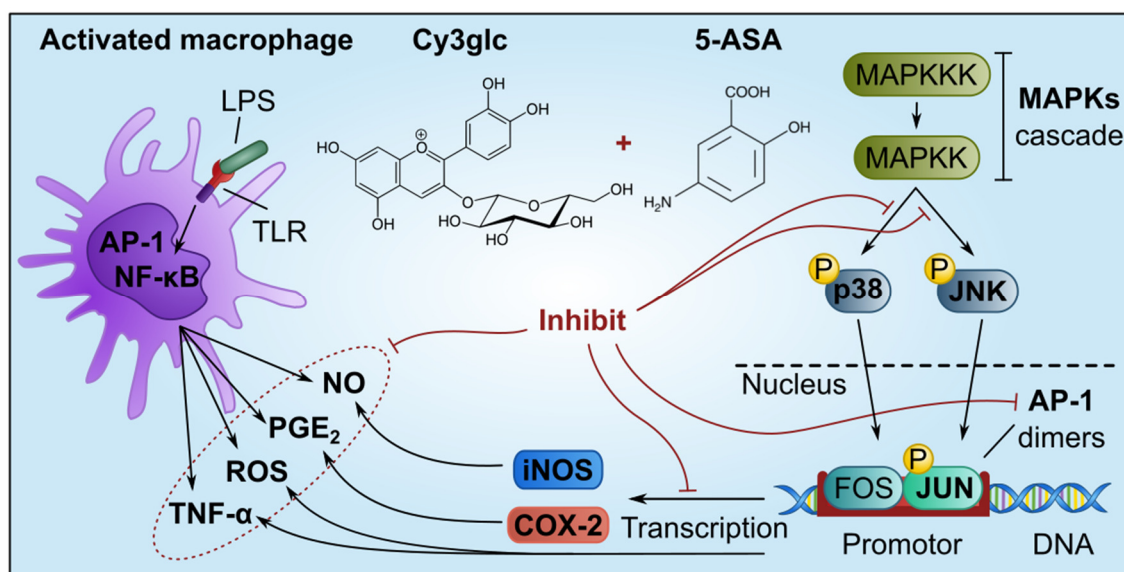
1. Abstract

The present study was aimed to investigate the anti-inflammatory action of cyanidin-3-glucoside (Cy3glc), an anthocyanin widely spread in diet, in comparison and association with 5-aminosalicylic acid (5-ASA), an anti-inflammatory reference drug in the context of inflammatory bowel diseases. The efficacy, action mechanism and interaction between Cy3glc and 5-ASA were assessed *in vitro*, using an *E. coli* LPS-activated RAW 264.7 macrophage cell line. Our data show a higher effectiveness of Cy3glc in counteracting LPS-induced nitric oxide ($^{\bullet}$NO) and prostaglandin E_2 (PGE_2) secretion, by inhibiting inducible nitric oxide synthase (iNOS) and cycloxigenase-2 (COX-2) expression, respectively, as compared to 5-ASA at the same concentration. In addition, the mixture (25 µM Cy3glc plus 25 µM 5-ASA) affords a better protection than 25 µM Cy3glc and a much better than 25 µM 5-ASA. Similar results were achieved regarding cellular reactive oxygen species (ROS) production. Although neither Cy3glc nor the mixture counteracted LPS-induced nuclear factor-κB (NF-κB) activation, Cy3glc plus 5-ASA strongly inhibited activator protein-1 (AP-1) translocation to the nucleus and prevented p38 mitogen-activated protein kinase and Jun N-terminal kinase (JNK) phosphorylation. Taken together these results suggest that the co-administration of Cy3glc and 5-ASA could be a potential strategy to control inflammation in patients with inflammatory bowel disease.

2. Introduction

Polyphenols have been reported to possess a wide range of relevant biological activities against diseases such as diabetes, cancer, cardiovascular and inflammatory diseases [1-3]. Although for several years, their biological activities have been closely related to their potent antioxidant properties, it is now clear that beyond such properties they modulate crucial signalling pathways and gene transcription, at very low concentrations, controlling several inflammatory cytokines and mediators [4, 5]. Previous work from our group have already demonstrated the impact of dietary anthocyanins, such as malvidin-3-glucoside and cyanidin-3-glucoside (cy3glc), on counteracting the expression of pro-inflammatory mediators in endothelial and epithelial intestinal cells, respectively [6-8].

Nevertheless, there is some controversy regarding the bioavailability of anthocyanins, since they seem to be not only absorbed in a low extent but also highly metabolized. In fact, it has been reported in an *in vitro* study that only around 3 to 4% of blueberry anthocyanins seem to be absorbed by human intestinal cells [9], which may be a limiting factor to their use in the context of inflammatory processes. However, this is not a true problem in the case of intestinal inflammatory bowel disease (IBD), once the intestine is the organ where dietary anthocyanins achieve the highest concentrations, and regular consumption of selected fruits such as berries, may lead to a high intake of these compounds [10].

IBD is a chronic, relapsing, idiopathic inflammation of the gastrointestinal tract (GI), which includes ulcerative colitis (UC) and Crohn's disease (CD), affecting approximately 1.5 and 2.2 million patients in USA and Europe, respectively. Since their incidence and prevalence have been clearly rising worldwide, IBD has been increasingly considered as an emerging global disease [11, 12]. Even though IBD etiology is not yet fully understood, it appears to be a multifactorial combination of genetic predisposition and environmental

triggers that can lead to an abnormal exacerbated immune response in the GI to a normally innocuous stimuli [13]. This leads to an upregulation of pro-inflammatory mediators, such as reactive oxygen and nitrogen metabolites, eicosanoids and chemokines, which play a crucial role in the disease onset and perpetuation of the inflammatory response [14]. In this context, innate immune cells such as macrophages have a key role, by migrating into target mucosal tissues and producing elevated levels of reactive species and pro-inflammatory cytokines, as compared to cells of healthy individuals [15, 16].

Treating IBD continues to be a challenge since it is not still available a specific causal treatment for this disease, and current therapeutic strategies aim to target the multiple inflammatory signalling cascades. Attempts to downregulate the uncontrolled immune response, mainly by using aminosalicylates, glucocorticoids, immunosuppressants and, more recently, biological drugs, have potential serious side effects that may limit their long-term use [17, 18]. As a result, the development of new effective and safer therapeutic alternatives remains a focus of interest and, in this context, the use of natural-derived products such as anthocyanins, may be a promising strategy. Despite the fact that these phytochemicals may be safer and promote health benefits [19-21], scientific evidences regarding its effectiveness and underlying action mechanisms need to be more elucidated.

Therefore, the aim of this work was to improve our knowledge on the use of Cy3glc, an anthocyanin widely-spread in our diet [22], as a natural anti-inflammatory agent and to explore a relatively new concept concerning the interaction between a natural product, and the synthetic drug 5-aminosalicylic acid (5-ASA) as a strategy to modulate intestinal inflammation. To achieve this goal, the study was carried out in an *in vitro* model of *E. Coli* LPS-induced inflammation in a RAW 264.7 macrophage cell line, and the action mechanisms were investigated by monitoring two of the most relevant signalling pathways controlling inflammation, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1).

3. Materials and methods

3.1. Reagents

General laboratory chemicals as well as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,7-dichlorofluorescein diacetate (DCFDA), 5-aminosalicylic acid (5-ASA) and lipopolysaccharide (LPS) (*Escherichia coli*, serotype 0111:B4) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Cyanidin-3-glucoside (Cy3glc) was obtained from Extrasynthèse (Genay, France).

Cell culture reagents, namely Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) pH 7.4, were purchased from Gibco-Invitrogen (Grand Island, NY, USA), except for penicillin and streptomycin, which were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Mouse monoclonal antibody against phosphorylated inhibitory κB-α (p-IκB-α) and β-actin and rabbit monoclonal antibody against inducible nitric oxide synthase (iNOS) and cycloxigenase-2 (COX-2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and against phosphorylated Jun N-terminal kinase (p-JNK), p-p38, and p-cJun were purchased from Cell Signaling Technology (Cell Signaling Technology Europe, Netherlands). Secondary antibodies were obtained from Abcam (Cambridge, UK).

3.2. Cell culture and treatment

Murine macrophage cell line RAW 264.7 was purchased from European Collection of Cell Cultures (Porton Down, Salisbury, UK). Cells were cultured in DMEM supplemented with 10% (v/v) FBS and 100U.mL^{-1} of penicillin and 100 μg.mL^{-1} streptomycin, on 75 cm^2 flasks, at 37ºC in a humidified atmosphere of 5% CO_2. Cells were counted using a

haemocytometer and the number of viable cells was determined by trypan blue dye exclusion.

Twenty-four hours prior to each experiment, cells were seeded in 60 mm wells ($1x10^6$ cells.mL^{-1}) for Western Blot analysis, and in 12-multiwells ($4x10^5$ cells.mL^{-1}) for all other assays. The medium was always replaced by fresh supplemented medium before cells treatment. Cells were treated with Cy3glc (25 µM), 5-ASA (25 µM) or both (25 µM Cy3glc plus 25 µM 5-ASA) twenty minutes before stimulation with LPS (1 µg.mL^{-1}). Thus, compounds and stimulus were present during the time of the experiment.

3.3. Nitric oxide production

Nitric oxide ($^{\bullet}$NO) production was evaluated by the amount of nitrite, a stable oxidized product of $^{\bullet}$NO, accumulated in the supernatants of cells submitted or not to treatment for 16 hours by a modified Griess method. Briefly, 100 µl of cell-culture medium was mixed with 100 µl of 1% (w/v) sulphanilamide in 5% (w/v) phosphoric acid for 5 min and 100 µl of 0.1% (w/v) naphthylethylenediamine dihydrochloride for an additional 5 min, at room temperature and protected from direct light. The absorbance was measured at 530 nm in a plate reader (Bio-TEK Synergy HT, Izasa S.A., Spain) and the concentration of nitrite was determined from a sodium nitrite standard curve. Fresh culture medium was used as a blank and all experiments were performed in triplicate.

3.4. Intracellular reactive oxygen species production

Intracellular reactive oxygen species (ROS) content was estimated by using the non-fluorescent probe 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) which permeates cell membrane and may be oxidized by ROS, yielding the 2′-7′-

dichlorofluorescein (DCF) [23]. Briefly, after cells treatments in 12-well platers (4×10^5 cells.mL^{-1}) for 16 hours, they were washed with PBS and new medium containing 1 μM DCFH-DA was added for 15 min at 37ºC, protected from direct light. After washing the cells, the fluorescence was measured using a plate reader (Bio-TEK Synergy HT, Izasa S.A., Spain) (excitation and emission wavelengths at 485 and 530 nm, respectively) and images were captured using an inverted fluorescence microscope coupled with a camera (Axiovert 40CFL coupled with Axiocam MRc5, Zeiss, USA) and a FITC filter.

3.5. Cell viability

Cell viability was assessed by the cellular dehydrogenase-dependent reduction of the yellow dye MTT to formazan, an insoluble intracellular purple product [24]. After incubation of the cells with Cy3glc or/and 5-ASA for 24 hours, the culture medium was removed and the cells were washed twice with PBS. MTT was added in the medium at a concentration of 0.5 mg.mL^{-1} for 1 hour at 37ºC. Finally, the supernatant was removed and the formazan crystals were dissolved in 500 μl of dimethyl sulfoxide (DMSO), and the absorbance was immediately read at 530 nm in a plate reader (Bio-TEK Synergy HT, Izasa S.A., Spain). Cell viability was expressed as a percentage of control cells, i.e., cells incubated for 24 hours but without compounds.

3.6. Prostaglandin E2 and TNF-alpha production

Prostaglandin E$_2$ (PGE$_2$) and tumour necrosis factor-alpha (TNF-α) production by RAW 264.7 was quantified by using competitive immunoassay kits, from Enzo Life Sciences (Farmingdale, New York) for PGE$_2$ (PGE$_2$ EIA kit) and from R&D Systems (Minneapolis, USA) for TNF-α. After cells treatment, under the specified conditions for

16 hours, aliquots of supernatant were collected and analysed according to the manufacturers' instructions. Determinations were performed in duplicate of four independent experiments and the results were normalized in terms of protein content, as evaluated by the Bradford method (Bio-Rad, USA).

3.7. Western blot analysis

The intracellular levels of p-IκB-α, iNOS, COX-2, p-JNK, p-p38, and p-cJun were assessed by Western-blotting. Briefly, following incubation under the specified conditions and for different time periods (8 min for p-IκB-α, 15 min for p-JNK and p-p38, 2 h for p-cJun, and 4 h for iNOS and COX-2), cells were washed twice with cold PBS. For total cellular protein extracts, washed cells were then scrapped and resuspended in ice-cold lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% (w/v) glycerol, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 1 mM PMSF, 1/100 (v/v) protease inhibitor cocktail) for 30 min, on ice. Lysates were subsequently centrifuged at 14000 rpm for 20 min at 4ºC and supernatants were collected and stored at -20ºC until used to explore iNOS, COX-2, p-JNK, and p-p38 expressions. For cytoplasmic proteins, the procedures were similar, but cells were lysed using an ice-cold buffer containing 10 mM Tris–HCl, 10 mM NaCl, 3 mM MgCl2, 0.5% Nonidet P-40 and 1% protease inhibitor cocktail, pH 7.5, for 10 min on ice. Lysates were then centrifuged at 5000 rpm for 5 min at 4ºC and supernatants (cytoplasmic extracts) were collected and stored at -20ºC until used to determine p-IκB-α. The pellets were used to extract the nuclear protein content, by resuspending them in ice-cold buffer containing 20 mM Hepes, 5 mM MgCl$_2$, 0.2 mM EDTA, 1 mM DTT, 300 mM NaCl, 20% (w/v) glycerol, and 1% protease inhibitor cocktail, pH 7.5 and left on ice for 30 min. The mixture was centrifuged at 14000 rpm for 20 min at 4ºC and the supernatants (nuclear extracts) collected and stored at -80ºC until used to explore p-cJun expression.

Cellular protein concentration was determined by using Bio-Rad protein assay dye (Bradford method), according to the manufacturer's specifications (Bio-Rad, USA). A range of 40-80 µg of reduced and denatured proteins were separated by electrophoresis on 10% SDS-polyacrylamide gel and blotted to polyvinylidene fluoride (PVDF) membranes (Amersham, Buckinghamshire, UK) for 2 h at 200 mA. After blocking non-specific sites with 5% (w/v) non-fat dried milk in TBS-T buffer [25mM Tris–HCl pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween 20] for 1 h at room temperature, membranes were incubated overnight at 4ºC with the target primary monoclonal antibodies, with gentle shaking. The membranes were then washed three times with TBS-T and further incubated for 1 h at room temperature with the phosphatase alkaline-labelled secondary antibodies. Immunoreactive complexes were detected by chemifluorescence after blots exposition to enhanced chemifluorescent reagent (Amersham Biosciences) in a Typhoon 9000 scanner (Amersham Biosciences). β-actin was used as protein loading control. Bands were analysed with the ImageQuant™ software from Amersham Biosciences (Molecular Dynamics).

3.8. Nuclear factor-κB DNA binding

To explore p65 subunit activation in the nucleus it was used an ELISA kit TransAM® from Active Motif (CA, USA). Briefly, after 30 min of treatment, cells were lysed in 300 µl of an ice-cold solution containing 10 mM Tris-HCl, 10mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 and 1% protease inhibitor cocktail, pH 7.5, for 10 min on ice. Lysates were centrifuged at 5000 rpm for 5 min at 4ºC and the supernatants (cytoplasmic extracts) were discarded. The pellet was dissolved in 30 µl of nuclear lysis buffer provided by Active Motif with 1% protease inhibitor cocktail, maintained in ice for another 10 min, and further centrifuged at 14000 rpm for 20 min at 4ºC, to obtain nuclear extracts. The supernatants

were collected and stored at -80ºC until use. NF-κB DNA binding was determined according to the manufacturer instructions, in 15 μg of protein per sample.

3.9. Fluorescence Confocal Microscopy

RAW 264.7 cells were seeded onto glass coverslips on 24 well plates and treated for 2 h, as described above. Then cells were washed twice with PBS and were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The blocking and permeabilization was performed simultaneously with PBS containing 0.1% saponin and 5% FBS for 30 min at room temperature. Cells were then incubated with a primary rabbit monoclonal antibody to p-cJun (1:250) overnight at 4ºC. After washing twice with PBS, cells were incubated with Alexa Fluor-488 conjugated secondary antibody (1:1000) for 1 h at room temperature. Finally, cells were again washed twice and the coverslips were mounted with glycerol and PBS containing the nucleic acid stain Hoechst (1 μg.mL^{-1}). Cells were examined under a confocal microscope (Ziess LSM 710) and representative pictures were taken.

3.10. Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) of at least three independent assays, each one performed in duplicate or triplicate. Differences between conditions were assessed using one-way analysis of variance (ANOVA), and Bonferroni´s test as post hoc. A value of $P<0.05$ was accepted as statistically significant.

4. Results

4.1. Cy3glc alone or in combination with 5-ASA decreases •NO and ROS production by LPS-stimulated RAW 264.7 cells in a concentration dependent way

To evaluate the potential anti-inflammatory action and to elucidate the underlying biochemical mechanisms of Cy3glc in comparison with 5-ASA and their eventual synergistic effect, the assay conditions were previously ascertained in LPS-stimulated RAW 264.7 cells, as a model of activated macrophages. It is well known that these activated cells produce and secrete high amounts of inflammatory mediators such as •NO and reactive oxygen species [25,26]. Thus, after cells incubation with several concentrations of LPS, the productions of •NO and ROS were evaluated by the Griess and DCFH-DA methods respectively, at two time points (16 h and 24 h) and the potential anti-inflammatory activity of several concentrations of Cy3glc and 5-ASA was tested in terms of their inhibitory effect on •NO production upon 16 h incubation. On basis of the results obtained (supplementary material) the LPS concentration of 1 $\mu g.mL^{-1}$ and two concentrations of Cy3glc and 5-ASA, 12.5 and 25 μM, were chosen for further experiments.

As shown in **Fig 1**, LPS stimulation led to a statistical significant increase in either •NO (**Fig 1A**) or ROS (**Fig 1B** and **C**) production, which was prevented by both selected concentrations of Cy3glc. In contrast, 5-ASA treatment at the same concentrations, did not present any significant effect, except at the concentration of 25 μM that led to a statistically significant small decrease in •NO production (**Fig 1A**). However, when in combination with Cy3glc the decrease in •NO and ROS production is statistically much higher. Moreover, in the concentration combination of 50 μM (25 μM Cy3glc and 25 μM 5-ASA), the inhibitory effects in both mediators were even higher than those with Cy3glc alone. The combination index calculated based on the Chou-Talalay method using the CompuSyn software was

lower than 1, suggesting a synergistic effect between Cy3glc and 5-ASA (supplementary material).
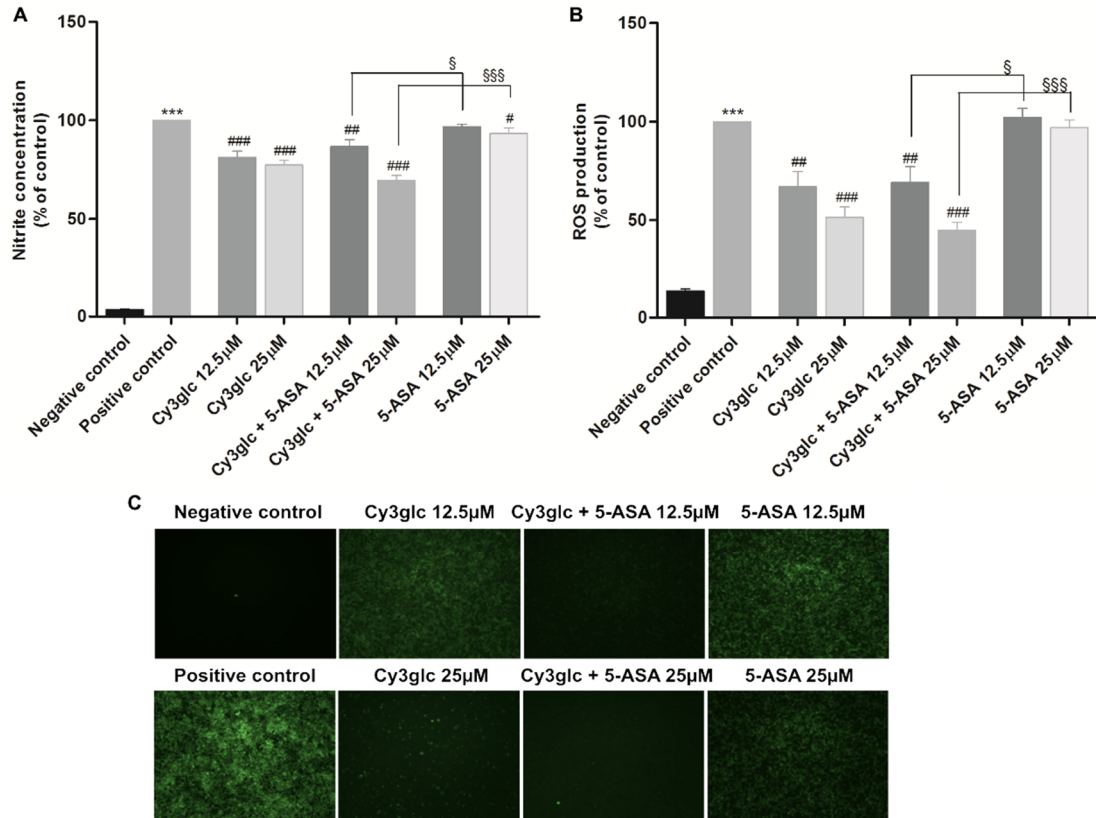
Figure 1: Effects of Cy3glc, 5-ASA or both on LPS-induced •NO and ROS production by RAW 264.7 cells. Cells were pre-incubated with Cy3glc and/or 5-ASA (12.5 and 25 μM) for 20 min and then were stimulated with LPS (1 μg.mL^{-1}) for 16 hours. (**A**) •NO production was evaluated in the supernatant by the Griess reaction and expressed as percentage of positive control. (**B**) ROS intracellular levels were determined in adherent cells by oxidation of DCFH-DA fluorescent probe and expressed in terms of fluorescence intensity relative to LPS-stimulated cells. (**C**) Representative images obtained by fluorescence microscopy (400x) are presented. Values are mean ± SEM of at least four independent experiments, each one assayed in triplicate. ***P<0.001 vs negative control (non-stimulated cells); #P<0.05, ##P<0.01, ###P<0.001 *vs* positive control (LPS-stimulated cells but without compounds); §P<0.05 and §§§P<0.05 combined effect *vs* 5-ASA *per se*.

The cytotoxicity of Cy3glc and 5-ASA was also evaluated upon 24 hours of RAW 264.7 cells incubation with the compounds by the MTT assay. As shown in **Fig 2**, neither Cy3glc nor 5-ASA *per se* or in combination affected cell viability. Also, no cellular morphological changes were observed by microscopic analysis (data not shown). On basis of these and the above assay results, the concentration of 25 µM was considered subtoxic for both Cy3glc and 5-ASA *de per se* and the same for 50 µM in combination (25 µM Cy3glc plus 25 µM 5-ASA). These concentrations were used in all the subsequent experiments.
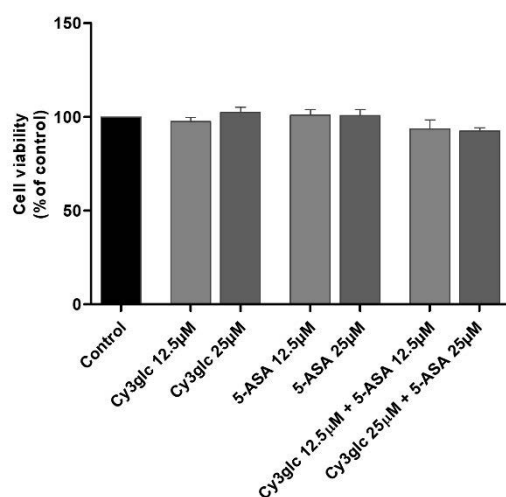
Figure 2: Effects of Cy3glc, 5-ASA or both on RAW 264.7 cells viability. Cell viability was assessed by MTT reduction, after 24 hours of cell incubation with the compounds and determined as percentage of control cells (without compounds). No statistical differences were observed.

4.2. Secretion of PGE$_2$ and TNF-alpha by LPS-stimulated macrophages is inhibited more efficiently by Cy3glc than by 5-ASA, being the mixture more effective than 5-ASA alone

To reinforce the ability of Cy3glc alone or in combination with 5-ASA to inhibit the secretion of pro-inflammatory mediators, the levels of PGE$_2$ and TNF-α produced by activated RAW 264.7 cells were also monitored.

Cells stimulation with 1 μg.mL^{-1} LPS for 16 h led to an increased production of PGE$_2$ (**Fig 3A**) and TNF-α (**Fig 3B**), which was significantly reduced by the treatment with 25 μM Cy3glc, in about 50% and 30%, respectively. 5-ASA, in similar concentration, showed no inhibitory effect on TNF-α production (**Fig 3B**) and a much lower reduction in PGE$_2$ secretion (about 26%) (**Fig 3A**). Combination of Cy3glc with 5-ASA significantly improved 5-ASA effect on both, PGE$_2$ and TNF-α productions, inducing a similar effect to that of Cy3glc alone in the first situation (**Fig 3A**). Regarding TNF-α, the mixture also significantly improved the effect of 5-ASA, although versus positive control it was not statistically significant (**Fig 3B**). In cells not exposed to the inflammatory stimulus, and incubated with Cy3glc, 5-ASA or both no increase in pro-inflammatory mediators' basal levels was observed (**Fig 3A** and **B**).
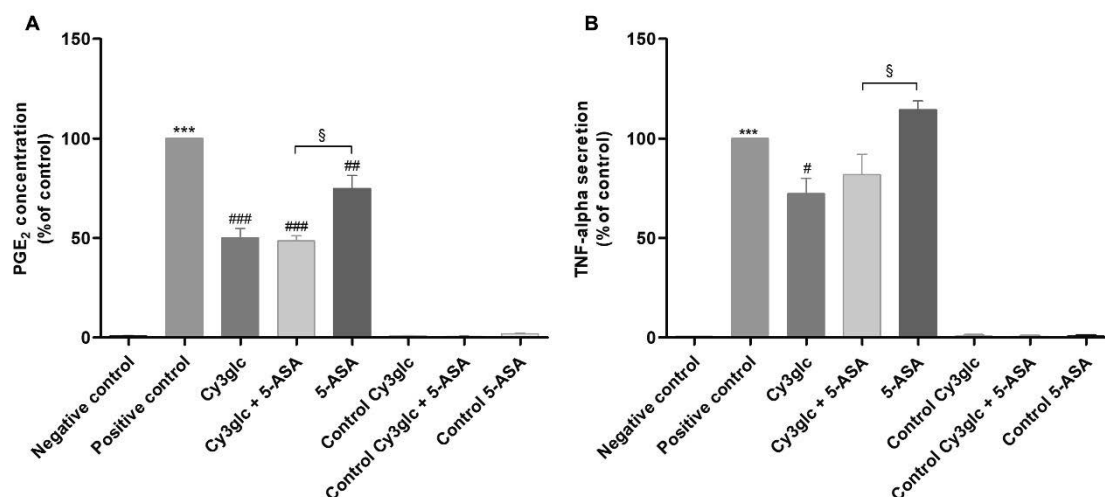
Figure 3: Effects of Cy3glc, 5-ASA or the combination of Cy3glc with 5-ASA on the production of PGE$_2$ and TNF-α in LPS-stimulated RAW264.7 macrophages. Cells were pre-incubated with Cy3glc (25 μM), 5-ASA (25 μM) or both, and then stimulated with LPS for 16 hours. PGE$_2$ (**A**) and TNF-α (**B**) production by cells was measured as described in section 2.6. Values are mean ± SEM of at least four independent experiments, each one assayed in triplicate. ***P<0.001 *vs* negative control (non-stimulated cells); #P<0.05, ##P<0.01, ###P<0.001 *vs* positive control (LPS-stimulated cells but without compounds); §P<0.05 *vs* 5-ASA.

4.3. Cy3glc and/or 5-ASA modulate LPS-induced expression of iNOS and COX-2 in RAW 264.7 cells

Bearing in mind that •NO and PGE$_2$ production are directly correlated with an overexpression of iNOS and COX-2 respectively [27,28], we further explored Cy3glc and/or 5-ASA ability to modulate both enzymes expression in RAW 264.7 macrophages.

Actually, iNOS (**Fig 4A**) and COX-2 (**Fig 4B**) were highly overexpressed after 4 h incubation with LPS, as assessed by Western-blotting. Treatment with 25 μM Cy3glc or 25 μM 5-ASA could strongly counteract both pro-inflammatory enzymes expressions. However, Cy3glc was more efficient than 5-ASA, decreasing by about 70% against 40% in the case of iNOS, and 50% against 23% in the case of COX-2. Regarding cells pre-treatment with both Cy3glc and 5-ASA in combination, the mixture afforded a better protection against both enzymes expression than Cy3glc alone and a much better protection than 5-ASA alone (in this case the inhibitory effect doubled). In non-stimulated

macrophages, the incubation with Cy3glc, 5-ASA or both in similar conditions did not change the expression of these pro-inflammatory enzymes (data not shown).
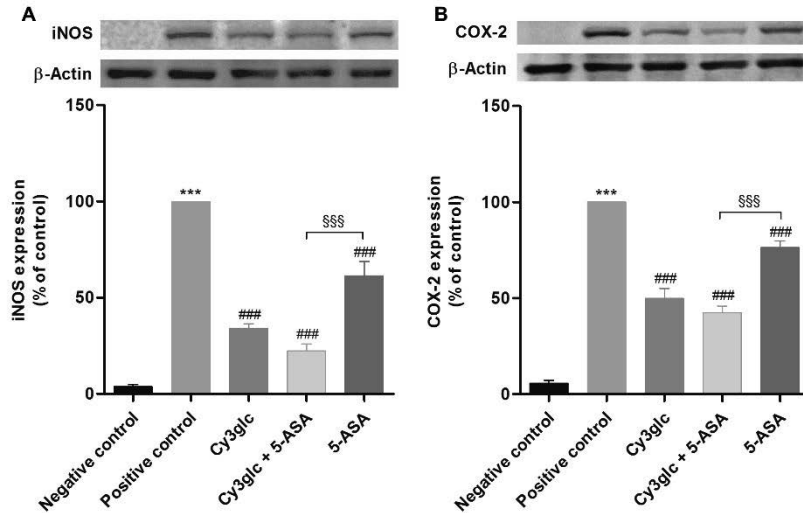
Figure 4: Effects of Cy3glc and/or 5-ASA on the LPS-induced expression of iNOS and COX-2 enzymes in RAW 264.7 cells. After the pre-incubation with Cy3glc and/or 5-ASA, cells were activated with LPS for 4 hours. Then, total extracts were collected and analyzed by Western blot for iNOS (A) and COX-2 (B) protein expressions. Results were normalized to endogenous b-actin and expressed in percentage of control, mean ± SEM of at least four experiments, and a representative blot is shown for each result. ***$P<0.001$ *vs* negative control (non-stimulated cells); ###$P<0.001$ *vs* positive control (LPS-stimulated cells but without compounds); §§§$P<0.001$ *vs* 5-ASA.

4.4. Cy3glc in combination with 5-ASA inhibits more efficiently LPS-induced AP-1 activation in RAW 264.7 cells via down-regulation of p38 and JNK MAPKs phosphorylation than Cy3glc or 5-ASA *de per se*

To investigate the mechanisms underlying the anti-inflammatory activity of Cy3glc in comparison with 5-ASA, their effects on the activities of the inflammation-mediated transcription factors NF-κB and AP-1 were evaluated.

It is well documented that macrophages stimulation by LPS leads to a rapid phosphorylation of IκB-α, the NF-κB inhibitor, followed by nuclear translocation of NF-κB

[29]. In our assay conditions, such activation was observed 8 min after LPS-stimulation by Western-blot analysis and is represented in **Fig 5A**. Cells pre-treatment with Cy3glc and/or 5-ASA, slightly decreased LPS-induced IκB-α phosphorylation but without statistical significance. As expected, 30 min after LPS-activation, p65 translocation to the nucleus occurred, as confirmed by NF-κB DNA binding and shown in **Fig 5B**. Similarly, the compounds in study *de per se* or in combination, were unable to counteract this effect.
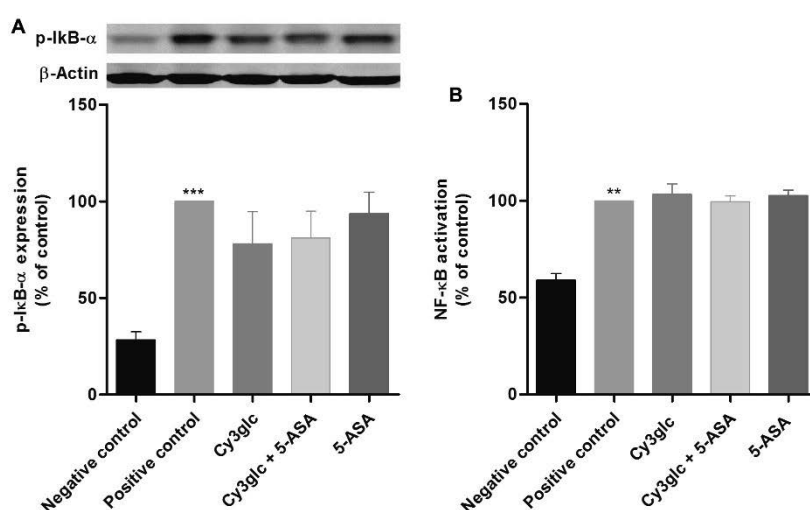
Figure 5: Effects of Cy3glc, 5-ASA or both on LPS-induced IκB-α phosphorylation and NF-κB activation in RAW 264.7 cells. After Cy3glc and/or 5-ASA pre-incubation, cells were further incubated with LPS for 8 min for IκB-α phosphorylation (**A**), and 30 min for NF-κB activation (**B**) analysis. IκB-α phosphorylation was evaluated in cytoplasmic extracts by Western blotting, as described in section 2.7. The results were normalized to endogenous β-actin and expressed in percentage of control. NF-κB activation was evaluated in nuclear extracts by a DNA-binding activity assay. The results were normalized to total protein and expressed in percentage of control, mean ± SEM of three independent experiments. **P<0.01, ***P<0.001 *vs* negative control (non-stimulated cells).

LPS stimulation together with an imbalanced ROS production are common triggers for MAPKs signalling pathway activation, upregulating the phosphorylation of MAPKs such as p38 and JNK [30,31]. The AP-1 family of transcription factors is a recognized downstream target of MAPK signalling pathway, and c-Jun subunit a direct target of JNK. When phosphorylated, c-Jun increases its transcriptional activity, thus regulating genes such as those encoding for iNOS and COX-2 [32].

Therefore, we next evaluated the effect of Cy3glc, 5-ASA and its combination on the phosphorylation of c-Jun. By Western blot analysis, it was possible to observe a great increase in the phosphorylated form of c-Jun in the cell nucleus (**Fig 6A**) after 2 h incubation with LPS, which was strongly suppressed (about 50%) only by the combination of Cy3glc plus 5-ASA. This effect was confirmed by nuclear protein immunofluorescent staining, and representative confocal pictures are shown in **Fig 6B**.
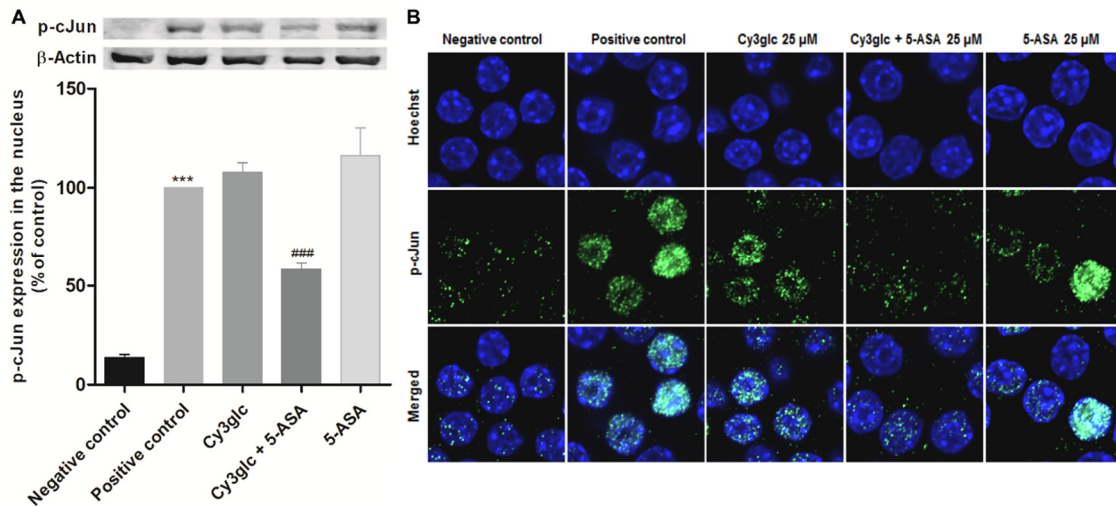
Figure 6: Effects of Cy3glc, 5-ASA or the combination of Cy3glc with 5-ASA on the activation of AP-1 subunit c-Jun in RAW 264.7 macrophages challenged by LPS. Cells were pre-incubated with 25 µM Cy3glc, 5-ASA or both (25 µM Cy3glc plus 25 µM 5-ASA) and then exposed to LPS for 2 hours. The levels of phosphorylated c-Jun were analyzed in nuclear extracts (**A**) as described in section 2.7. The results were normalized to endogenous β-actin and expressed as percentage of stimulated cells. Values are mean ± SEM of at least three independent experiments. $^{***}P<0.001$ *vs* negative control (non-stimulated cells); $^{###}P<0.001$ *vs* positive control (LPS-stimulated cells but without compounds). (**B**) Representative confocal microscopy pictures of non-stimulated (negative control), LPS-stimulated (positive control) and Cy3glc, 5-ASA or both pre-incubated RAW 264.7 macrophages. Simultaneous DNA labelling with Hoechst was performed to visualize the nuclear compartment.

To understand the molecular mechanism underlying AP-1 inhibition by the combination of Cy3glc plus 5-ASA in LPS-stimulated macrophages, the effect on the MAPKs signalling pathway was examined, in terms of p38 and JNK activation. In fact, in RAW 264.7 cytoplasmic extracts, after 15 min incubation with LPS it was possible to observe a great increase in the phosphorylated forms of p38 (**Fig 7A**) and JNK MAPKs (**Fig 7B**). Previous treatment with Cy3glc or 5-ASA inhibited LPS-elicited phosphorylation of p38 and JNK MAPKs in a similar extent (about 25%), whereas the combination Cy3glc plus 5-ASA induces the strongest inhibitory effect (about 50%).
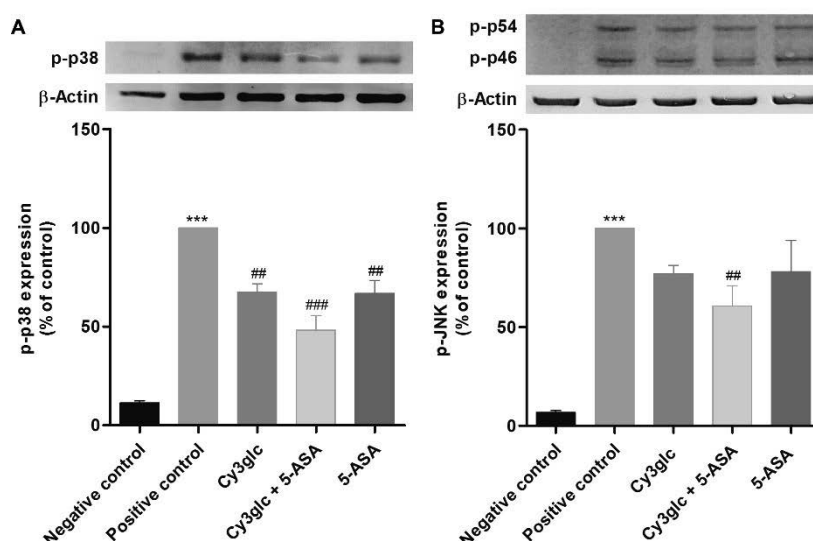
Figure 7: Effects of Cy3glc, 5-ASA or the combination of Cy3glc with 5-ASA on the phosphorylation of p38 and JNK in RAW 264.7 macrophages challenged by LPS. Cells were pre-incubated with 25 µM Cy3glc, 5-ASA or both (25 µM Cy3glc plus 25 µM 5-ASA) and then exposed to LPS for 15 min. The levels of phosphorylated p38 (**A**) and JNK (**B**) were analyzed in total cellular extracts as described in section 2.7. The results were normalized to endogenous β-actin and expressed as percentage of stimulated cells ± SEM, of at least three independent experiments. ***P<0.001 *vs* negative control (non-stimulated cells); ##P<0.01, ###P<0.001 *vs* positive control (LPS-stimulated cells but without compounds).

5. Discussion

Currently there is a substantial amount of evidence suggesting that many natural food components can modulate inflammation. This is particularly relevant in the context of chronic diseases where an association between inflammation mediated by inflammatory and immune cells is well established. Therefore, in this study we investigated the potential use of Cy3glc, one of the most abundant anthocyanins in our diet [22], as a strategy to modulate intestinal inflammation, using a macrophage cell line, RAW 264.7, as an inflammatory cell sensor. Moreover, we compared its efficiency with that of 5-aminosalicylic acid, the standard therapy for most IBD patients and explored their combination as a new therapeutic approach to mitigate inflammation.

Intestinal macrophages are key players during the inflammatory response and a central component of the pathogenesis of IBD. Defects in innate immune cells such as loss of tolerance to enteric commensal bacteria lead to the uncontrolled production of proinflammatory mediators [15]. Likewise, RAW 264.7 macrophage cell line, used as a model of inflammation, reacted to the bacterial LPS stimulation by increasing the secretion of several proinflammatory mediators, namely $^{\bullet}NO$, PGE_2, TNF-α and ROS.

In this model, we studied the effect and mechanism of Cy3glc in comparison and in combination with 5-ASA on LPS-stimulated $^{\bullet}NO$, ROS, PGE_2 and TNF-α. The results obtained showed that Cy3glc could significantly counteract all four mediators under study, in a much higher extent than 5-ASA, in similar concentrations and experimental conditions. This is particularly relevant since 5-ASA is a strong anti-inflammatory therapeutic agent commonly used as a first line drug in IBD patients. Furthermore, the combination of both compounds in the concentration combination of 50 µM (25 µM Cy3glc plus 25 µM 5-ASA) lead to an improvement of their individual efficacy in inhibiting the production of $^{\bullet}NO$ and ROS, even suggesting a synergistic effect between them.

In agreement with the results obtained in terms of $^{\bullet}$NO and PGE$_2$ production, Cy3glc demonstrated a much higher efficacy in reducing LPS-induced iNOS and COX-2 expressions than 5-ASA. Further, the simultaneous treatment with Cy3glc and 5-ASA improved their individual actions, particularly in reducing iNOS expression. This inhibitory effect is particularly relevant since inducible-derived $^{\bullet}$NO overproduction appears to contribute to the initiation and to the maintenance of intestinal inflammation, a condition that has been reported in IBD patients [33,34]. Also, recently it has been correlated with a very early onset of the disease [35]. Concerning COX-2, its overproduction has been largely described in the inflamed gut of IBD patients, being a target for several anti-inflammatory therapies, including 5-ASA, since this inducible form of cyclooxygenase is only overexpressed during inflammation [36,37].

In macrophages, LPS stimulation occurs via Toll-like receptor 4 (TLR4) activation, a pathogen-associated molecular pattern receptor, which triggers NF-κB signalling pathway [38]. This signalling pathway has been reported to be highly induced in the gut of IBD patients, and responsible for regulating the expression of relevant pro-inflammatory genes namely, those of iNOS and COX-2 [39,40]. In fact, in our assay conditions, RAW 267.4 macrophages rapidly reacted to LPS stimulation, inducing NF-κB activation, as observed by the fast and robust phosphorylation of IκB-α proteins, the NF-κB inhibitory molecules, with subsequent translocation to the nucleus and binding to DNA. However, according to previous results in human intestinal cells [8], treatment with either Cy3glc or 5-ASA *per se* or in combination, at the concentration used, did not efficiently prevent neither IκB-α phosphorylation, nor NF-κB activation.

Like NF-κB, AP-1 is another transcription factor involved in the expression of genes responsible for the inflammatory immune response, also regulating the expression of iNOS and COX-2 enzymes [32,41]. AP-1 is a homo- or hetero-dimeric protein complex composed of different Jun and Fos protein subfamilies. Phosphorylation of c-Jun increases

its transcriptional activity resulting in transcription of AP-1-driven target genes [42]. Therefore, we investigated whether Cy3glc and/or 5-ASA could prevent LPS-stimulated phosphorylation of c-Jun to comprise their anti-inflammatory activity. In the present study, neither 25 μM Cy3glc nor 25 μM 5-ASA revealed capacity to reduce the levels of phosphorylated c-Jun in the nuclear fractions of LPS-treated macrophages, as evaluated by Western-blot analysis, although some inhibitory effect has been visualized by confocal microscopy. However, together, in the concentration combination of 50 μM, they efficiently reduced phosphorylated c-Jun expression in the nucleus, demonstrating a real synergistic effect, and strengthening the observed effects on LPS-induced pro-inflammatory mediators.

Additionally, in macrophages, LPS stimulation triggers the activation of MAPKs such as p38 and JNK, upstream signaling molecules in the AP-1 pathway and with a vital role in AP-1 translocation [43,44]. In our experimental conditions, Cy3glc could counter the LPS-induced activation of these two MAPKs, by decreasing the phosphorylation of both p38 and JNK similarly to 5-ASA. Notably, the combination of Cy3glc with 5-ASA afforded an additional protection that may explain the greatest effect of the mixture on the suppression of AP-1 activation. Concerning MAPKs signalling activation, excessive LPS-induced ROS production may also aggravate the inflammatory response maintaining a feedback loop of activation [26]. Thus, the anti-oxidant efficacy of Cy3glc at countering ROS production may also account for the higher combined effect of Cy3glc and 5-ASA on the suppression of AP-1 transcriptional activation in LPS-stimulated cells.

6. Conclusion

In conclusion, our results show that the anthocyanin Cy3glc possesses relevant anti-inflammatory and anti-oxidant properties against LPS-induced inflammation when compared with 5-ASA, a commonly used anti-inflammatory drug in IBD. Moreover, they provide evidence about Cy3glc anti-inflammatory mechanism via inhibition of iNOS and COX-2 expressions, through suppression of nuclear AP-1 translocation, affecting JNK and p-38 MAPKs phosphorylation. In addition, our data highlight the synergistic interaction between Cy3glc and 5-ASA. These novel findings, regarding the simultaneous use of a polyphenol abundant in diet and a synthetic drug could be a good strategy for a robust control of inflammation in the pathology of chronic diseases, as is the case of IBD, and opens the possibility for using lower drug doses, thereby reducing adverse effects.

7. Supplementary material
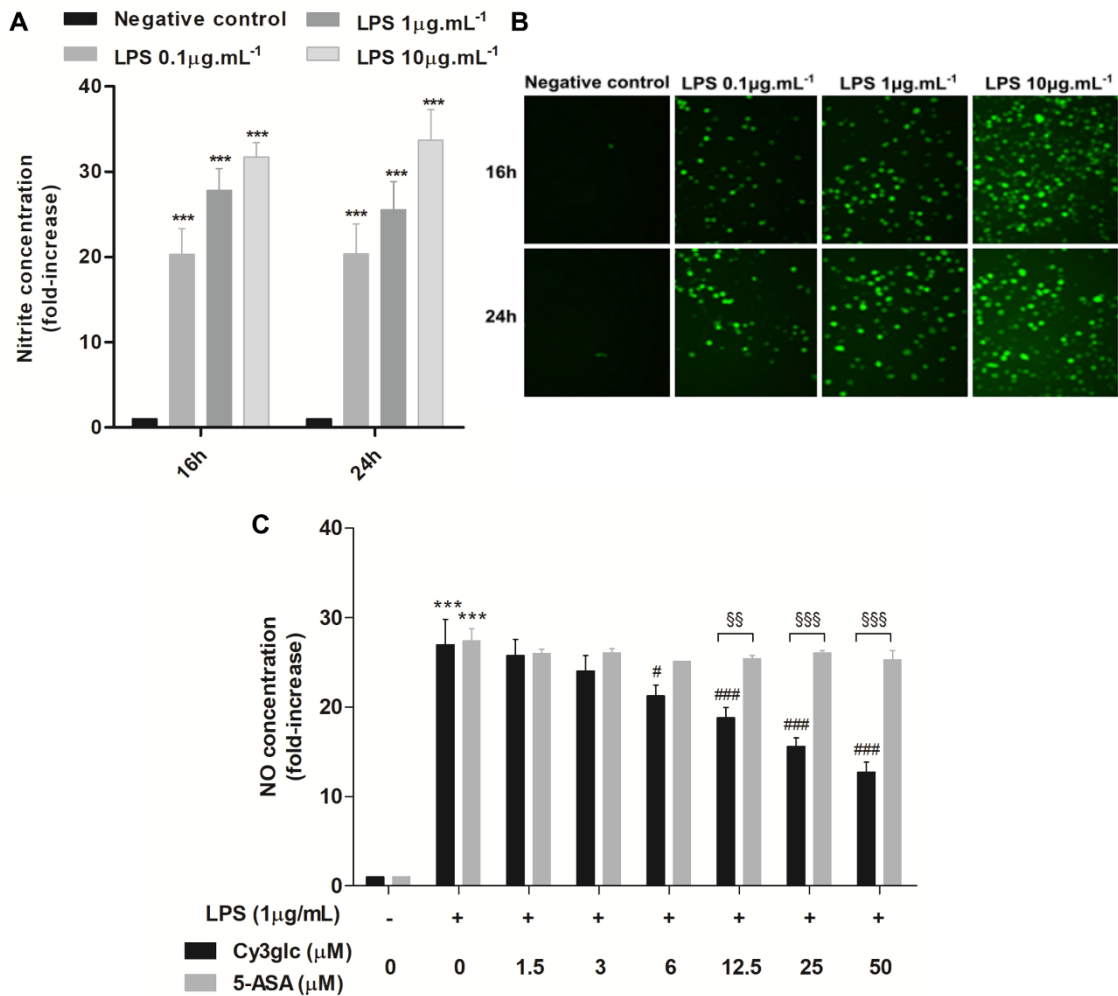
Sup. 1: Preliminary results to ascertain the assay conditions. LPS concentration and time dependent •NO production (**A**) and ROS production (**B**) and the effect of several concentrations of Cy3glc and 5-ASA on •NO production by LPS-activated macrophages (**C**). Values are mean ± SEM of at least three independent experiments, each one in duplicate. ***$P<0.001$ *vs* negative control (non-stimulated cells); #$P<0.05$, ###$P<0.001$ *vs* positive control (LPS-stimulated cells); §§$P<0.01$ and §§§$P<0.001$ Cy3glc *vs* 5-ASA.
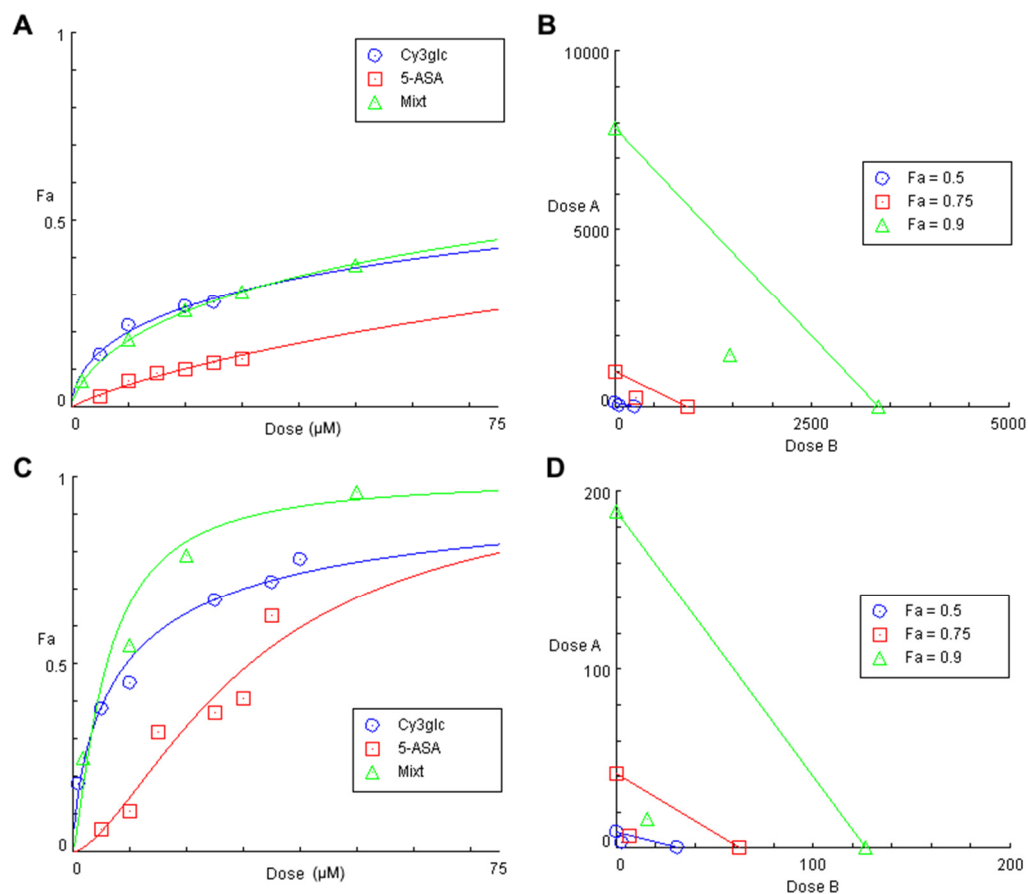
Sup. 2: Isobologram showing the interaction between Cy3glc and 5-ASA. The dose response curves for Cy3glc, 5-ASA and their mixture were traced in terms of nitrite (**A**) and ROS (**C**) reduction, by using CompuSyn software (version 1.0). In the isobologram method described by Chou and Talalay, the blue diagonal lines connecting the two 50% effective dose points is the theoretical line of additivity, for nitrite (**B**) and ROS (**D**) reduction, which indicate the synergistic effect of the mixture of cy3glc and 5-ASA.

8. References

1. Li D, Zhang Y, Liu Y, Sun R, Xia M (2015) Purified anthocyanin supplementation reduces dyslipidemia, enhances antioxidant capacity, and prevents insulin resistance in diabetic patients. *J Nutr* **145**: 742-748.

2. Giampieri F, Forbes-Hernandez TY, Gasparrini M, Alvarez-Suarez JM, Afrin S, et al. (2015) Strawberry as a health promoter: an evidence based review. *Food Funct* **6**: 1386-1398.

3. Sodagari HR, Farzaei MH, Bahramsoltani R, Abdolghaffari AH, Mahmoudi M, Rezaei N (2015) Dietary anthocyanins as a complementary medicinal approach for management of inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol* **9**: 807-820.

4. Esposito D, Chen A, Grace MH, Komarnytsky S, Lila MA (2014) Inhibitory effects of wild blueberry anthocyanins and other flavonoids on biomarkers of acute and chronic inflammation in vitro. *J Agric Food Chem* **62**: 7022-7028.

5. Biasi F, Astegiano M, Maina M, Leonarduzzi G, Poli G (2011) Polyphenol supplementation as a complementary medicinal approach to treating inflammatory bowel disease. *Curr Med Chem* **18**: 4851-4865.

6. Paixão J, Dinis TC and Almeida LM (2012) Malvidin-3-glucoside protects endothelial cells up-regulating endothelial NO synthase and inhibiting peroxynitrite-induced NF-kB activation. *Chem Biol Interact* **199**: 192-200.

7. Nunes C, Ferreira E, Freitas V, Almeida LM, Barbosa RM, Laranjinha J (2013) Intestinal anti-inflammatory activity of red wine extract: unveiling the mechanisms in colonic epithelial cells. *Food Funct* **4**: 373-383.

8. Serra D, Paixão J, Nunes C, Dinis TC and Almeida LM (2013) Cyanidin-3-glucoside suppresses cytokine-induced inflammatory response in human intestinal cells: comparison with 5-aminosalicylic acid. *PloS one* **8**: e73001.

9. Yi W, Akoh CC, Fischer J, Krewer G (2006) Absorption of anthocyanins from blueberry extracts by Caco-2 human intestinal cell monolayers. *J Agric Food Chem* **54**: 5651-5658.

10. Fang J (2014) Bioavailability of anthocyanins. *Drug Metab Rev* **46**: 508-520.

11. Malik TA (2015) Inflammatory bowel disease: historical perspective, epidemiology, and risk factors. *Surg Clin North Am* **95**: 1105-1122.

12. Vatn MH, Sandvik AK (2015) Inflammatory bowel disease. *Scandin J Gastroenterol* **50**: 748-762.

13. Loddo I, Romano C (2015) Inflammatory bowel disease: genetics, epigenetics, and pathogenesis. *Front Immunol* **6**: 551.

14. Algieri F, Zorrilla P, Rodriguez-Nogales A, Garrido-Mesa N, Banuelos O, et al. (2013) Intestinal anti-inflammatory activity of hydroalcoholic extracts of *Phlomis purpurea* L. and *Phlomis lychnitis* L. in the trinitrobenzenesulphonic acid model of rat colitis. *J Ethnopharmacol* **146**: 750-759.

15. Grainger JR, Konkel JE, Zangerle-Murray T, Shaw TN (2017) Macrophages in gastrointestinal homeostasis and inflammation. *Pflugers Archiv* **469**: 527-539.

16. Ohashi W, Hattori K, Hattori Y (2015) Control of macrophage dynamics as a potential therapeutic approach for clinical disorders involving chronic inflammation. *J Pharmacol Exp Ther* **354**: 240-250.

17. Devlin SM, Panaccione R (2010) Evolving inflammatory bowel disease treatment paradigms: top-down versus step-up. *Med Clin North Am* **94**: 1-18.

18. Triantafillidis JK, Merikas E, Georgopoulos F (2011) Current and emerging drugs for the treatment of inflammatory bowel disease. *Drug Des Devel Ther* **5**: 185-210.

19. Gasparrini M, Forbes-Hernandez TY, Giampieri F, Afrin S, Alvarez-Suarez JM, et al. (2017) Anti-inflammatory effect of strawberry extract against LPS-induced stress in RAW 264.7 macrophages. *Food Chem Toxicol* **102**: 1-10.

20. Chen T, Hu S, Zhang H, Guan Q, Yang Y, Wang X (2017) Anti-inflammatory effects of *Dioscorea alata* L. anthocyanins in a TNBS-induced colitis model. *Food Funct* **8**: 659-669.

21. Cassidy A, Rogers G, Peterson JJ, Dwyer JT, Lin H, Jacques PF (2015) Higher dietary anthocyanin and flavonol intakes are associated with anti-inflammatory effects in a population of US adults. *Am J Clin Nutr* **102**: 172-181.

22. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**: 727-747.

23. LeBel CP, Bondy SC (1990) Sensitive and rapid quantitation of oxygen reactive species formation in rat synaptosomes. *Neurochem Int* **17**: 435-440.

24. Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* **89**: 271-277.

25. Hwang SJ, Kim YW, Park Y, Lee HJ, Kim KW (2014) Anti-inflammatory effects of chlorogenic acid in lipopolysaccharide-stimulated RAW 264.7 cells. *Inflamm Res* **63**: 81-90.

26. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB (2010) Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* **49**: 1603-1616.

27. Feng GJ, Goodridge HS, Harnett MM, Wei XQ, Nikolaev AV, et al. (1999) Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: *Leishmania phosphoglycans* subvert macrophage IL-12 production by targeting ERK MAP kinase. *J Immunol* **163**: 6403-6412.

28. Kang SM, Lee J, Jin JH, Kim M, Lee S, et al. (2014) Synthesis and PGE(2) production inhibition of s-triazine derivatives as a novel scaffold in RAW 264.7 macrophage cells. *Bioorg Med Chem Lett* **24**: 5418-5422.

29. Wang S, Liu Z, Wang L, Zhang X (2009) NF-kappaB signaling pathway, inflammation and colorectal cancer. *Cell Mol Immunol* **6**: 327-334.

30. Zhong J, Kyriakis JM (2007) Dissection of a signaling pathway by which pathogen-associated molecular patterns recruit the JNK and p38 MAPKs and trigger cytokine release. *J Biol Chem* **282**: 24246-24254.

31. Racz B, Hanto K, Tapodi A, Solti I, Kalman N, et al. (2010) Regulation of MKP-1 expression and MAPK activation by PARP-1 in oxidative stress: a new mechanism for the cytoplasmic effect of PARP-1 activation. *Free Radic Biol Med* **49**: 1978-1988.

32. Coskun M, Olsen J, Seidelin JB, Nielsen OH (2011) MAP kinases in inflammatory bowel disease. *Clin Chim Acta* **412**: 513-520.

33. Guihot G, Guimbaud R, Bertrand V, Narcy-Lambare B, Couturier D, et al. (2000) Inducible nitric oxide synthase activity in colon biopsies from inflammatory areas: correlation with inflammation intensity in patients with ulcerative colitis but not with Crohn's disease. *Amino Acids* **18**: 229-237.

34. Sklyarov AY, Panasyuk NB, Fomenko IS (2011) Role of nitric oxide-synthase and cyclooxygenase/lipooxygenase systems in development of experimental ulcerative colitis. *J Physiol Pharmacol* **62**: 65-73.

35. Dhillon SS, Mastropaolo LA, Murchie R, Griffiths C, Thoni C, et al. (2014) Higher activity of the inducible nitric oxide synthase contributes to very early onset inflammatory bowel disease. *Clin Transl Gastroenterol* **5**: e46.

36. Singer II, Kawka DW, Schloemann S, Tessner T, Riehl T, Stenson WF (1998) Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease, *Gastroenterology* **115**: 297-306.

37. Sheibanie AF, Yen JH, Khayrullina T, Emig F, Zhang M, et al. (2007) The proinflammatory effect of prostaglandin E2 in experimental inflammatory bowel disease is mediated through the IL-23-->IL-17 axis. *J Immunol* **178**: 8138-8147.

38. Doyle SL, O'Neill LA (2006) Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. *Biochem Pharmacol* **72**: 1102-1113.

39. Ghosh S, Karin M (2002) Missing pieces in the NF-kappaB puzzle. *Cell* **s109**: 81-96.

40. Atreya I, Atreya R, Neurath MF (2008) NF-kappaB in inflammatory bowel disease. *J Intern Med* **263**: 591-596.

41. Hommes DW, Peppelenbosch MP, van Deventer SJ (2003) Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. *Gut* **52**: 144-151.

42. Vaiopoulos AG, Papachroni KK, Papavassiliou AG (2010) Colon carcinogenesis: learning from NF-kappaB and AP-1. *Int J Biochem Cell Biol* **42**: 1061-1065.

43. Guha M, Mackman N (2001) LPS induction of gene expression in human monocytes. *Cel Signal* **13**: 85-94.

44. Hess J, Angel P, Schorpp-Kistner M (2004) AP-1 subunits: quarrel and harmony among siblings. *J Cell Sci* **117**: 5965-5973.

Chapter 3

Comparison of anti-inflammatory activities of an anthocyanin-rich fraction from Portuguese blueberries (*Vaccinium corymbosum* L.) and 5-aminosalicylic acid in a TNBS-induced colitis rat model
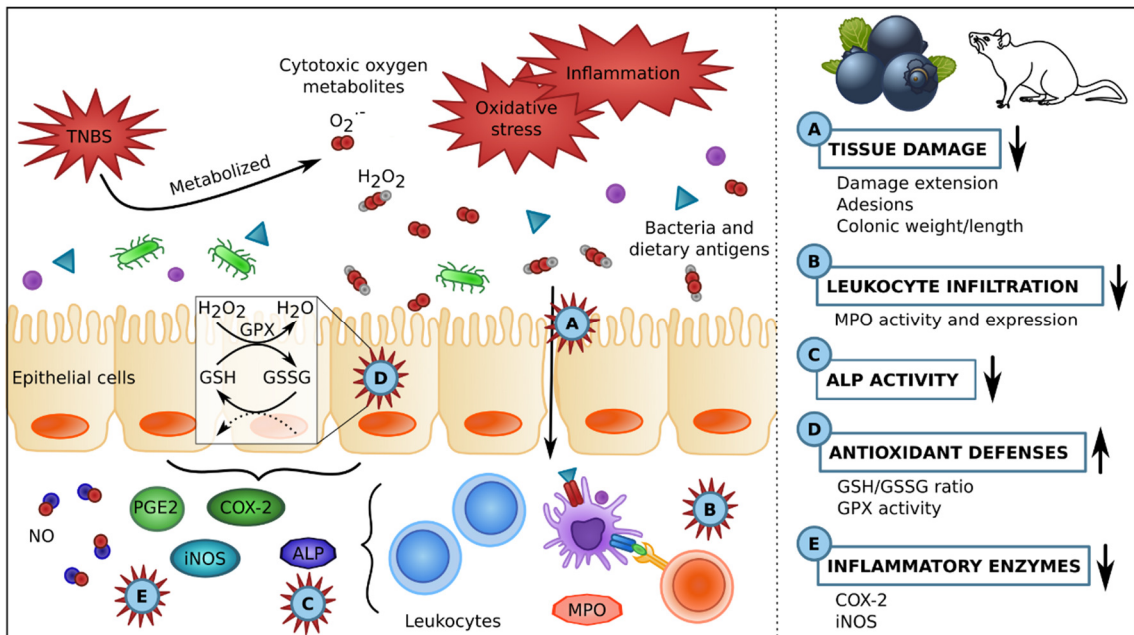
Sónia R. Pereira, Rita Pereira, Isabel Figueiredo, Victor Freitas, Teresa C. P. Dinis, Leonor M. Almeida (**2017**), *"Comparison of anti-inflammatory activities of an anthocyanin-rich fraction from Portuguese blueberries (Vaccinium corymbosum L.) and 5-aminosalicylic acid in a TNBS-induced colitis rat model"*, Published in PLoS One 12: e0174116.

1. Abstract

Despite the actual therapeutic approaches for inflammatory bowel disease (IBD), efficient and secure alternative options remain a research focus. In this context, anthocyanins seem promising natural anti-inflammatory agents, but their action mechanisms and efficacy as compared with established drugs still require more clarification. The main aim of this study was to compare the anti-inflammatory action of a chemically characterized anthocyanin-rich fraction (ARF), obtained from Portuguese blueberries (*Vaccinium corymbosum* L.), with that of 5-aminosalicylic acid (5-ASA), a first-line drug in IBD, in a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis rat model. Such fraction showed a high content and great molecular diversity of anthocyanins, with malvidin-3-galactoside and petunidin-3-arabinoside in the highest concentrations. After daily administration by intragastric infusion for 8 days, ARF, at a molar anthocyanin concentration about 30 times lower than 5-ASA, showed a higher effectiveness in counteracting the intestinal inflammation, as assessed by i) body weight variation and colon damage score, ii) reduction in leukocyte infiltration, iii) increase in antioxidant defenses and iv) by down-regulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in colon tissue homogenates. The strong inhibition of COX-2 expression seems to be a crucial anti-inflammatory mechanism common to both ARF and 5-ASA, but the additional higher abilities of anthocyanins to downregulate iNOS and to decrease leukocytes infiltration and to increase antioxidant defenses in colon may account for the much higher anti-inflammatory action of anthocyanins. These data may contribute to the development of a promising natural approach in IBD management.

2. Introduction

Inflammatory bowel disease (IBD) is a chronic and relapsing disorder of the gastrointestinal tract (GI), associated with an exacerbated intestinal immune response to an innocuous stimulus [1], leading to an upregulation of pro-inflammatory mediators which may trigger the onset and perpetuation of the disease [2].

Although several therapeutic strategies have been proposed [3], they are not free of adverse effects and so the development of new effective and safer ones remains a focus of interest. In this context, the use of natural products and dietary components has been gaining popularity for the treatment of inflammatory disorders [4–6], but scientific evidences regarding their efficacy and action mechanisms are still required.

Blueberries are among the fruits with potential health benefits associated with their high content in anthocyanins, a group of flavonoids widespread in human diet [7], which have been reported as potential therapeutic agents for several inflammatory diseases, including IBD [8,9]. Anthocyanins have been largely recognized by their potent antioxidant properties and ability to modulate crucial signalling pathways and gene regulation of several inflammatory enzymes and cytokines [5,9–13]. Therefore, beyond these modulatory roles, their antioxidant activity related to the capacity to scavenge reactive oxygen and nitrogen species or to activate cellular endogenous antioxidant systems [14,15], may be of major importance in countering the oxidative stress in IBD [16].

Despite the controversy regarding the bioavailability of anthocyanins [17,18], they can reach concentrations up to several hundred micromolar in the gastrointestinal tract [19], due to their abundance in diet and poor intestinal absorption. Recent studies have already shown that oral consumption of berry anthocyanins attenuates inflammation in mice models of colitis [20], but more information is required concerning their anti-inflammatory action mechanisms and efficacy as compared with standard anti-inflammatory drugs used in IBD patients.

100

Thus, in this context, we have already demonstrated, in an *in vitro* intestinal cell model, the higher anti-inflammatory activity of cyanidin-3-glucoside in comparison with 5-aminosalicylic acid (5-ASA) [13], a well-established anti-inflammatory drug in IBD. Now, in this work, we aimed to investigate the anti-inflammatory action of an anthocyanin-rich fraction (ARF) of Portuguese blueberries (*Vaccinium corymbosum* L.), in a TNBS-induced colitis rat model, in comparison with 5-ASA.

The efficacy of the treatments was firstly evaluated by body weight variations and extent of colonic mucosal injury. To gain a better insight into the anti-inflammatory mechanisms involved, we assessed in the colon tissue, i) the active myeloperoxidase (MPO) and the alkaline phosphatase (ALP) activity, as markers of inflammation, ii) the intracellular redox status, through evaluation of glutathione (GSH)/glutathione disulphide (GSSG) ratio and glutathione peroxidase (GPX) activity, and iii) the expression of the pro-inflammatory enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).

3. Materials and methods

3.1. Reagents

Laboratory chemicals, namely 5-aminosalicylic acid, 2,4,6-trinitrobenzenesulfonic acid solution (TNBS), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione reductase, L-glutathione reduced (GSH) and oxidized (GSSG) forms, β-NADPH, 5-sulfosalicylic acid, 2-vinylpyridine, 2,3-diaminonaphthalene (DAN), o-dianisidine dihydrochloride, hexadecyltrimethylammonium bromide (CTAB), protease inhibitor cocktail and general laboratory chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Microcon-10kDa centrifugal filters were obtained from Merck Millipore (Merck KGaA, Darmstadt, Germany).

Rabbit polyclonal antibody to iNOS was purchased from Cell Signalling Technology (Leiden, The Netherlands); rabbit polyclonal antibody to COX-2 was purchased from Abcam (Cambridge, UK); goat polyclonal antibody to MPO was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse monoclonal antibody to β-actin was purchased from Sigma-Aldrich (St Louis, Missouri, USA); anti-rabbit, anti-mouse and anti-goat IgG secondary antibodies were purchased from Abcam (Cambridge, UK).

3.2. Blueberry samples

The blueberries (*Vaccinium corymbosum* L, cultivar Bluecrop) were collected at the time of peak production, from the central region of Portugal. They were obtained from biological agriculture (Biogrêsso, Portugal) and kept frozen at -80ºC until use.

3.3. Preparation of anthocyanin-rich fraction

The anthocyanin-rich fraction was prepared according to Oszmianski, et al [21], as modified by Youdim (2002) [22]. Briefly, a total extract was obtained by homogenization of 30 g of the frozen fruits in 150 ml of methanol, acetone, water and formic acid mixture (40:40:20:0.1 v/v/v/v). Then, the extract was centrifuged at 2000g for 10 min and the supernatant was dried by rotatory evaporation at 40ºC under vacuum. The resulting residue was dissolved in deionized water and applied to an activated Sep-Pak C18 column (Waters Corporation, Milford, Massachusetts, USA). The column was then washed with 2 volumes of acidified water (0.01% HCl) to remove sugars and phenolic acids, and 2 volumes of ethyl acetate to elute other phenolic compounds. The anthocyanins were eluted only in the presence of acidified methanol (0.01% HCl) [23]. This fraction was then dried at low pressure, and further resolubilized in a 0.9% NaCl solution. The anthocyanin-rich extract was kept at -80ºC, under nitrogen, until use and will be further referred here as ARF.

3.4. Total phenols and total anthocyanins assays

The total content in phenolic compounds was assessed spectrophotometrically by the Folin-Ciocalteau reagent, as described by George *et al* [24]. Results were expressed as grams of gallic acid equivalents (GAE) per volume of ARF (L) and as milligrams of GAE per 100 g of blueberries.

Total monomeric anthocyanins in the ARF were estimated by a pH differential method described by Giusti et al [25], by using two buffer systems. The fraction was previously diluted at a ratio of 1:800 (v:v) either with 0.025 M KCl (pH 1) or with 0.4 M sodium acetate buffer (pH 4.5) and after an equilibration period (15 min), the absorbance spectrum of each solution was recorded between 400 and 700 nm in a Perkin Elmer

Lambda 45 spectrophotometer. The anthocyanin content was calculated in terms of malvidin-3-glucoside (Malv3glc) equivalent, using the maximum of absorbance measured around 530 nm, the molar absorptivity of 20200 and the molecular weight of 493.4. The results were expressed as grams of Malv3glc equivalents/L of ARF and as milligrams of Malv3glc equivalents/100 g blueberries.

3.5. HPLC-DAD

The extract was analysed by HPLC with direct injection of the samples (20 µL) (Hitachi L-2130) in a reverse phase C18 column with 250 x 4.6 mm i.d. (Merck KGaA, Darmstadt, Germany); the detection was performed at 511 nm, using a diode array detector (DAD) (Hitachi L-2455). The solvents used (mobile phase) were A: H_2O/HCOOH (9:1), and B: H_2O/CH3CN/HCOOH (6:3:1), in a gradient of 20-85% from solution B for 70 min at a flow rate of 1.0 mL/min. The column was then washed with 100% of solution B for 20 min. Before each use, the column was stabilized for the initial conditions for another 20 min [26].

3.6. Animals and experimental design

Four-week-old male Wistar rats were obtained from Charles River Laboratories (Barcelona, Spain), and maintained under standard conditions (temperature 24-25ºC, humidity 70-75%, lighting regimen of 12 h light/dark cycle), in plastic cages with free access to water and pellet food. Animal welfare and experimental procedures were carried out in accordance to the European Union regarding animal experimentation (Directive of the European Counsel 86/609/EEC) and approved by the Portuguese National Authority for Animal Health (Direcção-Geral de Alimentação e Veterinária - DGAV). All efforts were made to minimize the animals suffering and to reduce the number of animals used. An

early humane endpoint criteria was adopted: although weight loss was expected in this disease model, if this loss exceeded 20% of the initial weight, animals would be euthanized. The rats were randomly divided into four groups: (i) non-colitic control (n=10); (ii) TNBS-colitic control (n=10); (iii) TNBS-induced rats treated with ARF 10 mg.kg^{-1} (n=10); and (iv) TNBS-induced rats treated with 5-ASA 100 mg.kg^{-1} (n=10). The ARF and the therapeutic agent were administered solubilized in a mixture of 0.9% NaCl and 0.5% carboxymethylcellulose (vehicle), in a single dose by oral gavage, during 8 days after colitis induction by TNBS.

The dose of ARF used was chosen on basis of preliminary experiments, as the lowest to obtain safety and efficacy in the referred colitis rat model, and that of 5-ASA was as reported in literature [5,27] and taking into consideration the dose translation from animal to humans [28].

3.7. Induction of colitis

Colonic inflammation was induced as described by Siddiqui et al [29]. Rats were fasted overnight and were then anesthetized with ketamine (50 mg.kg^{-1}) and chlorpromazine (2.3 mg.kg^{-1}), to allow the rectal administration of TNBS. Briefly, the tip of a polyethylene catheter was advanced transanally 8 cm into the distal colon and a single dose of TNBS was instilled intraluminally (10 mg of TNBS dissolved in 0.25 mL of 50% ethanol) to induce colitis. The animals were maintained in a head-down position for approximately 60 seconds to prevent leakage of the infusate. Non-colitic control group was subjected to the same procedure, infusing a saline buffer instead of TNBS. After one day, the rats were treated for 8 days as assigned previously.

3.8. Macroscopic assessment and scoring of TNBS colitis severity

Rats were sacrificed by cervical dislocation under anaesthesia with ketamine (50 mg.kg^{-1}) and chlorpromazine (2.3 mg.kg^{-1}). The colon was excised, opened longitudinally and washed in saline solution. Afterwards, the colon segment was weighed and its length measured. Each colon was scored for macroscopically visible damage by two observers unaware of the treatment, according to the criteria reported by Bell *et al* [30] and described in (**Table 1**). Representative whole gut specimens were taken from the inflamed colon region, or its equivalent segment in the control group, and stored at -80ºC for subsequent biochemical measurements.

Table 1: Criteria for the colonic macroscopic damage score assessment.

Score	Criteria
0	No damage
1	Hyperemia, no ulcers
2	Linear ulcer with no significant inflammation
3	Linear ulcer with inflammation at one site
4	$\geq$2 sites of ulceration = inflammation
5	$\geq$2 major sites of ulceration and inflammation or one site of ulceration/inflammation extending >1cm along the length of the colon
6	If damage covers >2cm along the length of the colon, the score is increased by 1 for each additional centimeter of involvement

Criteria according to Bell *et al* [30].

3.9. Myeloperoxidase activity

Colonic mucosa was assayed for MPO activity as described by Stucchi *et al* [31], with slight modifications. Samples excised from each animal were thawed, and approximately 30-50 mg of mucosa was homogenized on ice with a Polytron tissue homogenizer in 4 mL of ice-cold 5 mM phosphate buffer (pH 6.0). To remove haemoglobin

and other blood products that could interfere with this assay, the homogenate was centrifuged at 30000g, for 30 min, at 4ºC and the supernatants discarded and the pellet resuspended in phosphate buffer, for three times. Finally, the pellet was solubilized in 10 volumes of ice-cold hexadecyltrimethylammonium bromide (0.5%) in phosphate buffer (50 mM, pH 6.0) and subjected to three cycles of freezing/thawing to extract the enzyme. The extract was allowed to stand at 4°C for 20 min and then centrifuged at 12500g for 15 min at 4ºC. MPO activity was evaluated in the supernatant, by mixing 10 µL of this supernatant with 2.99 mL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg.mL^{-1} of o-dianisidine dihydrochloride and 0.0005% of H_2O_2. The absorbance decrease of the reaction mixture was monitored at 460 nm for 2 min. Results were expressed as U MPO.mg^{-1} tissue, being one unit of MPO activity defined as the amount of enzyme that degrades 1 µmol of hydrogen peroxide per minute.

3.10. Alkaline phosphatase activity

ALP activity was measured in colon tissue samples by the method described by Bessey *et al* [32] as modified by Sanchez *et al* [33]. The samples were homogenized in cold saline solution (1:20 w/v) and centrifuged at 7000g for 10 min at 4ºC. The supernatants were used to determine spectrophotometrically the mucosal ALP activity, by mixing 100 µL of each sample with 1 mL of glycine (50 mM, pH 10.4), containing 5.5 mM p-nitrophenylphosphate and 0.5 mM magnesium chloride. After incubation for 30 min at 37ºC, 10 mL of 0.02 M sodium hydroxide were added to stop the enzymatic reaction, and the p-nitrophenol formed was measured at 405 nm. The protein content was determined by the Bradford reaction using the Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, California, USA), to express ALP activity as mU.g^{-1} of protein. One unit of ALP activity is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute.

3.11. Measurement of GSH/GSSG ratio

Total glutathione and GSSG were quantified in colon samples by an enzymatic recycling assay, essentially as described by Griffith *et al* [34]. The colon samples were previously treated by homogenization in extraction buffer containing 0.1% Triton X-100, 0.6% sulfosalicylic acid, 0.1 M KH_2PO_4 and 5 mM Na_2PO_4, pH 7.5), followed by centrifugation at 3000g, at 4ºC, for 10 min [35]. Total glutathione (GSH + GSSG) was assessed in the supernatant in a 96-well plate. Briefly, 20 µL of supernatant was added to 120 µL of freshly prepared 5 mM DTNB plus glutathione reductase (1:1 v/v) in 0.1 M potassium phosphate buffer and 60 µL of 2.4 mM β-NADPH, also prepared in the same phosphate buffer. The absorbance due to the yellow dianion formed by the reaction of thiol groups and DTNB, was immediately read at 412 nm, using a Synergy HT plate reader (Bio-Tek Instruments) and followed for 2 min, with reading intervals of 30 sec. For the GSSG determination, the procedure was similar but prior to the assay, the GSH was blocked by derivatization using 2-vinilpyridine, by incubating 100 µL of the tissue samples with 2 µL of this masking agent and 6 µL of triethanolamine, during 1 h at room temperature, in a fume hood. Colon glutathione content (total and oxidized) was calculated using concurrently run standard curves in nmol per mg of cellular protein, and expressed as GSH/GSSG ratio.

3.12. Measurement of glutathione peroxidase activity

The activity of GPX in colon samples was evaluated using a colorimetric assay kit purchased from Abcam (Cambridge, UK), which monitors GSH oxidation by recording the consumption of NADPH at 340 nm. Colonic tissue extraction and enzymatic activity were performed in accordance to the manufacturer's protocols and the results expressed in U per gram of colonic tissue.

3.13. Western-blot analysis of MPO, COX-2 and iNOS expressions

Colonic tissues were processed according to the method described by Sanchez-Fidalgo *et al* [36] slightly modified. Briefly, frozen colonic tissues were weighed and homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 8; 150 mM NaCl; 0.1% Triton X-100; 0.1% protease inhibitor cocktail; 1 mM PMSF; 0.5 mM EDTA; and 8 mM $MgCl_2$). Homogenates were incubated at 4ºC for 2 h with stirring, and then cell debris were removed by centrifugation (12000*g*, 20 min, at 4°C) and the supernatants (cytoplasmic extracts) were collected and stored at -80°C until use. Protein concentration was determined using the Bio-Rad protein assay kit. Aliquots of the supernatant, containing equal amount of reduced and denatured proteins (80 µg), were separated by SDS/PAGE electrophoresis in a 10% (v/v) acrylamide gel and further transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, UK) by electroblotting, using the Trans-Blot Turbo Transfer System of Bio-Rad (Hercules, California, USA). The membranes were blocked with skimmed milk in TBS-T buffer (20 mM Tris-HCl,150 mM NaCl, 0.1% Tween) to avoid non-specific binding. Afterwards the membranes were probed overnight, at 4ºC, with specific primary antibodies against MPO, COX-2, iNOS and β-actin. Each membrane was then washed three times with TBS-T and incubated with the respective alkaline phosphatase-conjugated secondary antibodies, for 1 h, at room temperature. Immunodetection was performed by chemifluorescence after blots exposition to enhanced chemifluorescent reagent in a Typhoon 9000 scanner (Amersham Biosciences, UK) and analysed with the ImageQuant™ software from Amersham Biosciences (UK). β-Actin was used as control for protein loading.

3.14. Statistical analysis

All results are expressed as the mean ± S.E.M. of at least 8 animals per group, and each sample assayed in duplicate. Differences between means were tested for statistical significance using a one-way or two-way analysis of variance (ANOVA), using Dunnett's multiple comparisons test as post hoc test. All statistical analysis was carried out with the GraphPad Prism version 5 (GraphPad Software, Inc., CA, USA) and values of $P<0.05$ were accepted as statistically significant.

4. Results

4.1. The blueberry extract showed a high content and a great diversity of anthocyanins

Anthocyanin analysis of the anthocyanin-rich fraction showed a high content of anthocyanins, about 10.28 ± 0.48 g/L, or 98.75 ± 5.71 mg/100g of fruit, in terms of Malv3glc. The total phenolic content of this fraction was 12.92 ± 1.59 g/L, or 119.96 ± 14.80 mg/100g of fruit, in terms of GAE. Moreover, using HPLC-DAD we could confirm that this fraction showed also a wide variety of anthocyanin molecules (15 peaks detected) as shown in **Fig 1**. The anthocyanins detected were, in descending order of amount, malvidin, petunidin, peonidin, delphinidin and cyanidin, conjugated with either galactose, glucose or arabinose, being malvidin-3-galactoside (20.93%) and petunidin-3-arabinoside (18.70%) the main anthocyanins detected (**Fig 1 inset**).

Total anthocyanins: 10.28 ± 0.48g.L^{-1}

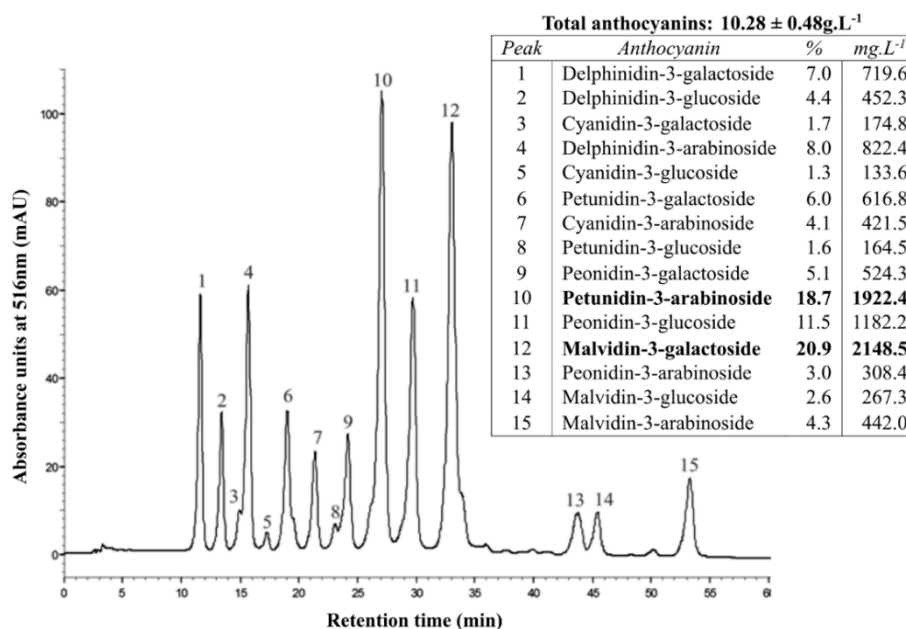| Peak	Anthocyanin	%	mg.L^{-1}
1	Delphinidin-3-galactoside	7.0	719.6
2	Delphinidin-3-glucoside	4.4	452.3
3	Cyanidin-3-galactoside	1.7	174.8
4	Delphinidin-3-arabinoside	8.0	822.4
5	Cyanidin-3-glucoside	1.3	133.6
6	Petunidin-3-galactoside	6.0	616.8
7	Cyanidin-3-arabinoside	4.1	421.5
8	Petunidin-3-glucoside	1.6	164.5
9	Peonidin-3-galactoside	5.1	524.3
10	**Petunidin-3-arabinoside**	**18.7**	**1922.4**
11	Peonidin-3-glucoside	11.5	1182.2
12	**Malvidin-3-galactoside**	**20.9**	**2148.5**
13	Peonidin-3-arabinoside	3.0	308.4
14	Malvidin-3-glucoside	2.6	267.3
15	Malvidin-3-arabinoside	4.3	442.0

Figure 1: HPLC-DAD chromatogram of blueberry anthocyanin-rich fraction. The peak identification and the contents of the different anthocyanins are indicated in the inset table.

4.2. The anthocyanin-rich fraction improved more efficiently than 5-ASA the recovery of body weight and the macroscopic colonic damage in TNBS-induced colitis in rats

During the study, the animals were constantly monitored for variations in body weight and general health. The inflammatory status in the TNBS-colitis control group was associated with severe anorexia and diarrhea, when compared to non-colitic control. Accordingly, as shown in **Fig 2A**, TNBS-colitis control group showed no significant weight gain with time, in contrast with the weight gain observed in healthy rats ($115.7 \pm 2.0\%$). Rats treated with ARF exhibited a significant recovery along the time, when compared with TNBS-colitis control group and the extent of recovery was clearly and statistically higher than that provided by 5-ASA, especially in the last two days. Notice that the observed benefits occurred at a concentration of anthocyanins about 30 times lower than that of 5-ASA (10 mg.kg^{-1} of ARF corresponds to 0.02 mmol.kg^{-1} in terms of Mal3glc, while 100 mg.kg^{-1} of 5-ASA corresponds to 0.65 mmol.kg^{-1}).

By the end of the experiment, the mortality in the TNBS-colitis control group was found to be very small (1/10), compared with the healthy, non-colitic control (0/10), indicating that the induced colonic injury was not fatal within 8 days, and neither the ARF nor 5-ASA, in the doses used, increased it. Additionally, neither TNBS nor the different treatments caused significant changes in the relative weights of the main organs (% of total body weight), such as liver, kidneys and heart (Supplementary Table) which are highly susceptible to drugs toxicity.
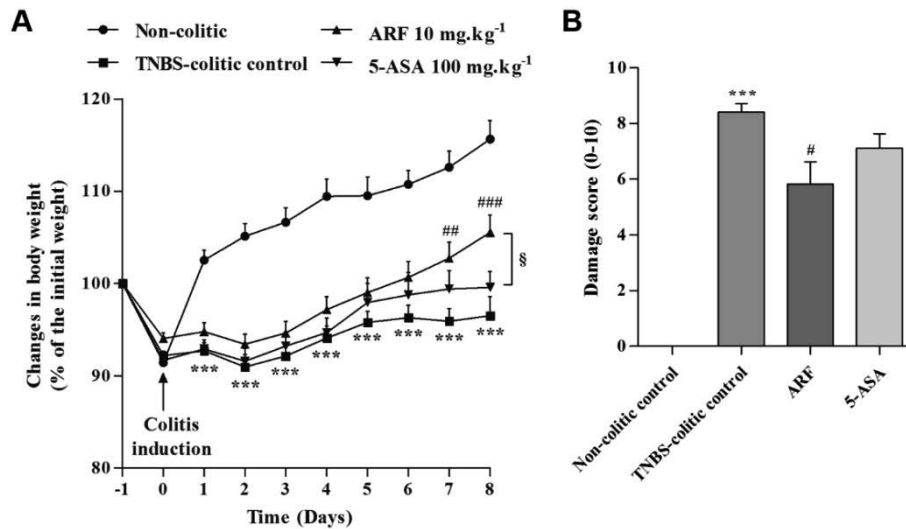
Figure 2: Effect of anthocyanin-rich fraction, as compared with 5-ASA, on body weight and on macroscopic colonic damage in rats with TNBS-induced colitis. After colitis induction, each group of animals was treated daily for 8 days with either ARF or 5-ASA and their effects on body weight were recorded overtime and expressed in terms of % of the initial weight (**A**). After colon collection (day 8 post TNBS-induced colitis), macroscopic colitis severity was blindly assessed and characterized in terms of damage score (**B**). Values are mean ± SEM of at least 8-9 animals per group. [***]$P<0.001$ *vs* non-colitic control; [#]$P<0.05$, [##]$P<0.01$, [###]$P<0.001$ *vs* TNBS-colitic control; [§]$P<0.05$ *vs* 5-ASA group.

The intracolonic administration of TNBS in ethanol induced colonic inflammation which was eight days later characterized by several events, namely: i) severe necrosis of the mucosa, typically extending 4-6 cm along the colon; ii) bowel wall thickening; iii) colon shortening; iv) hyperemia; and v) focal adhesions to adjacent organs, according to the scale represented in **Table 1**. Altogether, these data were correlated for each group of animals and shown in **Fig 2B**, indicating that the treatment with ARF counteracted the TNBS-induced damage score in a more efficient way than with 5-ASA. ARF also led to a very strong decrease in the colonic weight/length ratio (**Fig 3A**), being even more effective than 5-ASA, although in a much lower molar concentration than this drug. Representative colon pictures of each group are shown in **Fig 3B**.
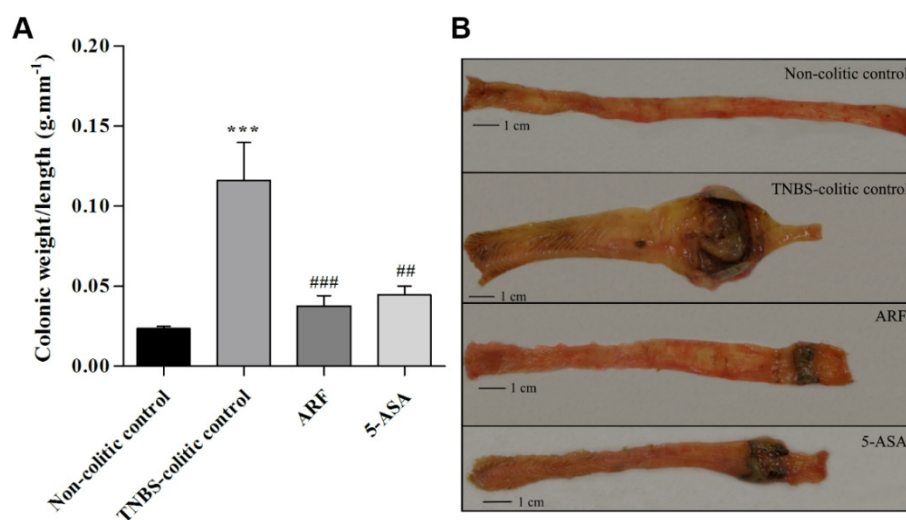
Figure 3: Effect of anthocyanin-rich fraction, as compared with 5-ASA, on the colon weight/length ratio of TNBS-induced colitic rats. On day 8, post TNBS-induced colitis, the colon of each animal was excised and evaluated in terms of weight and length. The colon weight vs length ratio for each group of animals is represented in graph **A**. Representative images of typical colons of each group are also shown (**B**). Values are mean ± SEM of at least 8–9 animals per group. [***]$P<0.001$ *vs* non-colitic control; [##]$P<0.01$, [###]$P<0.001$ *vs* TNBS-colitic control.

4.3. The anthocyanin-rich fraction inhibited the amount of active myeloperoxidase and the alkaline phosphatase activity, more efficiently than 5-ASA

The beneficial effects of our anthocyanin extract on TNBS-induced rat colitis were also studied biochemically in terms of well-established inflammatory markers. For this purpose, we analysed the MPO activity and its protein expression in colon sections from the different groups of rats, by the end of the experiment. The activity of this enzyme has been widely accepted as a strong marker of the inflammation degree, in terms of leukocyte infiltration in tissues [31]. As shown in **Fig 4**, TNBS led to a significant increase in either MPO activity (**A**) or its protein expression (**B**), when compared with the non-colitic control rats. Treatment with ARF, along 8 days after colitis induction, reduced significantly MPO activity (**Fig 4A**) and its protein expression (**Fig 4B**) in a higher extent than with 5-ASA, indicating a higher anti-inflammatory action.

Figure 4: Effect of anthocyanin extract administration on colon myeloperoxidase activity and expression as compared with 5-ASA, in TNBS-induced colitis. MPO activity (**A**) was measured in colon tissue homogenates and expressed in units of enzyme activity per mg of tissue. MPO protein expression (**B**) was assessed in colon tissue samples by Western blotting and expressed as fold increase relative to the non-colitic control group. Values are mean ± SEM from at least 8-9 animals per group, each one in duplicate. ***$P<0.001$ *vs* non-colitic control; #$P<0.05$, ##$P<0.01$, ###$P<0.001$ *vs* TNBS-colitic control.

Furthermore, we determined the ALP activity, since it has also been reported as a sensitive marker of inflammation in the intestine [33]. Results are shown in **Fig 5** and also indicated a significant capacity of anthocyanins to reduce this enzyme activity, in contrast with 5-ASA, whose effect was not statistically significant.
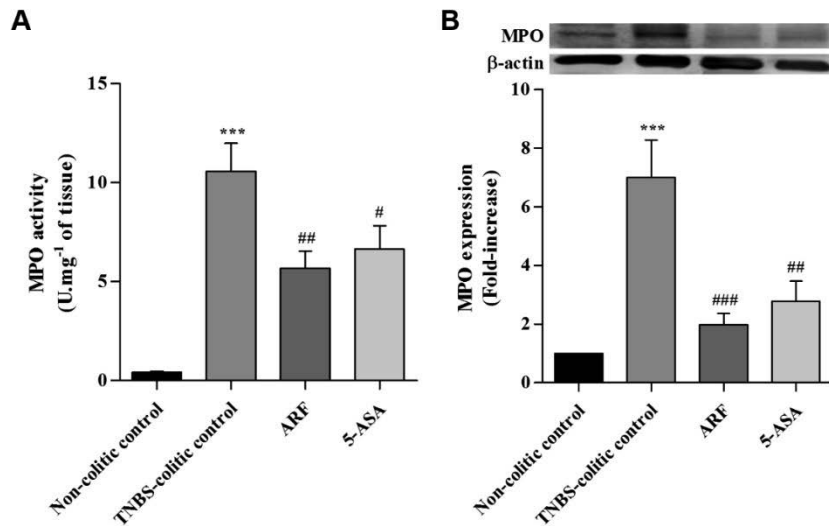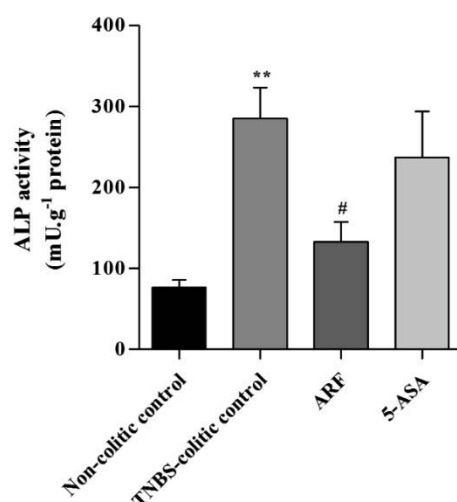
Figure 5: Effect of anthocyanin extract administration on colon alkaline phosphatase activity, as compared with 5-ASA, in TNBS-induced colitis. ALP activity was measured in colon tissue homogenates and expressed in units of enzyme activity per g of protein. Values are mean ± SEM from at least 8–9 animals per group, each one in duplicate. **P<0.01 *vs* non-colitic control; $^{#}$P<0.05 *vs* TNBS-colitic control.

4.4. The colonic antioxidant defenses were improved by the anthocyanin-rich fraction, more efficiently than by 5-ASA

An imbalance in the production of different reactive oxygen species, which may overwhelm the tissue antioxidant defenses, can be another molecular event involved in IBD [37]. Thus, to probe the colonic redox status, we decided to evaluate GSH content and GPX activity. In the TNBS-colitic control group, a great decrease (by about 65%) in the colonic GSH/GSSG ratio (**Fig 6A**), as well as in the GPX activity (by about 88%) (**Fig 6B**) was observed. ARF treatment significantly counteracted the induced decrease in GSH/GSSG ratio, indicating an improvement in the host antioxidant defenses, whereas 5-ASA was devoid of a significant effect, in the same experimental conditions (**Fig 6A**). Similar results were obtained for GPX activity (**Fig 6B**), showing a higher efficacy of anthocyanin fraction in recovering the antioxidant defenses, as compared with 5-ASA.
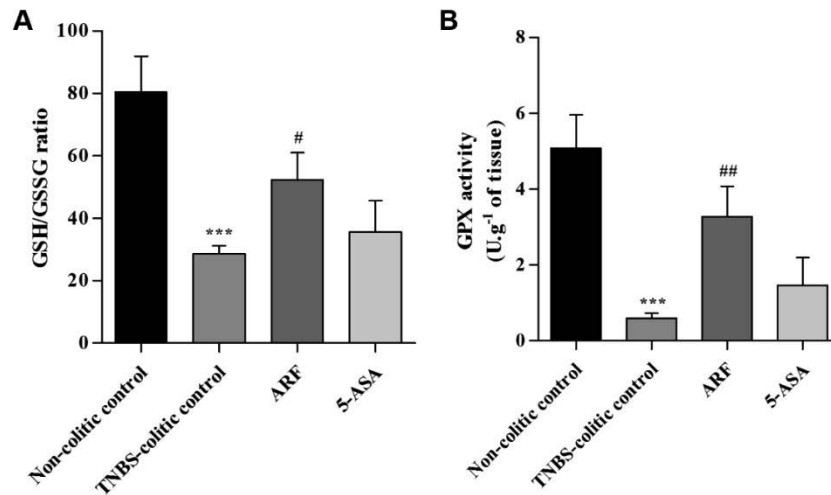
Figure 6: Effect of anthocyanin-rich fraction on colon GSH/GSSG ratio and GPX activity, as compared with 5-ASA, in TNBS-induced colitic rats. GSH/GSSG ratio and GPX activity were determined in samples of colonic tissue of each animal, as described in Materials and Methods. Statistics of GSH/GSSG ratio (**A**) and of GPX specific activity (**B**) for each group of animals are presented. Values are mean ± SEM obtained from at least 8-9 animals per group, each one in duplicate. [***]$P<0.001$ *vs* non-colitic control; [#]$P<0.05$, [##]$P<0.01$ *vs* TNBS-colitic control.

4.5. The anthocyanin-rich fraction strongly counteracted TNBS-stimulated expression of colon COX-2 and iNOS enzymes

The colonic inflammatory status was also characterized by a significant increase in the colonic COX-2 and iNOS protein expressions, relative to non-colitic control, as ascertained by Western blotting analysis from homogenates of inflamed tissue (**Fig 7**). Treatments with either ARF or 5-ASA strongly down-regulated COX-2 expression (**Fig 7A**), reverting it to control levels. This indicates that COX-2 inhibition is a relevant action pathway for anthocyanins, like for 5-ASA. Concerning the effects on iNOS expression, only the treatment with ARF could counteract drastically its increase in TNBS-induced colitis (by about 95%) whereas 5-ASA, in the dose used, did not show a significant effect (**Fig 7B**).
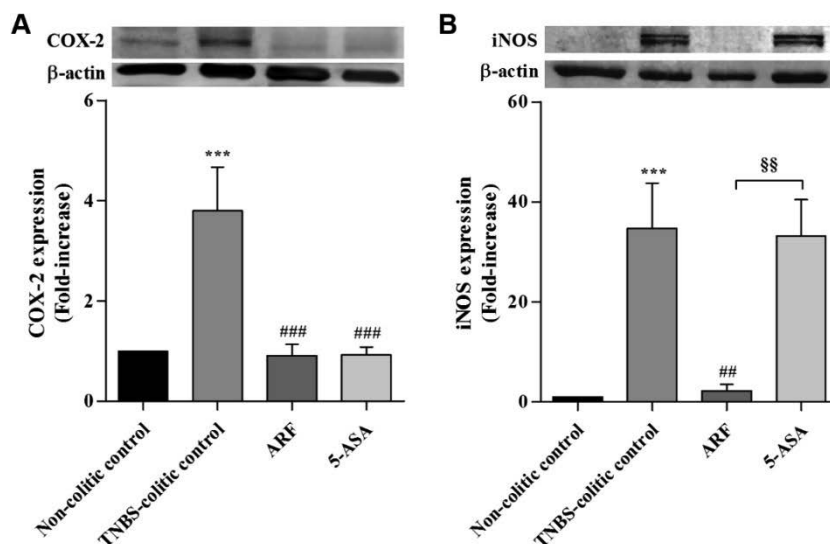
Figure 7: Comparison of the effects of anthocyanin-rich fraction and 5-ASA on COX-2 and iNOS protein expressions in colon tissue samples of colitic rats. COX-2 (**A**) and iNOS (**B**) protein expressions were evaluated by Western blotting in each animal of each group and expressed as fold increase relative to the non-colitic control group. Values are means ± SEM from of at least 8-9 animals per group, each one in duplicate. ***P<0.001 *vs* non-colitic control; ##P<0.01, ###P<0.001 *vs* TNBS-colitc control; §§P<0.01 *vs* 5-ASA group.

5. Discussion

The present study was undertaken mainly to evaluate the potential use of an anthocyanin-rich fraction obtained from Portuguese blueberry fruits for the treatment of intestinal inflammation, in a TNBS induced colitis rat model, in comparison with 5-ASA, the standard therapy for most IBD patients. Our data confirmed the anti-inflammatory action previously reported to blueberry anthocyanins [14,20] and showed, for the first time, that the protection afforded by this blueberry anthocyanin mixture in a colitis rat model was much more effective than that provided by 5-ASA, in similar experimental conditions.

The ARF used presented a high content and great variety of anthocyanins, which is in agreement with previously reported findings from different cultivars around the world [26,38,39]. However, the anthocyanin profile was somewhat different, with malvidin as the major class, instead of cyanidin, and malvidin-3-galactoside and petunidin-3-arabinoside in the highest concentrations (**Fig 1**). It is worthy of note that these two anthocyanins have been detected in higher amounts than others in the ileostomy fluids of test patients after blueberries consumption [40], suggesting their potential relevance in modulating the intestinal inflammatory process.

The results obtained in this rat model of colitis showed that either ARF (10 mg.Kg^{-1}) or 5-ASA (100 mg.Kg^{-1}) treatments counteracted the various signs of intestinal inflammation, including the very low rate of body weight gain, the colonic macroscopic damage score and the colon shortening, caused by TNBS (**Figs 2** and **3**). Nonetheless, the ARF treatment showed better improvements than 5-ASA, despite the much lower molar dose of anthocyanins used, indicating its higher effectiveness.

The intestinal anti-inflammatory potential of the anthocyanin-rich fraction was also demonstrated by the reduction of active MPO and by the inhibition of ALP activity. MPO is abundantly expressed in primary granules of neutrophils, thus, tissue-associated MPO activity and expression can be used as a fundamental and reliable biochemical marker of

leukocyte infiltration and of tissue recovery [41]. In agreement with the macroscopic results previously referred, ARF treatment significantly reduced, and more efficiently than 5-ASA, MPO activity and its expression stimulated by TNBS-induced colitis (**Fig 4A** and **4B**). Similarly, in what concerns intestinal ALP activity, ARF treatment demonstrated a better efficacy than 5-ASA in reducing this TNBS-induced enzyme activity (**Fig 5**). Currently, it is generally agreed that a tissue-nonspecific alkaline phosphatase isoform is essentially expressed in the colon and is upregulated in response to inflammation [33,42]. Also, in the inflamed colon, both colonocytes and infiltrating leukocytes account for the increased ALP activity. Moreover, it is known that this ALP isoform is specifically upregulated by oxidative stimulus [43]. Thus, since inflammation has been extensively associated with an increase in oxidative stress [37], the protection afforded by either anthocyanins or 5-ASA could be related to their well-known antioxidant properties [11,12,26,44].

On the other hand, TNBS-induced colitis has been associated with changes in antioxidant enzymes activity and in the levels of GSH [45], the major intracellular redox buffer, and decreases in GSH/GSSG ratio have been correlated with the severity of mucosal inflammation in IBD [46]. Our data showed a strong reduction in the intestinal GSH/GSSG ratio in the TNBS-induced colitis group, by about 65% (**Fig 6A**), indicating a severe decrease in intestinal redox status. In agreement with this, the intestinal GPX activity decreased drastically in the same group, by about 90% (**Fig 6B**). The relevance of this enzyme in the cellular antioxidant defense machinery is well known, by reducing hydroperoxides with the parallel oxidation of GSH to GSSG [16]. In this regard, the ARF treatment improved significantly either the GSH/GSSG ratio or the GPX activity in colon tissue, counteracting the TNBS-induced effects, in contrast with 5-ASA that did not significantly improve these oxidative stress markers (**Fig 6A** and **6B**). Thus, the higher beneficial effect of anthocyanins in alleviating the severity of TNBS-induced colitis may be related, in part, with the higher ability to strengthen colon antioxidant defenses.

Besides the increase in oxidative stress, the excessive production of pro-inflammatory mediators is a key player to the intestinal damage progression in IBD. Concerning such mediators, both iNOS and COX-2 enzymes seem to play a synergistic role in inflammation onset and severity [2]. Upregulation of these enzymes expression is known to be induced by activation of different signaling pathways, namely the nuclear factor κB (NF-κB) and the signal transducer and activator of transcription 1 (STAT1), whose expressions and activities have been reported to increase during intestinal inflammation [13,47,48]. This work demonstrated that the treatment with anthocyanins could counteract drastically the strong overexpression of both iNOS and COX-2 enzymes induced by TNBS, while 5-ASA only counteracted COX-2 overexpression. Actually, the strong inhibition of COX-2 expression seems to be a crucial common mechanism underlying the anti-inflammatory actions of 5-ASA and ARF (**Fig 7A**). This observation is highly relevant given that this inducible form of COX is known to be upregulated in the inflamed gut of IBD patients [49,50], being a target for several drugs used in IBD, including 5-ASA [51]. Regarding the effects on iNOS overexpression, the results obtained reinforced the much higher anti-inflammatory capacity of anthocyanins when compared to 5-ASA, as they inhibited almost completely the enzyme expression, in contrast with 5-ASA which did not show any significant effect (**Fig 7B**) despite being used in a much higher molar dose than that of anthocyanins.

In conclusion, our data show for the first time the stronger anti-inflammatory activity of an anthocyanin-rich fraction obtained from the blueberry *Vaccinium corymbosum* L., grown in Portugal, on TNBS-induced rat colitis, in comparison with 5-ASA, a reference anti-inflammatory drug in IBD. Such activity is complex, involving different mechanisms including decrease in leukocyte infiltration, increase in antioxidant defenses and down-regulation of proinflammatory enzymes. The strong inhibition of colon COX-2 expression seems to be a crucial anti-inflammatory mechanism common to 5-ASA and ARF, but the additional higher ability of anthocyanins to downregulate iNOS and to

decrease leukocytes infiltration and to increase antioxidant defenses in colon may account for the much higher anti-inflammatory action of ARF. Taken together, these results point to the efficacy of this anthocyanin mixture to counteract colitis severity and gather conditions to a faster recovery, being a contribution to the development of a promising natural therapeutic approach for intestinal inflammation.

6. Supporting information

Sup. Table: Statistics of liver, kidneys, and heart vs body weight (% of total body weight) for each group of animals. P-values were calculated using t-test analysis. $P^{(*)} > 0.05$, *vs* non-colitic control; $P^{(\#)} > 0.05$, *vs* TNBS-colitic control; Thus, no statistical significance was observed.

	Liver (%)	Right kidney (%)	Left kidney (%)	Heart (%)
Non-colitic control	3.94 ± 0.10	0.38 ± 0.01	0.36 ± 0.01	0.36 ± 0.02
TNBS-colitic control	4.20 ± 0.18; $P^{(*)} = 0.292$	0.40 ± 0.01; $P^{(*)} = 0.290$	0.38 ± 0.01; $P^{(*)} = 0.161$	0.40 ± 0.02; $P^{(*)} = 0.140$
ARF 10mg.kg^{-1}	4.46 ± 0.18; $P^{(\#)} = 0.321$	0.41 ± 0.01; $P^{(\#)} = 0.397$	0.40 ± 0.01; $P^{(\#)} = 0.515$	0.36 ± 0.02; $P^{(\#)} = 0.118$
5-ASA 100mg.kg^{-1}	4.33 ± 0.12; $P^{(\#)} = 0.599$	0.39 ± 0.01; $P^{(\#)} = 0.584$	0.38 ± 0.01; $P^{(\#)} = 0.738$	0.39 ± 0.02; $P^{(\#)} = 0.829$

7. References

1. Loddo I, Romano C (2015) Inflammatory bowel disease: genetics, epigenetics, and pathogenesis. *Front Immunol* **6**: 551.

2. Algieri F, Zorrilla P, Rodriguez-Nogales A, Garrido-Mesa N, Banuelos O, et al. (2013) Intestinal anti-inflammatory activity of hydroalcoholic extracts of *Phlomis purpurea* L. and *Phlomis lychnitis* L. in the trinitrobenzenesulphonic acid model of rat colitis. *J Ethnopharmacol* **146**: 750-759.

3. Lichtenstein GR, Hanauer SB, Sandborn WJ (2009) Management of Crohn's disease in adults. *Am J Gastroenterol* **104**: 465-483.

4. Serra D, Rufino AT, Mendes AF, Almeida LM, Dinis TC (2014) Resveratrol modulates cytokine-induced Jak/STAT activation more efficiently than 5-aminosalicylic acid: an in vitro approach. *PLoS One* **9**: e109048.

5. Liu L, Liu Z, Zhang T, Shi L, Zhang W, et al. (2015) Combined therapy with *Rheum tanguticum* polysaccharide and low-dose 5-ASA ameliorates TNBS-induced colitis in rats by suppression of NF-kappaB. *Planta Med* **81**: 705-712.

6. Farzaei MH, Rahimi R, Abdollahi M (2015) The role of dietary polyphenols in the management of inflammatory bowel disease. *Curr Pharm Biotechnol* **16**: 196-210.

7. He J, Giusti MM (2010) Anthocyanins: natural colorants with health-promoting properties. *Annu Rev Food Sci Technol* **1**: 163-187.

8. Lee SG, Kim B, Yang Y, Pham TX, Park YK, et al. (2014) Berry anthocyanins suppress the expression and secretion of proinflammatory mediators in macrophages by inhibiting nuclear translocation of NF-kappaB independent of NRF2-mediated mechanism. *J Nutr Biochem* **25**: 404-411.

9. Sodagari HR, Farzaei MH, Bahramsoltani R, Abdolghaffari AH, Mahmoudi M, et al. (2015) Dietary anthocyanins as a complementary medicinal approach for management of inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol* **9**:807-820.

10. Esposito D, Chen A, Grace MH, Komarnytsky S, Lila MA (2014) Inhibitory effects of wild blueberry anthocyanins and other flavonoids on biomarkers of acute and chronic inflammation in vitro. *J Agric Food Chem* **62**: 7022-7028.

11. Paixão J, Dinis TC, Almeida LM (2011) Dietary anthocyanins protect endothelial cells against peroxynitrite-induced mitochondrial apoptosis pathway and Bax nuclear translocation: an in vitro approach. *Apoptosis* **16**: 976-989.

12. Paixão J, Dinis TC, Almeida LM (2012) Malvidin-3-glucoside protects endothelial cells up-regulating endothelial NO synthase and inhibiting peroxynitrite-induced NF-kB activation. *Chem Biol Interact* **199**: 192-200.

13. Serra D, Paixão J, Nunes C, Dinis TCP, Almeida LM (2013) Cyanidin-3-glucoside suppresses cytokine-induced inflammatory response in human intestinal cells: comparison with 5-aminosalicylic acid. *PLoS One* **8**: e73001.

14. Li L, Wang L, Wu Z, Yao L, Wu Y, et al. (2014) Anthocyanin-rich fractions from red raspberries attenuate inflammation in both RAW264.7 macrophages and a mouse model of colitis. *Sci Rep* **4**: 6234.

15. Huang W, Zhu Y, Li C, Sui Z, Min W (2016) Effect of blueberry anthocyanins malvidin and glycosides on the antioxidant properties in endothelial cells. *Oxid Med Cell Longev* **2016**: 1591803.

16. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE (2014) Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev* **94**: 329-354.

17. Yi W, Akoh CC, Fischer J, Krewer G (2006) Absorption of anthocyanins from blueberry extracts by caco-2 human intestinal cell monolayers. *J Agric Food Chem* **54**: 5651-5658.

18. McGhie TK, Walton MC (2007) The bioavailability and absorption of anthocyanins: towards a better understanding. *Mol Nutr Food Res* **51**: 702-713.

19. Romier B, Schneider YJ, Larondelle Y, During A (2009) Dietary polyphenols can modulate the intestinal inflammatory response. *Nutr Rev* **67**: 363-378.

20. Wu LH, Xu ZL, Dong D, He SA, Yu H (2011) Protective effect of anthocyanins extract from blueberry on TNBS-induced IBD model of mice. *Evid Based Complement Alternat Med* **2011**: 525462.

21. Oszmianski J, Ramos T, Bourzeix M (1988) Fractionation of phenolic compounds in red wine. *Am J Enol Vitic* **39**: 259-262.

22. Youdim KA, McDonald J, Kalt W, Joseph JA (2002) Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults (small star, filled). *J Nutr Biochem* **13**: 282-288.

23. Rodriguez-Saona LE, Wrolstad RE (2001) Extraction, isolation, and purification of anthocyanins. *Current Protocols in Food Analytical Chemistry* **F**:F1:F1.1

24. George S, Brat P, Alter P, Amiot MJ (2005) Rapid determination of polyphenols and vitamin C in plant-derived products. *J Agric Food Chem* **53**: 1370-1373.

25. Giusti MM, Wrolstad RE (2001) Characterization and measurement of anthocyanins by UV-visible spectroscopy. *Current Protocols in Food Analytical Chemistry* **F**:F1:F1.2.

26. Faria A, Oliveira J, Neves P, Gameiro P, Santos-Buelga C, et al. (2005) Antioxidant properties of prepared blueberry (Vaccinium myrtillus) extracts. *J Agric Food Chem* **53**: 6896-6902.

27. Ancha HR, Kurella RR, McKimmey CC, Lightfoot S, Harty RF (2008) Luminal antioxidants enhance the effects of mesalamine in the treatment of chemically induced colitis in rats. *Exp Biol Med (Maywood)* **233**: 1301-1308.

28. Reagan-Shaw S, Nihal M, Ahmad N (2008) Dose translation from animal to human studies revisited. *FASEB J* **22**: 659-661.

29. Siddiqui A, Ancha H, Tedesco D, Lightfoot S, Stewart CA, et al. (2006) Antioxidant therapy with N-acetylcysteine plus mesalamine accelerates mucosal healing in a rodent model of colitis. *Dig Dis Sci* **51**: 698-705.

30. Bell CJ, Gall DG, Wallace JL (1995) Disruption of colonic electrolyte transport in experimental colitis. *Am J Physiol* **268**: G622-630.

31. Stucchi AF, Shofer S, Leeman S, Materne O, Beer E, et al. (2000) NK-1 antagonist reduces colonic inflammation and oxidative stress in dextran sulfate-induced colitis in rats. *Am J Physiol Gastrointest Liver Physiol* **279**: G1298-1306.

32. Bessey OA, Lowry OH, Brock MJ (1946) A method for the rapid determination of alkaline phosphates with five cubic millimeters of serum. *J Biol Chem* **164**: 321-329.

33. Sanchez de Medina F, Martinez-Augustin O, Gonzalez R, Ballester I, Nieto A, et al. (2004) Induction of alkaline phosphatase in the inflamed intestine: a novel pharmacological target for inflammatory bowel disease. *Biochem Pharmacol* **68**: 2317-2326.

34. Griffith OW (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* **106**: 207-212.

35. Rahman I, Kode A, Biswas SK (2006) Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat Protoc* **1**: 3159-3165.

36. Sanchez-Fidalgo S, Cardeno A, Villegas I, Talero E, de la Lastra CA (2010) Dietary supplementation of resveratrol attenuates chronic colonic inflammation in mice. *Eur J Pharmacol* **633**: 78-84.

37. Maor I, Rainis T, Lanir A, Lavy A (2008) Oxidative stress, inflammation and neutrophil superoxide release in patients with Crohn's disease: distinction between active and non-active disease. *Dig Dis Sci* **53**: 2208-2214.

38. Scalzo J, Stevenson D, Hedderley D (2013) Blueberry estimated harvest from seven new cultivars: fruit and anthocyanins. *Food Chem* **139**: 44-50.

39. Bunea A, Rugina D, Sconta Z, Pop RM, Pintea A, et al. (2013) Anthocyanin determination in blueberry extracts from various cultivars and their antiproliferative and apoptotic properties in B16-F10 metastatic murine melanoma cells. *Phytochemistry* **95**: 436-444.

40. Kahle K, Kraus M, Scheppach W, Ackermann M, Ridder F, et al. (2006) Studies on apple and blueberry fruit constituents: do the polyphenols reach the colon after ingestion? *Mol Nutr Food Res* **50**: 418-423.

41. Rosillo MA, Sanchez-Hidalgo M, Cardeno A, Aparicio-Soto M, Sanchez-Fidalgo S, et al. (2012) Dietary supplementation of an ellagic acid-enriched pomegranate extract attenuates chronic colonic inflammation in rats. *Pharmacol Res* **66**: 235-242.

42. Dahan S, Roth-Walter F, Arnaboldi P, Agarwal S, Mayer L (2007) Epithelia: lymphocyte interactions in the gut. *Immunol Rev* **215**: 243-253.

43. Lalles JP (2014) Intestinal alkaline phosphatase: novel functions and protective effects. *Nutr Rev* **72**: 82-94.

44. Dinis TC, Maderia VM, Almeida LM (1994) Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys* **315**: 161-169.

45. Nieto N, Torres MI, Fernandez MI, Giron MD, Rios A, et al. (2000) Experimental ulcerative colitis impairs antioxidant defense system in rat intestine. *Dig Dis Sci* **45**: 1820-1827.

46. Circu ML, Aw TY (2012) Intestinal redox biology and oxidative stress. *Semin Cell Dev Biol* **23**: 729-737.

47. Atreya I, Atreya R, Neurath MF (2008) NF-kappaB in inflammatory bowel disease. *J Intern Med* **263**: 591-596.

48. Schreiber S, Rosenstiel P, Hampe J, Nikolaus S, Groessner B, et al. (2002) Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. *Gut* **51**: 379-385.

49. Singer, II, Kawka DW, Schloemann S, Tessner T, Riehl T, et al. (1998) Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. *Gastroenterology* **115**: 297-306.

50. Chun KS, Surh YJ (2004) Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. *Biochem Pharmacol* **68**: 1089-1100.

51. Miyoshi J, Yajima T, Shimamura K, Matsuoka K, Okamoto S, et al. (2012) 5-aminosalicylic acid mediates expression of cyclooxygenase-2 and 15-hydroxyprostaglandin dehydrogenase to suppress colorectal tumorigenesis. *Anticancer Res* **32**: 1193-1202.

Chapter 4

Anthocyanin-rich fraction from Portuguese blueberries improves LPS-induced inflammation in co-cultured Caco-2 and RAW 264.7 cells: an *in vitro* model of gut inflammation
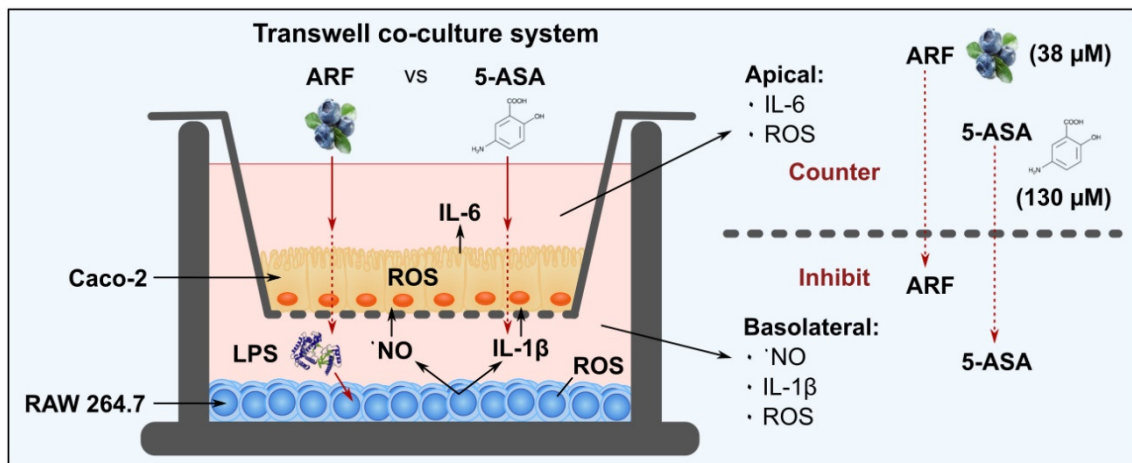
Preliminary results from ongoing work, which explores the action of the ARF extract on the close interaction between intestinal epithelial cells and macrophages, in an *in vitro* model of IBD.

1. Abstract

The use of polyphenols in inflammatory-related pathologies has been gaining popularity in recent years. Anthocyanins, as part of the most common dietary polyphenols, may be useful in the context of inflammatory bowel disease (IBD), a group of intestinal disorders that still need more efficient and safer therapeutic approaches. In this context, anthocyanins, highly available in human diet and recognized as health-promoting agents, have been a focus of interest. Thus, the aim of this work was to further explore the molecular anti-inflammatory action mechanisms of a characterized anthocyanin-rich fraction (ARF) from Portuguese blueberries, as compared with 5-aminosalicylic acid (5-ASA), a standard anti-inflammatory drug in IBD. For that, a co-culture system of intestinal epithelial Caco-2 cells and RAW 264.7 macrophage cells was used as a model of colitis. The compounds were pre-incubated in the apical side of a transwell plate containing a differentiated and polarized Caco-2 cell monolayer, for 3 h, followed by addition of lipopolysaccharide (LPS) to the basolateral side to stimulate the RAW 264.7 cells. After 24 h, the inflammatory process was evaluated in terms of nitric oxide, IL-6 and IL-1β contents in culture supernatants, as well as of intracellular reactive oxygen species (ROS). A monoculture of RAW 264.7 was used to explore the intestinal barrier interference. Our data showed that the production of pro-inflammatory mediators and ROS was significantly decreased by the anthocyanins mixture, in a higher extent than by 5-ASA.

2. Introduction

Inflammatory bowel disease (IBD) is a group of diseases characterized by chronic and relapsing inflammation of the gastrointestinal tract (GI). In patients with IBD, it seems to occur an exacerbated intestinal immune response towards a normally innocuous stimulus [1]. In this context, pro-inflammatory mediators are upregulated, being key players for the disease onset and progression [2]. The disease incidence and prevalence are increasing worldwide [3], but no curative treatment has been developed and current therapeutic strategies may cause adverse effects that impair their long-term use [4]. In this context, natural products and dietary compounds such as polyphenols have been drawing attention due to their anti-inflammatory and anti-oxidant potential [5,6]. Even though, it is still required to establish their efficacy and action mechanisms, so they can be included in IBD therapeutics.

Blueberries are among the fruits with the highest content in anthocyanins, a group of flavonoids widespread in our diet, with health-promoting benefits [7]. In agreement with other published results [8,9], we previously demonstrated the anti-inflammatory and antioxidant activity of an anthocyanin-rich fraction (ARF) prepared from Portuguese blueberries (*Vaccinium corymbosum* L.), in a TNBS-induced colitis rat model, in comparison with a conventional treatment for IBD, the 5-aminosalicylic acid (5-ASA) [10]. However, the molecular mechanisms underlying its activity as well as its interaction with the intestinal epithelial barrier still need clarification.

For that purpose, an *in vitro* model of intestinal inflammation closely resembling human colitis, similar to that used by Kim *et al* (2015) [11], was established. In this cellular system of intestinal epithelial cells (Caco-2 cells) and macrophages (RAW 264.7 cells) in co-culture, the inflammatory status was induced by macrophages stimulation in the basolateral compartment with lipopolysaccharide (LPS), and the effects of the

anthocyanin-rich fraction, in comparison with 5-ASA, added to the epithelial cells in the apical compartment (**Fig 1**), were investigated.

In fact, LPS-challenged RAW 264.7 cells produce elevated levels of pro-inflammatory mediators that in turn stimulate Caco-2 cells, mimicking the intimate relationship between intestinal barrier and immune cells during active IBD [12]. Thus, this model beyond elucidating about the mechanisms of ARF anti-inflammatory activity, also adds information about the bioavailability of anthocyanins, an yet controversial issue [13,14], once these compounds must cross the intestinal barrier to exert effects on the basolateral macrophage cells.

Therefore, in the present study, the chemically characterized anthocyanin-rich fraction from Portuguese blueberries was evaluated in terms of pro-inflammatory mediators' inhibition, namely nitric oxide, IL-6 and IL-1β cytokines and reactive oxygen species (ROS), by using the described *in vitro* co-culture model of IBD. Its activity was compared to that of 5-ASA, and the epithelial barrier interference was monitored by comparing the results with a RAW 264.7 cells monoculture submitted to similar experimental conditions.

3. Materials and methods

3.1. Reagents

Laboratory chemicals, namely 5-aminosalicylic acid (5-ASA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), lipopolysaccharide (LPS) (Escherichia coli, serotype 0111:B4), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), dimethylsulfoxide (DMSO), and general laboratory chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA).

Cell culture reagents namely Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) pH 7.4, were purchased from Gibco-Invitrogen (Grand Island, NY, USA). Penicillin and streptomycin were purchased from Sigma-Aldrich (St Louis, Missouri, USA).

3.2. Anthocyanin-rich fraction from Portuguese blueberries

The blueberries (*Vaccinium corymbosum L*, cultivar Bluecrop) were collected at the time of peak production, from the central region of Portugal. They were obtained from biological agriculture (Biogrêsso, Portugal) and kept frozen at -80ºC until use. The anthocyanin-rich fraction was prepared and characterized as previously reported [10]. The fraction content in anthocyanins is about $10g.L^{-1}$ (10 ± 0.48 $g.L^{-1}$) in terms of malvidin. The anthocyanins relative composition of the fraction determined by HPLC-DAD was malvidin-3-galactoside (20.9%), petunidin-3-arabinoside (18.7%), peonidin-3-glucoside (11.5%), delphinidin-3-arabinoside (8.0%), delphinidin-3-galactoside (7.0%), petunidin-3-galactoside (6.0%), peonidin-3-galactoside (5.1%), delphinidin-3-glucoside (4.4%), malvidin-3-arabinoside (4.3%), cyanidin-3-arabinoside (4.1%), peonidin-3-arabinoside

(3.0%), malvidin-3-glucoside (2.6%), cyanidin-3-galactoside (1.7%), petudin-3-glucoside (1.6%), and cyanidin-3-glucoside (1.3%) [10].

3.3. Cell culture

The human intestinal epithelial cell line (Caco-2) and the murine macrophage cell line (RAW 264.7) were obtained from European Collection of Cell Cultures (Porton Down, Salisbury, UK). Cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 U.mL^{-1} penicillin and 100 µg.mL^{-1} streptomycin, on 75 cm^2 flasks, at 37ºC in a humidified atmosphere of 5% CO_2. The medium was always replaced by fresh supplemented medium before cells treatment. Cells were counted using a haemocytometer and the number of viable cells was determined by trypan blue dye exclusion.

3.4. Co-culture system design

The co-culture system design was established as described by KM Kim *et al* (2015) [11]. In this system, human intestinal epithelial-like Caco-2 cells were seeded into a porous membrane of a transwell insert (1x10^5 cell/well; Corning Costar Corp., Cambridge, MA, USA) and allowed to differentiate and polarize for 21 days. During this time, cell culture medium was changed every 3 days. The RAW 264.7 macrophages were seeded in the basolateral chamber 24 h previous to each experiment in 12-multiwells (4x10^5 cells/well). After replacing the medium with fresh one, the inserts containing Caco-2 cells were added to the 12-multiwells containing the RAW 264.7 cells (**Fig 1**).

To evaluate the anti-inflammatory effect of the ARF and 5-ASA, these compounds were incubated separately in the apical side of the transwell system for 3h. The macrophage cells were then activated by the addition of 1 µg.mL^{-1} LPS into the basolateral

side. After 24h, the inflammatory process was evaluated in terms of •NO, IL-6 and IL-1β production in the culture supernatants, as well as of intra-cellular ROS. A monoculture of RAW 264.7 was used for comparison. Note that treatment and stimulus were not removed during the incubation time.
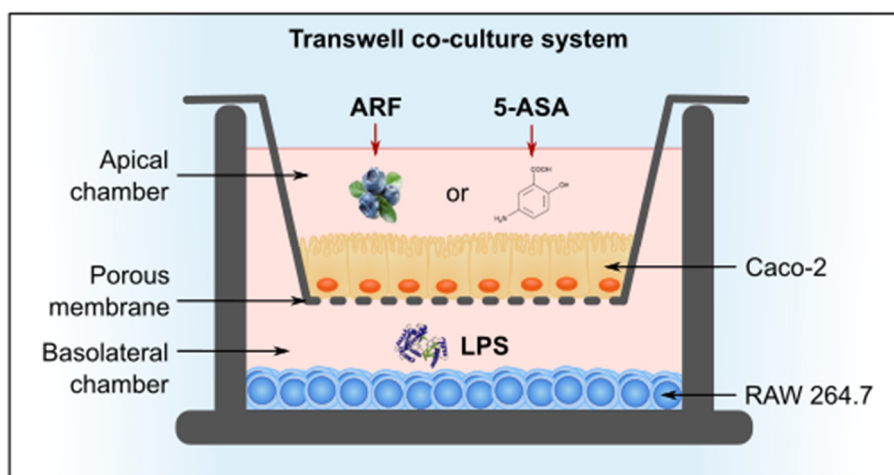

Figure 1: Schematic of the *in vitro* co-culture system in the transwell. The Caco-2 cells were seeded 21 days prior in 12-well inserts with a semipermeable membrane. The RAW 264.7 cells were seeded 24 h prior in 12-multiwells. Epithelial cells were pre-incubated with ARF or 5-ASA for 3 h, and then LPS was added to basolateral cells to induce activation.

3.5. Cell viability

Cell viability was assessed by the cellular dehydrogenase-dependent reduction of the yellow dye MTT to formazan, an insoluble intracellular purple product [15], in either Caco-2 or RAW 264.7 monocultures. After incubation of the cells with ARF or 5-ASA for 24 and 48 h, the culture medium was removed and the cells were washed twice with PBS. Then MTT was added in medium at a concentration of 0.5 mg.mL^{-1} for 1h at 37°C. Finally, the supernatant was removed, the formazan crystals were dissolved in 500 μl of DMSO and the absorbance was immediately read at 530 nm in a plate reader (Bio-TEK Synergy

HT, Izasa S.A., Spain). Cell viability was expressed as a percentage of control cells, i.e., cells incubated for 24 or 48 h but without extract or drug.

3.6. Nitric oxide production

The •NO production was evaluated in the supernatants of RAW 264.7 cells, either in co-culture or monoculture, by chemiluminescence, in gas phase, using the CLD 88 Analyser from Eco Medics (Switzerland). Briefly, after •NO production by the cell, it is oxidized to nitrite, accumulating in the medium. Then, the quantification of nitrite is achieved after reductive cleavage by an iodine-tri-iodide containing solution [45 mmol.L^{-1} potassium iodide (KI) and 10 mmol.L^{-1} iodine (I$_2$), in glacial acetic acid, kept at 56ºC in a septum-sealed reaction vessel continuously bubbled with nitrogen]. The reaction chamber design was similar to a commercially available unit (Sievers, Boulder, CO) built by Colaver (Colaver srl, Italy). The outlet of the gas stream was passed through a scrubbing bottle containing sodium hydroxide (1 mol.L^{-1}, at 0ºC) to trap traces of acid and iodine before transfer into the detector. The released •NO to the gas phase is then assessed by the chemiluminescent reaction with ozone (O$_3$), as described by M Feelisch et al (2002) [16]. This is a highly sensitive and selective method for bulk •NO quantification in biological samples. The concentrations were determined from a sodium nitrite standard curve. •NO signal output was samples at 2 Hz and peak integration was performed by using the EDAQ Power Chrom software.

3.7. Interleukine-6 and interleukine-1 beta production

The production of IL-6 in the apical side, and of IL-1β in the basolateral side of the transwell system or in monocultured RAW 264.7, were assessed by a competitive

immunoassay kits from Abcam (Cambridge, UK), for human- and mouse-derived interleukines respectively. After cells treatment, under the specified conditions, aliquots of supernatant were collected and analysed according to the manufacturers' instructions. Determinations were performed in duplicate of four independent experiments.

3.8. Intracellular reactive oxygen species production

The intracellular ROS content was estimated by using the non-fluorescent probe 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA), which permeates cell membrane and may be oxidized by ROS, yielding 2′-7′-dichlorofluorescein (DCF) [17]. Briefly, after cells treatments in either the transwell system or monoculture for 24 h, they were washed with PBS and new medium containing 1 μM DCFH-DA was added for 15 min at 37°C, protected from direct light. After washing the cells, the fluorescence was measured using a plate reader (Bio-TEK Synergy HT, Izasa S.A., Spain) (excitation and emission wavelengths at 485 and 530nm, respectively).

3.9. Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) of at least three independent assays, each one performed in duplicate or triplicate. Differences between conditions were assessed using one-way analysis of variance (ANOVA), and Bonferroni´s test as post hoc. A value of $P < 0.05$ was accepted as statistically significant.

4. Results

4.1. The ARF decreases nitric oxide and ROS production by LPS-stimulated RAW 264.7 cells in a concentration-dependent way

To evaluate the potential anti-inflammatory action of the ARF, several concentrations were tested in LPS-stimulated RAW 264.7 cells. This is a model of activated macrophages, described to produce and secrete high amounts of inflammatory mediators, such as $^{\bullet}$NO and ROS [18,19]. In accordance, incubation with LPS induced a high increase in the $^{\bullet}$NO secretion and intracellular ROS formation, as observed after 16 h of incubation. By treating the cells with 1-20 µg.mL^{-1} of ARF prior to LPS induction, it was observed a concentration-dependent reduction in either $^{\bullet}$NO (**Fig 2A**) or ROS (**Fig 2B**) levels. This reduction was more pronounced in terms of ROS production and particularly evident at the concentration of 20 µg.mL^{-1} of ARF.
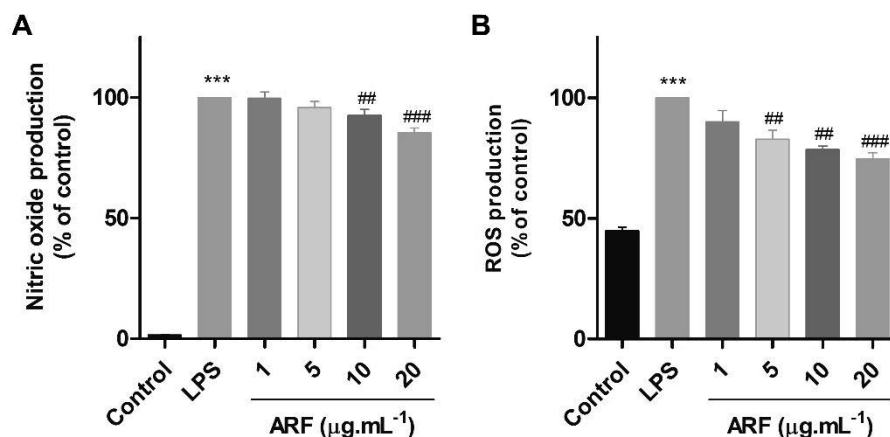

Figure 2: Effect of the ARF on $^{\bullet}$NO and ROS production by LPS-stimulated RAW 264.7 cells. RAW 264.7 were pre-incubated with crescent concentrations of ARF (1 to 20 µg.mL^{-1}) for 3h, and then further incubated 16 h with LPS (1 µg.mL^{-1}). The supernatants were collected to determine $^{\bullet}$NO concentration (**A**) by gas-phase chemiluminescence, and the cells were further incubated 15 min with DCF-DA to determine intracellular ROS production (**B**). ***P<0.001 vs negative control (non-stimulated cells); $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 *vs* positive control (LPS-stimulated cells without compounds).

Prior to these assays, the range of concentrations that could be used safely was ascertained by measuring the cytotoxicity of the ARF in RAW 264.7 and Caco-2 cells (**Fig 3**). The macrophage cells were cultured individually as monoculture and incubated with a range of concentrations of 1 to 6 µg.mL^{-1} for the fraction, during 24 (**Fig 3A**) and 48 h (**Fig 3B**). As evaluated by the MTT assay and presented in **Fig 3**, the highest concentrations of ARF (40 and 60 µg.mL^{-1}) reduced macrophages cellular viability, thus limiting the usage of higher concentrations. Therefore, 10 and 20 µg.mL^{-1} were the concentrations tested in the intestinal Caco-2 cells (**Fig 3C** and **D**) and also the concentrations selected to evaluate 5-ASA cytotoxicity. As shown in **Fig 3**, these concentrations did not alter the viability of both cell types. Also, no cellular morphological changes were observed by microscopic analysis (data not shown). Thus, the concentration of 20 µg.mL^{-1} (either ARF or 5-ASA) was established for the subsequent experiments.

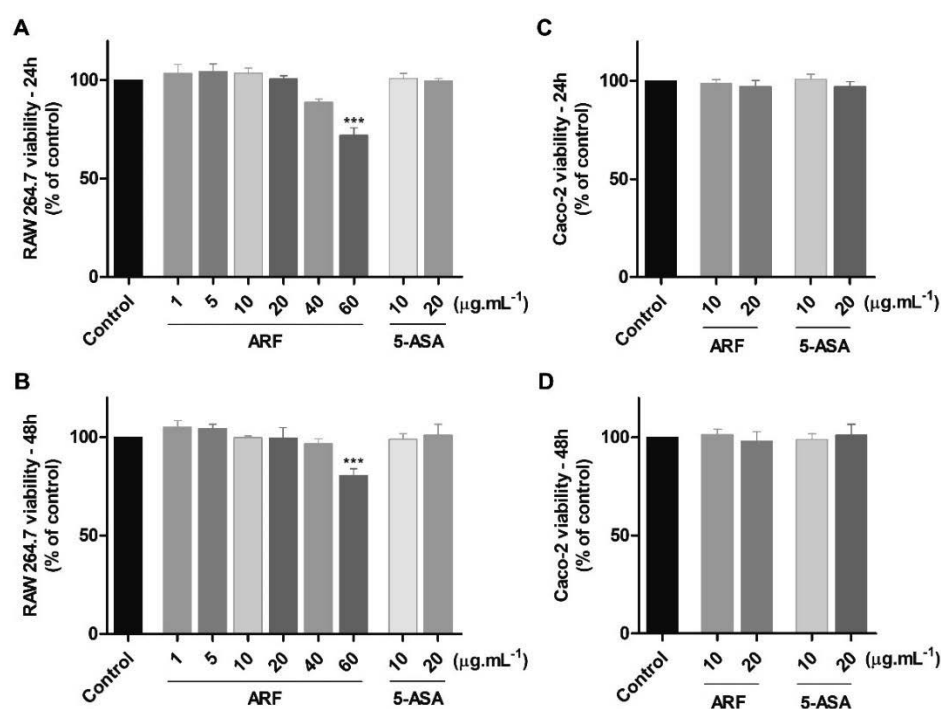**Figure 3: Effect of the ARF or 5-ASA on both RAW 264.7 and Caco-2 cells viability.** RAW 264.7 cells were incubated with ARF (1 to 60 µg.mL^{-1}) or 5-ASA (10 and 20 µg.mL^{-1}), for 24 h (**A**) and 48 h (**B**). Caco-2 cells were incubated with either ARF (10 and 20 µg.mL^{-1}) or 5-ASA (10 and 20 µg.mL^{-1}), for 24 h (**C**) and 48 h (**D**). After the incubation time, cells viability was determined by the MTT assay. Values are expressed as percentage of control (mean ± SEM of at least three experiments, each one assayed in triplicate). [***]P<0.001 *vs* control cells.

4.2. The ARF highly counters Caco-2 production of IL-6 and intra-cellular ROS induced by active macrophages

To explore the anti-inflammatory action of the ARF, it was used a co-culture system of intestinal epithelial cells and macrophages. In this model, the inflammation was induced by activation of the macrophage cell line on the basolateral compartment with LPS. This elicits the production of high levels of pro-inflammatory mediators by RAW 264.7 that, in turn, can activate the Caco-2 cells on the apical side of the system [11]. In accordance, the Caco-2 cells co-cultured for 24 h with active macrophages (LPS-induced RAW 264.7) show an increased secretion of IL-6 (**Fig 4A**) and intra-cellular production of ROS (**Fig 4B**). However, when Caco-2 cells are pre-incubated with the ARF, both IL-6 and ROS production are highly countered. When compared to 5-ASA action in similar conditions, the protection afforded by the blueberry fraction is more pronounced. Note that these results were obtained with an anthocyanin concentration about 3.4 times lower (20 µg.mL^{-1} of ARF corresponds to 38 µM, in terms of malvidin-3-galactoside) than that of 5-ASA (20 µg.mL^{-1} , *i.e.* 130 µM).
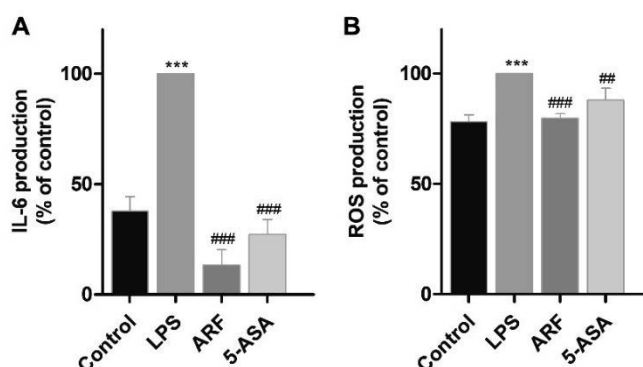

Figure 4: Effect of ARF, in comparison with 5-ASA, on the production of IL-6 and intra-cellular ROS by co-cultured apical Caco-2 cells. Caco-2 cells in the apical chamber were pre-incubated with either ARF or 5-ASA (20 µg.mL^{-1}) for 3 h. LPS (1 µg.mL^{-1}) was then added to the basolateral chamber and further incubated 24 h, at 37ºC. Afterwards, apical supernatants were collected to determine IL-6 by an ELISA kit (**A**). The cells were washed and further incubated with DCF-DA during 15 min, and the fluorescence was measured to determine the intracellular ROS content (**B**). [***]P<0.001 *vs* negative control (non-stimulated cells); [##]P<0.01 and [###]P<0.001 *vs* positive control (LPS-stimulated cells without compounds).

4.3. The ARF reduces more efficiently than 5-ASA inflammatory mediators and intracellular ROS produced by RAW 264.7 cells in the presence of an intestinal barrier

The potential anti-inflammatory action of the ARF was also explored in the basolateral compartment of the co-culture system. Following the same experimental design, after 24 h incubation with LPS, the basolateral supernatants were collected to determine the levels of $^\bullet$NO (**Fig 5A**), IL-1β (**Fig 5B**), and intracellular ROS generation (**Fig 5C**). As expected, LPS induced a significant increase in the production of these inflammatory mediators. Interestingly, pre-incubation with ARF only in the apical side of the co-cultured system, could reduce the production of these three mediators, especially IL-1β (**Fig 5B**), by the basolateral macrophages. In comparison, 5-ASA could only counter IL-1β secretion, but at a lower extent than the ARF.

Given that these effects were achieved in the basolateral chamber, while the ARF or 5-ASA were applied to the apical side, indicates that the compounds could pass the intestinal cells barrier, a critical aspect for the treatment of IBD. To strengthen this finding, a similar experiment was carried out in a monoculture of RAW 264.7 macrophages and the resulta are represented in the same graphs in parallel. In comparison, and given the direct contact of the cells with LPS and ARF or 5-ASA, the protection afforded by either ARF or 5-ASA was slightly higher in terms of $^\bullet$NO (**Fig 5A**) and ROS reduction (**Fig 5C**), but not in terms of IL-1β (**Fig 5B**).
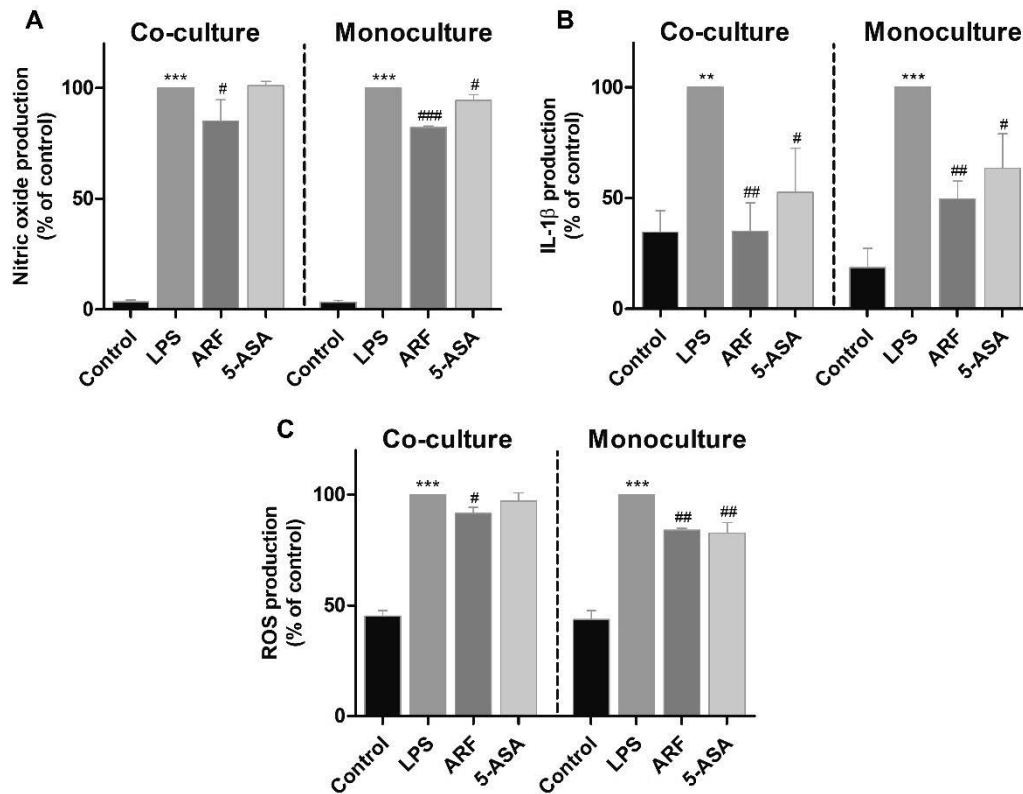
Figure 5: Effect of ARF, in comparison with 5-ASA, on the production of •NO, IL-1β and intra-cellular ROS, by RAW 264.7 cells in basolateral co-culture or in monoculture. Caco-2 cells in the apical chamber were pre-incubated with either ARF or 5-ASA (20 µg.mL^{-1}) for 3 h. LPS (1 µg.mL^{-1}) was then added to the basolateral chamber and further incubated 24 h, at 37ºC. Afterwards, the basolateral supernatants were collected to determine •NO (**A**), by chemiluminescence, and IL-1β (**B**), using an ELISA kit. The cells were washed and further incubated with DCF-DA during 15 min, and the fluorescence was measured to determine the intracellular ROS content (**C**). In parallel, RAW 264.7 cells in monoculture were pre-incubated with either ARF or 5-ASA (20 µg.mL^{-1}) for 3 h and then stimulated with LPS for 24 h. Also the levels of •NO (**A**) and IL-1β (**B**), as well as the intracellular ROS content (**C**) were evaluated **P<0.01 and ***P<0.001 *vs* negative control (non-stimulated cells); #P<0.05, ##P<0.01 and ###P<0.001 *vs* positive control (LPS-stimulated cells without compounds).

5. Discussion

The use of natural anti-inflammatory compounds is currently considered a promising alternative to conventional treatment, which are not devoid of side effects. In this context, anthocyanins, a group of polyphenols widely distributed in the Mediterranean diet, have been drawing attention given their antioxidant and anti-inflammatory properties [20-22]. In fact, their high consumption allied with their reported anti-inflammatory properties, may be of paramount relevance in intestinal inflammation management. In this context, previous work from our group demonstrated the anti-inflammatory and antioxidant activity of an anthocyanin-rich fraction prepared from Portuguese blueberries in an *in vivo* model of IBD [10]. Following that line of work, the present study was undertaken to further explore this ARF activity in terms of molecular signalling, using an *in vitro* gut inflammation model. Such model was established co-culturing intestinal epithelial cells (Caco-2) in the apical compartment and macrophage-like cells (RAW 264.7) in the basolateral compartment of a transwell co-culture system as shown in **Fig 1**.

In inflammatory disorders such as IBD, macrophage cells produce large amounts of pro-inflammatory mediators, playing a key role in the disease pathogenesis and progression [23]. Also, in this model, RAW 264.7 cells incubation with LPS induced the production of pro-inflammatory mediators namely, nitric oxide, IL-1β and intracellular ROS, as shown either in co-culture with Caco-2 or in monoculture.

Preliminary studies were conducted in monoculture to explore ARF efficacy at reducing nitric oxide and ROS production (**Fig 2**). The range of concentrations tested reduced the production of these mediators in a concentration-dependent manner, but the concentration of 20 µg.mL^{-1} was chosen taking in consideration its safety (**Fig 3**).

When using this model of intestinal inflammation, the stimulation of the intestinal epithelial cells is achieved by the action of the pro-inflammatory mediators produced by the basolateral macrophages cells, since both cells, epithelial and macrophages are not

in direct contact [11]. This interaction is real, given that epithelial cells significantly increase IL-6 and intracellular ROS production, although only macrophage cells contained in the basolateral compartment were incubated with LPS. Also, the apical cells incubation with ARF or 5-ASA mimics the intestinal lumen, because the effects observed in the basolateral compartment are indicative of the absorption and capacity of these compounds to pass the intestinal epithelial barrier. Therefore, the results obtained thus far demonstrate the ability of the ARF to counter the production of IL-6 (**Fig 4A)** and intracellular ROS (**Fig 4B**) by intestinal cells, to cross the intestinal barrier and then reduce the production of nitric oxide (**Fig 5A)**, IL-1β (**Fig 5B)**, and ROS (**Fig 5C**) by macrophage cells. When compared to 5-ASA, and as previously observed *in vivo*, the ARF exhibits higher efficacy to the concentration tested (20 $\mu g.ml^{-1}$) as evidenced in graphs. This efficacy is still more relevant in terms of molar concentration, once the ARF molar concentration is 3,4 times lower than that of 5-ASA. By using a monoculture of RAW 264.7 cells, it was possible to observe a small interference of the epithelial barrier, since in these conditions both the ARF and 5-ASA were slightly more efficacious.

Taking into consideration that the inhibition of pro-inflammatory mediators has been proposed as an effective strategy in the management of IBD [23,24], the results presented here support a relevant action of this blueberry anthocyanin mixture in this disease. Moreover, the anthocyanins most abundant in the ARF used, malvidin-3-galactoside and petunidin-3-arabinoside, were actually reported to reach the intestinal compartment of test patients after blueberries consumption [25].

In conclusion, our data indicate a relevant anti-inflammatory and anti-oxidant action of this blueberry anthocyanin mixture in a co-culture model of IBD, as compared with 5-ASA, encouraging us to continue the study in order to strengthen these results and to clarify the pathways involved in their mechanism of action.

6. References

1. Loddo I, Romano C (2015) Inflammatory Bowel disease: genetics, epigenetics, and pathogenesis. *Front Immunol* **6**: 551.

2. Pedersen J, Coskun M, Soendergaard C, Salem M, Nielsen OH (2014) Inflammatory pathways of importance for management of inflammatory bowel disease. *World J Gastroenterol* **20**: 64-77.

3. Malik TA (2015) Inflammatory bowel disease: historical perspective, epidemiology, and risk factors. *Surg Clin North Am* **95**: 1105-1122.

4. Grevenitis P, Thomas A, Lodhia N (2015) Medical therapy for inflammatory bowel disease. *Surg Clin North Am* **95**: 1159-1182.

5. Boussenna A, Joubert-Zakeyh J, Fraisse D, Pereira B, Vasson MP, et al. (2016) Dietary supplementation with a low dose of polyphenol-rich grape pomace extract prevents dextran sulfate sodium-induced colitis in rats. *J Med Food* **19**: 755-758.

6. Farzaei MH, Rahimi R, Abdollahi M (2015) The role of dietary polyphenols in the management of inflammatory bowel disease. *Curr Pharm Biotechnol* **16**: 196-210.

7. He J, Giusti MM (2010) Anthocyanins: natural colorants with health-promoting properties. *Annu Rev Food Sci Technol* **1**: 163-187.

8. Lee SG, Kim B, Yang Y, Pham TX, Park YK, et al. (2014) Berry anthocyanins suppress the expression and secretion of proinflammatory mediators in macrophages by inhibiting nuclear translocation of NF-kappaB independent of NRF2-mediated mechanism. *J Nutr Biochem* **25**: 404-411.

9. Sodagari HR, Farzaei MH, Bahramsoltani R, Abdolghaffari AH, Mahmoudi M, et al. (2015) Dietary anthocyanins as a complementary medicinal approach for management of inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol* **9**: 807-820.

10. Pereira SR, Pereira R, Figueiredo I, Freitas V, Dinis TC, et al. (2017) Comparison of anti-inflammatory activities of an anthocyanin-rich fraction from Portuguese blueberries (*Vaccinium corymbosum* L.) and 5-aminosalicylic acid in a TNBS-induced colitis rat model. *PLoS One* **12**: e0174116.

11. Kim KM, Kim YS, Lim JY, Min SJ, Ko HC, et al. (2015) Intestinal anti-inflammatory activity of *Sasa quelpaertensis* leaf extract by suppressing

lipopolysaccharide-stimulated inflammatory mediators in intestinal epithelial Caco-2 cells co-cultured with RAW 264.7 macrophage cells. *Nutr Res Pract* **9**: 3-10.

12. Grainger JR, Konkel JE (2017) Macrophages in gastrointestinal homeostasis and inflammation. *Pflugers Arch* **469**: 527-539.

13. Lila MA, Burton-Freeman B, Grace M, Kalt W (2016) Unraveling anthocyanin bioavailability for human health. *Annu Rev Food Sci Technol* **7**: 375-393.

14. Fernandes I, Nave F, Goncalves R, de Freitas V, Mateus N (2012) On the bioavailability of flavanols and anthocyanins: flavanol-anthocyanin dimers. *Food Chem* **135**: 812-818.

15. Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* **89**: 271-277.

16. Feelisch M, Rassaf T, Mnaimneh S, Singh N, Bryan NS, et al. (2002) Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. *Faseb j* **16**: 1775-1785.

17. LeBel CP, Bondy SC (1990) Sensitive and rapid quantitation of oxygen reactive species formation in rat synaptosomes. *Neurochem Int.* **17**: 435-440.

18. Hwang SJ, Kim YW, Park Y, Lee HJ, Kim KW (2014) Anti-inflammatory effects of chlorogenic acid in lipopolysaccharide-stimulated RAW 264.7 cells. *Inflamm Res* **63**: 81-90.

19. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB (2010) Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* **49**: 1603-1616.

20. Esposito D, Chen A, Grace MH, Komarnytsky S, Lila MA (2014) Inhibitory effects of wild blueberry anthocyanins and other flavonoids on biomarkers of acute and chronic inflammation in vitro. *J Agric Food Chem* **62**: 7022-7028.

21. Serra D, Paixão J, Nunes C, Dinis TC, Almeida LM (2013) Cyanidin-3-glucoside suppresses cytokine-induced inflammatory response in human intestinal cells: comparison with 5-aminosalicylic acid. *PLoS One* **8**: e73001.

22. Paixão J, Dinis TC, Almeida LM (2012) Malvidin-3-glucoside protects endothelial cells up-regulating endothelial NO synthase and inhibiting peroxynitrite-induced NF-kB activation. *Chem Biol Interact* **199**: 192-200.

23. Ohashi W, Hattori K, Hattori Y (2015) Control of macrophage dynamics as a potential therapeutic approach for clinical disorders involving chronic inflammation. *J Pharmacol Exp Ther* **354**: 240-250.

24. Ogata H, Hibi T (2003) Cytokine and anti-cytokine therapies for inflammatory bowel disease. *Curr Pharm Des* **9**: 1107-1113.

25. Kahle K, Kraus M, Scheppach W, Ackermann M, Ridder F, et al. (2006) Studies on apple and blueberry fruit constituents: do the polyphenols reach the colon after ingestion? *Mol Nutr Food Res* **50**: 418-423.

Chapter 5

Concluding remarks

1. Concluding remarks

The work presented in this thesis explores the potential use of common dietary anthocyanins for the treatment of intestinal inflammation, as *per se* or in combination with conventional drugs. The isolated anthocyanin Cy3glc clearly interferes with relevant inflammatory signalling pathways and seems to synergistically interact with 5-ASA, thus highlighting anthocyanins value as an adjuvant approach for the management of chronic inflammatory-related diseases such as IBD. Moreover, the anthocyanin mixture prepared from Portuguese blueberries exhibits relevant anti-inflammatory and antioxidant activities, either *in vivo* and *in vitro*, when compared to a current standard therapy.

To explore anthocyanins modulation of inflammatory signalling pathways closely related to IBD pathogenesis, a first part of work was conducted using a LPS-induced RAW 264.7 macrophage cell line, as an *in vitro* model of inflammation. This study was conducted with Cy3glc, one of the most abundant anthocyanins in the Mediterranean diet [1]. In fact, this isolated anthocyanin could counter all pro-inflammatory mediators tested ($^{\bullet}$NO, PGE_2 and TNF-α) and intracellular ROS, in a higher extent than 5-ASA, in similar experimental conditions. The inhibition of $^{\bullet}$NO and PGE_2 secretion was correlated with the inhibition of the pro-inflammatory enzymes iNOS and COX-2, respectively, but without interfering with NF-κB activation. Instead, this anthocyanin could downregulate AP-1 signalling pathway, clearly reducing the phosphorylated form of c-Jun in the nucleus, that could be related to the upstream down-regulation of p38 and JNK MAPKs. This work went even further to explore not only Cy3glc and 5-ASA comparative activities, but also their interaction. In fact, the combination of both compounds improved their individual effects in most parameters tested, even suggesting a synergistic effect.

Anthocyanins are widely distributed in the Mediterranean diet, and their consumption has been inversely correlated with chronic diseases incidence [2]. The blueberry fruits are rich in those compounds, that can reach the colon in high percentages,

despite the lower plasma bioavailability [3]. In agreement, the prepared Portuguese blueberry (*Vaccinium corymbosum* L.) fraction presented a high content and variety of anthocyanins, and could counter various signs of intestinal inflammation *in vivo*, in a colitis rat model. Administration of this anthocyanin mixture improved the body weight gain of those animals and countered the colonic damage extent, colon shortening and wall thickening caused by local TNBS instillation. These macroscopic effects were accompanied by a marked reduction of active MPO in the colonic tissue, a biomarker of leukocyte infiltration [4], and ALP activity, that is upregulated in response to inflammation and oxidative stress conditions [5]. Moreover, the extract improved the endogenous antioxidant defences, by improving both GSH/GSSG redox buffer and GPX enzyme activity. In terms of inflammatory mediators, both iNOS and COX-2 enzymes expressions were significantly inhibited. It is noteworthy that the beneficial effects observed in this study were far greater than those obtained with the reference 5-ASA treatment, although the much lower molar dose of anthocyanins used (32.5 times lower). The only exception seems to be COX-2 expression that was similarly countered by both ARF and 5-ASA, suggesting this as a shared anti-inflammatory mechanism.

The highest anti-inflammatory and antioxidant activity of this faction was further confirmed *in vitro*, by using an established co-cultured system of epithelial Caco-2 cells and murine RAW 264.7 macrophages [6]. The blueberry fraction, incubated only at the apical side of the transwell system, could counter the pro-inflammatory mediators produced in both apical and basolateral chambers, in a higher extent than 5-ASA. Therefore, the anthocyanins treatment could counter $^\bullet$NO and IL-1β secretion by LPS-induced basolateral macrophages, and IL-6 by the apical Caco-2 cells. Intracellular ROS were inhibited in both cell types. Again, these results were obtained with a much lower molar concentration of anthocyanins (3.4 times lower) compared to 5-ASA. The results obtained in the basolateral chamber may suggest that anthocyanins are able to cross the

intestinal barrier and exert their effects more deeply in the intestinal tissue. This may be corroborated by the lower leukocyte infiltration observed *in vivo*.

Even though we are aware that different anthocyanin structures account for different activities, the blueberry anthocyanin mixture may share the Cy3glc action mechanism on AP-1/MAPKs signalling modulation. In fact, the mixture and the single anthocyanin share the ability to counter iNOS and COX-2 enzymes expression, $^{\bullet}$NO and intracellular ROS production. On the other hand, although Cy3glc did not affect NF-κB activation in macrophage cells, it is still needed to explore such effect with the blueberry fraction, considering that it contains a mixture of 15 anthocyanins. Moreover, this work only explores the action of this fraction as a whole, thus leaving open for studying the individual potency of these anthocyanins or how they interact between them. Nonetheless, the results obtained with this work represent more closely the beneficial effects of an anthocyanin-rich diet for IBD management.

Taken together, the results presented in this thesis highlight the potential of the tested anthocyanins in countering intestinal inflammation and, at the same time, their ability to gather conditions for a faster recovery. The activity of these compounds, isolated or in association, is complex, interfering with several mechanisms closely related to IBD pathogenesis, namely by reducing immune cells infiltration, disrupting pro-inflammatory mediators' production and strengthening endogenous antioxidant defence mechanisms. The high anti-inflammatory and antioxidant activity achieved by these compounds arise from their capacity to modulate the activation of key signalling pathways, thus interfering with their target genes expression. These results confirm that anthocyanins are viable natural therapeutic candidates for intestinal inflammation in place of or in association with conventional treatments. Adding to their proved efficacy, they stand as a safer strategy for long-term use, that is especially relevant in chronic diseases such as IBD.

Although these results support the use of dietary anthocyanins for the management of IBD, much still needs to be done for them to actually be considered as part of the

therapeutic regimen. In addition to *in vitro* and *in vivo* studies, there is still missing data from human patients, either by using *ex vivo* biopsies or carefully elaborated clinical trials. Also, to take the most advantages of these compounds, it should be explored their efficacy when introduced directly in the diet versus standardized nutraceutical preparations. Therefore, data is still missing on anthocyanins individual efficacies and possible synergistic interactions, their metabolism and bioavailability. Furthermore, given the interaction observed with Cy3glc and 5-ASA, more studies should evaluate the interaction of anthocyanins with conventional therapies, since this may allow to reduce their dosages and inherent side effects.

2. References

1. Dugo P, Mondello L, Errante G, Zappia G, Dugo G (2001) Identification of anthocyanins in berries by narrow-bore high-performance liquid chromatography with electrospray ionization detection. *J Agric Food Chem* **49**: 3987-3992.

2. Smeriglio A, Barreca D, Bellocco E, Trombetta D (2016) Chemistry, pharmacology and health benefits of anthocyanins. *Phytother Res* **30**: 1265-1286.

3. Fang J (2014) Bioavailability of anthocyanins. *Drug Metab Rev* **46**: 508-520.

4. Hansberry DR, Shah K, Agarwal P, Agarwal N (2017) Fecal Myeloperoxidase as a biomarker for inflammatory bowel disease. *Cureus* **9**: e1004.

5. Lalles JP (2014) Intestinal alkaline phosphatase: novel functions and protective effects. *Nutr Rev* **72**: 82-94.

6. Kim KM, Kim YS, Lim JY, Min SJ, Ko HC, et al. (2015) Intestinal anti-inflammatory activity of *Sasa quelpaertensis* leaf extract by suppressing lipopolysaccharide-stimulated inflammatory mediators in intestinal epithelial Caco-2 cells co-cultured with RAW 264.7 macrophage cells. *Nutr Res Pract* **9**: 3-10.