U.PORTO

MESTRADO

TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Effect of H_2S in the reactivity of the rat colon in a refined model of TNBS-induced colitis

Daniela Menezes Pinto

M

2020

U.PORTO

Daniela Menezes Pinto. Effect of H_2S in the reactivity of the rat colon in a refined model of TNBS-induced colitis

Effect of H_2S in the reactivity of the rat colon in a refined model of TNBS-induced colitis

Daniela Menezes Pinto

M.ICBAS **2020**

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Daniela Menezes Pinto

Effect of H_2S in the reactivity of the rat colon in a refined model of TNBS-induced colitis

Dissertação de Candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientadora – Doutora Professora Manuela Morato

Afiliação – Laboratório de Farmacologia, Departamento das Ciências do Medicamento, LAQV@REQUIMTE, Faculdade de Farmácia da Universidade do Porto (FFUP)

Co-Orientadora – Professora Doutora Margarida Duarte Araújo

Afiliação – Departamento de ImunoFisiologia e Farmacologia, Instituto de Ciências Biomédicas Abel Salazar (ICBAS) da Universidade do Porto

This work was supported by Fundação para a Ciência e Tecnologia under the Partnership Agreement UIDB 50006/2020. Also, M.M. thanks GEDII (Grupo de Estudos da Doença Inflamatória Intestinal) for funding.

Acknowledgments

I come from a long journey with several challenges and many special people who always supported me.

I thank my parents, Angelita and Luiz Hosannah, for dreaming my dreams and giving me so much love, affection, support, and care. Without you I wouldn't have gotten this far. Thank you for believing in me.

To my dear sister, Renata, who even far away can be present in the most important moments. To my lovely stepmother, Valéria, for having a joy that overflows and spreads.

To my beloved grandmother, Heloísa, who has the best hug in the world and always welcomes me in the best way possible. To my uncles, Oscar, Marcelino, Artur for bringing a party to my heart and cheering my conquests. To my aunt Esmeralda, for being such a wonderful, concerned, caring person even from afar. To all my cousins who live in my heart.

I would also like to thank Dra. Manuela Morato and Dra. Margarida Araújo for the patience and guidance. Also, for all the availability, mentoring, knowledge, and for all the friendly support.

I like to thank my wonderful colleagues, Mariana Duarte and Marisa Monteiro, for all the team play and collaboration in the lab, also for the friendship, coffees and enthusiasm. To Maria do Céu, for all the help, patience, and instructions every day in the lab.

To my incredible boyfriend, Filipe Valente, for all the love, support, encouragement and understanding. Thank you for having been present, in good times as well as in every difficult step. Thank you for truly believing in me. Also, I would like to thank his amazing family.

To all my friends who were always present, thanks for every laugh, every advice, every hug. I appreciate you all, each one of you were remarkable and I wish you all the best.

I am grateful to everyone.

Abstract

Introduction: Despite toxicological concerns, endogenous hydrogen sulphide (H_2S) has physiological roles such as anti-inflammatory effects, namely in the intestine. But, high intestinal concentrations of H_2S have also been considered a trigger for the development of inflammatory bowel disease (IBD). However, there is still no data on the action of H_2S on intestinal motility in GI diseases, such as IBD. TNBS-induced colitis is the most frequently used experimental model of IBD in rats, but is associated with pain and discomfort.

Aims: To further refine the TNBS-induced model of colitis in rats, to increase welfare and reduce the percentage of rats developing severe colitis. Also, to understand the role of H_2S on colonic reactivity in this experimental model of IBD.

Methods: Protocols were approved by the institutional and national Animal Welfare Body. For induction of TNBS-induced colitis, male Wistar rats (16-18 weeks old; *n*=12) received a 30% ethanolic solution of TNBS (15mg/rat; 250 µL) rectally instilled. Pain relief and colonic motility were assured with metoclopramide (1mg/Kg, PO), and tramadol (20mg/kg, PO, once) and paracetamol (500 mg/kg, PO, SID). Animals were daily monitored for body weight, food and water intake, fecal peletts, and wellbeing. After 7 days, TNBS and control rats were euthanized by decapitation, the colon was excised and attributed a macroscopic score (MaS). Segments of the proximal (PC), middle (MC) and distal (DC) colon were mounted in organ baths and the effect of the H_2S donor, NaHS was tested over ACh-induced precontraction, on ACh-induced contraction, and directly on basal resting tone.

Results: According to the MaS, TNBS-induced colitis coursed with 4 animals with mild and 8 with moderate colitis (no rats developing severe colitis). TNBS-induced rats showed a similar pattern in all parameters analysed, although different from controls. The Welfare Score correlated with MaS. NaHS caused similar concentration-dependent relaxation of PC, MC and DC precontracted with ACh in control and TNBS-induced rats. In control rats, but not in TNBS-induced rats, NaHS decreased the contractile response to Ach. NaHS caused a concentration-dependent decrease in the basal resting tone of the PC and altered the pattern of spontaneous contractions in controls and TNBS-induced rats.

Conclusions: It was possible to refine the rat TNBS-induced model of IBD. In control conditions, NaHS relaxes the pre-contracted colon and attenuates ACh-mediated contractions. The mechanism underlying this effect is probably different and selectively compromised in the TNBS-induced rat, but further studies are needed to clarify these results.

Keywords: NaHS, hydrogen sulphide, colonic reactivity, IBD, TNBS-induced colitis

Resumo

Introdução: Apesar das preocupações toxicológicas, o sulfureto de hidrogênio (H_2S) endógeno pode ter efeitos anti-inflamatórios, principalmente no intestino. Mas, altas concentrações intestinais de H_2S também foram consideradas para o desenvolvimento da doença inflamatória intestinal (DII), não existindo ainda dados sobre a sua ação na motilidade intestinal nessa doença. A colite induzida por TNBS é o modelo experimental de DII mais frequentemente usado em ratazanas, mas está associada a dor e desconforto**.**

Objetivos: Refinar o modelo de colite induzida por TNBS em ratazanas, para aumentar o bem-estar e reduzir a porcentagem da colite severa. Além disso, compreender o papel do H_2S na reatividade do cólon neste modelo experimental de DII**.**

Métodos: Os protocolos foram aprovados pelo órgão institucional e nacional de bem-estar animal. Para a indução por TNBS, ratazanas Wistar machos (16-18 semanas de idade; *n*=12) receberam uma solução etanólica (30%) de TNBS (15mg / rato; 250 µL) instilada por via retal. O alívio da dor e a motilidade intestinal foram garantidos com metoclopramida (1mg/Kg, PO), tramadol (20mg / kg, PO, uma vez) e paracetamol (500 mg / kg, PO, SID). Os animais foram monitorizados diariamente relativamente ao peso corporal, ingestão de comida e água, pellets fecais e bem-estar. Após 7 dias, ambos os grupos foram sacrificados por decapitação, sendo o cólon excisado e atribuída uma pontuação macroscópica (MaS). Segmentos do cólon proximal (PC), médio (MC) e distal (DC) foram montados em banhos de órgãos e o efeito do dador de H_2S, o NaHS, foi testado na pré-contração induzida por ACh, na contração induzida por ACh e diretamente no tônus basal em repouso.

Resultados: De acordo com o MaS, a colite foi classificada como leve em 4 animais e moderada em 8 (nenhum animal desenvolveu colite severa). Ratazanas induzidas por TNBS mostraram um padrão semelhante em todos os parâmetros analisados, embora diferente dos controlos. A pontuação de bem-estar correlacionou-se com a MaS. O NaHS causou relaxamento à pré-contração por ACh de forma semelhante no colon dos controlos e nos animais induzidos por TNBS. Em animais controlos, mas não em ratazanas induzidas por TNBS, o NaHS diminuiu a resposta contrátil à ACh. O NaHS causou uma diminuição dependente da concentração no tônus basal de repouso do PC e alterou o padrão das contrações espontâneas em ambos os grupos.

Conclusões: Foi possível refinar o modelo de DII. Em condições controlo, o NaHS relaxa o cólon pré-contraído e atenua as contrações mediadas pela ACh. O mecanismo subjacente a esses efeitos é provavelmente diferente e seletivamente comprometido nos animais induzidos por TNBS, mas são necessários mais estudos para esclarecer esses resultados. **Palavras-chave:** NaHS, sulfureto de hidrogênio, reatividade colônica, IBD, colite induzida por TNBS

In the course of this work, the following original articles were published / submitted:

Characterization of ethyl acetate and n-butanol extracts of *Cymbopogon schoenanthus* and *Helianthemum lippii* and their effect on the smooth muscle of the rat distal colon

Nihed Djemam, Somia Lassed, Fatih Gül, Muhammed Altun, Marisa Monteiro, Daniela Menezes-Pinto, Samir Benayacheb Fadila Benayache, Djamila Zama, Ibrahim Demirtas, Manuela Morato. *Journal of Ethnopharmacology* Volume 252, 24 April 2020, 112613. https://doi.org/10.1016/j.jep.2020.112613

TNBS-Induced colitis in *Rattus norgevicus*: a categorization proposal

Mariana Ferreira-Duarte, Tiago Rodrigues-Pinto, Daniela Menezes-Pinto, Marisa Esteves-Monteiro, Salomé Gonçalves-Monteiro, Sara Capas-Peneda, Fernando Magro, Patrícia Dias-Pereira, Manuela Morato & Margarida Duarte-Araújo. *Experimental Animals* (2020).

Part of this work was presented in the following scientific meetings:

IJUP (Porto, 2020) – Poster Presentation

TNBS-induced colitis in rats: Can such an old model be refined?

Daniela Menezes-Pinto, Marisa Esteves-Monteiro, Mariana Ferreira-Duarte, Tiago Rodrigues-Pinto, Margarida Duarte-Araújo, Manuela Morato

Index

Figure index

Table index

List of abbreviations

3MP 3-Mercaptopiruvate
3MST Mercaptopiruvate Transferase
ACh Acetylcholine
ATP Adenosine triphosphate
Ca^{2+} Calcium
cAMP Cyclic adenosine monophosphate
CAT Cysteine aminotransferase
CBS Cystathione-b-synthase
CD Crohn's Disease
cGMP Cyclic guanosine monophosphate
CO Carbon monoxide
COVID Coronavirus Disease
CSE Cystathione ɣ-lyase
DC Distal colon
DNA Deoxyribonucleic acid
ENS Enteric nervous system
g Gram
GC Guanylyl cyclase
GI gastrointestinal
h Hours
H_2O Water
H_2S Hydrogen sulphide
IBD Inflammatory bowel disease
ICC Interstitial cells of Cajal
K_{ATP} ATP-sensitive potassium
l Liter
L-NNA NG-Nitro-L-arginine
MaS macroscopic score
MC Middle colon
min Minutes
ml Milliliter
µM Micromolar
mm Millimeter
mmol Milimolar
µL Microliter
MPO Myeloperoxidase
Na_2S Sodium sulphide
NaHS Sodium hydrosulphide
nM nanomolar
NO Nitric oxide
Nrf2 Nuclear factor erythroid 2-related factor 2
ODQ 1H-[1,2, 4]oxadiazolo [4,3,-a]quinoxalin-1-one
PC Proximal colon
PDE5 cGMP-specific phosphodiesterase 5
PKG cGMP-dependent protein kinase
PO Per os
ppm parts per milion
RNA Ribonucleic acid
ROS Reactive oxygen species
sGC Soluble Guanylyl cyclase
SID Once daily
SK_{Ca} Small conductance calcium-activated potassium
SRB Sulfate-reducing bacteria
TNBS 2,4,6-Trinitrobenzenesulfonic acid
TNF-α Tumor necrosis factor
TRPV1 Transient receptor potential vanilloid 1
TTX Tetradotoxin
UC Ulcerative Colitis

I. Introduction

1. Toxicological aspects of H_2S

Hydrogen sulphide (H_2S) is considered a toxic, colourless, flammable gas, soluble in water and that under normal conditions of pressure and temperature has a characteristic odour of rotten eggs (Beauchamp *et al*., 1984). For a long time, this gas was considered only a biological and environmental risk, being commonly called "sewage gas" (Jiang *et al.*, 2016). It can be found in nature in volcanic and swampy areas, and by human action in industrial areas such as paper mills, mining and oil refineries (Hessel *et al*., 1997). For that, workers in these areas are at greater risk of suffering occupational poisoning, which can eventually lead to death (Hessel *et al*., 1997; Goubern *et al*., 2007; Jiang *et al*., 2016).

The respiratory system is the main route for human exposure, both in workplaces and in the environment (Goubern *et al*., 2007). Due to its toxicity, H_2S is capable of irritating the eyes and act on the human nervous and respiratory systems (Jiang *et al*., 2016). Excess H_2S acts as a respiratory depressant producing hypoventilation, hypoxia, cyanosis and metabolic acidosis (Cao *et al*., 2019). Lethal poisoning occurs in concentrations of 1000 to 2000 ppm, due to paralysis of the respiratory centre and, consequently, cardio-respiratory arrest and death (Figure 1) (Roth, 2004). These concentrations are easily reached since despite its characteristic unpleasant odour, H_2S causes odour loss at levels higher than 150 ppm, due to fatigue of the olfactory system sensitive to the exposure (Beauchamp *et al*., 1984). The effects of controlled exposure to low concentrations of H_2S and whether the effects are completely reversible are still unclear, but in chronic exposure there is evidence of deleterious effects on the central nervous system and respiratory disorders (Hannah & Roth, 1991; Lim *et al*., 2016).

H_2S enters the body through the respiratory system and it is rapidly oxidized into compounds of lower toxicity, such as thiosulfate (Lim *et al*., 2016). There is no accumulation in the body and the catabolism occurs through oxidation, methylation and expiration (Cao *et al*., 2019). The elimination happens through urine, intestinal content and the expired air (Roth, 2004).

Intoxication is mainly due the inhibition of some enzymes such as Na^+/K^+ ATPase and cytochrome C oxidase, interfering with the electron transport chain (Goubern *et al*., 2007). Depending on the concentration, it can lead to death in minutes (Roth, 2004). Therefore, in the event of an accident involving leakage of this gas, the consequences can reach great proportions, endangering human life, the integrity of industrial heritage and the environment

(Jiang *et al*., 2016; Roth, 2004). When released, H_2S remains in the atmosphere for an average of 18 hours and during this time it can be converted into sulphur dioxide and sulfuric acid, which are toxic and corrosive metabolites (Chou *et al*., 2006).

Since H_2S exists as a gas, oral exposure is not likely to occur and the rate of absorption in the gastrointestinal tract is unknown. However, H_2S poisoning can be produced by soluble sulphide salts in the gastrointestinal tract, such as sodium sulphide (Na_2S) and sodium hydrosulphide (NaHS), also known as H_2S donors (U.S. Chemical Safety and Hazard Investigation Board, 2004).

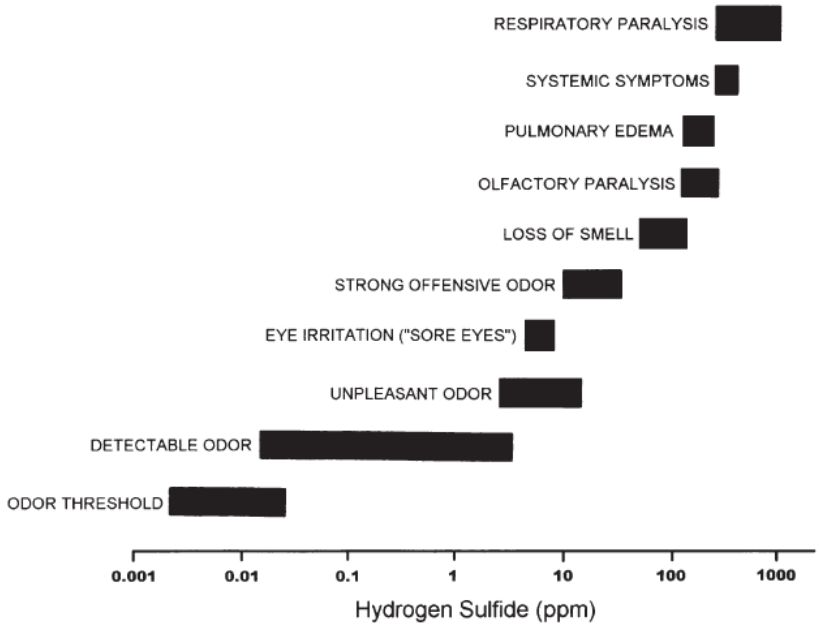

Figure 1. The effects of H_2S in concentration-dependent exposure (Roth, 2004).

2. Endogenous production of H_2S

Despite the initial toxicological concerns, several studies have demonstrated relevant physiological roles of H_2S, coupled with the discovery of its synthesis and endogenous presence in the body (Szabo, 2017). H_2S is produced both by endogenous enzymatic or non-enzymatic pathways and by naturally occurring gut microbiota (Medani *et al*., 2011).

2.1. Enzymatic production of H_2S

The endogenous production of H_2S in mammalian systems occurs mainly through an enzymatic pathway (Figure 2) that involves two vitamin B6 (pyridoxal-5-phosphate)-

dependent enzymes: cystathione-β-synthase (CBS) and cystathione γ-lyase (CSE) (Cao *et al*., 2019). The precursors of H_2S are L-cysteine, a non-essential amino acid that can be obtained from the diet or synthesized from methionine, and homocysteine, a sulfhydryl amino acid produced as an intermediate during the metabolism of methionine (Figure 2) (Flannigan *et al*., 2013).

Homocysteine is metabolized through transulfurization reactions with the participation of CBS and the production of cystathionine, which is hydrolysed by CSE to L-Cysteine and α-ketobutyrate (Vilaça *et al*., 2015). L-cysteine can then be metabolized by these enzymes generating H_2S, or it can be converted to 3-mercaptopiruvate (3MP) by cysteine aminotransferase (CAT), whose activity depends on the presence of α-ketobutyate (Flannigan *et al*., 2013). Then, mercaptopiruvate transferase (3MST), which is widely located in the mitochondria, can metabolize 3MP and generate H_2S (Figure 2) (Flannigan *et al*., 2013; Shibuya *et al*., 2009).

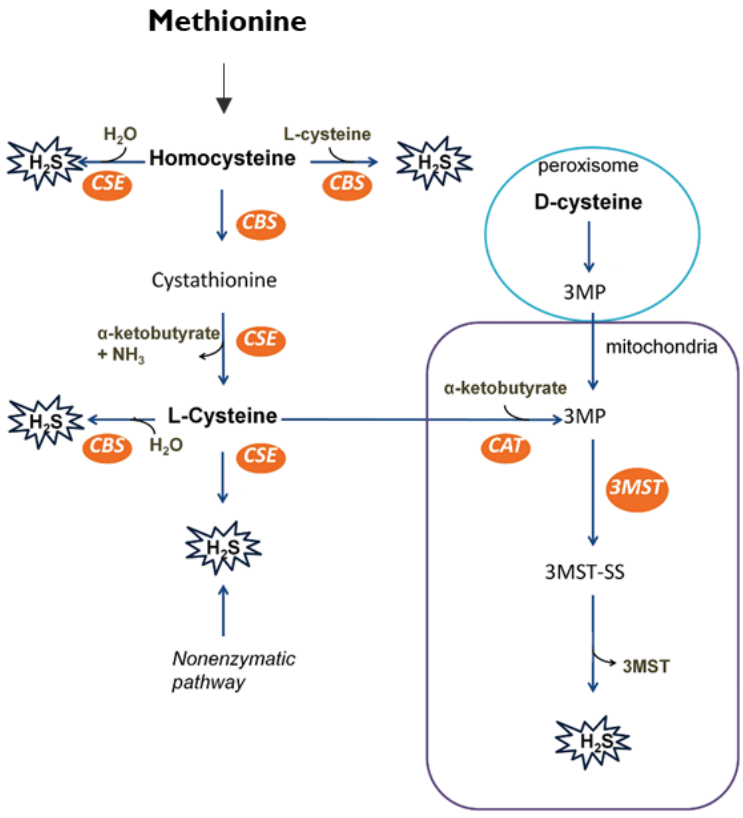

Figure 2. The main pathways for H_2S synthesis. L-cysteine is the main substrate for the generation of H_2S through the enzymes CBS / CSE and 3MST. Non-enzymatic production can occur, although it does not contribute significantly to total H_2S production (Adapted from Cao *et al*., 2019).

These H_2S-forming enzymes are widely distributed in the mammal organism, with CBS and 3MST being mainly expressed in the nervous system and CSE in several organs (Enokido *et al*., 2005; Shibuya *et al*., 2009). In the gastrointestinal (GI) tract, CBS/CSE were detected, by immunohistochemistry and western blot, throughout the colon of humans and rats (Gil *et al*., 2011; Martinez-Cutillas *et al*., 2015). They are expressed in neurons of submucosal and myenteric plexus, in some subclasses of interstitial cells of Cajal (ICC) (Schicho *et al*., 2006), and also in the epithelium and muscle wall of the rat colon (Liu *et al*., 2013). This makes endogenous H_2S physiologically present in different GI cells, being able to reach molecular targets in different systems (Gil *et al*., 2011; Liu *et al*., 2013), since these enzymes are responsible for most of the production of endogenous H_2S in mammalian cells.

The non-enzymatic pathway occurs under physiological conditions in erythrocytes and depends on iron and vitamin B6 for production of H_2S (Yang *et al*., 2019). This route corresponds to a very small portion of the total H_2S produced and the biological significance of this production is yet to be determined (Searcy & Lee, 1998; Yang *et al*., 2019).

2.2 Production of H_2S by the intestinal microbiota

Sulfate-reducing bacteria (SRB) are widely present in the lumen of the large intestine and H_2S is the final product of their metabolism (Dordević *et al*., 2020). Among SRB groups there are a variety of microorganisms, such as *Deltaproteobacteria* (mesophilic genera *Desulfovibrio*, *Desulfobacterium*), *Thermodesulfovibrio* (thermophilic gram-negative bacteria), *Desulfotomaculum* (gram-positive bacteria) and *Archaeoglobus* (Euryarchaeota) (Barton & Fauque, 2009). The most prevalent is the *Desulfovibrio* genera, representing 66% of colonic SRB (Gibson *et al*., 1993).

Consequently, H_2S can be present in high concentrations in the colon of healthy adult individuals, with a range from 0.3 to 3.4 mmol/l, but most of the H_2S is combined with the luminal content and, therefore, it is not able to reach the intestinal wall (Medani *et al*., 2011; Rowan *et al*., 2009). The low levels of free H_2S (μM range) in the human colon (measured by spectrophotometry) are rapidly oxidized to thiosulfate by the epithelial colon cells (Gil *et al*., 2013; Jørgensen & Mortensen, 2001; Mimoun *et al*., 2012). Also, the mitochondrial enzyme sulphide quinone reductase, highly expressed in the healthy colonic epithelium, consumes sulphide as a fuel, and H_2S is rapidly degraded (Lagoutte *et al*., 2010; Mimoun *et al*., 2012). Consequently, under physiological conditions, the concentration of H_2S produced by SRB that reaches the submucosa and the muscular layers of the intestine are rather low (Gil *et al*., 2013; Jørgensen & Mortensen, 2001; Mimoun *et al*., 2012). So, as the

enterocytes are important for detoxification of H_2S, when the intestinal barrier is preserved the level of H_2S is not able to modify colonic functions (Jimenez *et al*., 2017).

The intestinal microbiota is responsible for beneficial functions in mammals, such as metabolism and nutrition, protection and maintenance of mucosal integrity, and production of pharmacologically active signalling molecules (Boulangé *et al*., 2016). The knowledge of these functions has revolutionized the approach to certain chronic pathologies (Thursby & Juge, 2017). In the context of the present work, the disrupted equilibrium of gut microbiota can have a negative impact on health, due to inefficient detoxification of H_2S. Indeed, higher concentration of H_2S has been presented as one of the factors for triggering inflammatory bowel disease (IBD) and cancer (Teigen *et al*., 2019).

3. H_2S donors

Besides the endogenous production of H_2S, some inorganic salts such as Na_2S and NaHS, release H_2S almost instantaneously and are widely used to investigate H_2S-induced effects (Andruski *et al*., 2008; Stefano Fiorucci *et al*., 2005). Indeed, several activities attributed to H_2S have been demonstrated with these substances, such as decreased cytokine production, regulation of ion channels, neurogenic regulation, cardioprotective effects and the therapeutic actions of exogenous H_2S delivery combined with anti-inflammatory drugs (Song *et al*., 2014).

Less used are the slow liberation donors, including water-soluble molecules such as GYY4137 and tritone hydroxide anhydrate (ADT-OH) complexes (Wang, 2012). Moreover, there are naturally occurring compounds that can function as H_2S donors, including garlic diallyl disulfide and broccoli sulforaphane (Olson, 2011). Differently, the cysteine analogues, such as S-propil cysteine and other sulfonation molecules, have a stimulating effect on the endogenous production of H_2S (Wang, 2012).

4. Biological effects of H_2S

The understanding of H_2S gained a new perspective after Abe & Kimura (1996) first described the endogenous production of H_2S and its modulating role in the central nervous system (Abe & Kimura, 1996). After this, studies were carried out in order to explore its physiological and pathophysiological role in mammals, revealing an important effect in the nervous, cardiovascular, renal, reproductive, respiratory and digestive systems (Kamoun, 2004). Currently, H_2S is considered an endogenous gasotransmitter, along with nitric oxide (NO) and carbon monoxide (CO) (Vandiver & Snyder, 2012).

Many of the effects induced by H_2S have been demonstrated and reported to be mediated by protein S-sulfhydration (Ju *et al*., 2017), which involves the conversion of thiol groups (-SH) from cysteine to disulphide groups (-SSH) that mediate various cellular functions and metabolic pathways (Zhang *et al*., 2017). Among the main effects resulting from this H_2S-induced protein modification are the activation of ATP-sensitive potassium channels (K_{ATP}) and the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) (Islam *et al*., 2015). Activation of K_{ATP} channels is important for insulin secretion by β cells in the pancreas, neuronal protection and regulation of nociception and inflammation, which support the potential role of this gasotransmitter in several conditions, such as diabetes, neurodegenerative diseases and painful inflammatory conditions (Wallace & Wang, 2015). Nrf2 regulates the expression of several cytoprotective enzymes and plays an important role in antioxidant systems and oxidative stress response (Ju *et al*., 2017; Zhang *et al*., 2017). H_2S still interacts with other ion channels, such as voltage-dependent calcium channels and calcium-activated potassium channels, modulates the levels of second messengers, such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), and can influence (decrease or increase) intracellular calcium concentrations (Kabil *et al*., 2014).

Similar to other gaseous mediators, H_2S has anti-inflammatory properties (Fiorucci *et al*., 2005). It is a potent inhibitor of leukocyte adhesion to the vascular endothelium and has analgesic and antioxidant effects (Distrutti *et al*., 2010; Wallace *et al*., 1993), although the mechanism by which this molecule has these effects is not yet fully understood (Zanardo *et al*., 2006). The modulating role of H_2S in cardiovascular function is one of the most studied so far, especially its vasodilator activity (Lim *et al*., 2008). Hosoki *et al*., in 1997, demonstrated the presence of H_2S-producing enzymes in vessels and its vasorelaxation activity (Hosoki *et al*., 1997). Later, it was observed that the vasodilator effect induced by H_2S occurs through the activation of K_{ATP} channels and modulation of endothelial NO synthase activity (Pan *et al*., 2017; Zhao *et al*., 2001). H_2S induces a cardioprotective effect in several cardiac disorders, such as myocardial infarction, arrhythmia, cardiac hypertrophy, myocardial fibrosis and heart failure (Shen *et al*., 2015). This protection is mainly due to antioxidant and anti-apoptotic activities, in addition to activating K_{ATP} channels and preserving mitochondrial function (Shen *et al*., 2015).

In the GI system, H_2S plays a role in intestinal motility and inflammation (Linden, 2014). It has been reported an increased production of endogenous H_2S in the colon as a response to an injury (Wallace *et al*., 2009; Hirata *et al*., 2011). Also, exogenous H_2S has a protective role in models of intestinal ischemia, ethanol-induced gastritis (Chávez-Piña *et al*., 2010;

Liu *et al*., 2012; Medeiros *et al*., 2009), acetic acid-induced gastric ulcers, and colonic inflammation induced by 2,4,6-Trinitrobenzenesulfonic acid (TNBS) in rats (Kupai *et al*., 2017; Wallace *et al*., 2007).

In rats, H_2S modulates inflammation and contributes to the resolution of colitis, however further studies are needed to extrapolate to other species, such as humans (Kupai *et al*., 2017; Wallace *et al*., 2009). This characteristic has been studied for the expansion of therapeutics designed to release H_2S from nonsteroidal anti-inflammatory drug (NSAID) and to combat side effects, especially gastric ulcer (Fiorucci *et al*., 2007; Wallace *et al*., 2007).

5. Colon Motility and H_2S

The GI motility function requires both mixing/turning over and propulsion of luminal contents (Murthy, 2006). Digestive and absorptive functions are not constant throughout the GI tract and the intensity of mixing/turning over and the rates of propulsion vary among the organs, and in different parts of the same organ (Sanders *et al*., 2004). Colon is the last major organ in the GI tract and plays a critical role in regulating the frequency of defecation and consistency of stools (Murthy, 2006).

The intestinal smooth muscle is divided in longitudinal and circular muscular layer (Figure 3) (Murthy, 2006). Within each muscle bundle, the fibres are electrically connected through many communicating junctions, with low resistance to ions movement from one muscle cell to the other (Kumral & Zfass, 2018). These muscle bundles merge so that each muscle layer represents a network, functioning as a syncytium (Murthy, 2006; Kumral & Zfass, 2018). So when an action potential is fired at any point in the muscle mass, it propagates in all directions of the muscle (Kumral & Zfass, 2018). In addition, the enteric nervous system (ENS) is essential to control motility and GI secretion (Murthy, 2006). It is composed of two plexuses, the external plexus (between the longitudinal and circular muscular layers, called myenteric plexus) and the internal plexus (called submucosal plexus) (Figure 3) (Murthy, 2006).

The intestinal motility is mediated by the contractions of smooth muscle cells (circular and longitudinal) and, as in other muscle cells, are usually caused by a nervous impulse that spreads through the muscle fibre membrane (Webb, 2003). It then depends on the actin-myosin interaction, which is regulated by the intracellular concentration of calcium (Ca^{2+}) (Spencer & Hongzhen, 2020). Contraction is commonly initiated by opening the voltage-gated L-type Ca^{2+} channels during depolarization of the membrane (Webb, 2003). When

the light chain of myosin is phosphorylated, the myosin head interacts with an actin filament, resulting in smooth muscle contraction (Webb, 2003).

Smooth muscle relaxation occurs with hyperpolarization of the membrane and myosin light chain dephosphorylation (Spencer & Hongzhen, 2020). Dephosphorylation is enhanced when guanylyl cyclase (GC) is activated inside the smooth muscle cell (Murthy, 2006). This increases the production of cGMP, which stimulates cGMP-dependent protein kinase (Murthy, 2006; Webb, 2003), activating myosin light chain phosphatase. The dephosphorylation inhibits the interaction of the myosin head with actin, resulting in smooth muscle relaxation (Webb, 2003).

That said, there is evidence that H_2S is an important mediator of the GI motility (Martinez-Cutillas *et al*., 2015). It was demonstrated that NaHS, which is a H_2S donor, induces relaxation of the ileum of healthy rabbits, rats and humans (Gallego *et al*., 2008; Martinez-Cutillas *et al*., 2015; Nalli *et al*., 2017; Teague *et al*., 2002). Although the main effect of H_2S on smooth muscle is relaxation, in some tissues and in some species H_2S may have a contractile effect (Patacchini *et al*., 2004, 2005). H_2S has been shown to produce contractile responses in the urinary bladder of rats by activating the primary afferent neurons sensitive to capsaicin (receptor TRPV1) (Streng *et al*., 2008). Besides, in the stomach of guinea pig it was demonstrated that H_2S can exert both an excitatory effect producing tonic contraction (at low concentration of NaHS 0.1 to 0.3mM) and an inhibitory effect on spontaneous contractility (at high concentration of NaHS 0.3 to 1.0mM) (Zhao *et al*., 2009).

Exogenous delivery of H_2S affects smooth muscle contraction with data suggesting a relaxant role for this gasotransmitter in the GI tract (Jimenez *et al*., 2017). Isolated segments of rabbit, guinea pig, rat and human intestine exhibit relaxation and reduced acetylcholine (ACh) mediated contraction when exposed to NaHS (Dhaese *et al*., 2010; Gil *et al*., 2013; Martinez-Cutillas *et al*., 2015; Teague *et al*., 2002). The molecular mechanisms that contribute to H_2S-induced smooth muscle relaxation are not fully understood, and need more investigation (Medani *et al*., 2011). So far, some studies have demonstrated a partial contribution of the K_{ATP} channel in the hyperpolarization of smooth muscle and relaxation, and it has also been suggested a role for voltage-gated potassium channels, interaction with myosin light chain phosphatase, small conductance calcium-activated potassium channels (SK_{Ca}) and a possible synergy with the nitrergic pathway or nitrergic neuronal inputs (Distrutti *et al*., 2006; Gil *et al*., 2013; Matsunami *et al*., 2012; W. Zhao *et al*., 2001).

H_2S has also an inhibitory role on spontaneous rhythmic contractile activity in mechanical recording experiments in the colon (Gallego *et al*., 2008). Spontaneous rhythmic contractile activity occurs across species and in distinct gastrointestinal regions, but mostly in the colonic segment (Gallego *et al*., 2008; Zhao *et al*., 2009). These contractions are dependent on ICCs, which form a network and interpose in the smooth muscle layers, generating intrinsic pacemaker potentials (Figure 3) (Parajuli *et al*., 2010). H_2S inhibits pacemaker activity in isolated ICC of mouse small intestine and interacts with NO in regulating functional pacemaker activity (Yoon *et al*., 2011).

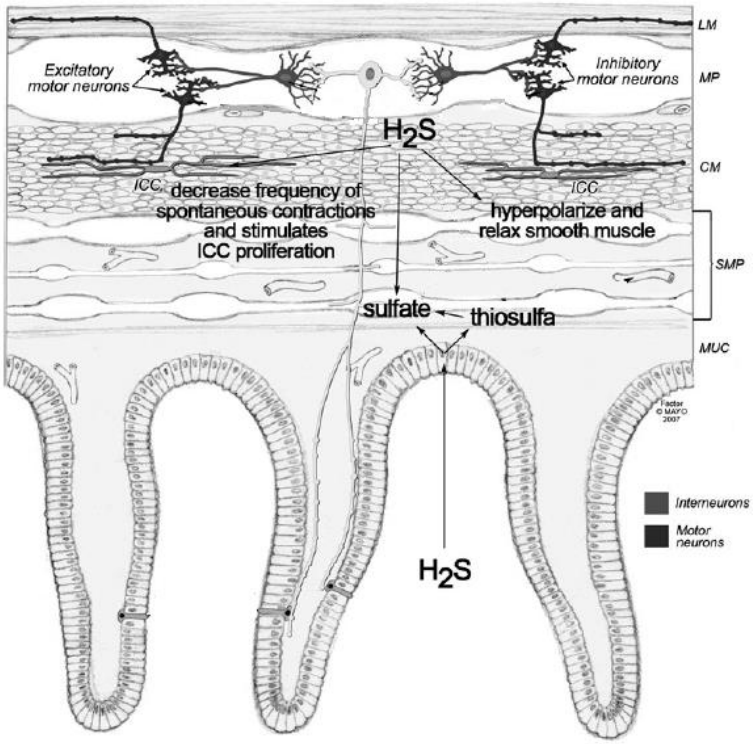

Figure 3. H_2S modulates gastrointestinal motility. The neural and muscular components of the peristaltic reflex are exemplified with known activities of H_2S on these components. Epithelial cells oxidize H_2S to thiosulfate and sulfate. ICC, interstitial cells of Cajal; LM, longitudinal muscle; MP, myenteric plexus; CM, circular muscle; SMP, submucosal plexus; MUC, mucosa (adapted from Linden, 2010).

Overall, the effects of H_2S in the GI system are complex and multiple, hence the need for more studies to investigate its action in a cellular level (Gil *et al*., 2013).

All studies concerning H_2S and motility so far were carried out on healthy individuals, both animals and humans, and there is still no data showing the action of H_2S on intestinal motility in GI diseases. Since this molecule has been studied for the treatment of colonic

inflammation, its action as antispasmodic molecule is still to be clarified (Chan & Wallace, 2013; Jimenez *et al*., 2017).

6. Inflammatory Bowel Disease

IBD includes two chronic and recurrent multifactorial conditions: Crohn's Disease (CD) and Ulcerative Colitis (UC). These two disorders have some similarities but they differ in terms of pathophysiological aspects, clinical manifestations and associated complications (Hanauer, 2006). CD and UC have a bimodal age distribution, with the highest incidence rates being observed in individuals aged between 15 and 30 years and a lower peak of incidence seen in patients aged between 50 and 70 years (Horn & Ufberg, 2011; Stange *et al*., 2008).

CD is a transmural inflammatory condition that can affect any region of the GI tract, but predominantly affects the terminal ileum and the perianal region (Lapaquette *et al*., 2012). It typically presents in a discontinuous form, reaching several portions of the GI and it can be associated with complications, such as fistulas and abscesses (Khor *et al*., 2011). CD can be clinically described as mild, moderate or severe (Hanauer, 2006). Mild disease is characterized by the absence of severe abdominal pain, intestinal obstruction, weight loss, or signs of systemic toxicity (fever, tachycardia, anaemia and an increase in the erythrocyte sedimentation rate) (Hanauer, 2006; Mowat *et al*., 2011). In moderate CD there are symptoms of systemic toxicity, weight loss, pain and tenderness in the abdominal region, nausea and vomiting without intestinal obstruction or significant anaemia (Hanauer, 2006; Mowat *et al*., 2011). The severe type of CD is characterized by high fever, cachexia, persistent vomiting, intestinal obstruction and abscess (Hanauer, 2006; Mowat *et al*., 2011; Singh *et al*., 2011).

Unlike CD, UC is characterized by diffuse inflammation of the colon mucosa, it affects the rectum (95% of cases) and the proximal portions of the colon, in a continuous manner, with no areas of normal mucosa between the affected parts (Kornbluth & Sachar, 2010). UC can be classified according to its anatomical location and extension into proctitis (inflammation restricted to the rectum), distal colitis (inflammation encompasses the sigmoid colon with or without the involvement of the descending colon), and pancolitis or total colitis (Baumgart & Sandborn, 2007). Also, UC can be classified by the severity of individual acute relapses and symptoms as: remission, mild, moderate and severe disease (Silverberg *et al*., 2005). Remission in UC is defined as a defecatory frequency equal to or less than 3 times a day, an endoscopically normal mucosa, and no retorrhagia, for example (Harbord *et al*., 2017).

6.1 Epidemiology of IBD

The two main forms of IBD are considered diseases of modern society and occur all over the world with a higher incidence and prevalence in the populations of developed countries (Sairenji *et al*., 2017). IBD affects men and women and represents a serious health issue since it affects young people and have frequent recurrences with highly serious clinical forms (Lamb *et al*., 2019).

IBD is more common in northern Europe and the United States, where 5,000 to 10,000 new cases are diagnosed per year (Lamb *et al*., 2019). An interesting aspect, although not yet clarified, is the increase in the incidence of IBD in countries whose socioeconomic conditions are being improved, such as, for example, in Latin American countries (Appleyard *et al*., 2004; Oliveira *et al*., 2010). This increased prevalence and incidence of IBD in developing countries might possibly be due to greater efficiency in identification of patients, coupled with greater access to diagnostic tools, and to environmental changes such as industrialization and adoption of a lifestyle similar to that of developed countries, where the incidence is higher (Gasparetto & Guariso, 2013; Ng *et al*., 2013).

6.2 Etiology and pathogenesis of IBD

Despite being known for more than five decades, CD and UC are considered idiopathic intestinal inflammatory disorders, since the triggering factors remain elusive (Mulder *et al*., 2014). A series of elements contribute to the development of mucosa inflammation, such as environmental causes, genetic factors, immunoregulatory defects, and exposure to microorganisms (Hanauer, 2006; Lapaquette *et al*., 2012).

The intestinal lumen, while harbouring possible pathogens and toxic substances that require a rapid response from the immune system, is also full of innocuous antigens present in food and commensal bacteria (Verma *et al*., 2013; Geremia & Arancibia-Cárcamo, 2017). That is, the intestinal lymphoid tissue should be able to react differently: initiate an inflammatory reaction to potentially harmful antigens and recognize harmless antigens, maintaining tolerance towards them (Geremia & Arancibia-Cárcamo, 2017). In IBD, the exacerbate growth of some type of bacteria population (SRB, for example) and dysbiosis may be one of the reasons for the intestinal uncontrolled reaction, with the intestine being unable to distinguish what is innocuous, and starting a chronic process of inflammation (Mulder *et al*., 2014; Geremia & Arancibia-Cárcamo, 2017).

Genetic and environmental factors such as stress, smoking, diet and medications can contribute to reducing the integrity of the intestinal mucosa, causing interaction between

microbiota products and cells of the immune system and inflammatory response (Verma *et al*., 2013). Failure to resolve the inflammatory response ends up making the process chronic, causing tissue damage and complications (Hanauer, 2006).

In active IBD, there is infiltration of innate (neutrophils, macrophages, dendritic cells, natural killer T cells) and adaptive (T and B lymphocytes) immune cells in the lamina propria of the gastrointestinal mucosa (Geremia & Arancibia-Cárcamo, 2017). These cells increase the tissue levels of TNF-α and pro-inflammatory interleukins, such as IL-17/IL-22 in CD and IL-13 in UC (Geremia & Arancibia-Cárcamo, 2017). Also, tissue damage and granuloma formation characteristic of IBD are the result of neutrophil migration and degranulation (Danese & Fiocchi, 2011). The infiltration of neutrophils in the tissue generates antimicrobial peptides and reactive oxygen species (ROS), leading to recruitment and activation of other cells, like macrophages (Burisch, 2014). Neutrophil migration can be monitored by the myeloperoxidase enzyme (MPO), whose increased activity is observed in several illnesses and inflammatory states such as atherosclerosis, tumours and degenerative diseases of the nervous system (Danese & Fiocchi, 2011).

6.3 H_2S and IBD

Since bacterial population may be one of the key factors for the etiology of IBD, SRB and high concentrations of H_2S have also been considered components for the development of the disease (Guo *et al*., 2016). Although, there is a lot of contradiction among the information about the role of H_2S in IBD (Medani *et al*., 2011). While some studies support that H_2S has an anti-inflammatory and favourable role in the resolution of ulcers in experimental IBD, other studies claim that SRB and H_2S toxicity may be candidates for causing UC (Levine *et al*., 1998; Pitcher *et al*., 2000; Wallace *et al*., 2009; Kupai *et al*., 2017). Some investigators demonstrated a significant increase in the population of SRB in patients with untreated and active UC, measured from fresh fecal samples and sulphate reducing activity (Pitcher *et al*., 2000). Others could not identify this alterations from rectal biopsies and real-time polymerase chain reaction, therefore no disease-related difference in SRB populations between patients with UC and controls was identified (Fite *et al*., 2004; Rowan *et al*., 2009).

Still, the inhibition of H_2S synthesis in healthy rats caused inflammation and mucosal damage in the small intestine and colon along with down-regulation of cyclooxygenase-2 messenger RNA expression and prostaglandin synthesis, and exacerbated colitis in rats with pre-existing disease, resulting in significant mortality (Wallace *et al*., 2009). Moreover, studies in different experimental models of colitis related the reduction of inflammatory mediators, such as TNF-α, to the use of H_2S donors or H_2S-releasing hybrids (Fiorucci *et*

al., 2006; Fiorucci *et al*., 2007; Wallace *et al*., 2009). Additionally, an exogenous H_2S donor could significantly decrease the extent of colonic inflammation in rats induced by TNBS (Kupai *et al*., 2017; Wallace *et al*., 2007). Also, endogenous and exogenous H_2S can have beneficial effects on the reestablishment microbiota biofilms and colonic mucus production, facilitating correction of microbiota biofilm dysbiosis and mucus layer reconstitution in inflammation in rats (Motta *et al*., 2015).
Therefore, in animal models of IBD, the beneficial anti-inflammatory effects of H_2S could prevail over the harmful/toxic effects, but more studies are needed to clarify those issues (Linden, 2014).

The concept that high concentrations of H_2S have deleterious effect on intestinal epithelial cells (Medani *et al*., 2011; Pitcher *et al*., 2000) is supported by studies that show genotoxicity and damage to colonocytes (Attene-Ramos *et al*., 2006; Moore *et al*., 1997). H_2S prevents the oxidation of short chain fatty acids in colonocytes, resulting in reduced absorption of sodium, reduced secretion of mucin and a shorter life of those cells (Moore *et al*., 1997). Also, when DNA repair is inhibited, H_2S can damage colonic cancer cells at concentrations of 250 μM, therefore in individuals with predisposing genetic background that compromises DNA repair, H_2S may lead to genomic instability and could trigger colorectal cancer and associated complications (Attene-Ramos *et al*., 2006). Additionally, in rat normal gastric epithelial cells a low concentration of NaHS (0.5 – 1mM) enhances hydrogen peroxide-induced toxicity, but a higher concentration (1.5 mM) protects the cells from its oxidative damage (Yonezawa *et al*., 2007). Despite these findings supporting toxic effects of H_2S, there is evidence of an adaptative metabolic response of colonic epithelial cells to sulphide inhibition of the activity of cytochrome C oxidase (a toxic event) (Leschelle *et al*., 2005). The reduction of respiration and proliferative activity of the colonic cells, without affecting the cell viability or ATP cell contents, contribute to preserve cell capability against the adverse effects of high concentrations of H_2S (Leschelle *et al*., 2005).

6.4 Animal models of IBD

Regarding the study of IBD, even though animal models are more time consuming and more expensive, they are still the ones that most resemble human pathology, allowing the histological and functional study of the injuries caused in the target organs and all the processes involved in IBD (Hibi *et al*., 2002; Jurjus *et al*., 2004). Animal experimental models of IBD include mainly mice and rats and can be divided into four groups: spontaneous inflammation, transgenic animals, induction by immune manipulation or chemical agent (Jones-Hall & Grisham, 2014).

The study of pathological mechanisms of IBD and possible therapeutic agents relies, in large part, on the use of experimental models of colitis induced by chemical agents such as oxazolone, Dextran Sodium Sulphate (DSS) or TNBS (Jones-Hall & Grisham, 2014). Oxazolone is applied directly to the rectal mucosa of mice or rats, usually after subcutaneous pre-sensitization, and induces an increase in the production of humoral immune response (Th2), cytokines and inflammation in the distal colon (Hibi *et al*., 2002). On the other hand, DSS is added to the drinking water of rats or mice (2 - 5% solution), for varying periods of time (often 5 - 7 days) (Randhawa *et al*., 2014). It has a toxic effect on the intestinal epithelial cells, increases the permeability of the mucosa and induces morphological changes compatible with UC (Randhawa *et al*., 2014). Regarding TNBS induced colitis, an alcoholic solution is intrarectally applied; ethanol causes disorganization of the intestinal barrier, while TNBS acts as a hapten, leading to a cellular immune response (Th1), with dense cellular infiltration, affecting all layers of the colon (Antoniou *et al*., 2016; Morris *et al*., 1989).

TNBS induced colitis in rats is one of the most common animal models of this disease, since it is easy to put into practice, is inexpensive and mimics the symptoms, morphological, histological and metabolic characteristics of human IBD (Jurjus *et al*., 2004) (Foligné *et al*., 2006). In previous protocols described by this research group Ferreira-Duarte and Rodrigues-Pinto were able to categorize TNBS-induced rats macro and microscopically into mild, moderate and severe colitis, while refining the induction protocol (Ferreira-Duarte, 2018; Rodrigues-Pinto, 2019).

II. Aims

In view of the above, the objective of this master's dissertation is to understand whether H_2S influences colonic reactivity in IBD.

Since we decided to use the TNBS-induced model of colitis, we first aimed at further refining this animal model, in order to reduce the percentage of rats that develop severe colitis, increasing their welfare.

In parallel, we decided to explore the role of H_2S in IBD, evaluating the reactivity of colonic smooth muscle of control and TNBS-induced rats.

III. Material and Methods

1. Animals and housing

All animals were raised and maintained in the rodent animal house facility of ICBAS-UP (024159/2017-DGAV Portugal). According to the European Union Directive 2010/63 and the Portuguese DL 113/2013, the project was approved by the competent authorities, both local (179/2017-ORBEAICBAS-UP) and national (003511/2018-DGAV). The study was performed according to European Guidelines for humane and responsible animal care, reported in accordance with the ARRIVE Guidelines.

Based on methods previously described by our group (Ferreira-Duarte, 2018; Rodrigues-Pinto, 2019), thirty male Wistar Han rats were raised and housed at ICBAS-UP rodent animal facility. Rats were housed in Sealsafe Plus GR900 cages, with Corncob ultra 12 bedding (Corncob ultra 12, Ultragene) and access to a laboratory rodent diet (4 RF 21, Mucedola S.r.I., Italy) and to sterile water *ad libitum*. The facilities had controlled lighting cycles (12-hour light/dark cycle), ventilation, temperature (20-24ºC) and relative humidity (40-60%). Animals were maintained with their littermates in groups of 2-3 *per* cage until colitis induction. From then on, they were housed individually.

2. Colitis induction and pain management

In this experimental protocol, twelve rats (16-18 weeks of age) underwent TNBS-induce colitis. One day before induction (day -1), all animals fasted for 12 hours and a prokinetic drug was administered (Metoclopramide 1mg/Kg, PO) to reduce the amount of fecal pellets in the intestines and facilitate the contact between the TNBS ethanolic solution and the colonic mucosa (Rodrigues-Pinto, 2019). During this fasting period rats had free access to both sterile water and a 5% sucrose solution to avoid hypoglycaemia (Nowland *et al*., 2011).

On the day of the induction (day 0), animals were placed individually in separate cages and were anesthetized, *ad effectum*, with isoflurane (Isoflo®, Esteve). Animals received 250 µL of an 30% ethanolic solution of TNBS (Sigma-Aldrich Inc, St. Lois, MO, USA) (15mg/rat) instilled rectally using a 7.6 cm ball-tipped needle (adapted from Morris *et al*., 1989). Then, in order to avoid expulsion of the solution and to guarantee a uniform distribution of the chemical, the rodents were kept upside down for 60 seconds. Metoclopramide (1mg/kg, PO, SID) was also administered to the TNBS group to enhance intestinal motility from day -1 to day 2 (four consecutive days). In addition, to ensure pain control, a synthetic opioid (Tramadol® 20mg/kg, PO, SID) was also administered. From that moment on, Tramadol was only given if the animals showed evident signs of discomfort. From day 1 towards,

paracetamol (500 mg/kg, PO, SID) was daily administered in a honey-based solution (300 µL) to ensure analgesic support without the anti-inflammatory activity (Figure 4) (Rodrigues-Pinto, 2019).

Every day, the weight of the: a) rats; b) fecal pellets; c) food and d) water was measured and a welfare supervision score sheet was completed by the research team.

During this protocol no mortality was observed.

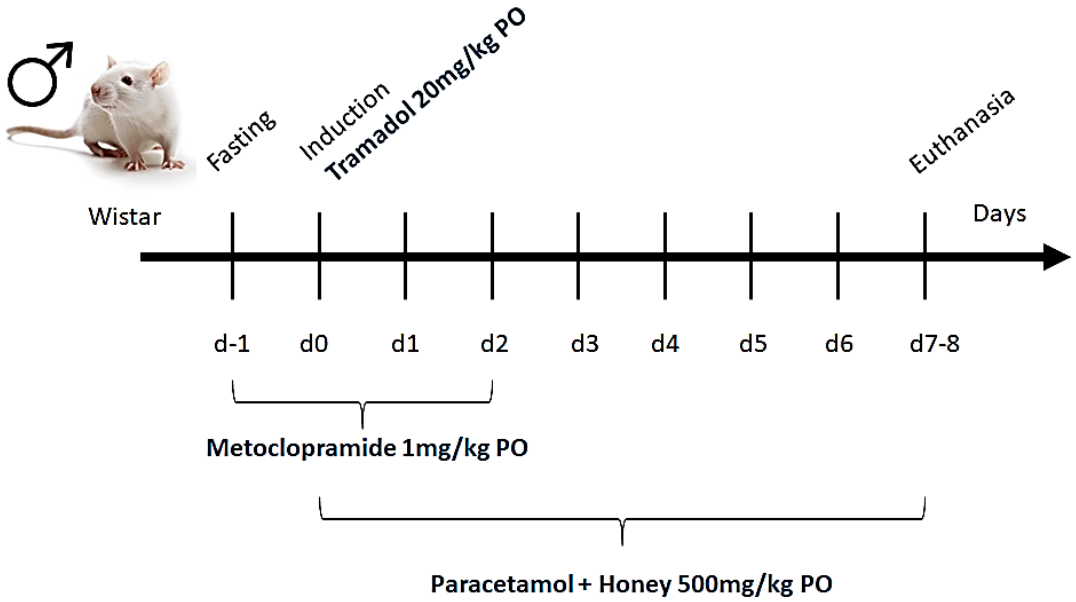

Figure 4. Schematic representation of drug administration during TNBS-induced colitis experimental protocol (from day -1 to day 7-8)

Part I – Refinement of the TNBS-model of colitis

3. Animal Welfare and clinical assessment

In order to improve the accuracy of animal welfare assessment, a supervision score sheet was completed daily (8:00 to 11:00 AM) by the research team. It included the evaluation of quantitative parameters (such as water and food intake, weight of the animal and number of fecal pellets), spontaneous behaviour of the animal (and some signs of discomfort) before and after handling, general health status and several particular signs such as: piloerection, posture (kyphosis) and locomotion, breathing, hydration, abdominal contractions, among others (Jirkof *et al*., 2019; Leung *et al*., 2016).

The quantitative parameters followed the criteria presented in Table 1. If a parameter reached the score 4, proper assessment by the veterinarian was required and human endpoints would be considered. The sum of all parameters corresponds to the Welfare Score, which could reach a maximum score of 43 points.

Table 1. Part of the scoring criteria of the supervision score sheet, regarding rat body weight, food / water intake and fecal pellets.

Body weight (%)	No weight loss	Weight loss between 1 and 6%	Weight loss between 7 and 14%	Weight loss between 15 and 19%	**Weight loss >20%**
Food intake (g)	<18g	12-18g	6-12g	<6g	**<6g from day 5**
Water intake (g)	Normal (20g/day - 40g/day)	Altered (**<**20 g/day or **>**40g/day)			
Fecal Pellets	>40 pellets/day	25-39 pellets/day and/or diarrhea	16-26 pellets/day	<15 pellets/day	**<15 pellets/day after day 5**
SCORE	0	1	2	3	**4**

4. Colon Macroscopic Evaluation

The macroscopic evaluation was performed as explained previously by Ferreira-Duarte, (2018) and Rodrigues-Pinto (2019). Briefly, 7 or 8 days after TNBS-induction, rats were euthanized by decapitation (small Decapitator, Harvard apparatus) in a separated room. The abdomen was opened by the midline and the general appearance of the colon, surrounding tissues and existence/extent of adhesions was observed. All colon was excised (from the cecum to the anus) and carefully cleaned of fecal content using Krebs-Henseleit solution [in mM: 118 NaCl, 4.8 KCl, 2.5 CaCl2.2H2O, 1.2 NaH2PO4.H2O, 1.2

MgSO4.7H2O, 25 NaHCO3, 0.02 Na2EDTA, 0.3 Ascorbic Acid and 11 glucose mono-hydrated]. Then, four 1 cm-length segments were cut: Two portions from the proximal colon (PC); one portion from the middle colon (MC) and other from the distal colon (DC) (Figure 5).

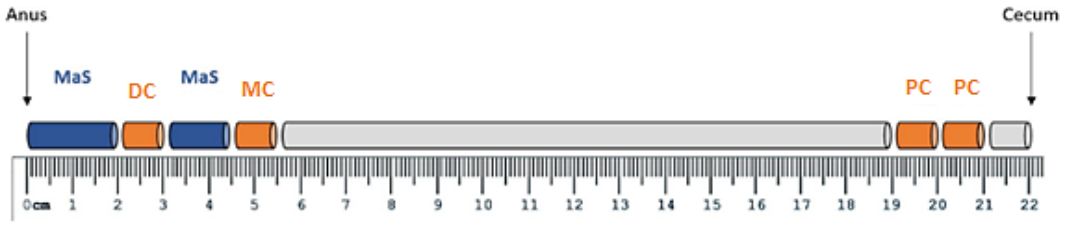

Figure 5. Schematic representation of colon segments removed from control and TNBS rats. Orange portions were used for functional study and blue portions for macroscopic evaluation (MaS). DC (distal colon); MC (middle colon); PC (proximal colon) (adapted from Ferreira-Duarte, 2018 and Rodrigues-Pinto, 2019).

After collecting the four portions of colon that were used in subsequent *in vitro* functional experiments, the colon was opened longitudinally through the non-mesenteric border, exposing the mucosa. The macroscopic evaluation of the damage caused by TNBS in the colon mucosa allowed the attribution of a macroscopic score (MaS), according to the criteria pointed out in Table 2. The mean of the MaS of both segments (portions in blue in figure 5) defined the final MaS, that could reach a maximum score of 12 points.

Table 2. Macroscopic Scoring Parameters (Ferreira-Duarte, 2018)

Parameter	**Macroscopic Score (MaS)**			
Colon adhesions	0=absent	1=mild/focal	2=moderate/zonal	3=severe/diffuse
Colon thickness	0=normal	1=mild increase	2=moderate	3=marked increased
Mucosal hyperemia	0=absent	1=mild	2=moderate	3=severe
Mucosal ulcers	0=absent	1=single	2=at one site	3=at more sites

As previously suggested by our group, TNBS-induced rats were then categorized according to their MaS, as having **mild colitis** (MaS=]0-4[), **moderate colitis** (MaS=[4-8[) or **severe colitis** (MaS=[8-12]) (Ferreira-Duarte, 2018; Rodrigues-Pinto, 2019).

Part II – Functional study: Effect of exogenous H_2S on the reactivity of colonic smooth muscle of control and TNBS-induced rats

5. H_2S Experimental protocols

For the functional study, we used control (*n*=18) and TNBS-induced animals (*n*=12). The four complete 1 cm-length segments of the colonic tissue collected from TNBS-induced rats were used (*n*=12) (Figure 5): PC, MC and DC.

a) Assemble of the colonic tissue

All colonic portions were placed individually in 10 ml organ baths filled with oxygenated (95% O2, 5% CO2) Krebs-Henseleit solution along their longitudinal axis. The system has a thermal circulator pump with water that maintained the temperature of each bath controlled at 37ºC. Each colonic segment was fixed to the bottom of the bath and to an isometrical transducer (UGO BASILE S.R.I., Italy, Model 7004) using a fine cotton thread, and was stretched passively to an initial resting tension of 1g. Isometric responses were recorded on a PowerLab system (ADInstruments).

After a 30 min equilibration period for the recovery of the tissue, colonic portions were triggered twice with 10μM of acetylcholine (ACh) (30 min apart with washing every 15 minutes in-between) to guarantee that the tissue was stabilized.

b) Protocols using NaHS

NaHS is a commonly used H_2S donor that provides a useful experimental source of H_2S, since it dissociates into hydrosulphide anion (HS^-) that then reacts with H^+ to form H_2S.

The functional study proceeded with 3 different protocols using NaHS. Each colonic portion was used for only a single protocol (1, 2 or 3). Adaptations were made from protocols already described for colonic reactivity in humans and rats, and the concentration range was 10μM to 10mM NaHS (adapted from Martinez-Cutillas *et al*., 2015 and Gallego *et al*, 2008).

Protocol 1 – effect of NaHS over ACh-induced precontraction. After a stable pre-contraction with ACh 3μM, we performed a cumulative concentration-response curve to NaHS (10μM to 10mM) by adding increasing concentrations every 10 minutes.

Protocol 2 – effect of NaHS on ACh-induced contraction. We performed a cumulative concentration-response curve to ACh (1nM to 10mM). Then, after a washing period of 30min (washes every 10 min), the tissues were incubated with 1mM NaHS for 10min and then, without washing, the cumulative concentration-response curve ACh (1nM to 10mM) was performed again. The concentration of 1mM NaHS was previously described as effective for incubation (Gil *et al*., 2013).

Protocol 3 – effect of NaHS on basal resting tone. We performed a cumulative concentration-response curve to NaHS (10μM to 10mM) by adding increasing concentrations every 10min.

At the end of the functional protocol, all tissues were stimulated with potassium chloride (KCl) 125 mM to register the maximum response. Finally, tissues were weighed right after the experiment and left overnight at room temperature, on a filter paper, to be weighed again after drying overnight.

6. Statistical analysis

All data are mean±S.E.M.; when stated, *n* refers to the number of experimental animals. Generally, a $p<0.05$ was considered statistically significant.

Statistical analysis for the refined TNBS-induced model was performed by pared/unpaired Student's t-test, one-way ANOVA or 2-way ANOVA. The 2-way ANOVA was used for comparisons between variables from control and experimental groups; unpaired Student's t-test was used for the MaS from control and TNBS-induced groups; and paired Student's t-test was used for correlation between MaS and welfare score.

For the functional study, each concentration-response curve was analysed by Graph Prism software 8.1.2 and the correspondent E_{max} and EC_{50} values obtained. E_{max} corresponds to the maximum contractile effect of the agonist and EC_{50} to the concentration of the agonist needed to obtain 50% of the maximum response. Then, the E_{max} (or the EC_{50}) values for control and TNBS-induced rats were compared by unpaired Student t test with Welch's correction for the protocols 1 and 3. Differently, for comparisons related to protocol 2, the paired t test was used between values obtained in the same tissue in the absence and presence of NaHS.

7. Drugs

Drugs used on the animal model were Metoclopramide (Primperam, oral solution 1mg/ml), Tramadol (Generis, oral solution 100mg/ml) and Paracetamol (Farmoz, tablets 1000mg).

Drugs used on the functional study were ACh, KCl and NaHS, obtained from Sigma-Aldrich, USA. The Krebs-Heinselet solution: in mM: 118 NaCl (from José Manuel Gomes dos Santos, Portugal); 11 monohydrated glucose; 4.8 KCl; 2.5 $CaCl_2.2H_2O$; 1.2 $MgSO_4.7H_2O$; 25 $NaHCO_3$, (all from Pancreac Quimica, Spain); 1.2 $NaH_2PO_4.H_2O$; 0.02 Na_2EDTA (both from MERCK, Germany) and 0.3 Ascorbic acid.

ACh and NaHS were dissolved in distilled water to the desired concentration. All preparations of NaHS used in the functional study were dissolved 10min before applying to the bath.

IV. Results

Part I – Refinement of the TNBS-induced model of colitis

1. Colon Macroscopic Evaluation

TNBS-induced animals usually lose the ability to form normal oval-shaped fecal pellets, being mostly pasty or slightly soft stool across the entire intestine (Figure 6A and 6B). It was possible to notice that as the colitis was worse, more adhesions were seen in surrounding structures. However, during this protocol, we did not see perforated ulcers, ascites or hemoperitoneum.

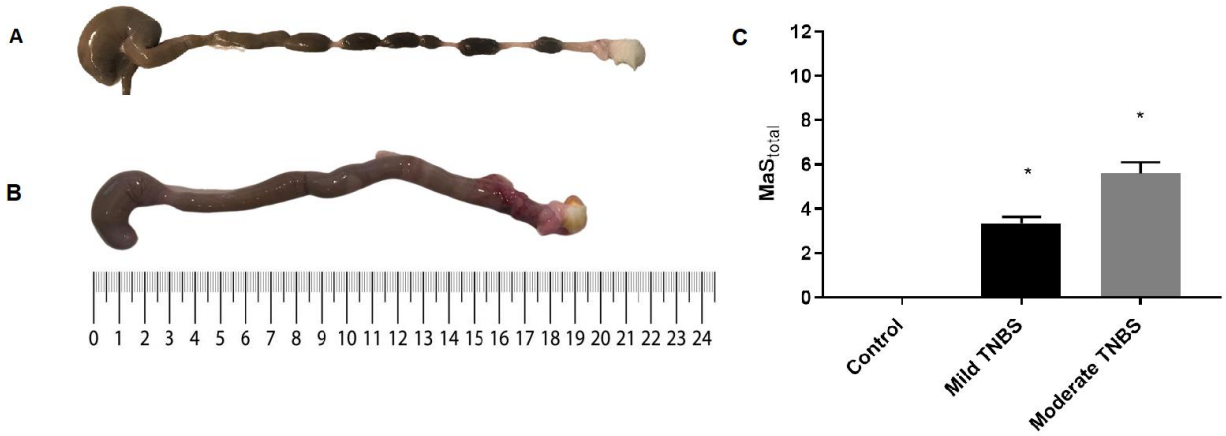

Figure 6. Representative photographs of the cecum to anus portion of (**A)** control and **(B)** TNBS-induced rats. **(C)** Graphic presentation of total macroscopic Score (MaS) of control and TNBS-induced rats. Control group (*n*=10), mild TNBS (*n*=4) and moderate TNBS (*n*=8). * p<0.05 – Mild and Moderate TNBS groups vs Control group (Unpaired t test).

Regarding MaS, TNBS-induced rats had higher and significantly different score than controls (Figure 6C). According to the colitis categorization based on the corresponding total MaS (Table 2), TNBS-induced group (*n*=12) was distributed across the categories of mild colitis (*n*=4) or moderate colitis (*n*=8), since no animal with severe colitis was detected in this protocol.

2. Physiological parameters

The physiological parameters of each animal were monitored daily throughout the protocol. The body weight change (%), water and food intake, and the number of fecal pellets were analysed. Those parameters are part of the consistent sighs proposed to predict colitis severity and corroborate the group's previous findings.

2.1 Body weight

Regarding the percentages of body weight change, rats with mild and moderate colitis showed a marked decrease starting from day 2 onwards and remain underweight from that day on. They differ from control animals from day 2 to 6 ($p<0.05$), but it is not possible to differentiate the weight change of rats with moderate colitis from that of rats with mild colitis (Figure 7A). This group of animals stopped losing weight at the end of the protocol, but still failed to return to their initial weight (Figure 7A).

2.2 Food and water intake

TNBS-induced rats with moderate colitis consumed less food in the first 48h of the protocol, but gradually increased it from day 3 forward (Figure 7B). On the other hand, rats with mild colitis stabilized their food intake in the first two days and then increased it (Figure 7B). Animals with colitis do not significantly differ from each other at any time during this experimental protocol, and both groups continued to recover their food intake until the end of the experiment. As so, rats with mild colitis were eating almost the same as control rats, at the end of the experimental protocol (day 6). Control animals maintained a significantly higher consumption of food when compared to animals with both mild and moderate colitis from day 1 to 3 ($p<0.05$). From day 4 forward only the TNBS-induced rats with moderate colitis differ from the control animals ($p<0.05$) (Figure 7B).

Regarding water consumption, it is possible to note that, overall, TNBS-induced rats drank more water than controls (Figure 7C). From day 1 to 2, water intake of control animals was significantly different from rats with moderate colitis ($p<0.05$), but from day 3 to 5 control rats were drinking less water than animals with mild or moderate colitis ($p<0.05$). On day 6, control animals differ only from the TNBS-induced rats with mild colitis ($p<0.05$) (Figure 7C).

2.3 Fecal pellets

As for the number of fecal pellets found in the cage, TNBS-induced rats with mild and moderate colitis eliminated less faeces than control animals, although in the second half of the experimental protocol that number gradually increased (Figure 7D). During the first 3 days it was possible to notice the difference between control rats and TNBS-induced rats with mild and moderate colitis ($p<0.05$). From day 4 onwards, animals with mild colitis were undifferentiated from the control animals and animals with moderate colitis were different from control animals ($p<0.05$) (Figure 7D).

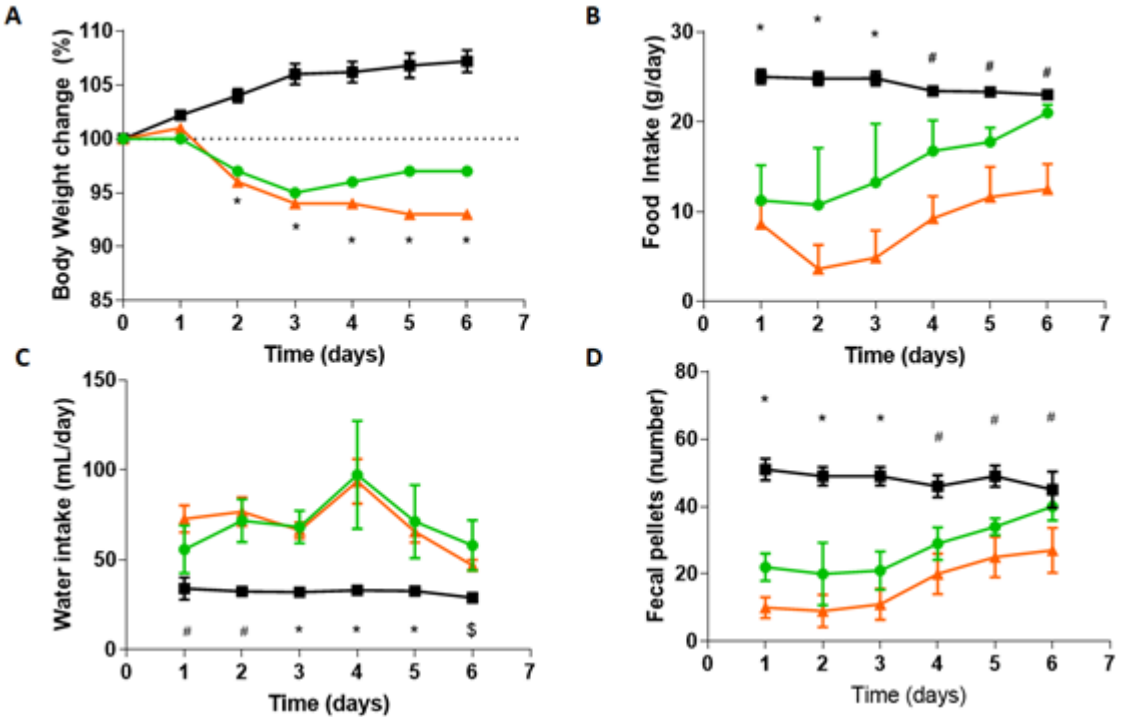

Figure 7. Time course of physiological parameters during the experimental protocol. **(A)** Body weight change from day 0 (%), **(B)** food intake (g/day), **(C)** fluid intake (mL/day) and **(D)** number of fecal pellets per day, of control rats and of TNBS-induced rats with mild colitis and moderate colitis. Control group (■; *n*=10), Mild (•; *n*=4), Moderate (▲; *n*=8). * p<0.05 – Mild and Moderate TNBS group vs Control group; # p<0.05 - Moderate TNBS group vs Control Group; $ p<0.05 – Mild TNBS group vs Control group.

3. Animal Welfare and clinical assessment

A modified supervision score sheet (Jirkof *et al*., 2019; Ferreira-Duarte, 2018) was daily completed to obtain the welfare score of each TNBS-induced rat, to increase the accuracy of animal welfare assessment.

TNBS-induced animals often presented with reduced grooming, hypokinesis, piloerection and slight kyphosis. Sometimes, signs of chromodacryorrhoea (reddish colour in the dorsal-cervical area with the deposit of porphyrin) were observed and, less frequently, alteration of locomotion and resting position. The welfare score of TNBS-induced rats was evaluated during the entire experimental protocol and it was possible to observe that the animals with mild and moderate colitis were different from controls from day 1 to the end of the protocol (p<0.5), but not statistically different from each other (Figure 8).

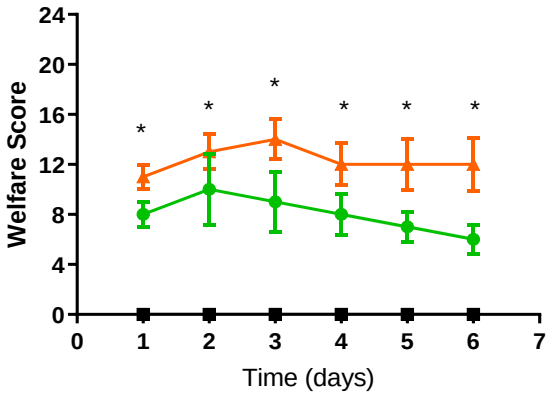

Figure 8. The welfare score of control (■; *n*=10), mild (•; *n*=4) and moderate (▲; *n*=8) TNBS-induced rats during the time course of the experimental protocol. Control group presented a score of zero, while TNBS-induced animals presented a positive score, between 4 and 20. * p<0.05 – Mild and Moderate TNBS groups vs Control group.

Considering the previous data, we tried to understand whether the welfare score correlated with macroscopic colitis evaluation (MaS). Already on day 1 (Figure 9A), it was possible to observe a significant correlation between those two variables, which increased until it reaching a maximum correlation of 87.96% on day 6 (r=0.8796, representing the correlation between the predicted values) (Figure 9F).

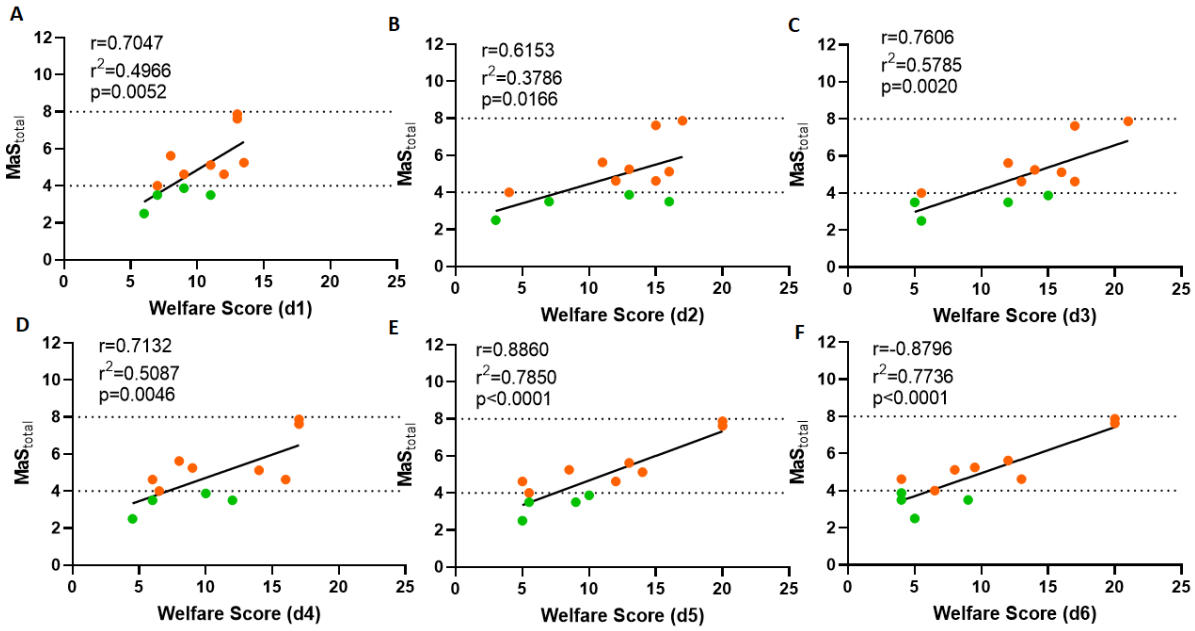

Figure 9. Correlation between total MaS and the welfare score of TNBS-induced animals, in the 6 days of the experimental protocol (A-F). • Represents animals with mild colitis (*n*=4) and • represents animals with moderate colitis (*n*=8).

Part II – Functional study: Effect of H_2S on the reactivity of colonic smooth muscle of control and TNBS-induced rats

4. H_2S Experimental protocols

In this protocol there were no animals with severe colitis, which showed in previous studies a very limited contractile capacity because of the extent of the injury, therefore all TNBS-induced animals in this protocol were used (Ferreira-Duarte, 2018; Rodrigues-Pinto, 2019).

a) Protocol 1 – effect of NaHS over Ach-induced precontraction

NaHS caused a concentration-dependent relaxation of PC, MC and DC precontracted with ACh in both control and TNBS-induced rats (Figure 11). A representative trace of this protocol is found in Figure 10.

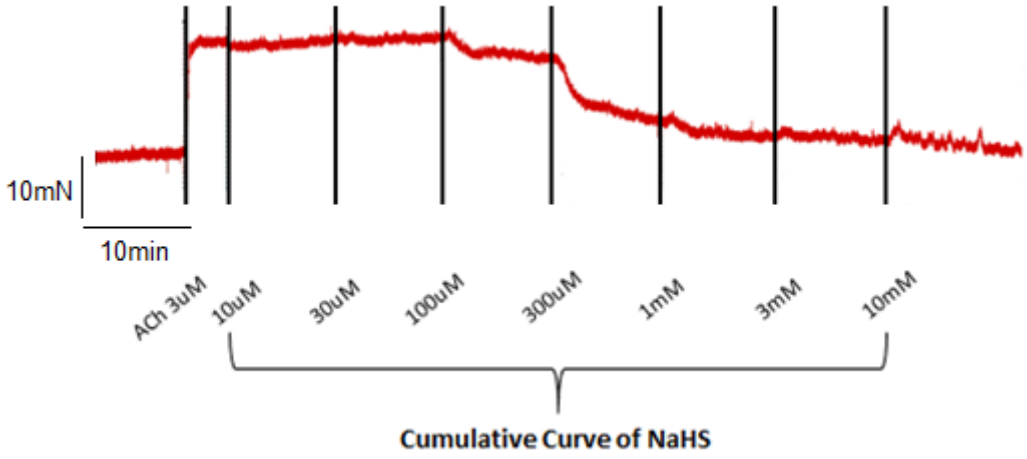

Figure 10. Representative trace of protocol 1 performed in the MC of a control rat after stabilization of the tissue.

There was no difference in the E_{max} or EC_{50} values for NaHS along the colon regions of control rats (Figure 10 and Table 3). The relaxant effect of NaHS on MC and DC in TNBS-induced animals was similar to that observed in control animals (Figure 10 and Table 3), and reached 100%, which means that smooth muscle tone returned to the initial basal tonus. However, we found some differences in the response to NaHS in the PC from TNBS-induced animals when compared to that found in control rats. Indeed, the relaxant response to NaHS in the PC of TNBS-induced rats was higher than that of control rats, but intriguingly, this was not statistically significant. The EC_{50} for NaHS in the PC was lower in tissues from TNBS-induced animals than in those from controls (Figure 10 and Table 3).

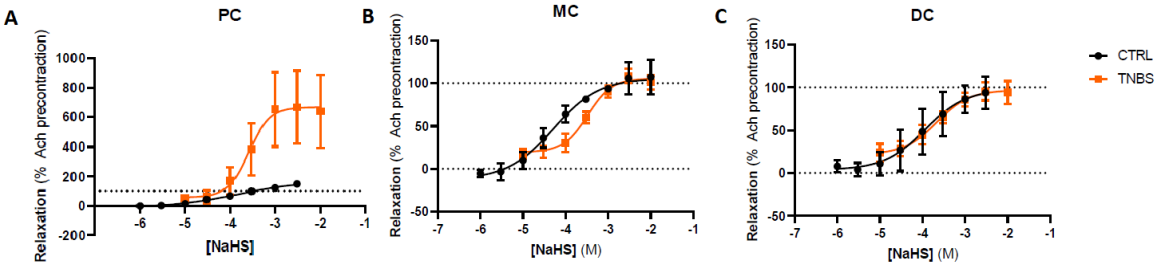

Figure 11. Concentration-response curves to NaHS upon a pre-contraction of ACh (3uM) in the PC **(A)** (control, *n*=6; TNBS-induced rats, *n*=5), MC **(B)** (control, *n*=6; TNBS-induced rats, *n*=5) and DC **(C)** (control, *n*=6; TNBS-induced rats, *n*=5). Control: black circles; TNBS-induced rats: orange circles.

Table 3. E_{max} (%) and EC_{50} (mM) values for NaHS upon a ACh-mediated contraction, in the PC, MC and DC of control and TNBS-induced rats.

	PC	MC	DC
	E_{max} (%)	**E_{max} (%)**	**E_{max} (%)**
Control	137.6±13.42	120.2±27.45	105.6±16.27
TNBS	671.8±257.1	108.4±11.27	108.9±15.12
	EC_{50} mM	**EC_{50} mM**	**EC_{50} mM**
Control	1.44±0.66	0.19±0.14	2.42±0.98
TNBS	0.27±0.03	0.36±0.07	0.34±0.08

b) Protocol 2 – effect of NaHS on ACh-induced contraction

We analysed the concentration-response curve of ACh in the absence and presence of NaHS (1mM) in PC, MC, and DC of control and TNBS-induced rats (Figure 13). A representative trace of this protocol is found in Figure 12. In control animals, the presence of NaHS was associated with lower E_{max} for Ach in the PC, MC and DC (Figure 13 and Table 4). In TNBS-induced animals, the contractile response to NaHS was similar in the absence and presence of NaHS (Figure 13 and Table 4). As for the EC_{50}, there were no differences associated with the presence or absence of NaHS (Figure 13 and Table 4).

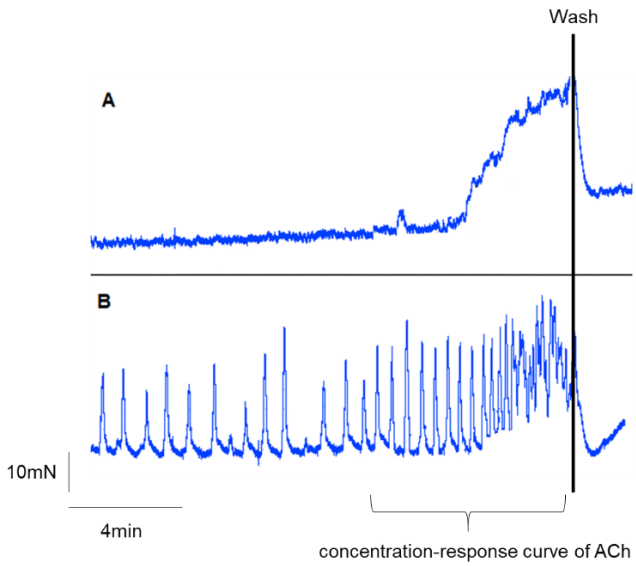

Figure 12. Representative trace of protocol 2 performed in the DC of a control rat after stabilization of the tissue. Concentration-response curve of ACh without NaHS (A) and with NaHS (B).

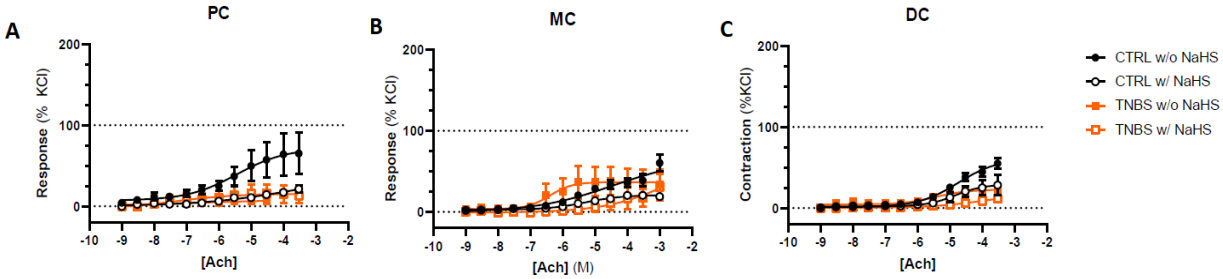

Figure 13. Concentration-response curves to ACh upon the presence or absence of the pre-incubation of NaHS (1mM) in the PC (A) (control, *n*=6; TNBS-induced rats, *n*=4), MC (B) (control, *n*=6; TNBS-induced rats, *n*=5) and DC (C) (control, *n*=6; TNBS-induced rats, *n*=5). Control without NaHS: black circles; Control with NaHS: black hollow circles TNBS-induced rats without NaHS: orange circles; TNBS-induced rats without NaHS: orange hollow circles.

Table 4. E_{max} (%) and EC_{50} (uM) values for ACh in the absence (w/o) and presence (w/) of NaHS, in the PC, MC and DC of control and TNBS-induced rats.

	PC	MC	DC
	E_{max} (%)	**E_{max} (%)**	**E_{max} (%)**
Control w/o NaHS	68.94 ±23.96	41.13 ±7.02	51.68 ±7.82
Control w/ NaHS	29.38 ± 9.34*	22.12 ±3.30*	19.79 ±3.94*
	E_{max} (%)	**E_{max} (%)**	**E_{max} (%)**
TNBS w/o NaHS	4.26±2.65	36.96 ± 16.53	24.21 ± 7.54
TNBS w/ NaHS	15.17±11.22	22.87 ± 12.66	13.78 ± 5.00
	EC_{50} µM	**EC_{50} µM**	**EC_{50} µM**
Control w/o NaHS	18.72 ±16.70	4.92 ±1.56	7.97 ±4.12
Control w/ NaHS	103.3 ±57.21	105.7 ±98.37	68.20 ±37.93
	EC_{50} µM	**EC_{50} µM**	**EC_{50} µM**
TNBS w/o NaHS	23.42±16.58	1.56 ± 0.57	6.14 ± 4.19
TNBS w/ NaHS	7.24±6.64	32.05 ± 25.82	31.55 ± 20.87

*p<0.05 control with NaHS vs control without NaHS

c) Protocol 3 – effect of NaHS on basal resting tone

NaHS caused a decrease in the basal resting tone of the PC of control animals, which was similar to that observed in the PC of TNBS-induced animals (Figure 14). The E_{max} for control and TNBS-induced animals was not significantly different (12.16±4.51% vs 30.07±9.18%, respectively, p>0.05), but the EC_{50} for control animals was higher than that found in TNBS-induced animals (7.71±2.02mM vs 0.57±0.07mM, respectively, p<0.05).

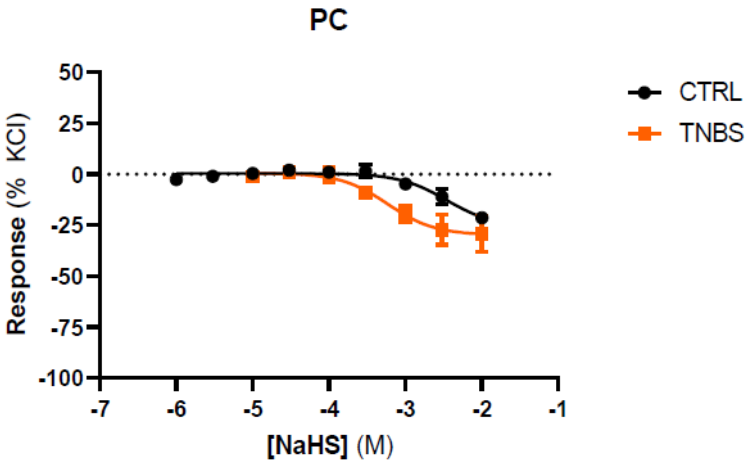

Figure 14. Concentration-response curves to NaHS on the basal tonus in the PC (control, *n*=4; TNBS-induced rats, *n*=6).

d) Effect of NaHS on spontaneous contractions

NaHS altered the pattern of spontaneous contractions in both control and TNBS-induced animals (Figure 15). In control animals it was possible to see a diverse type of changes after applying NaHS (1mM) to the bath, such as the appearance of high amplitude contractions or making them more frequent (Figure 15, red arrows). However, these alterations were not consistently observed (Figure 15 right trace, MC). In TNBS-induced animals, it was also observed an alteration in the pattern of spontaneous contractions although different than that observed in control rats (Figure 15 left trace).

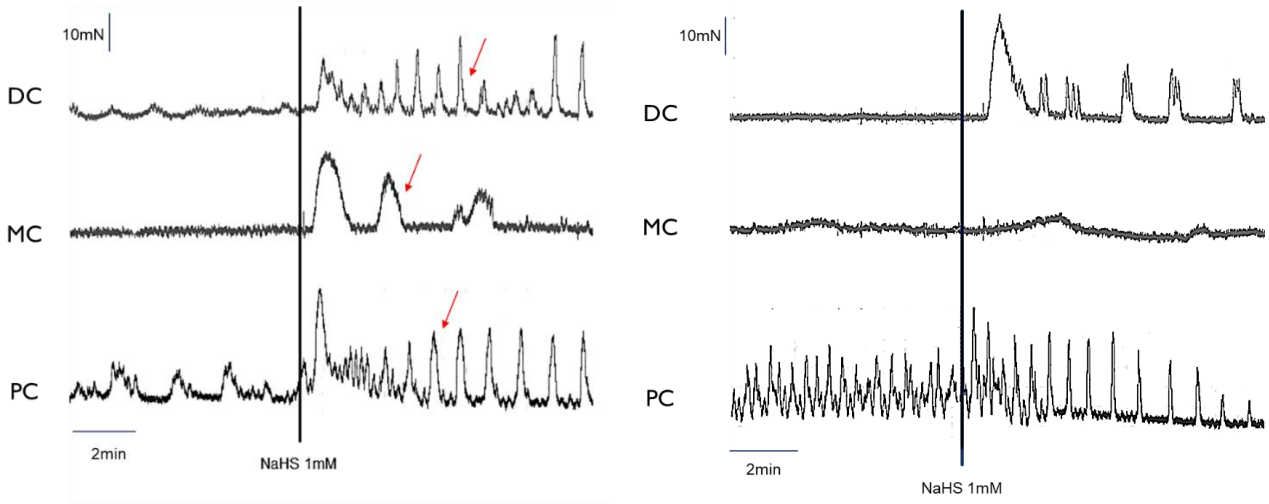

Figure 15. Spontaneous contraction pattern from DC, MC, and PC in control (left trace) and TNBS-induced (right trace) animals before and after incubation with NaHS 1mM. Red arrow showing the most notorious change.

V. Discussion

Part I – Refinement of the TNBS-model of colitis

The use of animals in research has been the subject of endless discussions, so their responsible use is of the utmost importance. The Laboratory Animal Science tries to implement this ideal, summarized by the 3 principles that William Russell and Rex Burch described in 1959: “Reduction, Replacement and Refinement”. These are the 3 R´s of animal experimentation, which aims to reduce the number of animals, minimize pain and discomfort, and seek alternatives for the replacement of *in vivo* testing (Cazarin *et al*., 2004). These concepts are important not only for ethical reasons, but also since increasing animal models’ replicability also favours science efficacy and quality. Therefore, in this master’s thesis we decided to continue to refine the TNBS-induced colitis model in rats, optimizing the use of animals, reducing their suffering and increasing experimental reproducibility with detailed data.

To induce colitis in rats, researchers have reported the use of different percentages of ethanol (from 30% to 50%) and several doses of TNBS (up to 150 mg/kg) compared with that described by Morris and colleagues (50% of ethanol and 100mg/kg TNBS) when they first established the model in 1989 (Cury *et al*., 2013; Foligné *et al*., 2006; Morris *et al*., 1989; Vermeulen *et al*., 2013; G. Zhao *et al*., 2014).
Also, some studies show a high mortality rate in induced animals (reaching 33% with the higher dose of TNBS), and so, our aim was to minimize this outcome (Foligné *et al*., 2006). We were able to fulfil that goal, since all TNBS-induced animals survived (12 rats), and their evolution was always accompanied by the administration of prokinetics and analgesics to minimize their discomfort.

In order to increase experimental reproducibility, our research team has previously suggested three categories to differentiate TNBS-induced rats with mild, moderate and severe colitis, according to the macroscopic evaluation of colonic segments. Indeed, this categorization resembles the existing categories for UC and CD, like the Montréal classification of UC for instance, that assesses disease activity and severity (Silverberg *et al*., 2005; Harbord *et al*., 2017). Thus, it is possible to classify UC as being in remission or as mild, moderate or severe, considering the frequency of defecation, presence of blood in the stool, and several other parameters (Silverberg *et al*., 2005). This classification enables medical doctors to implement the appropriate therapy and foresee the prognosis of the disease (Harbord *et al*., 2017).

Most of those parameters were also evaluated throughout the TNBS-induced colitis experimental protocol, and registered in the welfare scoring sheet. At the end of the protocol, a MaS-based classification helped to categorize the induced colitis as mild (*n*=4) or moderate (*n*=8). None of the rats induced during this period was classified as having severe colitis. This categorization improves accuracy of data obtained from studies on experimental colitis, increasing the effectiveness of using this model for preclinical studies with new drugs, that may be more or less effective for a certain type of colitis. Also, this categorization allows researchers to choose which type of colitis fits the purpose of their investigation, reducing experimental variability and supporting the application of humane endpoints precociously, if needed (Ferreira-Duarte, 2018).

As explained previously, in the last few years our research group has been trying to refine TNBS-induced colitis rat model. In the first protocol we used 20mg/kg of TNBS in a solution with 30% ethanol (Ferreira-Duarte, 2018); in the second protocol we used a 21% ethanolic solution with the same dose of TNBS (Rodrigues-Pinto, 2019), and in the present study colitis was induced by a 30% ethanolic solution with a TNBS dosage of 15mg/kg. In our hands, this TNBS solution offered the best results, as all animals developed mild or moderate colitis, meeting the project purpose.

However, although the last protocol was less harmful than the previous ones, the administration of several drugs was maintained to minimize the animals' discomfort. Pain management included the daily administration of paracetamol in a honey-based solution, and tramadol on the day of induction and whenever the animals showed signs of pain. For the management of symptoms associated with IBD, such as fever and abdominal pain, a good pharmacological option is paracetamol (Quintal, 2016). In these cases, NSAIDs, such as ibuprofen, naproxen and acetylsalicylic acid, are contraindicated as they interfere with the mucosa of the GI tract (Chamoun-Emanuelli *et al*. 2019). Paracetamol reduces fever, blocking the formation and release of prostaglandins in the nervous system and inhibits the action of endogenous pyrogens receptors of the hypothalamus, resulting in peripheral vasodilation, sweating and dissipation heat (Anderson *et al*., 1998). This drug causes analgesia without the characteristic anti-inflammatory effect that would counteract the desired colitis induction, which is why it was chosen for this protocol (Docherty *et al*., 2011). As for tramadol, its analgesic effect is partially related to the fact that it is a weak opioid, which also gives it some unwanted side effects such as nausea and, especially, constipation (Chou *et al*., 2009; Docherty *et al*., 2011). The inhibition of perception is due its action at the μ-opioid receptors, while the inhibition of transmission is due to the marked effect on the serotonergic and noradrenergic system, since it has the ability to interfere with the reuptake of serotonin and norepinephrine (Wilder-Smith, Bettiga, 1997). Constipation is

always a concern in rodents, but in this case, it could keep TNBS in contact with the colon mucosa for too long, causing intestinal perforation and subsequent peritonitis, which greatly worsens the animal's prognosis. Despite Tramadol having a minor effect on colonic transit, and no effect on upper gastrointestinal transit or gut smooth muscle tone (Wilder-Smith, Bettiga, 1997), we decided to counteract this risk and administer the prokinetic drug metoclopramide (D_2 dopamine receptor antagonist) on the day before colitis induction and on the following three days (Leppert, 2015). This prokinetic drug stimulates and coordinates esophageal, gastric, pyloric and duodenal motor activity, increasing the resting tension (tone) and the phasic contractile activity (Schulze-Delrieu, 1979).

Regarding colitis categorization, considering the MaS of each animal we previously reported that in the first protocol, Ferreira-Duarte (2018) and colleagues obtained 29.4% of TNBS-induced animals with mild colitis, 35.29% with moderate colitis and 35.29% with severe colitis. After, Rodrigues-Pinto and co-authors (2019), using the second protocol, were able to reduce the percentage of rats with severe colitis (10.53%), increasing the number of rats with mild (31.58%) and moderate colitis (57.89%). With the current protocol (3rd protocol) it was possible to further improve this distribution, as we no longer had rats with severe colitis, and had 33.33% of them developing mild and 66.67% moderate colitis (figure 6), probably due to the lower concentration of TNBS in the solution. These data represent a success in the refinement of the TNBS-induced colitis model, since no animals died or developed severe colitis. Not having animals with severe colitis not only fulfils the objective of Refinement (as these were the animals that showed the greatest discomfort) but also that of Reduction, because the extensive tissue damage made its use in contractility *in vitro* studies unfeasible. However, it is important to note that even in the same circumstances animals developed the disease differently in all protocols. The course of the colitis depends on the individualized immune response of each animal, as in humans with IBD, and that is also why it is important to categorize colitis in groups with similar conditions, to enhance translational research (Flamant & Xavier, 2018; Mowat *et al*., 2011).

In addition to improving the animal model itself, we also seek to describe it accurately, using a modified supervision score sheet that included physiological and welfare parameters. Regarding the physiological parameters (animal´s body weight, food and water intake, and excretion of fecal pellets), these are very rarely described in TNBS-induced colitis animal models (Cury *et al*., 2013; Foligné *et al*., 2006; Morris *et al*., 1989; Vermeulen *et al*., 2013; G. Zhao *et al*., 2014). The most reported parameter in the literature on this animal model is body weight (Cury *et al*., 2013; Fitzpatrick *et al*., 2010; Ferreira-Duarte, 2018; Rodrigues-Pinto, 2019). Body weight loss is partially caused by the direct effects of TNBS on the GI

tract (disruption of the mucosa causing various degrees of diarrhea and reduced fluid absorption), systemic inflammatory response and pain (Foligné *et al*., 2006). That may explain why the percentage of weight loss of TNBS-induced animals began 48h after induction and remained underweight during the remaining protocol. Both TNBS-induced categories were different from control animals. Moreover, animals with moderate colitis ate less and had fewer fecal pellets in the litter, compared to the control animals. Water intake is not a well-documented parameter, but our findings corroborate that TNBS-induced rats drank more water than control animals, probably because of the discomfort, the lack of appetite and to avoid dehydration (Motavallian-Naeini *et al*., 2012; Ferreira-Duarte, 2018; Rodrigues-Pinto, 2019). TNBS-induced rats with mild and moderate colitis showed a similar pattern in all of these parameters, so we could not predict TNBS categories *ante mortem* with them.

But, our modified supervision score sheet also included, in addition to the physiological parameters, a series of welfare parameters, like piloerection, posture (kyphosis), breathing, hydration, animal behaviour (before and after handling), and the occurrence of abdominal contractions. This allowed the researchers to easily and clearly distinguish the degree of discomfort experienced by each animal, considering the possibility of humane intervention. Animal welfare has always been a concern, as colitis is a painful condition. The results of this welfare scores showed us that the score from TNBS-induced rats was different from control animals since day 1, but animals with mild colitis did not differ from animals with moderate colitis. Throughout the protocol, the welfare score proved to be coherent and practical in assessing the general state of the animal. The correlation between the MaS and the welfare score was also made throughout the protocol. Starting from day 1, it was possible to notice a statistically relevant relation between these two variables. That is, the higher the welfare score is, the greater the chance the animal will develop moderate or even severe colitis. To our knowledge, this is the first time that the correlation between a welfare and an injury macroscopic score (MaS) is demonstrated.

Part II – Functional study: Effect of H_2S on the reactivity of colonic smooth muscle of control and TNBS-induced rats

Our study is the first to show the reactivity of H_2S on colonic smooth muscle in a refined model of colitis in rats. Every new discovery regarding H_2S helps the understanding of the physiological and pathophysiological aspects of this molecule. Because of H_2S complexity and multiple mechanisms, many questions remain open regarding its role in normal physiology, GI motility, and modulation in inflammatory diseases.

Studies have been carried out to explore the effects and mechanisms of action of H_2S in intestinal tissues from healthy subjects of different species, such as guinea pig, rats, mice, humans, and rabbits (Teague *et al*., 2002; Gallego *et al*., 2008; Gil *et al*., 2013; Ying Liu *et al*., 2013; Lu *et al*., 2014; Martinez-Cutillas *et al*. 2015; Nalli *et al*., 2017). Also, the endogenous production of H_2S, via CSE and CBS, has been demonstrated in different regions of the GI tract (Jimenez *et al*., 2017) and H_2S is gaining relevance as an important regulator of GI motility (Gallego *et al*., 2008; Jimenez *et al*., 2017; Teague *et al*., 2002; P. Zhao *et al*., 2009), being involved in many physiological functions including immune and inflammatory processes (Zanardo *et al*., 2006). The effects of H_2S on the intestinal smooth muscle have been shown to result in a relaxing/inhibitory effect, both in spontaneous contractions and in ACh-mediated contractions (Gallego *et al*., 2008; Gil *et al*., 2011; Martinez-Cutillas *et al*., 2015; Teague *et al*., 2002). H_2S seems to be a complex and multiple-acting molecule and its mechanism of action is still widely debated in the scientific community (Ying Liu *et al*., 2013; Lu *et al*., 2014; Nalli *et al*., 2017). But, further studies are needed to clarify the mechanism underlying the observed effects (Teague *et al*., 2002; Gallego *et al*., 2008; Gil *et al*., 2013; Ying Liu *et al*., 2013; Lu *et al*., 2014; Martinez-Cutillas *et al*. 2015; Nalli *et al*., 2017).
There are no studies yet published on the effect of exogenous H_2S on intestinal motility in IBD models, which implies a gap to be explored and deepened as this gasotransmitter is proposed as an anti-inflammatory and GI protective molecule (Wallace, 2009, Fiorucci *et al*., 2007). Our study is innovative and unique, being the first to test the effect of an H_2S donor in intestinal motility in a refined TNBS model in rats.

Since ACh is the main excitatory neurotransmitter of the ENC and a major regulator of GI motility (Costa *et al*., 1996; Duarte-Araújo *et al*., 2004), we decided to study the impact of H_2S over ACh-mediated pre-contraction and in ACh-mediated contraction. For that, we used NaHS, which is a H_2S donor. We performed two experimental protocols in control and

TNBS-induced rats to study the physiological role of H_2S but also to compare it with its pathophysiological role in TNBS-induced rats.

Our results suggest that NaHS can revert the established pre-contraction caused by ACh along the colon of control and TNBS-induced rats (in PC, MC and DC), with similar efficacy and potency. Interestingly, data in the PC suggests a higher relaxant capacity of NaHS in TNBS-induced rats than in controls, although the difference was not statistically different. When we looked to the raw data (not shown) we could observe that in all five PC samples of TNBS-induced rats, the relaxant effect of NaHS not only reverted ACh-induced pre-contraction but also surpassed the basal tone. However, the degree of this relaxation below the basal tone was extremely variable (from 146% to 1413%) and this might have contributed to the non-significant difference ($p=0.1063$ for the E_{max} and $p=0.1373$ for the EC_{50}). This numerically difference is quite intriguingly since the PC is the colonic region less affected by TNBS-induced inflammation. Alternatively, this might represent some compensatory mechanism that helps to control inflammation in the PC, as it has been shown that H_2S acts as a neuromodulator and an endogenous regulator of acute inflammation and pain (Zanardo *et al*., 2006).

Additionally, our results also suggest that H_2S physiologically opposes ACh-mediated contraction since incubation with the H_2S donor, NaHS, markedly attenuated the contraction induced by ACh in control animals. This result corroborates with those from studies carried out in the colon from control rats (male, Sprague-Dawley) and control mice wherein the response to carbachol (10μM) or bethanecol (30μM), two cholinesterase resistant parassympathomimetic drugs, was attenuated in the presence of NaHS (1mM and 300μM, respectively) (Gil *et al*., 2013; Gallego *et al*., 2008). Those studies also suggest that the effect of H_2S is mostly post-junctional, with no direct effect on the neuronal pathway, since it is insensitive to tetradotoxin (TTX) (Gallego *et al*., 2008; Gil *et al*., 2013). TTX is a neurotoxin that blocks the propagation of the nervous impulses, therefore H_2S is unlikely to be dependent on sodium channel mediated action potentials in neurons and, consequently, a non-neuronal source of H_2S may be present in the GI tract in healthy subjects (Jimenez *et al*., 2017). Studies using intracellular microeletrodes and mechanical recordings show that NaHS attenuates the cholinergic and tachykinetic response but does not affect the purinergic inhibitory junction potentials in the colon of healthy humans, rats and mice (Gallego *et al*., 2008; Martinez-Cutillas *et al*., 2015). Using a similar experimental design from ours, authors proved that this molecule could reverse the cholinergic contraction and inhibit the cholinergic neuromuscular transmission, similar to our results in control animals, at a possible specific target in the post-junctional pathways (Gil *et al*., 2013; Lu *et al*., 2014).

Our study shows that this antagonism of effect is compromised in TNBS-induced rats since the contraction induced by ACh was similar in the absence and presence of NaHS. Indeed, there was no difference in the contractile response to ACh when tissues were pre-incubated with NaHS. This difference between control and TNBS-induced rats concerning the H_2S-mediated antagonism of ACh-activated contractile machinery might be related to the H_2S levels associated with inflammation, and/or alteration in the mechanism responsible for H_2S-mediated relaxation, including alterations in signalling mechanisms. Intestinal inflammation, itself, is associated with increased production of H_2S. A study carried out in TNBS-induced rats showed an increased ability to synthesise H_2S in colon tissues, and the presence of the enzymes close to the injury, which suggests an increased endogenous production of this gas in colitis and reinforces its role in tissue recovery (Wallace *et al*., 2009). The increased H_2S content in the colonic mucosa of DSS-induced colitis, in mice, was associated with an increased colonic expression of CBS and CSE (Hirata *et al*., 2011). Also, inflammatory-mediated mucosal ulceration is followed by a significant decrease in sulphide quinone reductase expression, a key enzyme for oxidation of H_2S, which also contributes to the increase in H_2S concentration at sites of injury (Flannigan *et al*. 2013). This increased H_2S levels may represent an important response to inflammation due to its capacity to promote antioxidant/anti-inflammatory effects and repair (Fiorucci *et al*., 2007; Wallace *et al*., 2007, 2009). This is strengthen by the evidence that inhibitors of CSE and CBS lead to worsening of the disease, with a high mortality rate in these cases (Wallace *et al*., 2009). Taken the increased levels of H_2S associated with IBD described in the literature, one could expect to find a marked functional antagonism of NaHS over ACh-mediated contraction, which was not the case. However, those increased levels could also limit the superimposed relaxant effect of exogenously applied H_2S; this could also be interpreted as a protective mechanism, to maintain ACh-induced colonic contractions and associated mixing and propulsive forces. This would keep a proper intestinal function, including the renewal of microbiota, which is relevant for intestinal functions (Motta *et al*., 2015).

Other possible explanation for our results could be related to alterations in the cellular mechanisms associated with H_2S-mediated relaxation. In the colon of healthy rats and humans, H_2S-mediated relaxation partially involves K^+ channels and soluble guanylyl cyclase (sGC) but not prejunctional neuronal pathways (Gallego *et al*., 2008; Gil *et al*., 2013; Jimenez *et al*., 2017). Indeed, H_2S acts on the hyperpolarization of smooth muscles by opening K channels (K_{ATP}, SK_{CA}, and voltage-dependent K channels) (Gil *et al*., 2013; Ying Liu *et al*., 2013; P. Zhao *et al*., 2009) and stimulating cGMP (Nalli *et al*., 2017). This H_2S-mediated cGMP activation accounts for a positive interaction between H_2S and NO (Nalli *et al*., 2017). Release of NO from enteric neurons and activation of NO/soluble guanylyl cyclase (sGC)/ cGMP-dependent protein kinase (PKG) pathway in smooth muscle is

physiological during relaxation phase of peristalsis (Murthy, 2006). A recent study suggests that H_2S intensifies NO-induced relaxation in colonic smooth muscle by the inhibition of cGMP-specific phosphodiesterase 5 (PDE5) activity (Nalli *et al*., 2017) by an unknown mechanism. H_2S increases cGMP levels on simultaneous release of NO, activation of sGC, and generation of cGMP (Nalli *et al*., 2017). Furthermore, it is also suggested that NO/sGC/cGMP pathway increases the H_2S generation due to direct activation of CSE, involving PKG in this process (Nalli *et al*., 2017). Interestingly, in colitis, the cGMP pathway may be compromised (Brenna *et al*., 2015), as transcription factors, as well as several cGMP downstream mediators, were all significantly downregulated in both inflamed colonic IBD mucosa and TNBS colitis (Brenna *et al*., 2015), which could also contribute to the blunted H_2S-induced antagonism of ACh-induced contraction. This part of our results is in line with cGMP been altered in the IBD, along with the interaction with NO, and inflammatory alterations (Brenna *et al*., 2015).

One intriguingly achievement of our study is the different profile of response to H_2S that we observed between our protocols 1 and 2. We observed that when the H_2S donor (NaHS) was applied after the ACh-mediated pre-contraction, it relaxed the TNBS-induced colonic segments in a similar way as control colonic segments (eventually, even more in the PC of TNBS-induced animals). But, when the tissues were incubated with the H_2S donor (NaHS), it did not decrease the contractile response to ACh as observed in tissues from control animals. The mechanism of action of H_2S seems to be an intricate network that is only now starting to be studied. Overall, our results suggest that H_2S acts through different pathways since if a single relaxant pathway was possible, it should be activated in either protocol. Scientific reports show that H_2S may also interact with myosin light chain phosphorylation, Ca^{2+} channels, cAMP or act on the TRVP1 receptor interfering with contraction/relaxation in the GI tract (Matsunami *et al*., 2012; W. Zhao *et al*., 2001; Lu *et al*., 2014; Dunn *et al*., 2016). The effect of this molecule is very diverse due its sulphydration action on proteins, modulating a wide variety of cellular functions. Currently, there is a lack of experimental evidence that encompasses and proves all H_2S mechanisms. More studies should be conducted to ascertain limitations that inflammation may cause in the mechanism of action of H_2S and explore its molecular pathways.

Finally, the mechanisms triggered by NaHS might also depend on the time of exposure to it and this could contribute to our apparently inconsistent results. In protocol 1, NaHS was present for 10min for each concentration, therefore for 1 hour the colonic segment was exposed to NaHS. In 20 min (100μM of NaHS) we start to see the relaxant effect of the

concentration-response curve to NaHS. Differently, in protocol 2, NaHS was incubated for 10 min and that time could be not enough to induce transduction mechanisms in colitis.

Also, since H_2S is considered a toxic molecule with a biological role, the GI toxicity threshold is not yet known. Several studies present H_2S as genotoxic and connect it with chronic intestinal diseases such as IBD (Levine *et al*., 1998; Pitcher *et al*., 2000; Wallace *et al*., 2009; Kupai *et al*., 2017). The concentrations in the heathy GI tract are high and range from up to 1 mmol/L in the colon of mice and may reach 3.4 mmol/L in human stools, while in the human GI tract concentrations from 1 to 2.4mM are commonly presented (Rose *et al*., 2005). In mammalian blood, H_2S concentrations can reach 125 μM (Karunya *et al*., 2019), while in the brain the limit is 160 μM, although the catabolism may exceeded the rate of enzymatic release and the real concentration range nM, as so values higher than 200 μM exerts toxicity (Abe & Kimura, 1996; Dello Russo *et al*., 2000; Furne *et al*., 2008). Colitis can compromise the detoxification mechanisms by enterocytes and H_2S conjugation in stools, which can lead to high concentrations of H_2S at a deeper level of the intestinal wall (Flannigan *et al*. 2013). There are studies *in vivo* using a range of 0.1 to 5 mg/kg (PO) of NaHS in gastric mucosa of rats with no toxicity reported (Magierowski *et al*., 2017); also a range of 9.3 μM/kg to 600μM/kg (PO) of a H_2S donor in a model of TNBS-induced rats, with no signs of toxicity as well (Kupai *et al*., 2017); and in TNBS-induced animals a intracolonic application of NaHS of 30μM/kg were effective, with no toxicity shown (Wallace *et al*., 2009). This corroborates that the GI tract is probably more resistant to H_2S actions, and high concentrations of endogenous H_2S in inflammation may be a compensatory mechanism to recover the injury (Leschelle *et al*., 2005; Wallace, 2009).

Muscle basal tone is the state of elastic tension (slight contraction) that the muscle presents at resting conditions. Previous studies using intracellular microeletrode recording showed that in the resting membrane of MC of control rats, NaHS (0.1, 0.3 and 1mM) caused hyperpolarization and decreased the basal tonus in a dose dependent manner (Gil *et al*., 2013). Our finding with protocol 3 is in line with this previous findings published in control animals (Gil *et al*., 2013), since NaHS relaxed the basal tonus in rat PC, both in control and TNBS-induced animals. As speculated before, the PC of TNBS-induced rats could be more sensitive to the action of H_2S, and this is also seen in the result in the cumulative-curve response of NaHS in basal tonus, where it relaxes in a more potent way without any previous stimulus.

H_2S is also implicated in regulating spontaneous contractions in the intestine (Gallego *et al*., 2008; Gil *et al*., 2013). In our study, we found a diverse response to incubation with

NaHS (1mM) of the spontaneous pattern of contractions in control and TNBS-induced animals. The main response was the immediate appearance of high amplitude contractions. In the PC of TNBS-induced animals, we noticed an interesting change in pattern, wherein the high amplitude contractions became less frequent and almost faded away by the end of the incubation period (10min). Although we had a diverse result from this experiment, we have never seen a pattern similar to those described by Gil *et al*., (2013) which reported the inhibition of low-frequency (higher amplitude) contractions, without changing high-frequency (lower amplitude) contractions (Gil *et al*., 2013). H_2S influences pacemaker activity, namely by inhibiting pacemaker current in ICCs from the mouse small intestine (Parajuli *et al*., 2010). This inhibitory effect of H_2S seems to be potentiated by NO, since when both are present, lower doses of each gasotransmitter are required for the inhibitory effect to occur (Yoon *et al*., 2011). The inhibitory effect on spontaneous mechanical activity has also been reported to occur in the colon of rats in the presence of NaHS (1mM) (Gil *et al*., 2013). In line with this, inhibition of CSE or CBS increased spontaneous contractions in the colon, further supporting a role for H_2S as an endogenous signalling molecule in the regulation of gut motility (Gil *et al*., 2011; Martinez-Cutillas *et al*., 2015). The action of H_2S in spontaneous contractions in TNBS-induced animals is not reported in the literature and H_2S in inflammation may involve other cellular targets, such as enteric glial cells. These cells are highly responsive to microbial, luminal and inflammatory signals that regulate intestinal barrier function, immune responses, secretion and motility (Yu & Li, 2014). Also, NO regulates ion movement across the colonic wall through enteric nerves and enteric glia (MacEachern *et al*., 2011), thereby the direct or indirect role of H_2S toward those cells should be explore.

In TNBS-induced animals the changes in intestinal morphology and the dysfunction in the profile of frequency of rhythmic propulsive motor complexes (myogenic contractility increased) and rhythmic propagating ripples (spontaneous rhythmicity frequency decreased) are reported, indicating abnormal motility in 7 days of induction (Calabresi *et al*., 2019). These alterations may be attributed to the remodelling of the neural control of the circular smooth muscle responses during inflammation (Hosseini *et al*., 1999). These studies also indicate that the inflammation generates fibrosis and prominent thickening of the muscularis externa, and these may be related to the variation of contraction pattern (Calabresi *et al*., 2019; Hosseini *et al*., 1999). This fibrosis can compromise the enteric nervous system, leading to irreparable damage (Calabresi *et al*., 2019). Another possibility is that the alterations described in the literature are, at least in part, the result of increased tissue levels of H_2S. Consequently, as H_2S has important effects on the intestinal motility,

inflammation may alter the conditions and its mechanisms of action, not yet fully understood.

Our study is preliminary and pioneering for the action of H_2S in an IBD model. We have expanded the findings of this molecule in GI motility in control animals, combining the action of this gas in different colonic segments of TNBS-induced rats. H_2S in colitis seems to have an ambiguous action, and we still do not know if other pathways could be involved. More studies are necessary to explore the molecule and signal pathways.

VI. Future perspectives

As future perspectives for our group, we want to deepen the effects of exogenous H_2S on inflammation, increasing the sample in both cholinergic contraction and spontaneous contractions experiments. Also, we will perform a mechanical study to better understand the alterations found in the mechanism of action of H_2S on colitis. For that, we plan to test the role of K channels (using Glibenclamide and Apamin, which respectively block the K_{ATP} and SK_{Ca+} channels), cGMP/NO pathway (using GC blockers, such as ODQ, and NO synthesis blockers, such as L-NNA) both in control animals and in TNBS-induced rats. Also, in the literature, the effects of H_2S in colonic segments of control animals were insensitive to TTX, but there is no data showing if exogenous H_2S interacts with the neuronal path in inflammation, and we want to test this as well.

Furthermore, we want to explore the mechanical recordings with CSE and CBS blockers in control and inflammatory conditions. We will test the effect of two main inhibitors of those enzymes, L-propargylglycine (PAG, Sigma-Aldrich, USA), an inhibitor of CSE, and amino-oxyacetic acid (AOAA, Sigma-Aldrich, USA), an inhibitor of both CBS and CSE. It is our goal in the future to explore the ACh-mediated contraction and spontaneous contractions when the main source of endogenous H_2S is inhibited in colonic segments of TNBS-induced animals. Also, we want to implement the technique of immunohistochemistry or western blot for detection of those enzymes throughout the colon and compare their expression in the PC, MC and DC of control and TNBS-induced rats. We will also quantify inflammatory markers, such as tissue MPO to corroborate our macroscopic findings and colitis classification.

Much of these future perspectives could not be performed on time of this thesis due to the constrains associated with the present COVID-19 pandemic.

VII. Conclusions

In conclusion, our study corroborates the categorization of the TNBS-induced model of IBD in rats, providing detailed data and enabling future investigators to reproduce this model. In addition, the adaptations done in this animal protocol allowed us to have no animal with severe colitis, representing a success in the refinement approach. Therefore, it is possible to have a TNBS-induced model with minimal discomfort and no mortality.

In our functional study, we could conclude that NaHS relaxes and attenuates ACh-mediated contractions in the control rat colon. However, the mechanisms by which H_2S acts may be compromised in the TNBS-induced colitis, as the ACh contraction was not attenuated in the presence of NaHS. Yet, it caused a concentration-dependent relaxation to the precontracted rat colon in TNBS-induced animals, with an apparent sensitivity of the PC. Additionally, H_2S relaxed the basal tonus of the PC both in control and TNBS-induced animals and changed the spontaneous contractions pattern in the colon, in a non-standard way. More studies are needed regarding these results, especially because H_2S is a complex molecule with multiple interactions. We were able to test the reactivity of the colonic smooth muscle to the NaHS in a preliminary way, and more experiments are needed to further characterizer the exogenous H_2S role in the motility in inflamed tissue.

VIII. References

Abe, K., & Kimura, H. (1996). The possible role of hydrogen sulfide as an endogenous neuromodulator. *Journal of Neuroscience*, *16*(3), 1066–1071. https://doi.org/10.1523/jneurosci.16-03-01066.1996

Anderson, B. J., Holford, N. H. G., Woollard, G. A., & Chan, P. L. S. (1998). Paracetamol plasma and cerebrospinal fluid pharmacokinetics in children. *British Journal of Clinical Pharmacology*, *46*(3), 237–243. https://doi.org/10.1046/j.1365-2125.1998.00780.x

Andruski, B., McCafferty, D. M., Ignacy, T., Millen, B., & McDougall, J. J. (2008). Leukocyte trafficking and pain behavioral responses to a hydrogen sulfide donor in acute monoarthritis. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *295*(3), 814–820. https://doi.org/10.1152/ajpregu.90524.2008

Antoniou, E., Margonis, G. A., Angelou, A., Pikouli, A., Argiri, P., Karavokyros, I., Papalois, A., & Pikoulis, E. (2016). The TNBS-induced colitis animal model: An overview. *Annals of Medicine and Surgery*, *11*, 9–15. https://doi.org/10.1016/j.amsu.2016.07.019

Appleyard, C. B., Hernández, G., & Ríos-Bedoya, C. F. (2004). Basic epidemiology of inflammatory bowel disease in Puerto Rico. *Inflammatory Bowel Diseases*, *10*(2), 106–111. https://doi.org/10.1097/00054725-200403000-00007

Attene-Ramos, M. S., Wagner, E. D., Plewa, M. J., & Gaskins, H. R. (2006). Evidence that hydrogen sulfide is a genotoxic agent. *Molecular Cancer Research*, *4*(1), 9–14. https://doi.org/10.1158/1541-7786.MCR-05-0126

Barton, L. L., & Fauque, G. D. (2009). Biochemistry, Physiology and Biotechnology of Sulfate-Reducing Bacteria - Chapter 2. In *Advances in Applied Microbiology* (1st ed., Vol. 68, Issue 09). Elsevier Inc. https://doi.org/10.1016/S0065-2164(09)01202-7

Baumgart, D. C., & Sandborn, W. (2007). Infl ammatory bowel disease: clinical aspects and established and evolving therapies. *Gastroenterology 2*, *369*(3), 1641–1657. https://doi.org/10.1007/BF00604323

Beauchamp, R. O. J., Bus, J. S., Popp, J. A., Boreiko, C. J., & Andjelkovich, D. A. (1984). Critical review of the literature on hydrogen sulfide toxicity. *Crit. Rev. Toxicol*, *13*(1), 25–97.

Blachier, F., Davila, A. M., Mimoun, S., Benetti, P. H., Atanasiu, C., Andriamihaja, M., Benamouzig, R., Bouillaud, F., & Tomé, D. (2010). Luminal sulfide and large intestine mucosa: Friend or foe? *Amino Acids*, *39*(2), 335–347. https://doi.org/10.1007/s00726-009-0445-2

Boulangé, C. L., Neves, A. L., Chilloux, J., Nicholson, J. K., & Dumas, M. E. (2016). Impact of the gut microbiota on inflammation, obesity, and metabolic disease. *Genome Medicine*, *8*(1), 1–12. https://doi.org/10.1186/s13073-016-0303-2

Brenna, Ø., Bruland, T., Furnes, M. W., Granlund, A. V. B., Drozdov, I., Emgård, J., Brønstad, G., Kidd, M., Sandvik, A. K., & Gustafsson, B. I. (2015). The guanylate cyclase-C signaling pathway is down-regulated in inflammatory bowel disease. *Scandinavian Journal of Gastroenterology*, *50*(10), 1241–1252. https://doi.org/10.3109/00365521.2015.1038849

Burisch, J. (2014). Crohn's disease and ulcerative colitis: Occurrence, course and prognosis during the first year of disease in a European population-based inception cohort. *Danish Medical Journal*, *61*(1), 1–32.

Calabresi, M. F. F., Tanimoto, A., Próspero, A. G., Mello, F. P. F., Soares, G., Di, L. C., & Miranda, J. R. A. (2019). Changes in colonic contractility in response to inflammatory bowel disease: Long-term assessment in a model of TNBS-induced in fl ammation in rats. *Life Sciences*, *236*(September). https://doi.org/10.1016/j.lfs.2019.116833

Cao, X., Ding, L., Xie, Z., Yang, Y., Whiteman, M., Moore, P. K., & Bian, J. (2019). A Review of Hydrogen Sulfide Synthesis , Metabolism , and Measurement : Is Modulation of Hydrogen Sulfide a Novel Therapeutic for Cancer ? *Antioxidants & Redox Signaling*, *31*(1). https://doi.org/10.1089/ars.2017.7058

Carbajo, J. M., & Maraver, F. (2017). Sulphurous mineral waters: New applications for health. *Evidence-Based Complementary and Alternative Medicine*, *2017*. https://doi.org/10.1155/2017/8034084

Cave, N. J. (2003). Chronic inflammatory disorders of the gastrointestinal tract of companion animals. *New Zealand Veterinary Journal*, *51*(6), 262–274. https://doi.org/10.1080/00480169.2003.36380

Cazarin, K. C. C., Corrêa, C. L., & Zambrone, F. A. D. (2004). Redução, refinamento e substituição do uso de animais em estudos toxicológicos: uma abordagem atual. *Revista Brasileira de Ciências Farmacêuticas*, *40*(3), 289–299. https://doi.org/10.1590/s1516-93322004000300004

Chamoun-Emanuelli, A. M., Bryan, L. K., Cohen, N. D., Tetrault, T. L., Szule, J. A., Barhoumi, R., & Whitfield-Cargile, C. M. (2019). NSAIDs disrupt intestinal homeostasis by suppressing macroautophagy in intestinal epithelial cells. *Scientific Reports*, *9*(1), 1–15. https://doi.org/10.1038/s41598-019-51067-2

Chan, M. V., & Wallace, J. L. (2013). Hydrogen sulfide-based therapeutics and gastrointestinal diseases: Translating physiology to treatments. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *305*(7), 467–473. https://doi.org/10.1152/ajpgi.00169.2013

Chávez-Piña, A. E., Tapia-Álvarez, G. R., & Navarrete, A. (2010). Inhibition of endogenous hydrogen sulfide synthesis by PAG protects against ethanol-induced gastric damage in the rat. *European Journal of Pharmacology*, *630*(1–3), 131–136. https://doi.org/10.1016/j.ejphar.2009.12.017

Chen, Q. Q., Yan, L., Wang, C. Z., Wang, W. H., Shi, H., Su, B. Bin, Zeng, Q. H., Du, H. T., & Wan, J. (2013). Mesenchymal stem cells alleviate TNBS-induced colitis by modulating inflammatory and autoimmune responses. *World Journal of Gastroenterology*, *19*(29), 4702–4717. https://doi.org/10.3748/wjg.v19.i29.4702

Chou, R., Fanciullo, G. J., Fine, P. G., Adler, J. A., Ballantyne, J. C., Davies, P., Donovan, M. I., Fishbain, D. A., Foley, K. M., Fudin, J., Gilson, A. M., Kelter, A., Mauskop, A., O'Connor, P. G., Passik, S. D., Pasternak, G. W., Portenoy, R. K., Rich, B. A., Roberts, R. G., … Miaskowski, C. (2009). Clinical Guidelines for the Use of Chronic Opioid Therapy in Chronic Noncancer Pain. *Journal of Pain*, *10*(2), 113-130.e22. https://doi.org/10.1016/j.jpain.2008.10.008

Chou, S., Fay, M., Keith, S., Ingerman, L., & Chappell, L. (2006). TOXICOLOGICAL PROFILE FOR HYDROGEN SULFIDE. In *U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry*.

Costa, M., Brookes, S. J. H., Steele, P. A., Gibbins, I., Burcher, E., & Kandiah, C. J. (1996). Neurochemical classification of myenteric neurons in the guinea-pig ileum. *Neuroscience*, *75*(3), 949–967. https://doi.org/10.1016/0306-4522(96)00275-8

Cury, D. B., Mizsputen, S. J., Versolato, C., Miiji, L. O., Pereira, E., Delboni, M. A., Schor, N., & Moss, A. C. (2013). Serum calprotectin levels correlate with biochemical and histological markers of disease activity in TNBS colitis. *Cellular Immunology*, *282*(1), 66–70. https://doi.org/10.1016/j.cellimm.2013.04.004

Danese, S., & Fiocchi, C. (2011). Ulcerative colitis. *The New England Journal of Medicine*, *365*(18), 1713-25.

Danese, S., Sans, M., & Fiocchi, C. (2004). Inflammatory bowel disease: The role of environmental factors. *Autoimmunity Reviews*, *3*(5), 394–400. https://doi.org/10.1016/j.autrev.2004.03.002

Dello Russo, C., Tringali, G., Ragazzoni, E., Maggiano, N., Menini, E., Vairano, M., Preziosi, P., & Navarra, P. (2000). Evidence that hydrogen sulphide can modulate hypothalamo-pituitary-adrenal axis function: In vitro and in vivo studies in the rat. *Journal of Neuroendocrinology*, *12*(3), 225–233. https://doi.org/10.1046/j.1365-2826.2000.00441.x

Dhaese, I., Van Colen, I., & Lefebvre, R. A. (2010). Mechanisms of action of hydrogen sulfide in relaxation of mouse distal colonic smooth muscle. *European Journal of Pharmacology*, *628*(1–3), 179–186. https://doi.org/10.1016/j.ejphar.2009.11.024

Distrutti, E., Cipriani, S., Renga, B., Mencarelli, A., Migliorati, M., Cianetti, S., & Fiorucci, S. (2010). Hydrogen sulphide induces μ opioid receptor-dependent analgesia in a rodent model of visceral pain. *Molecular Pain*, *6*, 1–16. https://doi.org/10.1186/1744-8069-6-36

Distrutti, E., Sediari, L., Mencarelli, A., Renga, B., Orlandi, S., Antonelli, E., Roviezzo, F., Morelli, A., Cirino, G., Wallace, J. L., & Fiorucci, S. (2006). Evidence that hydrogen sulfide exerts antinociceptive effects in the gastrointestinal tract by activating KATP channels. *Journal of Pharmacology and Experimental Therapeutics*, *316*(1), 325–335. https://doi.org/10.1124/jpet.105.091595

Djemam, N., Lassed, S., Gül, F., Altun, M., Monteiro, M., Menezes-Pinto, D., Benayache, S., Benayache, F., Zama, D., Demirtas, I., & Morato, M. (2020). Characterization of

ethyl acetate and n-butanol extracts of Cymbopogon schoenanthus and Helianthemum lippii and their effect on the smooth muscle of the rat distal colon. *Journal of Ethnopharmacology*, *252*. https://doi.org/10.1016/j.jep.2020.112613

Docherty, M. J., Jones, R. C. W., & Wallace, M. S. (2011). Managing pain in inflammatory bowel disease. *Gastroenterology and Hepatology*, *7*(9), 592–601.

Doke, S. K., & Dhawale, S. C. (2015). Alternatives to animal testing: A review. *Saudi Pharmaceutical Journal*, *23*(3), 223–229. https://doi.org/10.1016/j.jsps.2013.11.002

Dordević, D., Jančíková, S., Vítězová, M., & Kushkevych, I. (2020). Hydrogen sulfide toxicity in the gut environment: Meta-analysis of sulfate-reducing and lactic acid bacteria in inflammatory processes. *Journal of Advanced Research*. https://doi.org/10.1016/j.jare.2020.03.003

Duarte-Araújo, M., Nascimento, C., Timóteo, M. A., Magalhães-Cardoso, T., & Correia-de-Sá, P. (2004). Dual effects of adenosine on acetylcholine release from myenteric motoneurons are mediated by junctional facilitatory A 2A and extrajunctional inhibitory A 1 receptors. *British Journal of Pharmacology*, *141*(6), 925–934. https://doi.org/10.1038/sj.bjp.0705697

Dunn, W. R., Alexander, S. P. H., Ralevic, V., & Roberts, R. E. (2016). Effects of hydrogen sulphide in smooth muscle. *Pharmacology and Therapeutics*, *158*, 101–113. https://doi.org/10.1016/j.pharmthera.2015.12.007

Dunn, W. R., Alexander, S. P. H., Ralevic, V., & Roberts, R. E. (2016). Effects of hydrogen sulphide in smooth muscle. *Pharmacology and Therapeutics*, *158*, 101–113. https://doi.org/10.1016/j.pharmthera.2015.12.007

Enokido, Y., Suzuki, E., Iwasawa, K., Namekata, K., Okazawa, H., & Kimura, H. (2005). Cystathionine β-synthase, a key enzyme for homocysteine metabolism, is preferentially expressed in the radial glia/astrocyte lineage of developing mouse CNS. *The FASEB Journal*, *19*(13), 1854–1856. https://doi.org/10.1096/fj.05-3724fje

Ferreira-Duarte, M. (2018). *Colonic reactivity to Angiotensin II in an experimental model of inflammatory bowel disease Mariana*. Universidade do Porto.

Fiorucci, S., Orlandi, S., Mencarelli, A., Caliendo, G., Santagada, V., Distrutti, E., Santucci, L., Cirino, G., & Wallace, J. L. (2007). Enhanced activity of a hydrogen sulphide-

releasing derivative of mesalamine (ATB-429) in a mouse model of colitis. *British Journal of Pharmacology*, *150*(8), 996–1002. https://doi.org/10.1038/sj.bjp.0707193

Fiorucci, S., Antonelli, E., Distrutti, E., Rizzo, G., Mencarelli, A., Orlandi, S., Zanardo, R., Renga, B., Di Sante, M., Morelli, A., Cirino, G., & Wallace, J. L. (2005). Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. *Gastroenterology*, *129*(4), 1210–1224. https://doi.org/10.1053/j.gastro.2005.07.060

Fiorucci, S., Distrutti, E., Cirino, G., & Wallace, J. L. (2006). The Emerging Roles of Hydrogen Sulfide in the Gastrointestinal Tract and Liver. *Gastroenterology*, *131*(1), 259–271. https://doi.org/10.1053/j.gastro.2006.02.033

Fite, A., Macfarlane, G. T., Cummings, J. H., Hopkins, M. J., Kong, S. C., Furrie, E., & Macfarlane, S. (2004). Identification and quantitation of mucosal and faecal desulfovibrios using real time polymerase chain reaction. *Gut*, *53*(4), 523–529. https://doi.org/10.1136/gut.2003.031245

Fitzpatrick, L. R., Meirelles, K., Small, J. S., Puleo, F. J., Koltun, W. A., & Cooney, R. N. (2010). A new model of chronic hapten-induced colitis in young rats. *Journal of Pediatric Gastroenterology and Nutrition*, *50*(3), 240–250. https://doi.org/10.1097/MPG.0b013e3181cb8f4a

Flamant, M., & Xavier, R. (2018). Inflammatory bowel disease: towards a personalized medicine. *Therapeutic Advances in Gastroenterology Review*, *11*, 1–15. https://doi.org/10.1177/https

Flannigan, K. L., Ferraz, J. G. P., Wang, R., & Wallace, J. L. (2013). Enhanced Synthesis and Diminished Degradation of Hydrogen Sulfide in Experimental Colitis: A Site-Specific, Pro-Resolution Mechanism. *PLoS ONE*, *8*(8), 1–9. https://doi.org/10.1371/journal.pone.0071962

Foligné, B., Nutten, S., Steidler, L., Dennin, V., Goudercourt, D., Mercenier, A., & Pot, B. (2006). Recommendations for improved use of the murine TNBS-induced colitis model in evaluating anti-inflammatory properties of lactic acid bacteria: Technical and microbiological aspects. *Digestive Diseases and Sciences*, *51*(2), 390–400. https://doi.org/10.1007/s10620-006-3143-x

Furne, J., Saeed, A., & Levitt, M. D. (2008). Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *295*(5), 1479–1485. https://doi.org/10.1152/ajpregu.90566.2008

Gallego, D., Clavé, P., Donovan, J., Rahmati, R., Grundy, D., Jiménez, M., & Beyak, M. J. (2008). The gaseous mediator, hydrogen sulphide, inhibits in vitro motor patterns in the human, rat and mouse colon and jejunum. *Neurogastroenterology and Motility*, *20*(12), 1306–1316. https://doi.org/10.1111/j.1365-2982.2008.01201.x

Gasparetto, M., & Guariso, G. (2013). Highlights in IBD epidemiology and its natural history in the paediatric age. *Gastroenterology Research and Practice*, *2013*. https://doi.org/10.1155/2013/829040

Geremia, A., & Arancibia-Cárcamo, C. V. (2017). Innate lymphoid cells in intestinal inflammation. *Frontiers in Immunology*, *8*(OCT), 1–13. https://doi.org/10.3389/fimmu.2017.01296

Gibson, G. R., Macfarlane, G. T., & Cummings, J. (1993). Leading article Sulphate reducing bacteria and hydrogen metabolism in the human. *Gut*, *34*, 437–439.

Gil, V., Gallego, D., & Jiménez, M. (2011). Effects of inhibitors of hydrogen sulphide synthesis on rat colonic motility. *British Journal of Pharmacology*, *164*(2 B), 485–498. https://doi.org/10.1111/j.1476-5381.2011.01431.x

Gil, V., Parsons, S. P., Gallego, D., Huizinga, J. D., & Jimenez, M. (2013). Effects of hydrogen sulphide on motility patterns in the rat colon. *British Journal of Pharmacology*, *169*(1), 34–50. https://doi.org/10.1111/bph.12100

Goubern, M., Andriamihaja, M., Nübel, T., Blachier, F., & Bouillaud, F. (2007). Sulfide , the first inorganic substrate for human cells. *The FASEB Journal • Research Communication*, *21*, 1699–1706. https://doi.org/10.1096/fj.06-7407com

Gregorakos, L., Dimopoulos, G., Liberi, S., & Antipas, G. (n.d.). *Angiology Hydrogen Sulfide Poisoning: Management and Complications*. 1123–1131.

Grider, J. R. (1993). Interplay of VIP and nitric oxide in regulation of the descending relaxation phase of peristalsis. *American Physiological Society*.

Guo, F. F., Yu, T. C., Hong, J., & Fang, J. Y. (2016). Emerging roles of hydrogen sulfide in inflammatory and neoplastic colonic diseases. *Frontiers in Physiology*, *7*(MAY), 1–8. https://doi.org/10.3389/fphys.2016.00156

Hanauer, S. B. (2006). Inflammatory Bowel Disease; Epidemiology,pathology, therapies.pdf. *Inflammatory Bowel Diseases*, *12*(January), 3–9.

Hannah, R. S., & Roth, S. H. (1991). Chronic exposure to low concentrations of hydrogen sulfide produces abnormal growth in developing cerebellar Purkinje cells. *Neuroscience Letters*, *122*, 225–228.

Harbord, M., Eliakim, R., Bettenworth, D., Karmiris, K., Katsanos, K., Kopylov, U., Kucharzik, T., Molnár, T., Raine, T., Sebastian, S., de Sousa, H. T., Dignass, A., & Carbonnel, F. (2017). Third European evidence-based consensus on diagnosis and management of ulcerative colitis. Part 2: Current management. *Journal of Crohn's and Colitis*, *11*(7), 769–784. https://doi.org/10.1093/ecco-jcc/jjx009

Hessel, P. A., Herbert, F. A., Melenka, L. S., Yoshida, K., & Nakaza, M. (1997). Lung Health in Relation to Hydrogen Sulfide Exposure in Oil and Gas Workers in Alberta, Canada. *AMERICAN JOURNAL OF INDUSTRIAL MEDICINE*, *557*, 554–557.

Hibi, T., Ogata, H., & Sakuraba, A. (2002). Animal models of inflammatory bowel disease. *Journal of Gastroenterology*, *37*(6), 409–417. https://doi.org/10.1007/s005350200060

Hine, C., Harputlugil, E., Zhang, Y., Ruckenstuhl, C., Cheon, B., Brace, L., Longchamp, A., Trevino-villarreal, J. H., Mejia, P., Keith, C., Wang, R., Gladyshev, V. N., Madeo, F., Mair, W. B., & James, R. (2015). Endogenous Hydrogen Sulfide Production Is Essential for Dietary Restriction Benefits. *Cell*, *160*(0), 132–144. https://doi.org/10.1016/j.cell.2014.11.048.Endogenous

Hirata, I., Naito, Y., Takagi, T., & Mizushima, K. (2011). *Endogenous Hydrogen Sulfide Is an Anti-inflammatory Molecule in Dextran Sodium Sulfate-Induced Colitis in Mice*. 1379–1386. https://doi.org/10.1007/s10620-010-1461-5

Holwerda, K. M., Karumanchi, S. A., & Lely, A. T. (2015). *Hydrogen sulfide : role in vascular physiology and pathology*. *24*(2), 170–176. https://doi.org/10.1097/MNH.0000000000000096

Horn, A. E., & Ufberg, J. W. (2011). Appendicitis, Diverticulitis, and Colitis. *Emergency Medicine Clinics of North America*, *29*(2), 347–368. https://doi.org/10.1016/j.emc.2011.01.002

Hosoki, R., Matsuki, N., & Kimura, H. (1997). The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochemical and Biophysical Research Communications*, *237*(3), 527–531. https://doi.org/10.1006/bbrc.1997.6878

Hosoki, R., Matsuki, N., & Kimura, H. (1997). The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochemical and Biophysical Research Communications*, *237*(3), 527–531. https://doi.org/10.1006/bbrc.1997.6878

Hosseini, J. M., Goldhill, J. M., Bossone, C., Eiro, V. P. I. N., & Donohue, T. S. (1999). *Progressive alterations in circular smooth muscle contractility in TNBS-induced colitis in rats*. *September 1998*.

Islam, K. N., Polhemus, D. J., Donnarumma, E., Brewster, L. P., & Lefer, D. J. (2015). Hydrogen Sulfide Levels and Nuclear Factor-Erythroid 2-Related Factor 2 (NRF2) Activity Are Attenuated in the Setting of Critical Limb Ischemia (CLI). *Journal of the American Heart Association*, *4*(5), 1–10. https://doi.org/10.1161/JAHA.115.001986

Jergens, A. E., & Simpson, K. W. (2012). Inflammatory bowel disease in veterinary medicine. *Frontiers in Bioscience - Elite*, *4 E*(4), 1404–1419. https://doi.org/10.2741/e470

Jiang, J., Chan, A., Ali, S., Saha, A., Haushalter, K. J., Lam, L. M., Glasheen, M., Parker, J., Brenner, M., Mahon, S. B., Patel, H. H., Ambasudhan, R., Lipton, S. A., Pilz, R. B., & Boss, G. R. (2016). Hydrogen Sulfide — Mechanisms of Toxicity and Development of an Antidote. *Scientific Reports*, *February* 1–10. https://doi.org/10.1038/srep20831

Jimenez, M., Gil, V., Martinez-Cutillas, M., Mañé, N., & Gallego, D. (2017). Hydrogen sulphide as a signalling molecule regulating physiopathological processes in gastrointestinal motility. *British Journal of Pharmacology*, *174*(17), 2805–2817. https://doi.org/10.1111/bph.13918

Jirkof, P., Rudeck, J., & Lewejohann, L. (2019). Assessing affective state in laboratory rodents to promote animal welfare—what is the progress in applied refinement research? *Animals*, *9*(12), 1–16. https://doi.org/10.3390/ani9121026

Jones-Hall, Y. L., & Grisham, M. B. (2014). Immunopathological characterization of selected mouse models of inflammatory bowel disease: Comparison to human disease. *Pathophysiology*, *21*(4), 267–288. https://doi.org/10.1016/j.pathophys.2014.05.002

Jørgensen, J., & Mortensen, P. B. (2001). Hydrogen sulfide and colonic epithelial metabolism: Implications for ulcerative colitis. *Digestive Diseases and Sciences*, *46*(8), 1722–1732. https://doi.org/10.1023/A:1010661706385

Ju, Y., Fu, M., Stokes, E., Wu, L., & Yang, G. (2017). H2S-mediated protein S-Sulfhydration: A prediction for its formation and regulation. *Molecules*, *22*(8), 1–11. https://doi.org/10.3390/molecules22081334

Jurjus, A. R., Khoury, N. N., & Reimund, J. M. (2004). Animal models of inflammatory bowel disease. *Journal of Pharmacological and Toxicological Methods*, *50*(2), 81–92. https://doi.org/10.1016/j.vascn.2003.12.002

Kabil, O., Motl, N., & Banerjee, R. (2014). H2S and its role in redox signaling. *Biochim Biophys*, *1844*(8), 1355–1366. https://doi.org/10.1038/jid.2014.371

Kamoun, P. (2004). Endogenous production of hydrogen sulfide in mammals. *Amino Acids*, *26*, 243–254. https://doi.org/10.1007/s00726-004-0072-x

Karunya, R., Jayaprakash, K. S., Gaikwad, R., Sajeesh, P., Ramshad, K., Muraleedharan, K. M., Dixit, M., Thangaraj, P. R., & Sen, A. K. (2019). Rapid measurement of hydrogen sulphide in human blood plasma using a microfluidic method. *Scientific Reports*, *9*(1), 1–11. https://doi.org/10.1038/s41598-019-39389-7

Khor, B., Gardet, A., & Xavier, R. J. (2011). Genetics and pathogenesis of inflammatory bowel disease. *Nature*, *474*(7351), 307–317. https://doi.org/10.1038/nature10209

Kornbluth, A., & Sachar, D. B. (2010). Ulcerative colitis practice guidelines in adults: American college of gastroenterology, practice parameters committee. *American Journal of Gastroenterology*, *105*(3), 501–523. https://doi.org/10.1038/ajg.2009.727

Kumral, D., & Zfass, A. M. (2018). Gut Movements: A Review of the Physiology of Gastrointestinal Transit. *Digestive Diseases and Sciences*, *0123456789*. https://doi.org/10.1007/s10620-018-5259-1

Kupai, K., Almási, N., Kósa, M., Nemcsók, J., Murlasits, Z., Török, S., Al-awar, A., Baráth, Z., Pósa, A., & Varga, C. (2017). H2S confers colonoprotection against TNBS-induced colitis by HO-1 upregulation in rats. *Inflammopharmacology*, *26*(2), 479–489. https://doi.org/10.1007/s10787-017-0382-8

Lagoutte, E., Mimoun, S., Andriamihaja, M., Chaumontet, C., Blachier, F., & Bouillaud, F. (2010). Oxidation of hydrogen sulfide remains a priority in mammalian cells and causes reverse electron transfer in colonocytes. *Biochimica et Biophysica Acta - Bioenergetics*, *1797*(8), 1500–1511. https://doi.org/10.1016/j.bbabio.2010.04.004

Lagoutte, E., Mimoun, S., Andriamihaja, M., Chaumontet, C., Blachier, F., & Bouillaud, F. (2010). Oxidation of hydrogen sulfide remains a priority in mammalian cells and causes reverse electron transfer in colonocytes. *Biochimica et Biophysica Acta - Bioenergetics*, *1797*(8), 1500–1511. https://doi.org/10.1016/j.bbabio.2010.04.004

Lamb, C. A., Kennedy, N. A., Raine, T., Hendy, P. A., Smith, P. J., Limdi, J. K., Hayee, B., Lomer, M. C. E., Parkes, G. C., Selinger, C., Barrett, K. J., Davies, R. J., Bennett, C., Gittens, S., Dunlop, M. G., Faiz, O., Fraser, A., Garrick, V., Johnston, P. D., … Hawthorne, A. B. (2019). British Society of Gastroenterology consensus guidelines on the management of inflammatory bowel disease in adults. *Gut*, *68*, s1–s106. https://doi.org/10.1136/gutjnl-2019-318484

Lapaquette, P., Brest, P., Hofman, P., & Darfeuille-Michaud, A. (2012). Etiology of Crohn's disease: Many roads lead to autophagy. *Journal of Molecular Medicine*, *90*(9), 987–996. https://doi.org/10.1007/s00109-012-0934-8

Leppert, W. (2015). Emerging therapies for patients with symptoms of opioid-induced bowel dysfunction. *Drug Design, Development and Therapy*, *9*, 2215–2231. https://doi.org/10.2147/DDDT.S32684

Leschelle, X., Goubern, M., Andriamihaja, M., Blottière, H. M., Couplan, E., Gonzalez-Barroso, M. D. M., Petit, C., Pagniez, A., Chaumontet, C., Mignotte, B., Bouillaud, F., & Blachier, F. (2005). Adaptative metabolic response of human colonic epithelial cells to the adverse effects of the luminal compound sulfide. *Biochimica et Biophysica Acta - General Subjects*, *1725*(2), 201–212. https://doi.org/10.1016/j.bbagen.2005.06.002

Leung, V., Zhang, E., & Pang, D. S. J. (2016). Real-Time application of the Rat Grimace Scale as a welfare refinement in laboratory rats. *Scientific Reports*, *6*(August), 1–12. https://doi.org/10.1038/srep31667

Levine, J., Ellis, C. J., Furne, J. K., Springfield, J., & Levitt, M. D. (1998). Fecal hydrogen sulfide production in ulcerative colitis. *American Journal of Gastroenterology*, *93*(1), 83–87. https://doi.org/10.1111/j.1572-0241.1998.083_c.x

Liang, Y. F., Zhang, D. D., Yu, X. J., Gao, H. L., Liu, K. L., Qi, J., Li, H. B., Yi, Q. Y., Chen, W. S., Cui, W., Zhu, G. Q., & Kang, Y. M. (2017). Hydrogen sulfide in paraventricular nucleus attenuates blood pressure by regulating oxidative stress and inflammatory cytokines in high salt-induced hypertension. *Toxicology Letters*, *270*, 62–71. https://doi.org/10.1016/j.toxlet.2017.02.004

Lim, E., Mbowe, O., Lee, A. S. W., & Davis, J. (2016). Effect of environmental exposure to hydrogen sulfide on central nervous system and respiratory function: a systematic review of human studies. *International Journal of Occupational and Environmental Health*, *3525*(May). https://doi.org/10.1080/10773525.2016.1145881

Lim, J., Liu, Y. H., Khin, E. S. W., & Bian, J. S. (2008). Vasoconstrictive effect of hydrogen sulfide involves downregulation of cAMP in vascular smooth muscle cells. *American Journal of Physiology - Cell Physiology*, *295*(5), 1261–1271. https://doi.org/10.1152/ajpcell.00195.2008

Linden, D. R. (2014). Hydrogen sulfide signaling in the gastrointestinal tract. *Antioxidants and Redox Signaling*, *20*(5), 818–830. https://doi.org/10.1089/ars.2013.5312

Liu, Y., Kalogeris, T., Wang, M., Zuidema, M. Z., Wang, Q., Dai, H., Davis, M. J., Hill, M. A., & Korthuis, R. J. (2012). Hydrogen sulfide preconditioning or neutrophil depletion attenuates Ischemiareperfusion-induced mitochondrial dysfunction in rat small intestine. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *302*(1), 44–54. https://doi.org/10.1152/ajpgi.00413.2010

Liu, Y., Luo, H., Liang, C., Xia, H., Xu, W., Chen, J., & Chen, M. (2013). Actions of Hydrogen Sulfide and ATP-Sensitive Potassium Channels on Colonic Hypermotility in a Rat Model of Chronic Stress. *PLoS ONE*, *8*(2). https://doi.org/10.1371/journal.pone.0055853

Lu, W., Li, J., Gong, L., Xu, X., Han, T., Ye, Y., Che, T., Luo, Y., Li, J., Zhan, R., Yao, W., Liu, K., Cui, S., & Liu, C. (2014). H2S modulates duodenal motility in male rats via activating TRPV1 and KATP channels. *British Journal of Pharmacology*, *171*(6), 1534–1550. https://doi.org/10.1111/bph.12562

MacEachern, S. J., Patel, B. A., Mckay, D. M., & Sharkey, K. A. (2011). Nitric oxide regulation of colonic epithelial ion transport: A novel role for enteric glia in the myenteric plexus. *Journal of Physiology*, *589*(13), 3333–3348. https://doi.org/10.1113/jphysiol.2011.207902

Magierowski, M., Magierowska, K., Hubalewska-Mazgaj, M., Sliwowski, Z., Pajdo, R., Ginter, G., Kwiecien, S., Brzozowski, T., Nagahara, N., & Wrobel, M. (2017). Exogenous and endogenous hydrogen sulfide protects gastric mucosa against the formation and time-dependent development of ischemia/reperfusion-induced acute lesions progressing into deeper ulcerations. *Molecules*, *22*(2), 1–16. https://doi.org/10.3390/molecules22020295

Mainier, F. B., & Viola, E. D. M. (2005). O Sulfeto De Hidrogênio (H2S) E O Meio Ambiente. *II Simpósio de Excelência Em Gestão e Tecnologia*, 612–618.

Martinez-Cutillas, M., Gil, V., Mañé, N., Clavé, P., Gallego, D., Martin, M. T., & Jimenez, M. (2015). Potential role of the gaseous mediator hydrogen sulphide (H2S) in inhibition of human colonic contractility. *Pharmacological Research*, *93*, 52–63. https://doi.org/10.1016/j.phrs.2015.01.002

Matsunami, M., Kirishi, S., Okui, T., & Kawabata, A. (2012). Hydrogen sulfide-induced colonic mucosal cytoprotection involves T-type calcium channel-dependent neuronal excitation in rats. *Journal of Physiology and Pharmacology*, *63*(1), 61–68.

Medani, M., Collins, D., Docherty, N. G., Baird, A. W., O'Connell, P. R., & Winter, D. C. (2011). Emerging role of hydrogen sulfide in colonic physiology and pathophysiology. *Inflammatory Bowel Diseases*, *17*(7), 1620–1625. https://doi.org/10.1002/ibd.21528

Medeiros, J. V. R., Bezerra, V. H., Gomes, A. S., Barbosa, A. L. R., Lima, R. C. P., Soares, P. M. G., Brito, G. A. C., Ribeiro, R. A., Cunha, F. Q., & De Souza, M. H. L. P. (2009). Hydrogen sulfide prevents ethanol-induced gastric damage in mice: Role of ATP-sensitive potassium channels and capsaicin-sensitive primary afferent neurons. *Journal of Pharmacology and Experimental Therapeutics*, *330*(3), 764–770. https://doi.org/10.1124/jpet.109.152801

Mimoun, S., Andriamihaja, M., Chaumontet, C., Atanasiu, C., Benamouzig, R., Blouin, J. M., Tomé, D., Bouillaud, F., & Blachier, F. (2012). Detoxification of H2S by differentiated colonic epithelial cells: Implication of the sulfide oxidizing unit and of the cell respiratory capacity. *Antioxidants and Redox Signaling*, *17*(1), 1–10. https://doi.org/10.1089/ars.2011.4186

Molodecky, N. A., Soon, I. S., Rabi, D. M., Ghali, W. A., Ferris, M., Chernoff, G., Benchimol, E. I., Panaccione, R., Ghosh, S., Barkema, H. W., & Kaplan, G. G. (2012). Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology*, *142*(1), 46-54.e42. https://doi.org/10.1053/j.gastro.2011.10.001

Monjok, E. M., Kulkarni, K. H., Kouamou, G., McKoy, M., Opere, C. A., Bongmba, O. N., Njie, Y. F., & Ohia, S. E. (2008). Inhibitory action of hydrogen sulfide on muscarinic receptor-induced contraction of isolated porcine irides. *Experimental Eye Research*, *87*(6), 612–616. https://doi.org/10.1016/j.exer.2008.09.011

Moore, J. W. E., Millard, S., Babidge, W., Rowland, R., & Roediger, W. E. W. (1997). Hydrogen sulphide produces diminished fatty acid oxidation in the rat colon in vivo: Implications for ulcerative colitis. *Australian and New Zealand Journal of Surgery*, *67*(5), 245–249. https://doi.org/10.1111/j.1445-2197.1997.tb01956.x

Morris, G. P., Beck, P. L., Herridge, M. S., Depew, W. T., Szewczuk, M. R., & Wallace, J. L. (1989). Hapten-Induced Model of Chronic Inflammation and Ulceration in the Rat Colon. *Gastroenterology*, *96*(2), 795–803. https://doi.org/10.1016/S0016-5085(89)80079-4

Motavallian-Naeini, A., Andalib, S., Rabbani, M., Mahzouni, P., Afsharipour, M., & Minaiyan, M. (2012). Validation and optimization of experimental colitis induction in rats using 2, 4, 6-trinitrobenzene sulfonic acid. *Research in Pharmaceutical Sciences*, *7*(3), 159–169.

Motta, J. P., Flannigan, K. L., Agbor, T. A., Beatty, J. K., Blackler, R. W., Workentine, M. L., Da Silva, G. J., Wang, R., Buret, A. G., & Wallace, J. L. (2015). Hydrogen sulfide protects from colitis and restores intestinal microbiota biofilm and mucus production. *Inflammatory Bowel Diseases*, *21*(5), 1006–1017. https://doi.org/10.1097/MIB.0000000000000345

Mowat, C., Cole, A., Windsor, A., Ahmad, T., Arnott, I., Driscoll, R., Mitton, S., Orchard, T., Rutter, M., Younge, L., Lees, C., Ho, G. T., Satsangi, J., & Bloom, S. (2011). Guidelines for the management of inflammatory bowel disease in adults. *Gut*, *60*(5), 571–607. https://doi.org/10.1136/gut.2010.224154

Mulder, D. J., Noble, A. J., Justinich, C. J., & Duffin, J. M. (2014). A tale of two diseases: The history of inflammatory bowel disease. *Journal of Crohn's and Colitis*, *8*(5), 341–348. https://doi.org/10.1016/j.crohns.2013.09.009

Murthy, K. S. (2006). Signaling for Contraction and Relacation in Smooth Muscle of the Gut. *Annu. Rev. Physiol*, *68*, 345–74. https://doi.org/10.1146/annurev.physiol.68.040504.094707

Nalli, A. D., Bhattacharya, S., Wang, H., Kendig, D. M., Grider, J. R., & Murthy, K. S. (2017). Augmentation of cGMP/PKG pathway and colonic motility by hydrogen sulfide. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *313*(4), G330–G341. https://doi.org/10.1152/ajpgi.00161.2017

Ng, S. C., Bernstein, C. N., Vatn, M. H., Lakatos, P. L., Loftus, E. V., Tysk, C., O'Morain, C., Moum, B., & Colombel, J. F. (2013). Geographical variability and environmental risk factors in inflammatory bowel disease. *Gut*, *62*(4), 630–649. https://doi.org/10.1136/gutjnl-2012-303661

Nowland, M. H., Hugunin, K. M. S., & Rogers, K. L. (2011). Effects of short-term fasting in male Sprague-Dawley rats. *Comparative Medicine*, *61*(2), 138–144.

Ohama, T., Hori, M., & Ozaki, H. (2007). Mechanism of abnormal intestinal motility in inflammatory bowel disease: How smooth muscle contraction is reduced? *Journal of Smooth Muscle Research*, *43*(2), 43–54. https://doi.org/10.1540/jsmr.43.43

Oliveira, F. M., Emerick, A. P. do C., & Soares, E. G. (2010). Aspectos epidemiológicos das doenças intestinais inflamatórias na macrorregião de saúde leste do estado de Minas Gerais. *Ciencia e Saude Coletiva*, *15*(SUPPL. 1), 1031–1037. https://doi.org/10.1590/s1413-81232010000700009

Olson, K. R. (2011). The therapeutic potential of hydrogen sulfide: Separating hype from hope. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *301*(2). https://doi.org/10.1152/ajpregu.00045.2011

Owoyele, B. V., Oladejo, R. O., Ajomale, K., Ahmed, R. O., & Mustapha, A. (2014). Analgesic and anti-inflammatory effects of honey: The involvement of autonomic receptors. *Metabolic Brain Disease*, *29*(1), 167–173. https://doi.org/10.1007/s11011-013-9458-3

Pan, L., Qin, M., Liu, X., & Zhu, Y. (2017). *The Role of Hydrogen Sulfide on Cardiovascular Homeostasis : An Overview with Update on Immunomodulation*. *8*(September), 1–13. https://doi.org/10.3389/fphar.2017.00686

Parajuli, S. P., Choi, S., Lee, J., Kim, Y. D., Park, C. G., Kim, M. Y., Kim, H. Il, Yeum, C. H., & Jun, J. Y. (2010). The inhibitory effects of hydrogen sulfide on pacemaker activity of interstitial cells of Cajal from mouse small intestine. *Korean Journal of Physiology and Pharmacology*, *14*(2), 83–89. https://doi.org/10.4196/kjpp.2010.14.2.83

Patacchini, R., Santicioli, P., Giuliani, S., & Maggi, C. A. (2004). Hydrogen sulfide (H 2 S) stimulates capsaicin-sensitive primary afferent neurons in the rat urinary bladder. *British Journal of Pharmacology (2004)*, *March*, 31–34. https://doi.org/10.1038/sj.bjp.0705764

Patacchini, R., T, P. S., Giuliani, S., & Maggi, C. A. (2005). Pharmacological investigation of hydrogen sulfide (H 2 S) contractile activity in rat detrusor muscle. *European Journal of Pharmacology*, *509*, 171–177. https://doi.org/10.1016/j.ejphar.2005.01.005

Pitcher, M. C. L., Beatty, E. R., & Cummings, J. H. (2000). The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. *Gut*, *46*(1), 64–72. https://doi.org/10.1136/gut.46.1.64

Quintal, J. B. (2016). *Doença Inflamatória Intestinal: Cuidados Farmacêuticos*. Universidade de Coimbra.

Randhawa, P. K., Singh, K., Singh, N., & Jaggi, A. S. (2014). A review on chemical-induced inflammatory bowel disease models in rodents. *Korean Journal of Physiology and Pharmacology*, *18*(4), 279–288. https://doi.org/10.4196/kjpp.2014.18.4.279

Reiffenstein, R. J., Hulbert, W., & Roth, S. H. (1992). Toxicology of hydrogen sulfide. *Pharmacol. Toxicol.*, *1984*(5), 109–134.

Rodrigues-Pinto, T. (2019). *Contribution of non-neuronal cells to Angiotensin II- mediated contraction in a refined model of rat colitis induced by 2,4,6-trinitrobenzene sulfonic acid.* Universidade do Porto.

Roth, S. H. (2004). Toxicological and Environmental Impacts of Hydrogen Sulfide. In *Signal Transduction and the Gasotransmitters: NO, CO, and H2S in Biology and Medicine* (pp. 293–313).

Rowan, F. E., Docherty, N. G., Coffey, J. C., & O'Connell, P. R. (2009). Sulphate-reducing bacteria and hydrogen sulphide in the aetiology of ulcerative colitis. *British Journal of Surgery*, *96*(2), 151–158. https://doi.org/10.1002/bjs.6454

Sairenji, T., Collins, K. L., & Evans, D. V. (2017). An Update on Inflammatory Bowel Disease. *Primary Care - Clinics in Office Practice*, *44*(4), 673–692. https://doi.org/10.1016/j.pop.2017.07.010

Sanders, K. M., Koh, S. D., Ördög, T., & Ward, S. M. (2004). Ionic conductances involved in generation and propagation of electrical slow waves in phasic gastrointestinal muscles. *Neurogastroenterol Motil*, *16*, 100–105.

Schicho, R., Krueger, D., Zeller, F., Von Weyhern, C. W. H., Frieling, T., Kimura, H., Ishii, I., De Giorgio, R., Campi, B., & Schemann, M. (2006). Hydrogen Sulfide Is a Novel Prosecretory Neuromodulator in the Guinea-Pig and Human Colon. *Gastroenterology*, *131*(5), 1542–1552. https://doi.org/10.1053/j.gastro.2006.08.035

Schicho, R., Krueger, D., Zeller, F., Von Weyhern, C. W. H., Frieling, T., Kimura, H., Ishii, I., De Giorgio, R., Campi, B., & Schemann, M. (2006). Hydrogen Sulfide Is a Novel Prosecretory Neuromodulator in the Guinea-Pig and Human Colon. *Gastroenterology*, *131*(5), 1542–1552. https://doi.org/10.1053/j.gastro.2006.08.035

Schulze-Delrieu, K. (1979). Metoclopramide. *Gastroenterology*, *77*(4), 768–779. https://doi.org/10.1016/0016-5085(79)90236-1

Searcy, D. G., & Lee, S. H. (1998). Sulfur reduction by human erythrocytes. *Journal of Experimental Zoology*, *282*(3), 310–322. https://doi.org/10.1002/(SICI)1097-010X(19981015)282:3<310::AID-JEZ4>3.0.CO;2-P

SHAH, V., GREG, L., GREG, G., & GIANRICO, F. (2004). Nitric Oxide in Gastrointestinal Health and Disease. *GASTROENTEROLOGY*, *116*, 903–913. https://doi.org/10.1053/j.gastro.2003.11.046

Shen, Y., Shen, Z., Luo, S., Guo, W., & Zhu, Y. Z. (2015). The cardioprotective effects of hydrogen sulfide in heart diseases: From molecular mechanisms to therapeutic potential. *Oxidative Medicine and Cellular Longevity*, *2015*. https://doi.org/10.1155/2015/925167

Shibuya, N., Tanaka, M., Yoshida, M., Ogasawara, Y., Togawa, T., Ishii, K., & Kimura, H. (2009). 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. *Antioxidants and Redox Signaling*, *11*(4), 703–714. https://doi.org/10.1089/ars.2008.2253

Silos-Santiago, I., Hannig, G., Eutamene, H., Ustinova, E. E., Bernier, S. G., Ge, P., Graul, C., Jacobson, S., Jin, H., Liong, E., Kessler, M. M., Reza, T., Rivers, S., Shea, C., Tchernychev, B., Bryant, A. P., Kurtz, C. B., Bueno, L., Pezzone, M. A., & Currie, M. G. (2013). Gastrointestinal pain: Unraveling a novel endogenous pathway through uroguanylin/guanylate cyclase-C/cGMP activation. *Pain*, *154*(9), 1820–1830. https://doi.org/10.1016/j.pain.2013.05.044

Silverberg, M. S., Satsangi, J., Ahmad, T., Arnott, I. D. R., Bernstein, C. N., Brant, S. R., Caprilli, R., Colombel, J. F., Gasche, C., Geboes, K., Jewell, D. P., Karban, A., Loftus, E. V., Peña, A. S., Riddell, R. H., Sachar, D. B., Schreiber, S., Steinhart, A. H., Targan, S. R., … Warren, B. F. (2005). Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Canadian Journal of Gastroenterology = Journal Canadien de Gastroenterologie*, *19 Suppl A*(September). https://doi.org/10.1155/2005/269076

Singh, S., Blanchard, A., Walker, J. R., Graff, L. A., Miller, N., & Bernstein, C. N. (2011). Common Symptoms and Stressors Among Individuals With Inflammatory Bowel Diseases. *Clinical Gastroenterology and Hepatology*, *9*(9), 769–775. https://doi.org/10.1016/j.cgh.2011.05.016

Smith, N. W., Shorten, P. R., Altermann, E. H., Roy, N. C., & McNabb, W. C. (2019). Hydrogen cross-feeders of the human gastrointestinal tract. *Gut Microbes*, *10*(3), 270–288. https://doi.org/10.1080/19490976.2018.1546522

Song, Z. J., Ng, M. Y., Lee, Z. W., Dai, W., Hagen, T., Moore, P. K., Huang, D., Deng, L. W., & Tan, C. H. (2014). Hydrogen sulfide donors in research and drug development. *MedChemComm*, *5*(5), 557–570. https://doi.org/10.1039/c3md00362k

Spencer, N. J., & Hongzhen, H. (2020). Enteric nervous system: sensory transduction, neural circuits and gastrointestinal motility. *PhysioloNat Rev Gastroenterol Hepatol.*, *17*(6), 338–351. https://doi.org/10.1016/j.physbeh.2017.03.040

Stange, E. F., Travis, S. P. L., Vermeire, S., Reinisch, W., Geboes, K., Barakauskiene, A., Feakins, R., Fléjou, J. F., Herfarth, H., Hommes, D. W., Kupcinskas, L., Lakatos, P. L., Mantzaris, G. J., Schreiber, S., Villanacci, V., & Warren, B. F. (2008). European evidence-based Consensus on the diagnosis and management of ulcerative colitis: Definitions and diagnosis. *Journal of Crohn's and Colitis*, *2*(1), 1–23. https://doi.org/10.1016/j.crohns.2007.11.001

Streng, T., Axelsson, H. E., Hedlund, P., Andersson, D. A., Jordt, S. E., Bevan, S., Andersson, K. E., Högestätt, E. D., & Zygmunt, P. M. (2008). Distribution and Function of the Hydrogen Sulfide-Sensitive TRPA1 Ion Channel in Rat Urinary Bladder. *European Urology*, *53*(2), 391–400. https://doi.org/10.1016/j.eururo.2007.10.024

Suchodolski, J. S., & Steiner, J. M. (2003). Laboratory Assessment of Gastrointestinal Function. *Clinical Techniques in Small Animal Practice*, *18*(4), 203–210. https://doi.org/10.1016/S1906-2867(03)00000-0

Szabo, C. (2017). Hydrogen sulfide, an enhancer of vascular nitric oxide signaling: Mechanisms and implications. *American Journal of Physiology - Cell Physiology*, *312*(1), C3–C15. https://doi.org/10.1152/ajpcell.00282.2016

Takagi, T., Naito, Y., Hirata, I., & Yoshikawa, T. (2011). Role of hydrogen sulfide in colitis. *Gas Biology Research in Clinical Practice*, 73–80. https://doi.org/10.1159/000321943

Teague, B., Asiedu, S., & Moore, P. K. (2002). The smooth muscle relaxant effect of hydrogen sulphide in vitro: Evidence for a physiological role to control intestinal contractility. *British Journal of Pharmacology*, *137*(2), 139–145. https://doi.org/10.1038/sj.bjp.0704858

Teigen, L. M., Geng, Z., Sadowsky, M. J., Vaughn, B. P., Hamilton, M. J., & Khoruts, A. (2019). Dietary factors in sulfur metabolism and pathogenesis of ulcerative colitis. *Nutrients*, *11*(4), 1–20. https://doi.org/10.3390/NU11040931

Thursby, E., & Juge, N. (2017). Introduction to the human gut microbiota. *Biochemical Journal*, *474*(11), 1823–1836. https://doi.org/10.1042/BCJ20160510

U.S. Chemical Safety and Hazard Investigation Board. (2004). NaHS Safety Bulletin. In *CSB Safety Bulletin* (Issue Noviembre).

Vandiver, M. S., & Snyder, S. H. (2012). Hydrogen sulfide: A gasotransmitter of clinical relevance. *Journal of Molecular Medicine*, *90*(3), 255–263. https://doi.org/10.1007/s00109-012-0873-4

Verma, G., Marella, A., Shaquiquzzaman, M., & Alam, M. (2013). Immunoinflammatory responses in gastrointestinal tract injury and recovery. *Acta Biochimica Polonica*, *60*(2), 143–149. https://doi.org/10.18388/abp.2013_1964

Vermeulen, W., Joris G., D. M., Heiko U., D. S., Bult, H., Moreels, T. G., Pelckmans, P. A., & De Winter Benedicte, Y. (2013). Role of TRPV1 and TRPA1 in visceral hypersensitivity to colorectal distension during experimental colitis in rats. *European Journal of Pharmacology*, *698*(1–3), 404–412. https://doi.org/10.1016/j.ejphar.2012.10.014

Vilaça, C. de O., Freitas, M. R. G. de, Nascimento, O. J. M. do, Orsini, M., Leite, M. A. A., & Souza, J. A. de. (2015). Metabolismo da homocisteína em doenças neurológicas. *Revista Brasileira de Neurologia*, *51*(3), 73–78.

Wallace, J. L., McKnight, W., Miyasaka, M., Tamatani, T., Paulson, J., Anderson, D. C., Granger, D. N., & Kubes, P. (1993). Role of endothelial adhesion molecules in NSAID-induced gastric mucosal injury. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *265*(5 28-5). https://doi.org/10.1152/ajpgi.1993.265.5.g993

Wallace, J. L., Dicay, M., McKnight, W., & Martin, G. R. (2007). Hydrogen sulfide enhances ulcer healing in rats. *The FASEB Journal*, *21*(14), 4070–4076. https://doi.org/10.1096/fj.07-8669com

Wallace, J. L., Vong, L., McKnight, W., Dicay, M., & Martin, G. R. (2009). Endogenous and Exogenous Hydrogen Sulfide Promotes Resolution of Colitis in Rats. *Gastroenterology*, *137*(2), 569-578.e1. https://doi.org/10.1053/j.gastro.2009.04.012

Wallace, J. L., & Wang, R. (2015). Hydrogen sulfide-based therapeutics: Exploiting a unique but ubiquitous gasotransmitter. *Nature Reviews Drug Discovery*, *14*(5), 329–345. https://doi.org/10.1038/nrd4433

Wang, R. (2012). Physiological implications of hydrogen sulfide: A whiff exploration that blossomed. *Physiological Reviews*, *92*(2), 791–896. https://doi.org/10.1152/physrev.00017.2011

Webb, R. C. (2003). Smooth muscle contraction and relaxation. *American Journal of Physiology - Advances in Physiology Education*, *27*(1–4), 201–206. https://doi.org/10.1152/advan.00025.2003

Wilder-Smith, C. H., & Bettiga, A. (1997). The analgesic tramadol has minimal effect on gastrointestinal motor function. *British Journal of Clinical Pharmacology*, *43*(1), 71–75. https://doi.org/10.1111/j.1365-2125.1997.tb00035.x

Yang, J., Minkler, P., Grove, D., Wang, R., Willard, B., Dweik, R., & Hine, C. (2019). Non-enzymatic hydrogen sulfide production from cysteine in blood is catalyzed by iron and vitamin B6. *Communications Biology*, *2*(1), 1–14. https://doi.org/10.1038/s42003-019-0431-5

Yonezawa, D., Sekiguchi, F., Miyamoto, M., Taniguchi, E., Honjo, M., Masuko, T., Nishikawa, H., & Kawabata, A. (2007). A protective role of hydrogen sulfide against oxidative stress in rat gastric mucosal epithelium. *Toxicology*, *241*(1–2), 11–18. https://doi.org/10.1016/j.tox.2007.07.020

Yoon, P. J., Parajuli, S. P., Zuo, D. C., Shahi, P. K., Oh, H. J., Shin, H. R., Lee, M. J., Yeum, C. H., Choi, S., & Jun, J. Y. (2011). Interplay of Hydrogen Sulfide and Nitric Oxide on the Pacemaker Activity of Interstitial Cells of Cajal from Mouse Small Intestine. *Chonnam Medical Journal*, *47*(2), 72. https://doi.org/10.4068/cmj.2011.47.2.72

Yu, Y. B., & Li, Y. Q. (2014). Enteric glial cells and their role in the intestinal epithelial barrier. *World Journal of Gastroenterology*, *20*(32), 11273–11280. https://doi.org/10.3748/wjg.v20.i32.11273

Zanardo, R. C. O., Brancaleone, V., Distrutti, E., Fiorucci, S., Cirino, G., Wallace, J. L., Zanardo, R. C. O., Brancaleone, V., Distrutti, E., Fiorucci, S., Cirino, G., & Wallace, J. L. (2006). Hydrogen sulfide is an endogenous modulator of leukocyte-mediated

inflammation. *The FASEB Journal*, *20*(12), 2118–2120. https://doi.org/10.1096/fj.06-6270fje

Zhang, D., Du, J., Tang, C., Huang, Y., & Jin, H. (2017). H2S-induced sulfhydration: Biological function and detection methodology. *Frontiers in Pharmacology*, *8*(SEP), 1–13. https://doi.org/10.3389/fphar.2017.00608

Zhao, G., Li, J., Wang, J., Shen, X., & Sun, J. (2014). Aquaporin 3 and 8 are down-regulated in TNBS-induced rat colitis. *Biochemical and Biophysical Research Communications*, *443*(1), 161–166. https://doi.org/10.1016/j.bbrc.2013.11.067

Zhao, P., Huang, X., Wang, Z. yu, Qiu, Z. xun, Han, Y. fei, Lu, H. li, Kim, Y. chul, & Xu, W. xie. (2009). Dual effect of exogenous hydrogen sulfide on the spontaneous contraction of gastric smooth muscle in guinea-pig. *European Journal of Pharmacology*, *616*(1–3), 223–228. https://doi.org/10.1016/j.ejphar.2009.05.014

Zhao, W., Zhang, J., Lu, Y., & Wang, R. (2001). The vasorelaxant effect of H2S as a novel endogenous gaseous KATP channel opener. *EMBO Journal*, *20*(21), 6008–6016. https://doi.org/10.1093/emboj/20.21.6008

Zheng, Y., Ji, X., Ji, K., & Wang, B. (2015). Hydrogen sulfide prodrugs-a review. *Acta Pharmaceutica Sinica B*, *5*(5), 367–377. https://doi.org/10.1016/j.apsb.2015.06.004