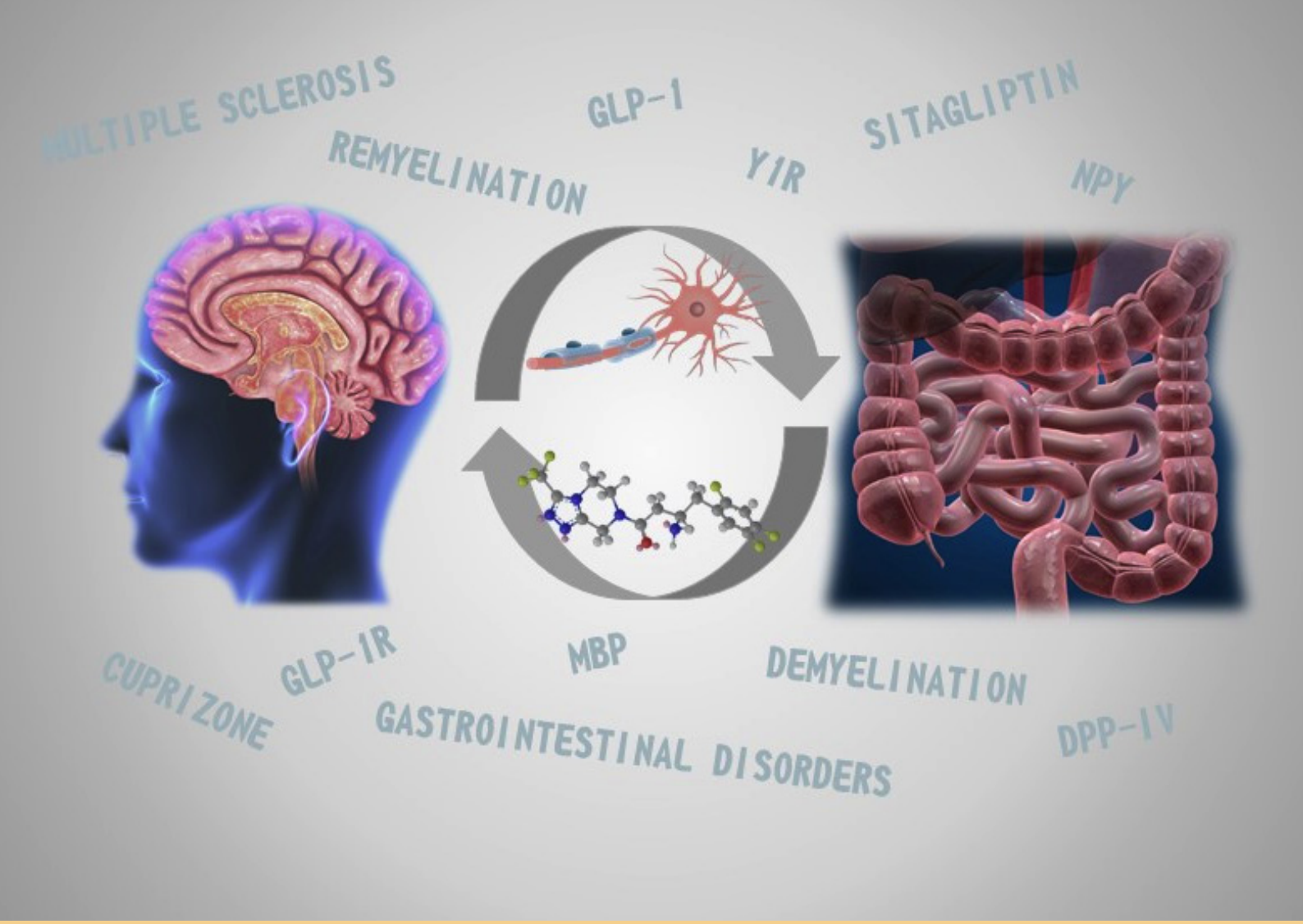

Beatriz de Oliveira Martins

Effects of incretin-based therapies on the gastrointestinal motility of an animal model of Multiple Sclerosis

Dissertação para obtenção ao grau de Mestre em Farmacologia Aplicada sob a orientação da Professora Doutora Sónia Silva Santos e pelo Doutor Flávio Reis e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2018

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Universidade de Coimbra

UNIVERSIDADE D COIMBRA FACULDADE DE FARMÁCIA

Effects of incretin-based therapies on the gastrointestinal motility of an animal model of Multiple Sclerosis

Dissertation presented to University of Coimbra as a requirement for the degree of MSc in Applied Pharmacology and realized under scientific orientation of Professor Doctor Sónia Silva Santos from Pharmacology and Pharmaceutical Care group from the Faculty of Pharmacy of the University of Coimbra and from Pharmacology and Experimental Therapeutics Laboratory of Coimbra Institute for Clinical and Biomedical Research (iCBR) from the Faculty of Medicine of the University of Coimbra and Doctor Flávio Reis and from Pharmacology and Experimental Therapeutics Laboratory of Coimbra Institute for Clinical and Biomedical Research (iCBR) from the Faculty of Medicine of the University of Coimbra

Beatriz de Oliveira Martins

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Realized by Beatriz Martins and Margarida Esteves

”Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less”

Marie Curie

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Abbreviatures

5-HT	5-Hydroxytryptamine
aa	Amino acid
ACh	Acetylcholine
ADA	Adenosine deaminase
ANS	Autonomic nervous system
APC	Antigen-presenting cells
AT-EAE	Adoptive transfer experimental autoimmune encephalomyelitis
ATP	Adenosine triphosphate
BBB	Blood-brain-barrier
BCA	Bicinchoninic acid
cAMP	Cyclic adenosine monophosphate
CD26	Cluster of differentiation 26
CNS	Central nervous system
CPZ	Cuprizone
CSF	Cerebrospinal fluid
DHODA	Dehydroorotate dehydrogenase
DIS	Dissemination in space
DIT	Dissemination in time
DM	Diabetes Mellitus
DMT	Disease-modifying therapies
DPP-IV	Dipeptidyl-peptidase IV
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECF	Chemifluorescence enhancer substrate
EDTA	Ethylenediamine tetracetic acid
EMA	European medicines agency
Emax	Maximum contractile response
ENS	Enteric nervous system
ER	Endoplasmatic reticulum
Ex3	Exendin-3
FDA	Food and drug administration
FR	Frequency-contractile response

GABA	γ – aminobutyric acid
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
GLP-1R	Glucagon-like peptide 1 receptor
GLP-1RA	Glucagon-like peptide 1 receptor agonists
GPCR	G protein-coupled receptor
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICC	Interstitial cells of Cajal
IP3	Inositol triphosphate
IPANs	Intrinsic primary afferent neurons
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MHV	Mouse hepatitis virus
MLCK	Myosin light-chain kinase
mN	Millinewtons
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
n	Number of animals
NK	Natural Killer
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
Nrf2	Nuclear grown factor (erythroid-derived 2)-like 2
OPC	Oligodendrocyte precursor cells
PBS	Phosphate buffered solution
PC 1/3	Prohormone convertase 1/3
PKA	Protein kinase A
PLP	Proteolipid protein
PNS	Peripheral nervous system
PP	Pancreatic polypeptide

PPMS	Primary Progressive Multiple Sclerosis
PYY	Peptide YY
RNS	Reactive nitrogen species
ROS	Reactive oxidative species
RRMS	Relapsing-remitting Multiple Sclerosis
s	Number of isolated ileum segments
S.E.M.	Standard error of mean
sDPP-IV	Soluble dipeptidyl-peptidase IV
SDS	Sodium dodecyl sulphate
SPMS	Secondary Progressive Multiple Sclerosis
TGF-β	Transforming growth factor β
T_H1	$CD4^+$ T helper 1 cell
T_H17	$CD4^+$ T helper 17 cell
TMEV	Theiler's murine encephalomyelitis virus
TNF-α	Tumour necrosis factor α
T_{reg}	Regulatory lymphocyte
VIP	Vasoactive intestinal peptide
Y1R	Y1 receptor
Y2R	Y2 receptor
Y4R	Y4 receptor
Y5R	Y5 receptor

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory immune-mediated disease of the central nervous system (CNS) characterized by myelin sheath degradation (demyelination), gliosis and consequent neuroaxonal degeneration and disruption of neuronal signaling. In addition to central degeneration, MS also involves dysregulation of the enteric nervous system (ENS), which leads to gastrointestinal (GI) complications, namely constipation. Since therapies currently used in MS are only effective on reducing the frequency of relapses and the most progressive forms of the disease are still practically untreatable, there is the need to find new neuroprotective and anti-inflammatory therapies that can be useful in progressive stages of MS. Incretin-based therapies, namely dipeptidyl-peptidase IV (DPP-IV) inhibitors, seem to be a good alternative not only due to its proliferative, neuroprotective and anti-inflammatory properties, mainly because of consequent increased levels of Glucagon-like peptide 1 (GLP-1) and neuropeptide Y (NPY), but also to their metabolic effects at a peripheral level that can counteract some of the GI symptoms associated to the disease. However, both GI dysfunction and DPP-IV/GLP-1/NPY pathways remain practically uncharacterized in some animal models of induced-MS, such as cuprizone (CPZ)-induced demyelination mouse model.

Taking all these into account, the main aim of this study was to evaluate the effects of Sitagliptin, a DPP-IV inhibitor, in the intestinal motility of the CPZ animal model, using the ileum as the target region.

For this, 10-week old C57BL/6 mice were fed with an oral solution of CPZ (0.2%) for five weeks (CPZW5), followed by CPZ withdraw, in half of them, during two more weeks (CPZW7). Two control animal groups were also analyzed in the same time points (CTRLW5 and CTRLW7). Another two animal groups of seven-week CPZ experiment were also analyzed, being administered, through a jelly vehicle, with two weeks of treatment with Sitagliptin (CPZ+Sita(T)) or seven weeks of a preventive treatment of Sitaglitpin (CPZ+Sita(P)).

The demographic and biochemical profile of all animal groups, where parameters like body weight, glycemia and thymus weight were analyzed, allowed to successful characterize and confirm the animal model of induced-MS. Additionally, MBP (myelin basic protein) ileal levels allowed to confirm that CPZ intoxication also induced demyelination in the ENS. The efficacy of Sitagliptin administration were confirmed through the DPP-IV activity assay in serum samples, since this activity was reduced by approximately 80% on both animal groups treated with Sitagliptin.

Functional studies performed on isolated ileum from all animal groups confirmed that CPZ intoxication alters the intestinal contractile response to electrical field stimulation in an inhibitory way, which turns this a good animal model to study GI dysfunctions associated with MS, specially constipation. Moreover, the Sitagliptin treatment reverted the inhibitory effects of CPZ intoxication, since interfered with the intestinal motility in an excitatory way. The preventive treatment, in its turn, normalized the contractile response, tending to reach control group motility. This normalization appears to involve the inhibitory effects of GLP-1, not only because the blockade of GLP-1 receptor (GLP-1R) with Exendin-3 lead to an increase on intestinal motility only on groups submitted to Sitagliptin administration, but also because GLP-1R density was significantly higher in the preventive group. Finally, functional studies performed in the presence of Y1 receptor (Y1R) antagonist, BVD-10, allowed to conclude that this receptor only contributed in an inhibitory way to intestinal motility in a disease state, due to a possible alteration on Y1R expression or NPY levels.

With this study, we can conclude that CPZ animal model reflects the most frequent GI dysfunction present in MS patients and the Sitagliptin appears to be a good therapeutical option, not only because of their neuroprotective and anti-inflammatory effects, but also due to their contribution to ameliorate the GI dysregulation, improving the lifestyle of MS patients.

Keywords: Multiple Sclerosis, Gastrointestinal dysfunction, DPP-IV, GLP-1, GLP-1R, NPY, Y1R, Sitagliptin

Resumo

A Esclerose Múltipla (EM) é uma doença inflamatória crónica imuno-mediada do sistema nervoso central (SNC) caracterizada pela degradação da bainha de mielina (desmielinização), gliose, consequente degeneração axonal e interrupção da neurotransmissão. Para além da degeneração a nível central, a EM também envolve desregulação do sistema nervoso entérico (SNE), que leva a complicações gastrointestinais (GI), essencialmente obstipação. Uma vez que as terapêuticas atualmente utilizadas para o tratamento da EM são apenas eficazes na redução da frequência dos surtos, as formas progressivas da doença continuam sendo praticamente incuráveis, existindo a necessidade de encontrar novas terapêuticas que sejam neuroprotetoras e anti-inflamatórias e que ao mesmo possam ser utilizadas nas formas progressivas da doença. As terapêuticas baseadas em incretinas, nomeadamente os inibidores da dipeptidil-peptidase IV (DPP-IV), aparentam ser uma boa alternativa, não só devido às suas propriedades proliferativas, neuroprotetoras e anti-inflamatórias, essencialmente pelo aumento dos níveis do peptídeo semelhante ao glucagon 1 (GLP-1) e do neuropeptídeo Y (NPY), mas também por causa dos seus efeitos a nível periférico que compensam alguns sintomas GI associados à EM. No entanto, tanto a disfunção GI como a via DPP-IV/GLP-1/NPY permanecem por caracterizar em alguns modelos animais de EM induzida, nomeadamente o modelo animal de desmielinização induzida por administração de cuprizona (CPZ).

Tendo em conta tudo isto, o principal objetivo deste estudo foi avaliar os efeitos da Sitagliptina, um inibidor da DPP-IV, na motilidade intestinal do modelo animal de CPZ, usando o íleo como região alvo.

Para isto, murganhos da estirpe C57BL/6 com 10 semanas de idade foram alimentados oralmente com uma solução de CPZ (0,2%) durante cinco semanas (CPZW5), seguido da sua suspensão por mais duas semanas (CPZW7). Dois grupos de animais foram usados como controlo, sendo analisados nos mesmos dois espaços temporais (CTRLW5 e CTRLW7). Além disso, mais dois grupos de animais submetidos a sete semanas de experiência foram também utilizados, sendo administrados de Sitagliptina oralmente, através de um veículo gelatinoso, durante as últimas duas semanas (CPZ+Sita(T)) ou preventivamente durante as sete semanas de experiência (CPZ+Sita(P)).

O perfil demográfico e bioquímico de todos os grupos de animais, onde parâmetros tais como o peso corporal, a glicemia e o peso do timo foram avaliados, permitiram caracterizar e confirmar o modelo animal de EM induzida. Além disso, os níveis de MBP (proteína básica de mielina) no íleo permitiram confirmar que a intoxicação com CPZ também induziu

desmielinização no SNE. A eficácia da administração da Sitagliptina foi confirmada através do ensaio de atividade da DPP-IV realizado em amostras de soro, no qual esta atividade se encontrava reduzida aproximadamente 80% em ambos os grupos de animais tratados com Sitagliptina.

Os estudos funcionais realizados em íleo isolado deste modelo animal comprovaram o comprometimento da resposta contráctil à estimulação elétrica de campo no pico de desmielinização, o que torna este um bom modelo animal para estudar disfunções GI associadas à EM, essencialmente obstipação. O tratamento com Sitagliptina reverteu este efeito sobretudo quando utilizada como tratamento preventivo, com o qual se igualou a situação controlo. Para esta normalização parece ser determinante o GLP-1 uma vez que os níveis proteicos de GLP-IR analisados por *Western Blotting* estavam significativamente aumentados no grupo sujeito ao tratamento preventivo. Estes resultados do estudo molecular foram reforçados pelos estudos funcionais na presença do antagonista seletivo do GLP-IR Exendin-3 que confirmaram o efeito inibitório mediado pelo GLP-1R. No entanto, este recetor não parece contribuir para a resposta contrátil basal à estimulação do campo elétrico nos grupos controlo e animais CPZ não submetidos à Sitagliptina. Por outro lado, estudos funcionais realizados na presença do antagonista seletivo do recetor Y1 (Y1R), o BVD-10, permitiu concluir que este recetor apenas contribui de forma significativa para a motilidade intestinal na presença da patologia, devido a uma possível alteração da expressão do Y1R ou dos níveis de NPY, que necessita de futura investigação.

Com este estudo, foi possível concluir que o modelo animal de CPZ reflete a disfunção GI mais frequente em doentes com EM e que a Sitagliptina aparenta ser uma boa opção terapêutica, não só pelos seus efeitos neuroprotetores e anti-inflamatórios, mas também pelos seus efeitos na desregulação GI, melhorando assim a qualidade de vida relacionada com a saúde destes doentes.

Palavras-chave: Esclerose Múltipla, Disfunção Gastrointestinal, DPP-IV, GLP-1, GLP-1R, NPY, Y1R, Sitagliptina

CHAPTER I

INTRODUCTION

1 Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) caused by an autoimmune response to self-antigens in a susceptible individual. It is related to focal lymphocyte infiltration across the blood-brain-barrier (BBB), which leads to myelin sheath degradation (demyelination), gliosis and consequent neuroaxonal degeneration and disruption of neuronal signaling. In most of the patients, the early course of the disease is characterized by episodes of reversible neurological deficits but over time this is followed by progressive neurological deterioration. MS has a heterogenous presentation with different clinical manifestations like sensory and visual disturbances, motor impairments, fatigue, pain and cognitive deficits and is diagnosed on the basis of the medical history and physical exam with supporting tests, such as magnetic resonance imaging (MRI) of the brain and examination of the cerebrospinal fluid (CSF) (Compston and Coles, 2008; Dendrou, Fugger and Friese, 2015; Goldenberg, 2012).

1.1 Epidemiology and risk factors

MS affects approximately 2.5 million people worldwide and is a leading cause of disability in young adults. Usually begins in early adulthood, aged between 20 and 40 years, affecting twice as many women as men. Its prevalence varies according to ethnicity and geographic location, since North Europe and North America have the highest values of prevalence (108 and 140 per 100,000 inhabitants, respectively) (Figure 1). In Portugal, the prevalence of MS is 56 per 100,000 inhabitants (Gitto, 2017; MS International Federation, 2013).

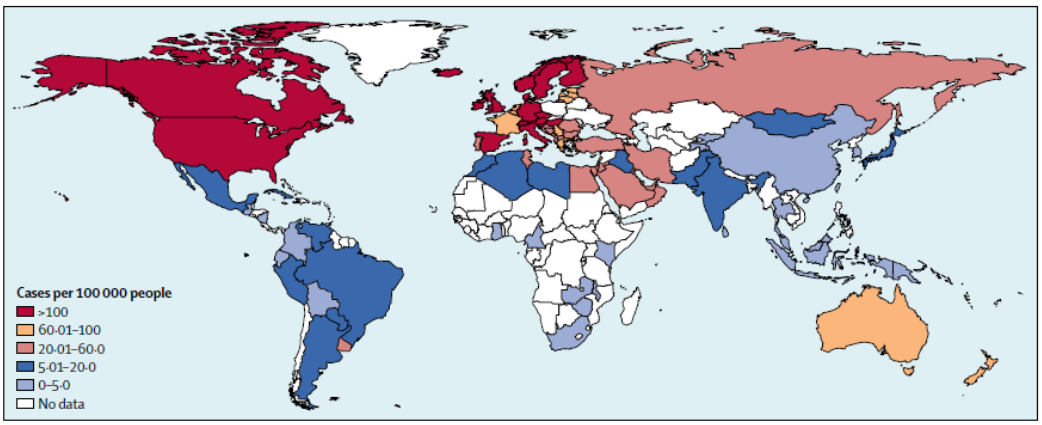

Figure 1: Global prevalence of MS. (Taken from *Thompson et al.,* 2018)

Until today, the precise causes of MS are still unknown, but it appears to be triggered by a combination of genetic susceptibility and environmental factors (Goldenberg, 2012). Epidemiological studies and disease concordance in twins or family members have revealed that there is a strong genetic factor in the onset of the disease. In fact, siblings of affected individuals have a 10- to 20-fold higher risk of developing MS (2-4%) when compared to general population's risk (0.2%), with monozygotic twins having even a higher risk (30%). This genetic susceptibility mostly resides within the human leukocyte antigen (HLA) system, that is the genetic complex encoding the major histocompatibility complex (MHC), being the HLA-DRB1*15:01 allele the one that presents the strongest effect. These genetic alterations implicate central tolerance mechanisms, as well as peripheral differences in effector T-cell function due to altered cytokine production and responsiveness (Dendrou, Fugger and Friese, 2015; Gitto, 2017; Nylander and Hafler, 2012; Thompson *et al.*, 2018).

Since genetic predisposition do not completely explains the risk increase, interactions with the environment are likely to have a significant role on the susceptibility of developing MS. In fact, several agents have recently been identified as environmental risk factors. The ones that are best characterized are Epstein-Barr virus (EBV) infection, low levels of vitamin D/sun exposure and smoking. The presence of these risk factors, especially during adolescence, contribute to an increased risk of MS. However, other factors like adolescent obesity, shift work and gut microbiota alterations also seem to be associated with greater risk (Alfredsson and Olsson, 2018; Michel, 2018).

1.2 Clinical features

1.2.1 MS forms and symptoms

The course of the disease and the symptomatology of MS are very heterogenous, ranging from relatively mild neurological symptoms to a rapidly evolving and debilitating disease. However, despite this heterogenicity, it is possible to recognize three disease subtypes: relapsing-remitting MS (RRMS), secondary-progressive MS (SPMS) and primary-progressive MS (PPMS).

RRMS, the most common form affecting approximately 85% of patients, is a biphasic disease course marked by alternating episodes of neurological disability and recovery. It is characterized by an initial episode of neurological dysfunction, normally called clinically isolated syndrome, followed by a remission period when symptoms improve or disappear. In this form,

relapses coincide with focal CNS inflammation and demyelination and are associated with the appearance of new lesions or reactivation of the old ones in the brain, brainstem and spinal cord. Following this course, improvement during each remission wanes as disability accumulates and, within 20-25 years, approximately 70% of RRMS patients develop a SPMS where inflammatory lesions are no longer characteristic, and a progressive neurological decline is accompanied by CNS atrophy (decrease of brain volume and increase of axonal loss). The transition from RRMS to SPMS occurs when axonal loss exceeds the compensatory capacity of the CNS. In addition, 10-15% of MS patients will experience instead a PPMS that is characterized by progressive decline in neurological function from the onset and the absence of relapses and recovery periods (Figure 2) (Dendrou, Fugger and Friese, 2015; Dutta and Trapp, 2015).

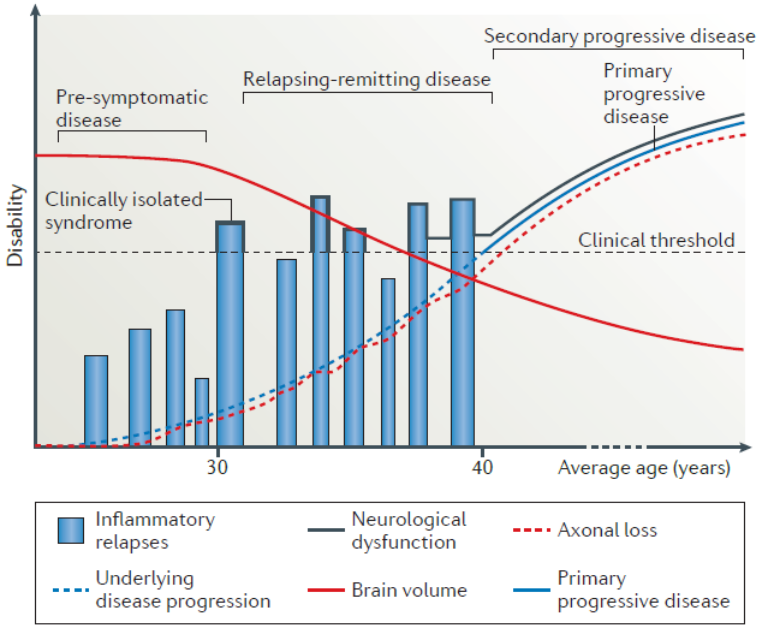

Figure 2: MS forms and clinical course. Approximately 85% of patients are affected by RRMS that is characterized by flare-ups (relapses) of symptoms followed by periods of remission (black line). After one or two decades, about 70% of RRMS patients present an irreversible progression form of clinical disability, named SPMS. 10-15% of MS patients are affected by PPMS and experience a progressive decline from the onset with and absence of relapses (blue line). All of these patients develop an important atrophy of the CNS, with decrease of brain volume (red line) and increase of axonal loss (pointed red line). (Taken from *Dendrou et al.,* 2015)

Initial clinical symptoms found in MS patients are weakness or diminished dexterity in one or more limbs, paresthesias (numbness and tingling), dysesthesias (burning and "pins and needles"), optic neuritis (mononuclear visual loss), diplopia (double vision), gait instability and ataxia. As the disease progresses, clinical symptoms tend to aggravate and begins to appear others like fatigue, heat sensitivity, vertigo, Lhermitte's sign (an electric shock-like sensation

down the spine and into the limbs evoked by neck flexion), and hemifacial weakness or pain. Cognitive deficits also tend to worsen, especially in advanced cases, and include memory loss, impaired attention, problem-solving difficulties, slowing information processing and shifting between cognitive tasks. Depression is present in approximately 60% of MS patients during the course of the illness (Goldenberg, 2012; Hauser and Oksenberg, 2006). These patients also present several autonomic dysfunctions like sweating abnormalities, bladder disfunction, cardiovascular autonomic dysfunction, gastrointestinal dysregulation (especially constipation but also some cases of fecal incontinence) and sexual dysfunction (Pintér *et al.*, 2015).

1.2.2 Diagnosis

Besides MS has heterogenous clinical and imaging manifestations, which differ between patients and change over time, the diagnostic is based on the integration of clinical, imaging and laboratory findings and was formalized as the McDonald Criteria. These criteria emphasize the need to demonstrate dissemination of lesions in space (DIS) and in time (DIT) to distinguish MS from monophasic self-limiting diseases such as acute disseminated encephalomyelitis. MRI of the CNS is essential to support, supplement or even replace some clinical criteria, allowing earlier diagnosis with increase certainty of successive versions of the diagnostic criteria (Polman *et al.*, 2011; Thompson *et al.*, 2018). The last revision of these criteria, made in 2017, continue to be applied primarily to patients experiencing a typical clinical isolated syndrome but implemented some changes taking into account abnormalities of the cerebrospinal fluid (CSF) and using MRI protocols for baseline and follow-up scans (Thompson *et al.*, 2017).

1.3 MS pathogenesis

1.3.1 Basic aspects of the nervous system

The nervous system is involved in most of the organic functions and is subdivided in two systems: the CNS and the peripheral nervous system (PNS). The CNS consists of the brain and the spinal cord. The PNS consists of the nerves and ganglia outside brain and spinal cord, being that it can also be subdivided in sensory neurons and motor neurons; this last division can be separated in somatic nervous system and autonomic nervous system (ANS). The ANS is responsible for the involuntary movements (cardiac muscle, for example), with the digestive

movements being controlled by the enteric nervous system (ENS), a group of neurons that are responsible for controlling the gastrointestinal (GI) tract.

The nervous system has neurons (the fundamental unit) and non-neuronal cells. Neurons receive, process and transmit action potentials and are organized to form a complex network that perform the nervous system functions. The non-neuronal cells, also called glia cells, can be divided into macroglia (oligodendrocytes, Schwann cells and astrocytes) and microglia. Astrocytes give structural support to neurons and blood vessels and play an important role in regulating which blood substances reach the neurons. Oligodendrocytes form the myelin sheath around CNS neurons, whereas Schwann cells perform the same function in the PNS. A single oligodendrocyte is able to myelinate several neurons while a Schwann cell is only able to myelinate one neuron. Microglia consists of specialized macrophages that are able to phagocyte, protecting the neurons (Seeley, Stephens and Tate, 2003).

The myelin sheath is a membrane that is wrapped around the nerve axon in a spiral fashion and acts like an electrical isolator, facilitating conduction in axons. In fact, action potentials flow more rapidly along the myelinated neurons than over the unmyelinated ones. In myelinated neurons, the excitable axonal membrane is exposed to the extracellular space only at short portions of the axon that were left uncovered by myelin (nodes of Ranvier). In these nodes, where sodium channels are localized, the electrical resistance is low, thereby facilitating depolarization, generating local currents and, in turn, triggering saltatory conduction (Figure 3) (Brady *et al.*, 2005; Compston and Coles, 2008).

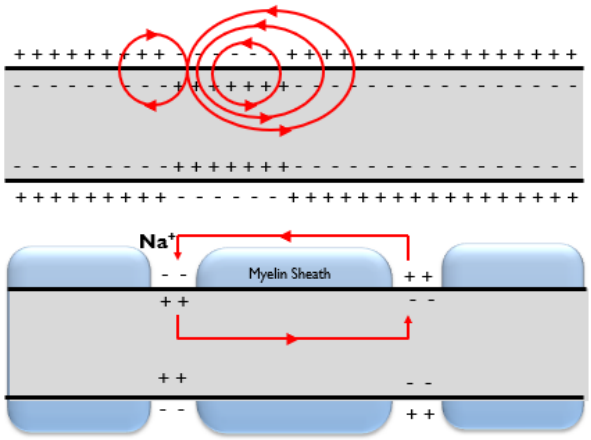

Figure 3: Flow of local action currents along unmyelinated (upper illustration) and myelinated (bottom illustration) fibers. In unmyelinated neurons, the circuits flow through the adjacent piece of membrane while in myelinated fibers the circuit flow jumps to the next node. (Adapted from *Brady et al.*, 2005)

1.3.2 Main pathologic features and types of MS plaques

The pathologic hallmarks of MS are confluent demyelinating areas present in the white and grey matter of the brain and spinal cord, called plaques. They are characterized by variable gliosis and inflammation, indicating a loss of myelin sheath and oligodendrocytes. The characteristics of these plaques, like their location, number, size and shape, vary among patients and, besides they are present throughout all CNS, they have a predilection for optic nerves (leading to optic neuritis), subpial spinal cord, brainstem, cerebellum and periventricular white matter regions.

MS lesions evolve differently during early and chronic phases of the disease. In the early stages (relapsing-remitting disease), there are some relative axonal preservation since the demyelinating areas can be partially repaired through a mechanism called remyelination. However, as the disease progresses the levels of demyelination overlap the remyelination, resulting in gradual axonal and neuronal loss, which is correlated with brain atrophy, ventricular enlargement and consequent patient disability (Dendrou, Fugger and Friese, 2015; Popescu and Lucchinetti, 2012).

Early active plaques show a profound pathological heterogenicity among different MS patients, suggesting that the targets of injury (myelin and/or oligodendrocytes) and the mechanisms of demyelination may be different in subgroups of the disease and at different stages of MS development. Thus, MS lesions can be classified into four major patterns of immunopathology, based on protein loss, plaque extent and topography, oligodendrocyte destruction, immunoglobulin deposition and complement activation.

Pattern I and pattern II, found in 15% and 58% of the patients, respectively, share several characteristics since both have sharply demarcated perivascular lesions presenting active demyelination with equal loss of myelin components and dense infiltration of T cell and myelin-laden macrophages. However, in pattern II active lesions it is also possible to observe immunoglobulin deposition and complement activation on myelin and within macrophages, implying that this type of lesion may be induced by antibody- and complement-mediated mechanisms. In pattern I lesions, there is no immunoglobulin and complement activation, suggesting that demyelination and tissue damage are induced by toxic factors produced by activated macrophages.

Pattern III lesions can be found in 26% of MS patients and are ill-defined lesions that present active demyelination with oligodendrocyte apoptosis, T cell infiltration, macrophage and microglia activation and preferential loss of the peri-axonal myelin components (like

myelin-associated glycoprotein (MAG)). There is no evidence of immunoglobulin or complement activation and it is clear a pronounced loss of oligodendrocytes at the active plaque border that extends into the apparently normal peri-plaque white matter. Since oligodendrocytes are responsible for the axonal myelination, remyelinated plaques are absent. This hypoxia-like injury may be driven by mitochondrial disfunction.

Finally, pattern IV lesions are found in only 1% of MS patients and are characterized by a profound nonapoptotic death of oligodendrocytes in peri-plaque white matter suggesting that exists a primary metabolic oligodendrocyte disorder that turns them extremely vulnerable to the toxic action of inflammatory mediators (Popescu and Lucchinetti, 2012; Popescu, Pirko e Lucchinetti, 2013).

1.3.3 Immunopathogenesis of MS

The BBB is a membrane barrier that is responsible for separating the brain tissue from the circulating blood components and is formed by endothelial cells connected by tight junctions. In the CNS, blood capillaries are structurally different from the other tissues' capillaries, being surrounded by the basement membrane, pericytes and the endfeet of astrocytes. Healthy BBB works like a protective mediator of the brain, preventing the entry of xenobiotics, toxic metabolites and immune cells into the CNS and keeping the homeostasis. However, changes in the BBB' delicate balance is one of the most important features present in the onset of MS. In fact, the breakdown of the BBB leads to autoreactive T and B cells infiltration and consequent formation of acute inflammatory lesions. However, it is not clear whether the invasion of the CNS by T cells and B cells is the initiating event of MS, or whether is secondary to the activation of microglia and macrophages and the local-release of self or foreign antigens (Hemmer *et al.*, 2006; Mcfarland and Martin, 2007; Ortiz *et al.*, 2014).

Thymus is responsible for the establishment of the central tolerance, deleting most of the autoreactive T cells that can be formed. However, some of the autoreactive T cells can escape from these control process and be released into the periphery. When the peripheral tolerance mechanisms are also broken, these autoreactive T cells can be activated and then they upregulate adhesion molecules (such as α4β1 integrin) that allow these T cells to cross BBB and establish an inflammatory response against myelin (Figure 4).

This peripheral tolerance breakdown is due to defects on regulatory mechanisms that are normally maintained by regulatory lymphocytes (T_{reg}). Although the number of T_{reg} cells in peripheral blood and cerebrospinal fluid (CSF) is not different between MS patients and healthy controls, there is a loss of functional suppression by T_{reg} in response to autoreactive effector

T cells. Dysregulation of these interactions between regulatory and effector cells will culminate into emergence of autoreactive adaptive immune cells that are capable of infiltrating and promoting damage within the CNS.

MS is considered primarily a T-cell-mediated disease because of the similarities that has been observed in several studies involving animal models of the disease, namely experimental autoimmune encephalomyelitis (EAE) (described on Section 1.5.1), which is typically induced by $CD4^+$ T cells. Myelin-specific autoreactive T cells can be found in blood and CSF of MS patients, but they can also be detected in healthy controls. However, myelin-reactive T cells are more active in MS patients and have a memory phenotype, when compared to healthy controls that present T cells with a resting naïve phenotype.

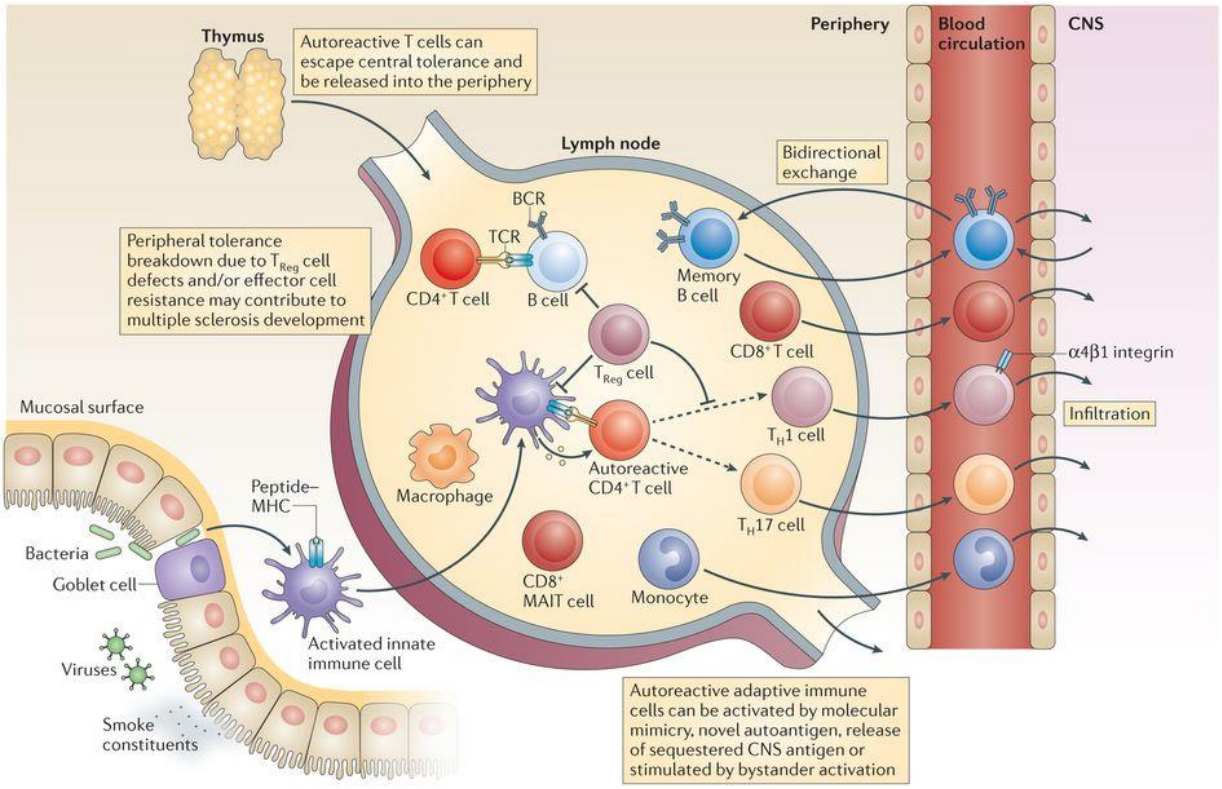

Figure 4: Immune system dysregulation outside CNS. BCR – B cell receptor; TCR – T cell receptor; $CD8^+$ MAIT – $CD8^+$ mucosa-associated invariant T cell. (Taken from *Dendrou et al.*, 2015)

Both $CD4^+$ and $CD8^+$ T cells have been described in MS lesions with $CD4^+$ T cells being more concentrated in the perivascular cuff and $CD8^+$ T cells widely distributed within the parenchyma. T-helper 1 (T_H1) and T_H17 cells are the main $CD4^+$ T cell subtype implicated in the disease, probably being the main inducers of the disease activity, whereas $CD8^+$ cells are probably more relevant for CNS tissue damage. In fact, cells expressing IFN-γ and IL-17 (cytokines produced by T_H1 and T_H17 cells, respectively) appear to effectively cross the BBB

and accumulate within the brain. Besides that, these T cells can also be reactivated by antigens presented on antigen-presenting cells (APCs), such as dendritic and microglial cells, and locally release several inflammatory mediators that will attract macrophages to the lesion sites.

$CD8^+$ T cells can be found in white and grey matters cortical demyelinating lesions, outnumbering $CD4^+$ T cells. In fact, axonal damage is closely related with the number of $CD8^+$ T lymphocytes infiltrating the lesion. In addition, these cells can also secrete inflammatory mediators and directly attack cells that express HLA class I, such as neurons and oligodendrocytes, causing neuronal damage.

Concerning to B cells, their role in MS pathogenesis has been receiving increased attention, since clonally expanded B cells can be found in the meninges, parenchyma and CSF. The importance of humoral immunity is also correlated with the fact that the local synthesis of antibodies in CSF of MS patients, demonstrated by the intrathecal production of oligoclonal immunoglobulins, is part of a diagnostic criteria for the disease. The number of these cells vary through the disease progression and they can participate in the demyelinating process with the involvement of the complement, or directly leading to antibody-mediated phagocytosis.

In addition to lymphocytes involvement, cells from the innate immune system, such as macrophages, astrocytes and microglia, also participate in MS pathogenic process. Macrophages can release a range of neurotoxic inflammatory mediators, such as cytokines, chemokines and reactive oxygen species (ROS), which predominantly damage oligodendrocytes and neurons. Reactive astrocytes are responsible to induce gliosis in the lesion border. Microglia can be pathogenic or protective (or both), since it has been described that microglia infiltrate MS lesions removing myelin debris and inflammatory by-products, being its role still uncertain (Comabella and Khoury, 2012; Compston and Coles, 2008; Dendrou, Fugger and Friese, 2015; Hemmer *et al.*, 2006; Mcfarland and Martin, 2007; Nylander and Hafler, 2012; Racke, 2009; Reich, Lucchinetti and Calabresi, 2018).

1.3.4 Remyelination

As previously described, axonal/neuronal loss is one of the hallmarks of MS and it is responsible for the chronic progression, occurring as a consequence of demyelination. In contrast, myelin repair of acute inflammatory lesions in early stages of the disease is associated with functional recovery and clinical remittances. Being that, remyelination therapies would represent a powerful protective intervention, restoring saltatory conduction, boosting axonal survival and consequently changing the natural history of the disease (Palavra, Reis and Almeida, 2014).

Remyelination is the default spontaneous process in which new myelin sheaths are generated around demyelinated axons in the adult CNS. This phenomenon, that is not only found in inactive lesions but also in lesions with ongoing demyelinating activity, is able to restore conduction properties of the axons, thereby restoring neuronal function. Since repair processes are especially characteristic of the early stage disease, remyelinated lesions may begin to appear, as shadow plaques, within a month or two after active demyelination. However, remyelination rate decreased with MS progression, appearing to be limited by oligodendrocyte density in a late stage of the disease (Chari, 2007; Podbielska *et al.*, 2013).

Spontaneous or intrinsic remyelination requires endogenous oligodendrocytes precursor cells (OPCs) that are widespread throughout the CNS, in both white matter and grey matter. When an injury occurs, after their activation, OPCs proliferate and migrate into the lesion site once they are very sensitive to several attractive and repellent cues, such as netrin 1, semaphorins and fibroblast grown factor 2. Then, recruited OPCs will differentiate into remyelinating oligodendrocytes, a process that involves three different steps: 1) contact with the previously demyelinated axon; 2) expression of myelin genes and production of myelin membrane; 3) wrap and compact to form the myelin sheath. These processes allow to generate functional myelin sheaths that can restore conduction properties to neurons. Schwann cells are also able to remyelinate damaged axons in the PNS (Chari, 2007; Palavra, Reis and Almeida, 2014; Plemel, Liu and Yong, 2017).

The cause of remyelination failure is multifactorial and include the presence of extrinsic inhibitors in the lesion, insufficient pro-regenerative factors and an impaired intrinsic capacity in oligodendrocyte lineage cells. Nevertheless, remyelination failure can be distinguished by two different phases: impaired recruitment of OPCs into the lesion site and an inability to differentiate or mature into remyelinating oligodendrocytes. These recruitment and differentiation abnormalities will lower the remyelination capacity for people with MS (Plemel, Liu and Yong, 2017).

Nowadays, treatment of MS is broadly based in anti-inflammatory therapies that can leave to notable adverse effects and have not yet been able to show capable to prevent progression of the disease, which is characterized by extensive axonal degeneration. Therefore, therapies that can promote myelin regeneration in MS are considered a promising therapeutic strategy, since they may help not only restore optimal circuit function but also serve to protect axons and neurons from degeneration (Cole, Early and Lyons, 2017).

1.4 Disease-modifying therapies

In the past years, treatment development has been extremely active and several disease-modifying therapies (DMTs) have been discovered and approved by Food and Drug Administration (FDA) and by European Medicines Agency (EMA) for patients with RRMS (Figure 5). The main goal of these agents in MS patients include shortening the duration of acute exacerbations, decreasing their frequency and providing symptomatic relief. The first-line DMTs are interferons β 1a and 1b, glatiramer acetate, teriflunomide and dimethyl fumarate (Thompson *et al.*, 2018; Vidal-jordana, 2018).

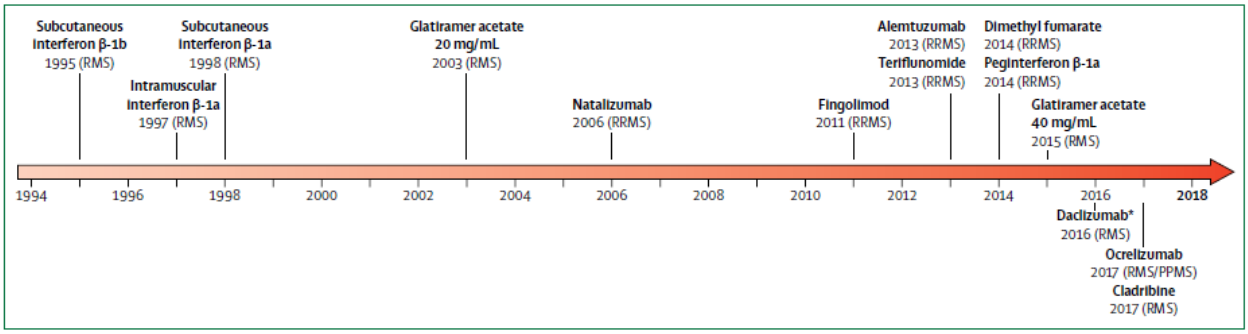

Figure 5: Disease-modifying therapies for multiple sclerosis and their year of discovery or licensing. RMS – Relapsing multiple sclerosis. * Daclizumab was withdrawn for use in the treatment of MS in March 2018, because of reports of adverse events including inflammatory encephalitis and meningoencephalitis. (Taken from *Thompson et al.*, 2018)

The first medication approved for MS was interferon β-1b, followed by interferon β-1a. The first one is administered by subcutaneous injection and the second can be administered by subcutaneous or intramuscular route. These two are naturally occurring cytokines secreted by immune cells that inhibit viral replication via several immunomodulating and antiviral activities. In fact, the mechanism of action of both interferons β is still unknown, however they exhibit multiple immunomodulatory effects like curtail T-cell trafficking and redress T_H1-T_H2 imbalance, that is in favor of T_H1 responses in MS patients. These drugs are indicated for the treatment of RRMS, considering that they have been shown to reduce the incidence of relapses by approximately one-third. More recently, in 2014, a pegylated interferon was approved by FDA, peginterferon β-1a, that has the advantage of only be administered once every two weeks (Goldenberg, 2012; Luzzio and Dangond, 2018; Vidal-Jordana, 2018).

Glatiramer acetate is a synthesized polypeptide mixture consisting of L-glutamic acid, L-lysine, L-alanine and L-tyrosine. It was approved by FDA for the reduction of frequency relapses in patients with RRMS, via subcutaneous injection. Although the precise mechanism of this drug is unknown, it theoretically modifies some of the immune processes, inducing and activating glatiramer acetate-specific suppressor T cells in the periphery (Goldenberg, 2012; Luzzio and Dangond, 2018).

Natalizumab is a recombinant humanized immunoglobulin (IgG_4) monoclonal antibody administered by intravenous infusion and is indicated as monotherapy for the treatment of patients with RRMS who have not responded to a first-line DMTs or who have very active disease. Besides its precise mechanism of action has also not been fully defined, it is known that natalizumab binds to the adhesion molecule α4β1 integrin, inhibiting its adherence to its receptors (Luzzio and Dangond, 2018; Vidal-Jordana, 2018).

Fingolimod is the first orally administered DMT approved for relapsing forms of MS that is also able to reduce the frequency of clinical exacerbations and delay the accumulation of physical disability. Fingolimod is a sphingosine-1-phosphate receptor modulator that is metabolized by sphingosine kinase to its active metabolite, called fingolimod-phosphate. Besides its mechanism of action being incomplete understood, the active metabolite is responsible for blocking the migration of lymphocytes from lymph nodes, thereby reducing the number of lymphocytes in peripheral blood that, in turn, may reduce the lymphocyte migration into the CNS (Goldenberg, 2012; Luzzio and Dangond, 2018; Vidal-jordana, 2018).

Teriflunomide, approved by FDA for the treatment of patients with relapsing forms of MS, is an oral immune-modulating drug that inhibits *de novo* synthesis of pyrimidine. This will block the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH) and reduce DNA synthesis, which leads to a cytostatic effect on proliferating B and T cells (Goldenberg, 2012; Vidal-Jordana, 2018).

Alemtuzumab is a humanized monoclonal antibody against CD52, a broadly expressed cell-surface molecule of immune cells. Is administered intravenously and its action promotes antibody-dependent lysis. This monoclonal antibody is also prescribed to patients with RRMS, but it is reserved to those who have an inadequate response to two or more DMTs (Luzzio and Dangond, 2018; Vidal-Jordana, 2018).

Dimethyl fumarate, one of the first-line DMTs, is an activator of nuclear grown factor (erythroid-derived 2)-like 2 (Nrf2) pathway that is orally administered to patients with RRMS. Monomethyl fumarate (MMF), its active metabolite, reduces the release of inflammatory cytokines and activates antioxidant pathways, which can reduce the proportion of patients

who relapsed by approximately 50% (Goldenberg, 2012; Luzzio and Dangond, 2018; Vidal-Jordana, 2018).

Ocrelizumab is a humanized anti-CD20 monoclonal antibody that targets mature B lymphocytes. The adhesion to its target produces a selective depletion of specific B cells (pre-B cells, mature B cells and memory cells). Is administered intravenously and is the unique DMT that, despite being prescribed to relapsing forms of MS, is also approved for adult patients with PPMS (Goldenberg, 2012; Vidal-Jordana, 2018).

Finally, cladribine is a synthetic purine analog that, when orally administered, enters the cell via the purine nucleoside transporters and is subsequently phosphorylated. Is relatively selective for lymphocytes and, when its active metabolite accumulates, cellular metabolism is disrupted and DNA is damaged, causing death of B and T cells (Luzzio and Dangond, 2018; Vidal-Jordana, 2018).

As just described, existing DMTs only act by preventing disease's symptoms and are prescribed essentially to patients with RRMS, existing only one that has been shown to slow progression in patients with PPMS. No curative therapies for MS are currently available. Being that, there is an urgent need to deeply study and understand the mechanism of the disease to find more DMTs that can act in progressive forms of the disease and with fewer and not so severe adverse effects. To this end and tacking into account that is extremely difficult to access human CNS, the study in animal models is the key to fully understand the disease and to approach future treatments.

1.5 Animal models of MS

MS is a complex and heterogeneous neurological illness that involves a complex interaction between two of the most intricate biological system, immune system and CNS. Due to the large number of molecular mechanisms, variability of the disease among patients and uncertain etiology, the use of animal models to study this disease has been critical to understand its pathogenesis and develop new therapies. Although there is no single animal model that can capture the entire spectrum of heterogenicity of human MS, there is a large number of animal models that can mimic some clinical and pathological aspects of the disease. The available models are: EAE, an autoimmune model; Theiler's murine encephalomyelitis virus (TMEV) and mouse hepatitis virus (MHV), that are viral-autoimmune models; CPZ and lysophosphatidylcholine (Lysolecithin) or ethidium bromide, that are toxic models; and some genetic models (Gudi *et al.*, 2014; Mix *et al.*, 2010; Palumbo and Pellegrini, 2017; Procaccini *et*

al., 2015; Ransohoff, 2012; Torkildsen *et al.*, 2008). In this section we will dissect the main features of the EAE model, the most widely used inflammatory model, and the CPZ model, which we have used in this word to study demyelination and remyelination

1.5.1 Experimental autoimmune encephalomyelitis

EAE, developed in the 1930's, is the most widely used experimental model of MS that presents the key pathological features of the disease: inflammation, demyelination, axonal loss and gliosis. Because of its complex neuropharmacology, many of the drugs that are currently used by patients with MS were developed, tested or validated on the basis of EAE studies (Constantinescu *et al.*, 2011).

EAE can be induced through two distinct protocols: active induction or passive induction, both activating primarily $CD4^+$ T cells. Active EAE, the most common protocol, is induced through the administration of myelin-derived peptides which, in turn, cause an immune reaction against specific antigenic myelin proteins, such as myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). The resulting phenotype depends on the genetic background of the animal species and strains used and on the antigen source. For example, immunization of SJL/J mice with $PLP_{139-151}$ induces a relapsing-remitting disease course, while immunization of C57BL/6 mice (the most favored mice for transgenic experiments) with MOG_{35-55} triggers chronic-progressive EAE. Passive EAE, also known as adoptive transfer EAE (AT-EAE), can be induced by transferring pathogenic, myelin-specific $CD4^+$ T cells generated in donor animals immunized with myelin antigens (Mix *et al.*, 2010; Palumbo and Pellegrini, 2017; Procaccini *et al.*, 2015; Robinson *et al.*, 2014).

EAE has unequivocal value as a model of the inflammatory aspects of the disease, as well the autoimmunity, cytokine biology and immunogenetics aspects. Since there are several EAE protocols that allow to study different aspects of MS, this model can mimic the clinical symptoms and the pathology of MS. Nonetheless, there are also several limitations to the use of this animal model because it provides very few information about MS progression; is difficult to study remyelination; has a relative lack of involvement of the brain, since EAE affects predominantly spinal cord; and there are not many studies analyzing extensively the role of B cells and $CD8^+$ T cells, despite their importance in MS (Gudi *et al.*, 2014; Procaccini *et al.*, 2015; Ransohoff, 2012).

1.5.2 Cuprizone model

Cuprizone (CPZ, bis-cyclohexanone-oxaldihydrazone), a chemical compound that was obtained by the condensation of oxalylhydrazide with cyclohexanone, is a copper chelating agent that has peculiar neurotoxic properties when orally administered in mice, causing oligodendroglial cell death with subsequent demyelination (Figure 6). After a constant demyelination, a spontaneous remyelination occurs with withdraw of CPZ, thus making this model excellent for the study of several factors that can prevent demyelination and stimulate remyelination (Gudi *et al.*, 2014; Torkildsen *et al.*, 2008).

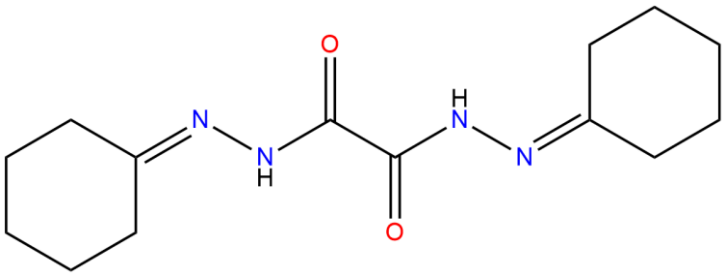

Figure 6: Cuprizone (CPZ) molecular structure. CPZ ($C_{14}H_{22}N_4O_2$) has a molecular weight (MW) of 278.356 g/mol, owns 2 H donors (N-H-), 4 H acceptors (=N-, =O), 2 routable bounds (N-N(H)) and 0 formal charge. CPZ is slightly soluble in water and extremely soluble in ethanol. (National Center for Biotechnology Information. PubChem Compound Database; CID=9723, http://pubchem.ncbi.nlm.gov/compound/9723 (accessed at March 10, 2018); Image taken from: http://en.chembase.cn/molecule-103437.html (accessed at March 10, 2018))

During the past decades, CPZ model has been applied on several mouse strains, but currently this model is mainly used on C57BL/6 strain since they develop de- and remyelination with high reproducibility that is accompanied by microgliosis and astrogliosis. The standard protocol is feeding 8-10-week-old C57BL/6 males with a diet of chow or water mixed with 0.2% (w/w) CPZ for 4-6 weeks to induce acute demyelinating lesions. At week 5 is possible to observe a complete demyelination in multiple brain structures like hippocampus, cerebellum and corpus callosum, that is the most studied structure. Spontaneous remyelination starts after the CPZ withdrawal from the diet due to the proliferation and differentiation of OPCs. Chronic demyelinated lesions can be observed after feeding mice with 0.2% CPZ continuously for 12 weeks. In this case, the ability to remyelination is limited (Praet, Guglielmetti and Berneman, 2014).

Although it is widely accepted that CPZ disrupts the metabolism of oligodendrocytes leading to their death and consequent demyelination, the underlying mechanisms are not fully

understood. What is known is that Cu plays an important role in several cellular processes and its concentration must be tightly regulated within the cell, otherwise neurodegeneration can occur as consequence of a disturbance on its homeostasis. Since CPZ is as a copper-chelating compound it affects Cu homeostasis. The plausible hypothesis is that the neurotoxic properties are due to a disturbance of cellular respiration that will lead to oligodendrocytes apoptosis. This disturbance in energy metabolism is corroborated by the formation of megamitochondria in the liver and in oligodendrocytes of mice treated with CPZ. The presence of this megamitochondria is related to the increase of oxidative stress in oligodendrocytes, with consequent high levels of ROS/RNS (reactive nitrogen species) and shortage of ATP, which can lead to a disruption of endoplasmatic reticulum (ER) proper function. Together, oxidative stress and ER stress reduce amino acid levels that leads to disturbed myelin lipids and protein synthesis and eventually to myelin sheet disintegration. This process, if remaining for a period of a few days, will result in oligodendrocyte apoptosis; however, a second hit of immune system is required to effectively induce extensive oligodendrocyte apoptosis by week 4 of CPZ treatment. Although in this model the BBB remains intact, one of the most important changes is the infiltration of neutrophils into the CNS lesion already after 1 week of treatment that, in combination with microglia, are the main inducers of oligodendrocyte apoptosis. Additionally, astrocytes and microglia play an important role on metabolic perturbations once they clean cellular and myelin debris and excess fluid as an attempt to restore the homeostasis. The clearance of all these debris together with the secretion of neurotrophic factors is extremely important for an effective remyelination (Kipp *et al.*, 2009; Praet, Guglielmetti and Berneman, 2014).

Remyelination starts at week 3 of CPZ treatment, coinciding with the beginning of OPC accumulation and with microglia and astrocytes infiltration. The proliferation and migration of OPCs toward the lesion site are followed by the differentiation of OPCs into OLGs at week 6, with newly formed OLGs becoming fully mature (myelinating OLGs). This maturation process only occurs after the withdrawal of CPZ because, unlike OLGs, OPCs survive to CPZ treatment, since their metabolism is much slower and they are much less susceptible to oxidative stress compared to mature OLGs.

Since dysfunction of immune system appears to be a major component of MS pathology, the available MS therapies nowadays focus only on the control or suppression of immune-mediated mechanisms. However, since the pathology of MS lesions, as well as the individual course of disease, are extremely heterogeneous, and the currently available therapies are only effective in RRMS, there is a need to develop new MS therapies that include

immunomodulatory, protective and regenerative components. Therefore, CPZ seems to be an excellent model for studying pathology and therapy for MS since this is a well-established and reproductible model with predictable kinetics of de- and remyelination. This model has a simple oral induction protocol with a non-immune mediated demyelination, primarily induced by oligodendroglial damage associated with mitochondrial dysfunctions, as seen in MS pattern III and IV lesions (Gudi *et al.*, 2014; Kipp *et al.*, 2009).

2 Dipeptidyl peptidase IV, GLP-1 and NPY pathways

Incretin-based therapies, including glucagon-like peptide 1 (GLP-1) agonists and dipeptidyl peptidase IV (DPP-IV) inhibitors, are a drug class currently used for the treatment of diabetic patients. However, due to their neuroprotective and anti-inflammatory properties, several studies have been performed using these drugs as a possible treatment of neurodegenerative diseases, namely MS.

2.1 Dipeptidyl peptidase IV

DPP-IV, also known as T-cell activation antigen cluster of differentiation (CD) 26 or adenosine deaminase (ADA)-binding protein, is a type-II transmembrane glycoprotein with ubiquitous expression, which acts as a cell surface serine protease, selectively cleaving dipeptides from proteins containing proline or alanine in the N-terminal penultimate position (Kim, Yu and Lee, 2014). Only oligopeptides in the *trans* conformation can bind to the active site.

DPP-IV is a member of the serine peptidase/prolyl oligopeptidase gene family. The 70-kb human gene is located on the long arm of chromosome 2 (2q24.3) and comprises 26 exons encoding a 766-amino acid (aa) protein (Mulvihill and Drucker, 2014). Being a transmembrane protein, the 110 kD DPP-IV monomer consists mainly of 4 domains: a short cytoplasmic domain (1-6), a transmembrane domain (7-28), a flexible stalk segment (29-39) and the extracellular domain (40-766). More specifically, the extracellular domain can be separated in three regions – a highly glycosylated region, a cysteine-rich region and a catalytic region. DPP-IV can be organized as a monomer, homodimer, or even as homotetramer on the surface of cells. However, only dimerized forms present enzymatic activity, being this the predominant form of DPP-IV (Röhrborn, Wronkowitz and Eckel, 2015). Additionally, to the transmembrane form, DPP-IV also presents a soluble circulating form (sDPP-IV) with 727 aa. The sDPP-IV

lacks the intracellular tails and transmembrane regions and represents a substantial proportion of DPP-IV activity in human serum (Figure 7-A) (Mulvihill and Drucker, 2014). In crystal structure of DPP-IV shows a N-terminal eight-bladed β-propeller domain and a C-terminal α/β-hydroxylase domain (Figure 7-B). This last domain contains the catalytic triad that consists of Ser360, Asp708 and His740 (Hiramatsu *et al.*, 2003; Rasmussen *et al.*, 2003).

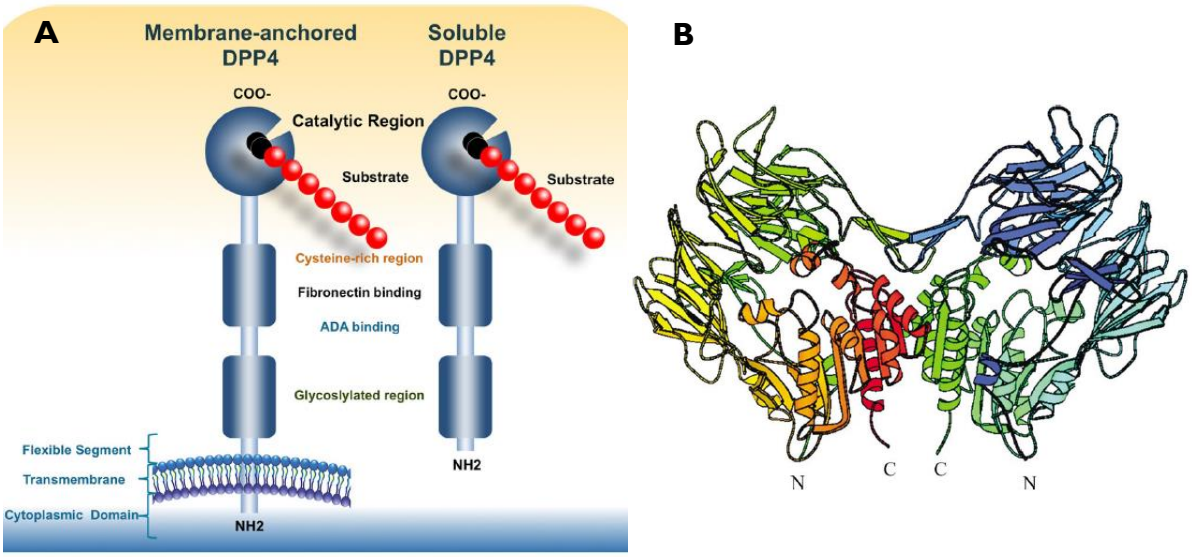

Figure 7: DPP-IV structure. A) Schematic representation of the membrane-bound monomer (left) and soluble DPP-IV (right). Both forms share many domains including glycosylated region, ADA binding domain, fibronectin bonding domain, cysteine-rich domain and catalytic domain. Only membrane-bound DPP-IV presents a cytoplasmic domain, a transmembrane domain and a flexible segment (Taken from *Mulvihill et al*, 2014). B). Crystal structure of DPP-IV in a homodimer form, with each subunit composed by two domains, an eight-bladed β-propeller domain and a α/β-hydroxylase domain. (Taken from *Hiramatsu et al.*, 2003)

DPP-IV is a protein that exhibits a large number of biological functions, including protease activity, association with ADA, interaction with extracellular matrix, cell surface co-receptor activity mediating viral entry and regulation of intracellular signal transduction coupled to control of cell migration and proliferation (Mulvihill and Drucker, 2014). However, its principal role is the enzymatic function. Peptides with N-terminal penultimate proline or alanine and up to 80 residues have been listed as substrates of DPP-IV- This includes regulatory peptides like glucose-dependent insulinotropic peptide (GIP), GLP-1, vasoactive intestinal peptide (VIP), chemokines, neuropeptides [such as NPY, substance P and peptide YY (PYY)], and others (Drucker, 2007; Kim, Yu and Lee, 2014).

DPP-IV is widely expressed in many cells, including T cells, B cells, natural killer (NK) cells, subsets of macrophages, hematopoietic stem cells and hematopoietic progenitor cells. It

is also present in several tissues such as kidney, small and large intestine, prostate and liver (Kim, Yu and Lee, 2014; Matteucci and Giampietro, 2009).

2.2 Glucagon-like peptide 1

GLP-1 is an endogenous 30-aa incretin produced by and secreted from enteroendocrine L cells, found in the jejunum, ileum and colon. This incretin is produced in response to nutrient intake by posttranslational proteolytic cleavage of proglucagon by prohormone convertase 1/3 (PC1/3), with GLP-1 corresponding to positions 78-108 of the human proglucagon precursor. Bioactive GLP-1 in circulation exists as GLP-1_{7-37} and GLP-1_{7-36} amide, being the last one the most abundant form of active GLP-1 in human plasma. This hormone is quickly degraded by DPP-IV producing GLP-1_{9-36} amide and/or GLP-1_{9-37}, which results in an apparent half-life in plasma of approximately 2 min (Campbell and Drucker, 2013; Gallwitz *et al.*, 1994; Larsen and Holst, 2005; Tasyurek *et al.*, 2014). GLP-1 has innumerous pleotropic effects in several organs, such as pancreas, liver, kidney, adipose tissue, muscle, nervous system, GI system and cardiovascular system, and can do it through both receptor-dependent and -independent pathways (Pabreja *et al.*, 2014).

GLP-1 receptor (GLP-1R) is a 463 aa heptahelical G-protein-coupled receptor (GPCR), characterized by an extracellular N-terminal domain responsible for high affinity binding of endogenous peptide ligands, three conserved disulphide bridges and several glycosylation sites. Activation of this receptor stimulates cyclic adenosine monophosphate (cAMP) formation and activation of protein kinase A (PKA) pathway, which in pancreatic β-cells results in the opening of voltage-gated Ca^{2+} channels and influx of Ca^{2+}. Increase in cytoplasmic Ca^{2+} leads to insulin secretion from β-cells thus determining their incretin effect (glucose-stimulated insulin secretion). Besides its insulinotropic effect, GLP-1 also promotes β-cell proliferation, differentiation and regeneration, insulin gene synthesis, islet cell mass increase and anti-apoptotic pathways (Drucker, 2006; Pabreja *et al.*, 2014; Tasyurek *et al.*, 2014). Besides pancreatic β-cells, GLP-1R is also widely detected in other cells and organs including the kidney, lung, heart, hypothalamus, endothelial cells, neurons, astrocytes and microglia, suggesting that GLP-1 might have additional roles other than glucose-lowering effects. In fact, several studies have demonstrated that incretin-based therapies also present anti-inflammatory effect by reducing the production of inflammatory cytokines (Lee and Jun, 2016).

2.2.1 GLP-1 on gastrointestinal system

Besides action on control of post-prandial glucose, GLP-1 also present important functions in GI motility. One of the most relevant roles of GLP-1 in GI system is the slowing of gastric emptying by mechanisms that include antral inhibition and stimulation of pyloric motility (Marathe *et al.*, 2011). In a previous study performed by our research group it was shown that a GLP-1-based therapy induced an increase in the gastric fundus tonus, which can explain the reduction of food intake experienced by patients treated with incretin-based therapies (Carrêlo, 2016). In terms of small and large intestine, GLP-1 also appears to inhibit their motility (Marathe *et al.*, 2011). Additionally, this hormone may contribute to the "ileal brake", a mechanism that optimizes nutrient absorption in the proximal small intestine by inhibiting upper GI motor activity, if unabsorbed nutrients reach the ileum. This mechanism can also explain the regulatory role of GLP-1 in the control of the appetite and energy intake (Amato *et al.*, 2010; Steinert, Beglinger and Langhans, 2015).

The mechanism by which GLP-1 inhibits GI motility appears to involve, in some cases, communication between the CNS and the PNS, through vagal afferent innervation, or involving direct activation of GLP-1R in the CNS. The presence of GLP-1R in several brain areas, namely arcuate nucleus and other hypothalamic regions and in neurons of myenteric plexus (ENS), corroborates the hypothesis that this peptide modulate visceral functions by its action in the CNS (Bucinskaite *et al.*, 2009). Several studies have been published in order to evaluate these mechanisms, being possible to determine, for example, that in small and large intestine GLP-1 reduces the GI motility acting through nitric oxide (NO) production (Amato *et al.*, 2010).

2.2.2 GLP-1 pathway in neurodegenerative diseases

GLP-1 is peripherally produced by enteroendocrine L cells, as previous described, but is also produced within the CNS. Central GLP-1 expression occurs in the hypothalamus, cortex, hippocampus, striatum, substantia nigra, brain stem and supraventricular zone. Peripheral GLP-1 can also communicate with the brain by crossing the BBB or via sensory afferent neurons. Additionally, GLP-1R is primarily confined to large output neurons, in particular on pyramidal and dentate granule neurons, as well as Purkinje cells (where it localized to dendrites and/or near synapses). This suggests that GLP-1 signaling exerts neuroprotective and neurotrophic effect, with possible positive implications for the treatment of neurodegenerative diseases (Campbell and Drucker, 2013; Duarte *et al.*, 2013; Fedele, Ricciarelli and Rebosio, 2016).

Progressive neurodegenerative diseases are associated with chronic inflammatory response in the brain. This feature contributes to further neurodegenerative effects via the activation of immune cells in the brain, such as microglia, that can release neurotoxic factors like pro-inflammatory cytokines and free oxygen radicals. GLP-1 (and GLP-1 mimetics) not only present neuroprotective properties, but also have anti-inflammatory effects. This can be explained by signaling pathways associated GLP-1R, since its activation leads to increased levels of cAMP and activation of PKA and other downstream kinases that are related to growth factor signaling. All these pathways explain the GLP-1 effects on neuroprotection, neuronal development and memory formation (Figure 8) (Hölscher, 2014).

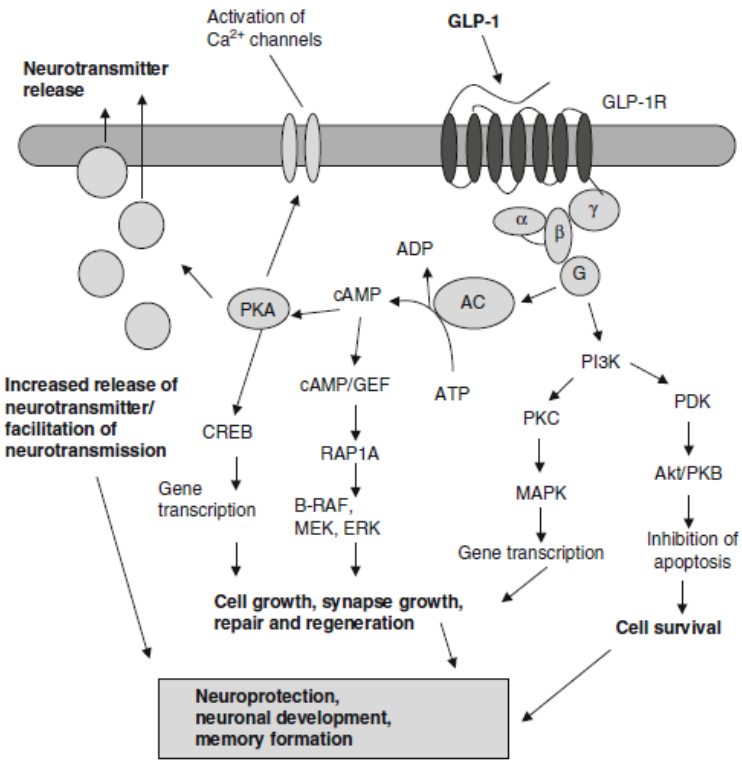

Figure 8: Activity of GLP-1 in neurons. Activation of GLP-1R activates adenylyl cyclase and increases cAMP levels. This activates PKA and other downstream kinases that are related to grown factors signaling. All these pathways explain the GLP-1 effects on neuroprotection, neuronal development and memory formation. ADP – adenosine diphosphate; ATP – adenosine triphosphate; cAMP – cyclic adenosine monophosphate; CREB – cAMP response element-binding protein; ERK – extracellular signal-regulated kinase; G – guanine nucleotide-binding protein; GEF – guanosine exchange factor; GLP-1 – glucagon-like peptide 1; GLP-1R – GLP-1 receptor; MAPK – mitogen-activated protein kinase; PDK – Phosphoinositide-dependent kinase; PI3K – phosphatidylinositol 3-kinase; PKA – protein kinase A; PKB – protein kinase B; PKC – protein kinase C. (Taken from *Hölscher*, 2014)

Despite clear differences between the most common neurodegenerative brain diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and MS, they share some

degenerative mechanisms and pathological features, like apoptosis, chronic inflammatory response, reduced neurogenesis, synaptic failure and neuronal death. Being that, GLP-1 can be a potential target that can be useful in a range of neurodegenerative conditions (Holscher, 2012). In fact, some studies with GLP-1 analogues were able to conclude that GLP-1R activation have profound effects on memory formation and synaptic plasticity within the brain. Additionally, GLP-1 and GLP-1R agonists (GLP-1RA) also exert a protective role in mouse models of AD. In these cases, GLP-1 protected against the cellular apoptosis induced by β-amyloid and had the ability to reduce both β-amyloid and amyloid precursor protein levels (Seino and Yabe, 2013). In PD animal models, several studies have shown very impressive protective effects of GLP-1RA. Actually, they were able to promote adult neurogenesis *in vitro* and *in vivo*, normalize dopamine imbalance and increase the number of cells positive for markers of dopaminergic neurons in substantia nigra (Holscher, 2012).

In conclusion, GLP-1 based therapies may be useful as a therapeutic approach of neurodegenerative disorders given their anti-inflammatory and neuroprotective properties.

2.3 Neuropeptide Y

NPY is a 36 aa peptide that was first isolated from porcine brain in 1982 (Tatemoto, 1982). It possesses an amidated C-terminal residue and several tyrosine residues (which are normally abbreviated by the letter Y) included in both ends of the molecule (Silva *et al.*, 2005). The NPY gene is located on human chromosome 7 at the locus 7p15.1. NPY synthesis occurs in the endoplasmatic reticulum, starting from a 97-aa precursor protein, named pre-pro-NPY. The cleavage of this peptide by a signal peptidase originates pro-NPY (69 aa protein), which is further processed by a prohormone converting enzymes resulting in NPY (1-39) and a C-terminal flanking peptide of NPY. The NPY (1-39) is then processed by carboxypeptidase H and peptidylglycine α-amidating monooxygenase in order to obtain the mature 36-aa C-terminally amidated peptide, being the amine group an essential requirement for receptor binding and biological activity (Walther, Mörl and Beck-Sickinger, 2011).

The NPY family consists not only of NPY, but also of PYY and pancreatic polypeptide (PP). Despite of structural differences between these three polypeptides, they share a common hairpin-like three-dimensional structure (PP-fold), a 36 aa structure and an amidated C-terminal (Cabrele and Beck-Sickinger, 2000). Moreover, NPY exhibits a 70% homology with PYY and 50% homology with PP (Brothers and Wahlestedt, 2010). The peptides of these family act as hormones and/or neurotransmitters/neuromodulators. NPY acts more as a

neurotransmitter/neuromodulator, being expressed in multiple neuronal brain systems and in the enteric neurons. PP and PYY act more like neuroendocrine hormones and are localized in endocrine cells in the ileum, colon and rectum; additionally, PP is also found in endocrine cells of the pancreatic islets of Langerhans (El-Salhy and Hausken, 2016).

NPY is one of the most abundant neuropeptides in the mammalian brain, being widely distribute within the CNS and PNS. Concerning the CNS, high levels of NPY can be detected in several brain regions, such as hypothalamus, thalamus, hippocampus, cerebral cortex and brainstem, as well as in spinal cord. Peripherally, NPY is found in sympathetic neurons, where it co-exists with noradrenaline (NA) and ATP (Walther, Mörl and Beck-Sickinger, 2011), and in enteric neurons (Holzer, Reichmann and Farzi, 2012). NPY is involved in a variety of physiological process, including endocrine and cardiovascular function, regulation of feeding, axon guidance, neurogenesis, anxiety, stress, circadian rhythm, memory retention, pain, among others. NPY is also involved in inflammation and immune responses (Walther, Mörl and Beck-Sickinger, 2011).

The pleotropic action of NPY is accomplished by the multiplicity of NPY receptors, with at least 5 subtypes of transmembrane GPCRs (Y1, Y2, Y4, Y5 and Y6) being identified so far. However, only four subtypes of receptors are functional in humans (Y1, Y2, Y4 and Y5), since Y6 is functional only in other mammals (Duarte-Neves, Almeida and Cavadas, 2016). The Y1, Y2 and Y5 receptor subtypes preferentially bind NPY and PYY, whereas Y4 is primarily activated by PP (Silva *et al.*, 2005). Activation of G-protein complex by NPY results in

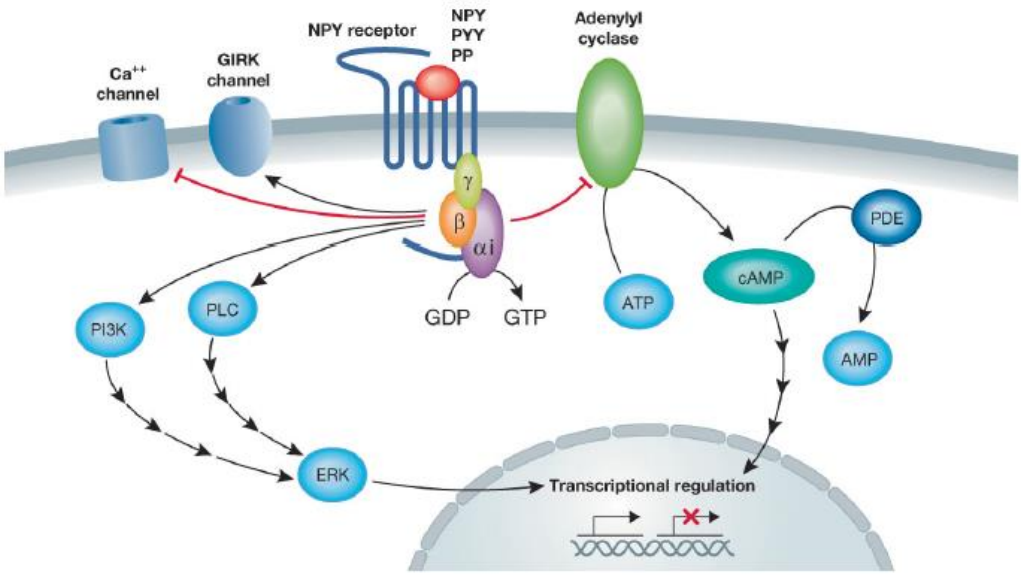

Figure 9: Overview of NPY receptors signal transduction. NPY receptors couple to the G-protein signaling cascade, leading to the inhibition of adenylyl cyclase. Furthermore, the activation of the G-protein complex can also lead to decreased Ca^{2+} channel activity and enhanced G-protein coupled inwardly rectifying potassium (GIRK) currents. (Taken from *Brothers et al*, 2010)

decreased cAMP production in the cells. Furthermore, this activation can also lead to the modulation of Ca^{2+} and K^{+} channels (Brothers and Wahlestedt, 2010). Other signaling routes activated by NPY may involve protein kinase C (PKC), mitogen-activated protein kinase (MAPK), inositol triphosphate (IP3) and production and release of Ca^{2+} from intracellular stores (Figure 9) (Malva *et al.*, 2012).

The human Y1 receptor (Y1R) consists of 384 aa and it was the first receptor being cloned in the NPY family. This receptor is widely distributed in the CNS, primarily in cerebral cortex, hippocampus, amygdala, thalamus and hypothalamus (Silva *et al.*, 2005). Peripherally, Y1R is found in adipose tissue, blood vessels, vascular smooth muscle cells (Walther, Mörl and Beck-Sickinger, 2011) and GI tract (Jackerott and Larsson, 1997). The most important Y1-receptor mediated effects of NPY are vasoconstriction and anxiolysis (Cabrele and Beck-Sickinger, 2000).

The human Y2 receptor (Y2R) contains 381 aa and is expressed in several brain regions, such as hippocampus, thalamus, hypothalamus and brain cortex. Y2R can also be found in PNS, particularly in parasympathetic, sympathetic and sensory neurons, as well as in the intestine and certain blood vessels. The main effects associated with this receptor are related to suppression of neurotransmitter release (Walther, Mörl and Beck-Sickinger, 2011).

The Y4 receptor (Y4R), a 375-aa protein, is the only subtype with high affinity for PP and moderate affinity for PYY and NPY. This receptor is mainly expressed on GI tract, including intestine, colon and pancreas; however, it can also be found in heart, skeletal muscle and thyroid gland. In the CNS it is found in low expression levels (Walther, Mörl and Beck-Sickinger, 2011). Activation of Y4R induces inhibition of pancreatic secretion and gall bladder contraction (Cabrele and Beck-Sickinger, 2000).

The Y5 receptor (Y5R) is the only that presents two isoforms – a long isoform with 455 aa and a short isoform with 445 aa. Both isoforms present similar pharmacological profile, being mainly expressed in CNS, particularly in hypothalamus. Here, its activation is responsible for induce food intake (Walther, Mörl and Beck-Sickinger, 2011).

As discussed above, NPY is implicated in the modulation of important features of neuronal physiology, including calcium homeostasis and neurotransmitter released. Indeed, NPY not only protects neurons from excitotoxic cell death but also promote neuronal differentiation via Y1R. Additionally, NPY stimulates neuroproliferation and neuronal survival, induces autophagy that result in the clearance of disease-causing aggregate-prone proteins and attenuates neuroinflammation. Being that, NPY system seems to have therapeutic potential in several neurodegenerative diseases, since it can attenuate pathologic mechanisms that lead to

neurodegeneration and can directly or indirectly contribute to the generation of new neurons, survival, and functional remodeling of brain cells (Duarte-Neves, Almeida and Cavadas, 2016; Malva *et al.*, 2012).

2.3.1 NPY on gastrointestinal system

As discussed above, NPY and its receptors are present in GI tract, more specifically through all brain-gut axis. NPY can be found in enteric neurons, primary afferent neurons, several pathways throughout the brain and sympathetic neurons, being the enteric neurons the major source of NPY in the GI tract. In this system, NPY is located in interneurons and descending inhibitory motoneurons of the myenteric plexus, where it is co-localized with VIP and NO synthase (NOS). In the submucosal plexus, NPY is expressed in noncholinergic secretomotor neurons. Additionally to enteric neurons, NPY can also be found in postganglionic sympathetic neurons, where is colocalized with NA and ATP (Holzer, Reichmann and Farzi, 2012).

One of the major functions of NPY in the PNS is related with the control of blood flow, vascular tone and blood pressure, acting as a potent vasoconstrictor. Moreover, on GI system, this peptide has inhibitory effects on pancreatic and GI secretion, as well as on motility (Vona-Davis and McFadden, 2007). In addition, NPY receptors are involved in other roles of the GI tract, such as adaptation to diet, electrolyte balance, nutrient/water intake, intestinal growth and gastric emptying (Brothers and Wahlestedt, 2010). Together with PYY and similarly to GLP-1, NPY also contribute to the "ileal break" observed in the GI tract after fat ingestion (Vona-Davis and McFadden, 2007).

In the GI tract, NPY exert its functions through Y1R, Y2R and Y5R subtypes receptors. Specifically, Y1R was identified in nerve cell bodies of the submucosal and myenteric plexuses throughout the rat intestine. Apparently, NPY mainly exert its functions on the GI tract through this receptor, exerting a proinflammatory action. Nevertheless, NPY system appears to protect against distinct behavioral disturbances caused by peripheral immune challenge, attenuating the acute sickness response and preventing the development of long-term depression. In fact, some studies detected that gastric lesion formation and gastric secretion (that contribute to mucosal injury) can be reduced by the administration of Y1R selective agonists (Holzer, Reichmann and Farzi, 2012; Vona-Davis and McFadden, 2007). Furthermore, in other GI diseases, such as constipation/diarrhea and inflammatory bowel disease (IBD), the use of NPY receptor modulators may also present some therapeutical potential. For example, in the treatment of diarrhea, a Y1R selective agonist might promote gut health, by increasing

water absorption. On the other hand, Y1R knockout mice have a lower susceptibility to IBD, an antagonist for this receptor might be the best therapeutical option in the treatment of IBD and/or constipation (Brothers and Wahlestedt, 2010).

2.4 DPP-IV inhibitors

As explained above, DPP-IV is responsible for maintaining physiological glucose homeostasis, mainly due to the regulation of incretin hormones levels. Additionally, patients with type 2 diabetes mellitus (T2DM) exhibit DPP-IV overexpression, as well as an incretin deficit/defect. These features took researchers and pharmaceutical companies to search for drugs that were able to enhance incretins bioavailability by inhibiting DPP-IV activity. The incretin-based therapies are a new class of anti-diabetic drugs available for the treatment of diabetic patients and include GLP-1 agonists (namely Liraglutide) and DPP-IV inhibitors (Tasyurek *et al.*, 2014).

Nowadays, there are several DPP-IV inhibitors approved by FDA, including Sitagliptin, Vildagliptin, Saxagliptin and Alogliptin and others that are still in development. They are small molecules that are rapidly absorbed following oral dosing. They reach their maximum plasma concentration between 1-2 hours and have a bioavailability higher than 80%. After administration, it is possible to observe over 80% inhibition of DPP-IV activity for the full 24-h period, which result in increased levels of incretins and other DPP-IV substrates, such as NPY and PYY (Martin *et al.*, 2011).

The main application of this drug class is in T2DM, being associated with long-term control of HbA_{1C} and improvement in β-cell function, as well as improvement of cardiovascular function (Kazakos, 2011). Furthermore, DPP-IV inhibitors also have neurotrophic and immune regulating functions, which has aroused interest in the application of these drugs for the management of neuroinflammatory/neurodegenerative diseases (Al-Badri *et al.*, 2018).

2.4.1 Sitagliptin

Sitagliptin, whose chemical structure is represented on figure 10, was the first compound of the DPP-IV inhibitors class to be introduced in the market (Januvia®, Merck Pharmaceuticals, USA). It is a highly potent and selective inhibitor of DPP-IV enzyme, with a bioavailability of approximately 87% and a half-life between 8-14 hours. Sitagliptin is 38% bound to plasma protein and undergoes metabolism via CYP3A4 and CVP2C9. Its elimination is

mainly renal, with 75% of an oral dose found in the urine as unchanged drug. Nowadays, Sitagliptin is indicated in the treatment of T2DM, being currently approved in 42 countries. The recommended dose is 100mg once daily and can be prescribed in monotherapy or combined with other anti-diabetic drugs, such as metformin (Badyal and Kaur, 2008; Bhavya and Madhussudan, 2013). Sitagliptin is prescribed for the therapy of T2DM, since its action is mediated by increasing levels of the incretin hormones (GLP-1 and GIP). Thus, insulin secretion is stimulated and glucagon secretion is inhibited, allowing the regulation of the postprandial and fasting levels of glucose. Additionally, sitagliptin has also been shown to be effective in reducing HbA_{1C} (Gallwitz, 2007).

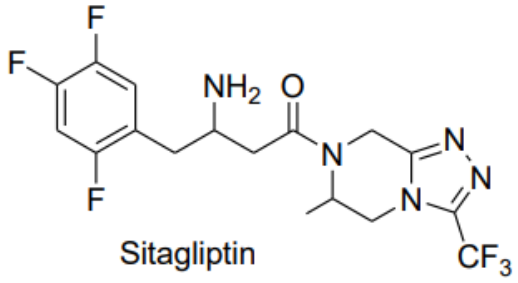

Figure 10: Chemical structure of Sitagliptin, a DPP-IV inhibitor. The chemical structure of Sitagliptin is (2*R*)-4-oxo-4[3-(trifluoromethyl)-5,6-dihydro[1,2,4]-triazolo-[4,3]-pyrazin-7-(8H)-yl]-1-(2,4,5-trifluorophenyl)-butan-2-amine. (Taken from *Bhavya et al,*2013)

In terms of neurodegenerative diseases, Sitagliptin has not been extensively studied. However, some studies have demonstrated the potential benefit of this drug, which appears to promote neuroprotection and neuroregeneration (Badawi *et al.*, 2017; Gault, Lennox and Flatt, 2015).

2.4.2 DPP-IV inhibitors and MS

DPP-IV inhibitors have been attracted a major interest as a potential target for the development of anti-inflammatory therapies in MS based on the findings of several studies that report a co-localization of DPP-IV with myelin-reactive T cell lines. In fact, T cell clones of MS patients express significantly higher levels of DPP-IV compared to resting peripheral blood T cells. Based on these facts, in studies involving mice with EAE, the administration of a DPP-IV inhibitor stimulated T-cell clones, inhibited the production of IL-4, interferon-γ and tumour necrosis factor (TNF)-α and caused a reduction of pro-inflammatory cytokines production. Along with the increase in the levels of the immune suppressive transforming growth factor beta 1 (TGF-β1) and reduced T-cell proliferation, these results suggest that inhibition of DPP-

IV activity may suppress the inflammatory response associated with MS (Al-Badri *et al.*, 2018; Steinbrecher *et al.*, 2001; Yazbeck, Howarth and Abbott, 2009).

Other studies involving DPP-IV substrates, namely NPY, also suggest a neuroprotective action of this peptide that depend on DPP-IV activity. Not only NPY, via Y1R, has an accelerative effect on oligodendrocytes myelination (Hashimoto *et al.*, 2010), but also present an inflammatory effect mediated by the same receptor that is dependent on inhibition of DPP-IV (Dimitrijević *et al.*, 2008). Recent studies with GLP-1 agonists also reported that increasing the levels of this peptide has neuroprotective effects, since it delays the onset of EAE-induced MS, improves the clinical signs of the disease and reduces the immune responses associated with MS pathology (DellaValle *et al.*, 2016; Lee *et al.*, 2018).

In summary, given the anti-inflammatory and neuroprotective properties of DPP-IV substrates and DPP-IV inhibitors, the use of this class of drugs may be beneficial in the treatment of MS.

3 Gastrointestinal system

The GI system is one of the most complex and important organ systems and includes not only the GI tract but also the accessory organs. The GI tract, which is 7-9 m in the adult, comprise the mouth, pharynx, esophagus, stomach, small intestine and large intestine; the accessory organs, that are the salivary glands, liver, gallbladder and pancreas, secrete substances into the alimentary canal via connecting ducts that play an essential role in chemical digestion. The overall function of the GI system is to take in nutrients and process them into molecular forms that are then transferred, along with salts and water to the body's internal environment, separating the ones that can be used by the body from the eliminate wastes (Hammer and McPhee, 2014; Vander, Sherman and Luciano, 2001).

3.1 Structure of GI tract wall

The GI tract wall has a general structure that is very similar from the midesophagus to the anus. Most of its tube's luminal surface is highly convoluted, being formed by specialized structures named villi and microvilli, that greatly increase the surface area available for absorption. The first are finger-like projections extending from the luminal surface of the small intestine and are covered with single layer of epithelial cells that, in turn, have small projections

in their surface membranes, called microvilli. These epithelial cells are linked together along the edges of their luminal surfaces by tight junctions.

In terms of structure, the GI wall is composed by four layers, namely mucosa, submucosa, muscularis externa and serosa, each layer containing a dominant tissue type that performs specific functions in the digestive process (Figure 11). The precise structure of some of these layers, especially the mucosa, can vary from one region to the next, along the GI tract. Nevertheless, the mucosa, which lines the lumen of the GI tract, is the most secretory and absorptive layer. It is composed by the combination of three more specific layers: epithelium, lamina propria and muscularis mucosae. The epithelium consists of a single layer of exocrine cells, that secrete mucus into the lumen, and endocrine cells, that release hormones into the blood, and is supported by the lamina propria. This is a layer of connective tissue containing blood vessels, nerve fibers and numerous lymph nodules, which are important in protecting against disease. External to the lamina propria there is a layer of smooth muscle, named muscularis mucosae, that is responsible for the formation of villi in certain portions of the GI tract. Beneath the mucosa, there is a relatively thick and highly vascular layer of connective tissue, called submucosa. This layer is composed by larger blood and lymphatic vessels and a nerve plexus of the ENS, termed the submucosal (Meissner) plexus, that is particularly important for control of secretion in the small and large intestines. Moreover, the muscularis externa is composed of an inner and outer layer of circular and longitudinal smooth muscle that is responsible for segmental contraction and peristaltic movement through the GI tract.

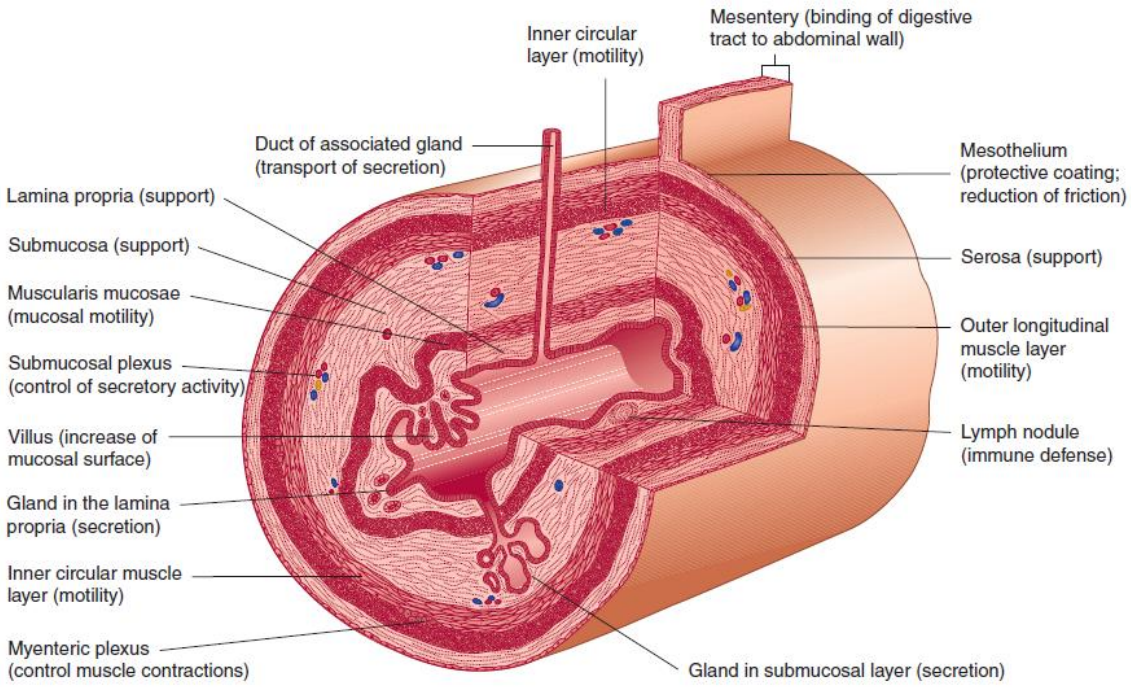

Figure 11: Schematic structure of a portion of the digestive tract showing the several components of the gastrointestinal wall. (Taken from Ha*mmer et al*, 2014)

Between these two layers of smooth muscle, there is the myenteric (Auerbach) plexus, a division of the ENS that includes fibers and ganglia of both sympathetic and parasympathetic nervous system. Finally, surrounding the outer surface of the GI tube, there is a thin layer of squamous mesothelial cells and connective tissues, named serosa, that supports the GI tract in the abdominal cavity (Fox, 2011; Hammer and McPhee, 2014; Vander, Sherman and Luciano, 2001).

Anatomically, the small intestine is specifically divided in three portions: duodenum, jejunum and ileum. Is in the latter part of the GI tract that most of the digestion and absorption process occur. Concretely the ileum, which is connected to the large intestine by ileocecal valve, is the local of the intestine where bile salts, vitamin B_{12}, water and electrolytes are absorbed (Hammer and McPhee, 2014).

3.2 Intestinal smooth muscle contraction

Motility is a process that is responsible to move and mix the secretions and luminal contents from mouth to anus, underlined by the coordinated contractions of smooth muscle cells. The intestinal smooth muscle cells are present in the inner cellular layer and in the outer longitudinal layer of the muscularis externa. Concerning to their morphology, they are fusiform cells packed together in bundles by connective tissue sheaths. Between smooth muscle cells, gap junctions allow the quickly spread of action potentials from cell to cell so that the contraction of bundles occurs synchronously.

In the small intestine, two types of contractions are present: peristalsis and segmentation. The first, present throughout all GI tract, are characterized by propulsive movements that are much weaker in the small intestine than in the esophagus and stomach. On the other hand, segmentation represents the major contractile activity in the small intestine and is characterized by alternated muscular constrictions of the lumen. Occurring simultaneously at different intestinal segments, segmentation movements are responsible for mixing the intestinal contents with digestive enzymes and mucus. The segmentation occurs automatically and is initiated by electrical activity generated by a pacemaker activity that is produced by unique modified smooth muscle cells, often associated with autonomic nerve endings. These cells, named interstitial cells of Cajal (ICCs), are mesenchymal cells that form an extensive network of stellate cells in the muscle layers of the stomach and intestine that, not only have the capacity to generate the basal electrical rhythm underlying phasic contractions of the smooth muscle layers, but also transmit information from enteric neurons to the smooth

muscle cells (Fox, 2011; Hammer and McPhee, 2014; Hansen, 2003; Vander, Sherman and Luciano, 2001; Webb, 2003).

Slow waves, the basal electrical rhythm set by ICCs, spread by way of gap junctions between ICCs in stomach and intestines, being able to spread only a short distance and thus must be regenerated by the next pacemaker region (segmentation contractions in the intestine). The production of slow waves varies along the length of the intestine, being faster at the proximal end of the intestine than at the distal end, creating a gradient of pressure that allows the chyme to be forced downwards.

The slow waves are responsible for depolarizing the adjacent smooth muscle cells, that have a resting membrane potential in the range of -40 to -80 mV because of the relative conductances of K^+, Na^+ and Cl^- ions. When the slow wave depolarization reaches a threshold value, it triggers spiked action potentials that depolarize the smooth muscle cells membranes and induce an influx of Ca^{2+} ions into the cytoplasm through voltage-sensitive Ca^{2+} channels. Contrary to what happens in the skeletal muscle, the Ca^{2+} released from sarcoplasmic reticulum is less important than that coming from extracellular fluid, that is responsible for sustained contraction. Smooth muscle contraction can be triggered by receptor activation by hormones or neurotransmitters or by the action of ICCs. The extent of the contraction depends on the extent of depolarization, since the greater the depolarization, the more Ca^{2+} will enter the cell and the stronger will be the smooth muscle contraction.

Already in the cytoplasm, Ca^{2+} combines with the Ca^{2+}-binding protein calmodulin, forming the Ca^{2+}-calmodulin complex. This complex activates myosin light-chain kinase (MLCK), an enzyme that uses ATP to catalyze the phosphorylation of myosin light chains, which allows the establishment of cross bridges between myosin and actin filaments, inducing tension and shortening and, finally, producing contraction. Thus, in smooth muscle, the intensity of the contractile response is primarily regulated by the phosphorylation of myosin.

On the other hand, the relaxation of smooth muscle occurs due to the closure of the Ca^{2+} channels, which leads to a decrease of intracellular Ca^{2+} by the action of Ca^{2+}-ATPase active transport pumps. In this case, Ca^{2+} -calmodulin complexes dissociate from MLCK, inactivating this enzyme. As a consequence, myosin dephosphorylation also occurs by myosin phosphatase, inhibiting the cross bridge with actin (Fox, 2011; Hammer and McPhee, 2014; Hansen, 2003; Vander, Sherman and Luciano, 2001; Webb, 2003).

3.3 Regulation of intestinal motility

The GI tract processes, like motility and secretion, depend on two innervation components: extrinsic innervation by parasympathetic and sympathetic nerves and intrinsic innervation by ENS. Besides the neuronal control, hormonal and paracrine control are also important for the regulation of GI functions.

Extrinsic innervation is composed by parasympathetic and sympathetic fibers from the ANS (both myelinated (Chawla, 2016)) and is responsible for transmitting sensory information to the brain and spinal cord via vagal and spinal (splanchnic and pelvic) afferents, respectively. This bidirectional communication between the brain and the gut, also known as brain-gut axis, can regulate the function of the ENS or directly control the activity of other cell types. Parasympathetic activity, originated by vagus nerve, generally stimulate motility and secretions of GI tract, using acetylcholine (ACh) as the main neurotransmitter. In contrast, sympathetic nerves are often inhibitory of GI functions, reducing peristalsis and secretory activity, and norepinephrine is their major postganglionic neurotransmitter.

Intrinsic innervation is mainly regulated by ENS (Figure 12), the third division of ANS, that is an extensive reflex control system for digestive functions, extending from the esophagus to the rectum. Is formed by small aggregations of nerve cells, the enteric ganglia, neuronal connections between these ganglia and nerve fibers that are responsible for supplying effector tissues, like the muscle of the GI wall, blood vessels and enteroendocrine cells. The ENS is mainly responsible for the control of GI functions like motility and secretion, in an independent way of extrinsic innervation (reason why it is also known as "mini-brain"). This system contains as many neurons as the spinal cord (200-600 million neurons), and its neurons are organized into ganglia that are interconnected by the two above described plexuses – the myenteric and the submucosal plexus. Enteric neurons can be classified according to its function, being organized into three main neuronal classes – sensory neurons, interneurons and motor neurons. The sensory neurons, also known as intrinsic primary afferent neurons (IPANs), detect distortion of the mucosa, chemical changes in the lumen, tension changes in the muscle and compressive forces on enteric ganglia, reacting to these signals to initiate appropriate reflex control functions. The IPANs are connected to each other, with interneurons or directly with motor neurons. Interneurons are organized into ascending and descending neurons, forming chains that extend along the GI tract. Enteric motor neurons are divided into several types – muscle motor neurons, secretomotor neurons, vasomotor neurons and neurons innervating enteroendocrine cells. Muscle motor neurons can be excitatory or

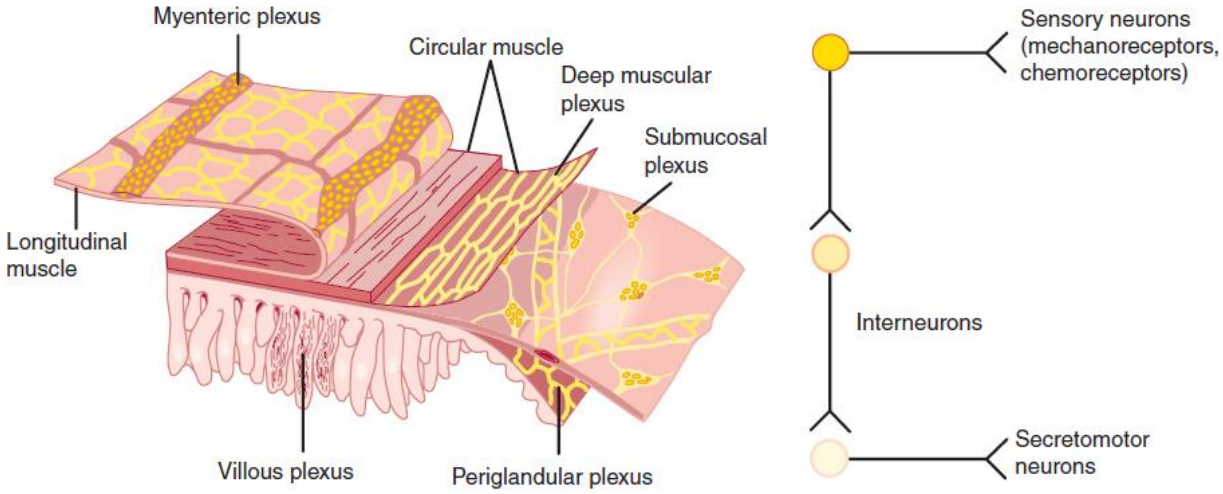

Figure 12: The enteric nervous system (ENS). In the left image, ENS of the small intestine shows that enteric neurons are organized in two nerve plexuses, the submucosal plexus and the myenteric plexus. In the right image, is possible to observe that ENS includes sensory neurons, interneurons and motor neurons. Complete reflex arcs exist within the ENS. (Taken from Ha*mmer et al*, 2014)

inhibitory and innervate the longitudinal and circular smooth muscle as well as the muscularis mucosae. Secretomotor and vasomotor neurons control secretions and blood flow, respectively. Additionally, secretomotor neurons can be divided into cholinergic and non-cholinergic neurons, with ACh and vasoactive intestinal polypeptide (VIP) as their neurotransmitter, respectively (Furness, 2000, 2012, Furness *et al.*, 2013, 2014; Greenwood-Van Meerveld, Johnson and Grundy, 2017; Hammer and McPhee, 2014; Olsson and Holmgren, 2011; Vander, Sherman and Luciano, 2001).

The neurochemical signaling within the ENS is extremely complex. Several transmitters can be released from enteric neurons to performed excitatory or inhibitory activity. The primary transmitters of the excitatory neurons are ACh, serotonin [5-hydroxytryptamine (5-H)] and substance P, while the inhibitory neurons have NA, VIP, NPY and NO as main transmitters (Furness, 2000).

3.4 Gastrointestinal dysfunction in MS

MS, as a neurodegenerative disease, also involves dysregulation of the ANS. This dysregulation leads to various clinical symptoms, including GI dysfunction. The prevalence of GI dysfunction in MS patients is higher than in the general population, with approximately 70% of the patients complaining about function loss in the GI domain. The most frequently reported symptoms are constipation, faecal incontinence or the combination of both and dysphagia. Gut

dysfunction is a source of considerable psychosocial disability, since these disturbances substantially reduce the quality of life of affected patients. It is also a clinical challenge to physicians due to the variability of clinical presentation and inconsistent data on diagnosis and treatment, making the gut management in MS patients essentially empirical (Pintér *et al.*, 2015; Wiesel *et al.*, 2001).

According to the most recent studies, constipation is the most common gut problem in MS patients, affecting 37% of the patients. Faecal incontinence is also present in this population, affecting 15% of the patients (Levinthal *et al.*, 2013). In some cases, both dysfunctions can coexist in the same patient. The presence of both GI alterations correlates strongly with the severity of the disease, with the patients with severe impairment presenting higher frequency of constipation and/or faecal incontinence. Etiology of GI symptoms is multifactorial, with constipation appearing because of poor dietary habits, physical inactivity or, more important, due to spinal cord lesion. Faecal incontinence appears most likely because of sphincter dysfunction, but factors like general muscle weakness, spasticity and impaired motility can also contribute to incontinence problems. Polypharmacy and pelvic floor incoordination are also factoring that contribute to gut dysfunction.

In fact, MS can affect the extrinsic neurological control of GI and sphincter function. The loss of myelin in the brain and spinal cord, which can cause damage to autonomic nerve pathways, alters colonic motility due to impaired parasympathetic and sympathetic input. This may result in reduced or lost sensation and control over muscle function, leading to faecal incontinence, or more often in slower transit and constipation (National Multiple Sclerosis Society, 2014; Pintér *et al.*, 2015; Trust, 2011; Wiesel *et al.*, 2001).

There are nonpharmacological and pharmacological treatments available for constipation. Concerning to nonpharmacological approach, increasing physical activity, fluids ingestion, biofeedback[1] and mechanical evacuation are some options for MS patients with constipation. Pharmacological management includes bulking agents, osmotic and stimulant laxatives, rectal stimulants and prokinetic agents. In more severe cases, colostomy may be the only option. Regarding faecal incontinence, the therapeutical options are fewer. Antimotility drugs, biofeedback, sacral nerve stimulation and surgical techniques like sphincter repair, dynamic graciloplasty and artificial bowel sphincter are the currently available options for these patients (Luzzio and Dangond, 2018; Pintér *et al.*, 2015).

[1] Biofeedback consists in conscious modification of body physiological processes having received a signal about body function. These techniques include attention to rectal and pelvic floor function, in addition to bowel retraining and use of medication. The treatment varies depending on whether constipation or faecal incontinence is the predominant symptom (Wiesel *et al.*, 2000).

More recently, some studies have been conducted concluding that gut microbiome is a source of chronic inflammation that can theoretically lead to the development of MS, taking into account the relevance of brain-gut axis (Colpitts *et al.*, 2017; Mirza and Mao-draayer, 2017; Wunsch *et al.*, 2017). On the other hand, by itself, this theory may explain some GI symptoms that MS patients often present.

Considering the prevalence of GI dysfunction in MS patients, the routine assessment of these patients should include screening for these symptoms, since gut dysfunction has been insufficiently characterized. Furthermore, finding the best therapeutical option will increase the quality of life of these patients, that often become isolated because of bladder and gut dysfunction.

CHAPTER II

OBJECTIVES

Objectives

Since incretin-based therapies, more specifically Sitagliptin, a DPP-4 inhibitor, can hypothetically promote remyelination by increasing the bioavailability of GLP-1 and NPY, peptides with recognized proliferative and anti-inflammatory properties, and since the percentage of constipation is so high in patients with MS, the general aim of this study was to evaluate the effects of Sitagliptin treatment, in the intestinal motility of an animal model of MS induced by CPZ intoxication, using the ileum as the target region.

To accomplish the main purpose, specific approaches have been devised as follow:

- Characterize the animal model of CPZ-induced MS, evaluating the demographic and biochemical profile of C57BL/6 mice subjected to CPZ, such as body weight, glycemia, water and food intake.
- Confirm the induced demyelination by cuprizone and remyelination by Sitagliptin, through determination of the density of MBP (myelin basic protein) through Western Blotting.
- Confirm the efficacy of Sitagliptin administration and treatment through determination of the DPP-4 activity in serum samples collected from the animal model.
- Through functional studies, evaluate the effect of CPZ-induced MS as well as the effect of Sitagliptin treatment in the ileum contractile response to electrical field stimulation, performing non-cumulative frequency-contractile response curves in ileum segments isolated from the cuprizone animal model.
- In order to evaluate the exact role of GLP-1 and NPY in intestinal motility in the presence of the disease, pharmacologically characterize the contractile response of the ileum isolated from the CPZ animal model by using a selective antagonist of GLP-1R (Exendin-3) and a selective antagonist of Y1R (BVD-10).
- Determine the density of GLP-1R in isolated ileum of control animals and animals subjected to CPZ treated with Sitagliptin, through Western Blotting.

CHAPTER III

MATERIALS AND METHODS

1 Animal model

In this study, C57BL/6 male mice with 8 weeks age, obtained from Charles River Laboratories (Barcelona, Spain) were used. Mice were housed in the vivarium of iCBR (Coimbra Institute for Clinical and Biomedical Research), Faculty of Medicine, University of Coimbra, under controlled temperature (22±1°C) and relative humidity (50-60%) and a 12-h light 12-h dark cycle. The animals were housed in pairs and fed *ad libitum* with distilled water and maintenance rodent chow (containing 18.5% protein, 3% lipids, 6% fiber and 3.2% minerals - 4FR21, Mucedola, Italy). All procedures involving animals were performed according to the National and European Communities Council Directives of Animal Care.

1.1 In vivo study

After 2 weeks of acclimatization, animals were randomly divided into six groups, according to the Figure 13: one group with five weeks of cuprizone treatment and its control – control (CTRL W5) and cuprizone (CPZ W5) – and three groups with seven weeks of cuprizone and/or sitagliptin treatment and their control – control (CTRL W7), five weeks cuprizone (CPZ W7), five weeks cuprizone also treated with Sitagliptin during the last two weeks [CPZ + Sita (T)] and five weeks cuprizone also treated with Sitagliptin during all seven weeks [CPZ + Sita (P)]. Both control groups received tap water for drinking and the other four groups received tap water mixed with 0.2% (w/v) CPZ (*C9012* Sigma-Aldrich, Sintra, Portugal) during the first five weeks; in the animal groups subjected a seven-week experiment period, CPZ was removed from the diet in the last two weeks, allowing spontaneous remyelination to occur (Figure 13). All animals were fed maintenance rodent chow.

The two animal groups treated with Sitagliptin (Januvia®, MSD, Portugal) received a dose of 50mg/kg/day, orally, through a jelly vehicle optimized from a previous protocol (Zhang, 2011); all other groups received only the jelly vehicle. All groups were submitted to a 1-week training before the beginning of the experiment, to ensure that all animals eat the entire jelly during the treatment period. The animals were sacrificed with a postprandial period of 2 hours, after receiving the last dose of Sitagliptin or vehicle.

Several biochemical parameters were evaluated in all animal groups. Body weight and food intake were evaluated once a week; water intake was evaluated twice a week; and glycemia was evaluated every two weeks (at the beginning of the treatment, at week three,

week five and on the sacrifice day). Glycemia was monitored with a glucometer (Accu-Chek Aviva, Roche, USA).

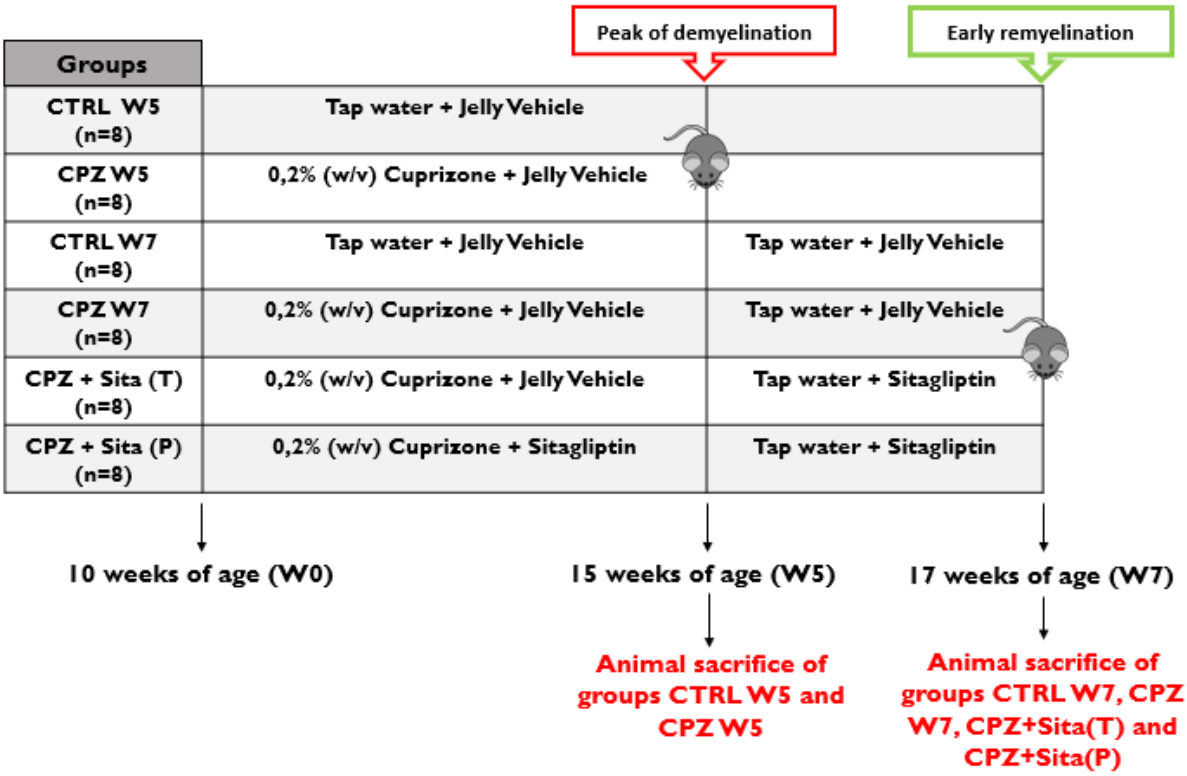

Figure 13: Experimental protocol of the *in vivo* study.

1.2 In vitro study

For the *in vitro* study, the animals were randomly divided into two groups: the control (CTRL) group, which continued to receive tap water for drinking, and the CPZ group, which received tap water mixed with 0.2% (w/v) CPZ for five weeks. All animals were fed with maintenance rodent chow. Weight and glycaemia of both animal groups were also monitored as well as food and water intake, following the same time protocol previously described for the *in vivo* study.

1.3 Sample collection

At the end of the treatment, animals were anaesthetized first in a saturated chamber with isoflurane (IsoFlo®, Abbott) and then with 150mg/kg of a solution of ketamine chloride (1g/ml; Imalgene®) in chlorpromazine 2.5% (Largactil®) through an intraperitoneal injection for blood collection through heart puncture. Blood was collected to serum tubes (BD *Vacutainer* SST II

Advance) and then centrifuged at 3500rpm during 15 minutes at 4°C and stored at -20°C. Posteriorly, the animals were sacrificed by cervical dislocation and the ileum and thymus were isolated. The thymus was washed, weighted and frozen at -80°C. The ileum was immediately immersed in a cold, carbogen aerated (95%O_2 / 5%CO_2) Krebs-Henseleit solution of the following composition (mmol/L): NaCl 118.67; KCl 5.36; $MgSO_4 \cdot 7H_2O$ 0.57; $CaCl_2$ 1.90; $KH_2PO_4 \cdot 2H_2O$ 0.90; $NaHCO_3$ 25.0; glucose 11.1; pH 7.4). Then the ileum was washed and the intestinal content as well as the portions of the adjacent mesentery were removed. The ileum was then divided into several segments of approximately 15mm to be immediately used in functional studies; other portions of the ileum were frozen at -80°C for subsequent lysis and homogenization for molecular studies.

2 Functional studies

2.1 Experimental protocol

Ileum segments were suspended on stainless steel hooks, between platin electrodes (Panlab, Barcelona, Spain) connected with an electrical stimulator (Grass SD9 Stimulator), under a basal tension in 15mL organ baths filled with Krebs-Henseleit solution aerated with 95%O_2 / 5%CO_2 and maintained at 37°C. The basal tension in each experiment was that which occurred spontaneously in tissue submitted to the optimal tension of 9.8mN, after the equilibrium state has been reached, following the organ assembly. Quiescent tissues with little or no spontaneous activity were not used.

Following a 1-hour equilibration period with periodic washings, phasic isometric contractions were recorded with a Letica Scientific Instruments isometric transducer connected to a four-channel polygraph (Polygraph 4006; Panlab, Letica Scientific Instruments, Barcelona, Spain). Non-cumulative frequency-contractile response (FR) curves were obtained submitting the ileum segments to increasing frequencies of 1, 2, 4, 8 and 16 Hz (100 V, 5 ms pulse width) of electrical field stimulation (Figure 13), in order to activate intrinsic ileum nerves. In ileum segments isolated from animal groups treated with Sitagliptin, a second FR curve were performed in the absence and presence of Exendin-3 (Ex3), a selective antagonist of GLP-1R, added to the organ bath 7 minutes before each electrical stimulation frequency. In the *in* vitro study, in ileum segments isolated from both CTRL and CPZ groups, a second FR curve was also performed in the absence and presence of Ex3 or BVD-10, a selective antagonist of NPY1R, also added to the organ bath 7 minutes before each electrical stimulation

frequency. In each assay control segments were used with the appropriate solvent of each drug.

All ileum segments were subjected to 100μM of exogenous acetylcholine (ACh) to directly induce the maximum contraction of the smooth muscle and, in this way, be possible to compare results from different experiments.

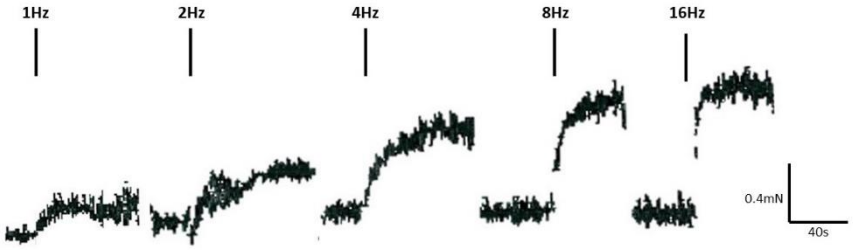

Figure 14: Image representative of a non-cumulative frequency-contractile response (FR) curve of an isolated ileum from a control (CTRL) animal (C57BL/6).

2.2 Reagents and drugs used

All reagents used in the preparation of the physiological Krebs-Henseleit solution were purchased from Panreac (Barcelona, Spain) and were *pro analysi* products. The following drugs were used for functional studies: Exendin-3 and BVD-10 (Tocris Bioscience, Bristol, UK), and acetylcholine hydrochloride (Sigma-Aldrich, St. Louis, USA). The solutions were prepared with the solvents listed by the manufactures.

2.3 Data processing and statistical analysis

The maximum contractile response (Emax) of all animal groups to the first FR curve was determined. Results were expressed in terms of millinewtons (mN) of tension. To determine the statistical differences between the Emax values of all groups, ANOVA (Analysis of variance) was used followed by Tukey's multiple comparison test and Student's *t* test was used for unpaired data from CTRL and CPZ groups of the in vitro study. Contractile responses to electrical stimulation, performed in the presence of Ex3 or BVD-10, were expressed as a percentage of the maximum contraction obtained in the first FR curve (control curve) of the respective segments. Final results were expressed as a percentage of control segment response of each assay. Differences between the Emax values in the presence and in the absence of both antagonists were also evaluated by Student's *t* test for unpaired data. Values

of $P < 0.05$ were considered to indicate significant differences. Results were presented as mean ± standard error of mean (SEM) of the number of isolated ileum segments (s) *per* the number of animals (n). All statistical analyses were performed using *GraphPad Prism* PC Software and IBM SPSS Statistics Software.

3 Biochemical parameters

3.1 Serum DPP-4 activity

In order to measure the serum activity of DPP-IV, a continuous fluorometric assay was employed using a fluorogenic substrate (H-Gly-Pro-AMC.HBr by BACHEM, Bubendorf, Switzerland), which is cleaved by the enzyme to release the fluorescent aminomethylcoumarin (AMC). Briefly, 20 µl of serum sample was mixed with the assay buffer (50 mM Glycine, pH 8.7, 1 mM EDTA). After 5-min preincubation at room temperature, the reaction was initiated by the addition of the substrate H-Gly-Pro-AMC.HBr to a final concentration of 200 µM. The final reaction volume for each well was 100 µl. The release of AMC was monitored at 360 nm (excitation) and 460 nm (emission) wavelengths (microplate reader Synergy HT, BioTek, Winooski, VT, USA) every 5 min for a total of 60 min. For comparison of DPP-IV activity between samples, data was plotted as relative fluorescence units versus time for each sample. The time range over which the reaction was linear was determined. A trend line for these data points was obtained and the slopes determined.

3.2 Protein expression by western blotting

3.2.1 Protein extraction from mice ileum and BCA assay

Ileum sections of approximately 100mg were cut into small pieces and homogenized by mechanical dissociation using a Potter-Elvehjem, at 4°C, in 300uL of RIPA lysis buffer [50mM Tris HCl (pH 8.0); 150mN NaCl; 1.0% NP-40; 0.5% (w/v) sodium deoxycholate (DOC); 0.1% (w/v) sodium dodecyl sulphate (SDS); 2mN ethylenediamine tetracetic acid (EDTA)], supplemented with phosphatase inhibitor cocktail tablets in EASYpacks (PhosSTOP™, Roche, USA) and of protease inhibitor cocktail tablets in EASYpacks (cOmplete™ mini, Roche, USA). After incubation on ice for 1h the lysates were sonicated and then centrifuged at 13000rpm for 15min at 4°C.

After the centrifugation, the resulting supernatant fraction (corresponding to total extract) was collected and protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Pierce Biotechnology, Rockfor, IL, USA). This method, in which reduction of Cu^{2+} to Cu^{+} by the protein in alkaline environment, allows a selective and highly sensitive colorimetric detection of Cu^{+} using a single reagent consisting of BCA. The reaction product of this assay shows a purple coloration resulting from the chelation of two BCA molecules with a Cu^{+} ion. This complex is soluble in water and exhibits strong absorbance at 562 nm, so the absorbance can be measured between 540 and 590 nm. For this assay, absorbance reading was performed at 570 nm using Gen5 Data Analysis Software.

Finally, samples were denatured with sample buffer [0.5M Tris HCl (pH 6.8); 10%(w/v) SDS; 30% (v/v) glycerol; 0.6M DTT; 0.01% (w/v) bromophenol blue] for 30 minutes at 70°C.

3.2.2 Polyacrylamide gel electrophoresis and immunodetection

For the western blot analysis, 60μg of protein were loaded per lane and separated by electrophoresis on SDS-12% polyacrylamide gel in running buffer (125mM Tris-base; 100mM glycine and 0.5% (w/v) SDS; pH 8.3), at 100 V for 15 minutes and at 140 V until the marker reaches the desired level. After electrophoresis, proteins were electro-transferred to 0.45μm polyvinylidene difluoride Amersham™ Hybond™ PVDF membranes (GE Healthcare, USA), in transfer buffer (100mM CAPS; pH 11) during 90 minutes at 320 A and 4°C. After transfer, in order to avoid non-specific bonds, membranes were blocked with a 5% (w/v) non-fat milk in phosphate buffered saline (TBS – 1.37M NaCl, 27mM KCl, 18mM KH_2PO_4, 100mM $Na_2HPO_4 \cdot 2H_2O$ – containing 0.1% (v/v) Tween-20 (TBS-T)) for 1 hour with agitation, at room temperature. Membranes were then incubated with primary antibodies (Table 1), diluted in TBS-T/5% non-fat milk, overnight at 4°C.

Membranes were washed every 20 minutes with PBS-T for 1 hour and then incubated with adequate secondary antibody (Table 1) with agitation for 1 hour at room temperature. After secondary antibody incubation, membranes were washed again every 20 minutes with PBS-T for 1 hour. In the end of this procedure, the intensity of the bands was detected by chemifluorescence enhancer substrate (ECF) (GE Healthcare Life Sciences, USA) in the Fluorescence Image Analyzer Typhoon FLA 900 detector (GE Healthcare Bio-Sciences).

To confirm equal protein loading and sample transfer, membranes were re-incubated with mouse anti-GAPDH or mouse anti-tubulin antibodies. In order to normalize data,

GAPDH and tubulin were used as loading proteins. The optical density of the bands was quantified by densitometry, using the Image Quant 5.0 Software.

Table 1: Primary and secondary antibodies used for Western Blotting analysis.

Antibody	***Molecular Weight (kDa)***	***Dilution***	***Company***
Rabbit Polyclonal Anti-GLP-1R	53	1:200	Bioss Antibodies (bs-1559R)
Rat Monoclonal Anti-MBP	23	1:100	Novus Biologicals (NB600-717)
Mouse Monoclonal Anti-GAPDH	38	1:1000	MilliPore (MAB374)
Mouse Monoclonal Anti-α-tubulin	52	1:1000	Cell Signaling Technology (2125)
Goat Anti-Rabbit	-	1:5000	GE Healthcare (NIF1317)
Goat Anti-Rat	-	1:5000	Abcam (ab97054)
Goat Anti-Mouse	-	1:5000	Sigma-Aldrich (A3562)

3.3 Data processing and statistical analysis

To determine the statistical differences between all the study groups, ANOVA was used followed by Tukey's multiple comparison test. Values of $p < 0.05$ were considered to indicate significant differences. Results were presented as mean ± standard error of mean (SEM) of the number of animals (n). All statistical analyses were performed using *GraphPad Prism* PC Software and IBM SPSS Statistics Software.

CHAPTER IV

RESULTS

1 Characterization of the animal model of MS

1.1 Demographic and biochemical profile

To characterize the animal model of MS induced by cuprizone intoxication, demographic and biochemical profiles were evaluated, including body weight, water and food intake, post prandial *ad libitum* glycemia and thymus weight.

Concerning the body weight, as expected, the animal group at five weeks of CPZ treatment (CPZW5) and its respective control group (CTRLW5) are the ones who gained less weight, presenting a low percentage of weight gain, measured in comparison with the baseline values. Regarding the groups submitted to the seven-week experience, the five weeks cuprizone (CPZW7) group and five weeks cuprizone plus Sitagliptin during the last two weeks [CPZ+Sita (T)] were the groups that presented a low percentage of weight gain compared to CTRLW7 and CPZ+Sita (P), although without statistical difference. CTRLW7 and CPZ+Sita (P) groups presented a higher statistically significant difference than CPZW7, when compared to CPZW5. Nevertheless, this suggests a body weight gain in groups where CPZ was removed from the diet in the last two weeks of the experiment (Figure15).

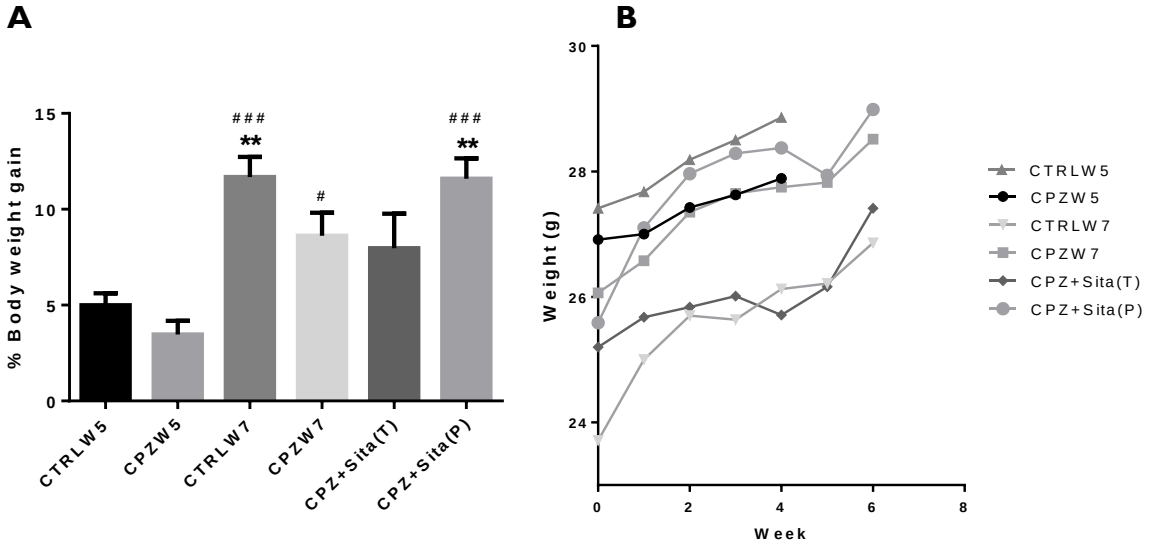

Figure 15: Percentage of body weight gain (A) and evolution of body weight during experiment (B) from all animal groups. Results are expressed as mean ± S.E.M of 8 animals *per* group. Statistical differences were evaluated by ANOVA and Turkey's multiple comparison test. ** p <0.01 vs CTRLW5; # p <0.05 and ### p <0.005 vs CPZW5.

In terms of water and food intake during the experiment (Figure 16-A and 16-B), only the food intake presented a significant reduction in CTRLW7 and CPZ+Sita (T) groups compared to CPZW7 and CPZ+Sita (P) ($p<0.01$). Regarding the glycemia values (Figure 16-C), that were measured three times on the 5-week experiment groups (at the beginning, at 3rd week and at the final) and four times on the 7-week experiment groups (at the beginning, at 3rd week, at 5th week and at the final), there were no significant differences between the groups.

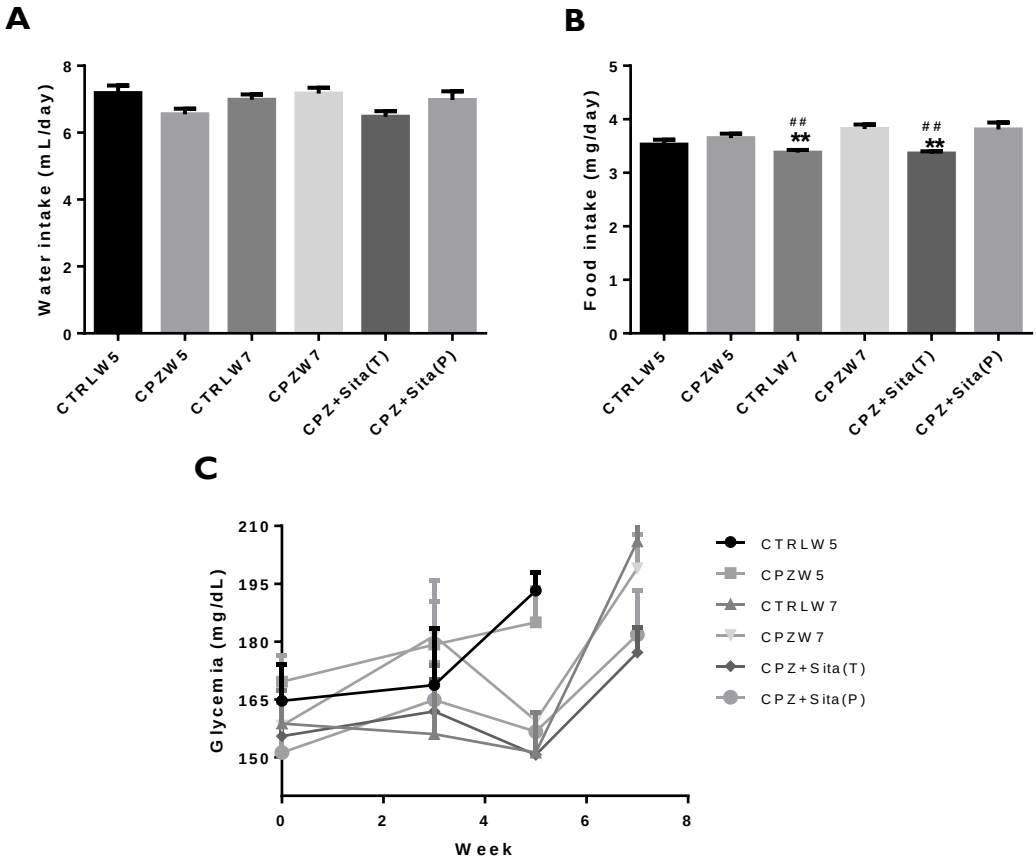

Figure 16: Demographic and biochemical profiles data of the animal model of MS. A – Water intake expressed in mL/day; B – Food intake expressed in mg/day; C – Post prandial *ad libitum* glycemia values of all animal groups at the beginning of CPZ treatment, at 3rd week, at 5th week and at the final of the experiment. Results are expressed as mean ± S.E.M. of 8 animals *per* group. Statistical differences were evaluated by ANOVA and Turkey's multiple comparison test. ** p <0.01 vs CPZW7; ## p <0.01 vs CPZ+Sita (P).

Lastly, there was differences of thymus weight between some groups, which is in line with the fact that MS is an autoimmune disease (Figure 17). In fact, a statistically significant difference was observed between the CPZW7 and the control groups at week 5 and 7 ($p <0.05$ and $p <0.005$, respectively), suggesting the development of thymus atrophy in the animals of treated with CPZ during 7 weeks.

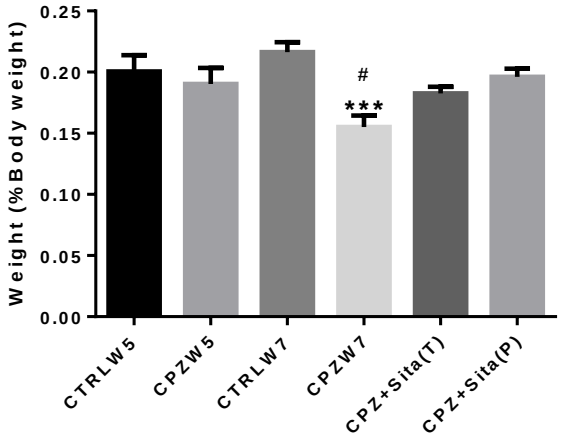

Figure 17: Thymus weight of all animal groups expressed in percentage of body weight. Results are expressed as mean ± S.E.M. of 8 animals *per* group. Statistical differences were evaluated by ANOVA and Turkey's multiple comparison test. *** p <0.005 vs CTRLW7 and # p <0.05 vs CTRLW7.

1.2 Serum DPP-IV enzymatic activity

The serum DPP-IV activity was quantified to confirm the inhibitory effect of Sitagliptin. As expected, it is possible to observe a statistically significant lower DPP-IV activity in the groups treated with Sitagliptin, when compared to controls (CTRL) and to the non-treated groups CPZW5 and CPZW7, thus confirming the efficacy of Sitagliptin administration and treatment (Figure 18).

Since both control groups (CTRLW5 and CTRLW7) had no differences in terms of treatment during the entire experiment other than age, they will be presented together from now on.

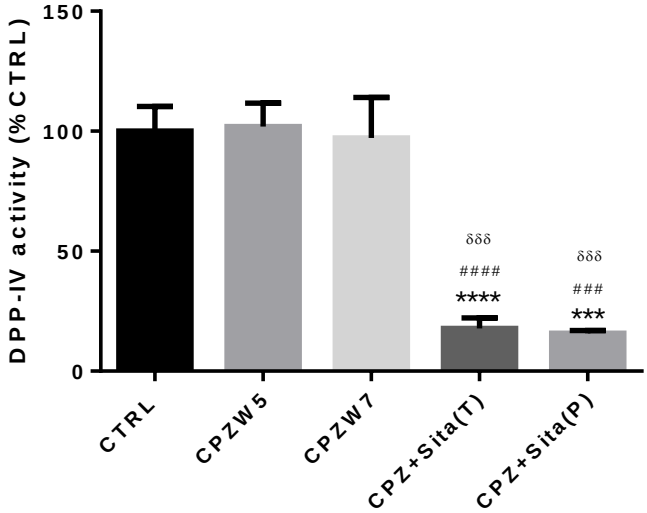

Figure 18: Serum DPP-IV activity in the animal groups under evaluation. Results are expressed as mean ± S.E.M. of 6-8 animals *per* group. Statistical differences were evaluated by ANOVA and Turkey's multiple comparison test. *** p <0.005 and ****p <0.001 vs CTRL; ### p <0.005 and ##### p <0.001 vs CPZW5; δδδ p <0.005 vs CPZW7.

1.3 Effect of CPZ treatment in the MBP ileal levels

To confirm the induction of MS by using CPZ administration, the levels of MBP were evaluated by Western Blotting in ileum cell lysates from all animal groups (Figure 19). The results showed a statistically significant decrease in MBP levels in CPZW5 animals compared to the control group (CPZW5 = 65.50±5.68% vs CTRL = 100.00±5.48%; p <0.01) and a partial recovery of MBP levels in the other three groups of animals, where CPZ treatment was suspended in the last two weeks of the experiment allowing spontaneous remyelination potentiated by Sitagliptin, as expected.

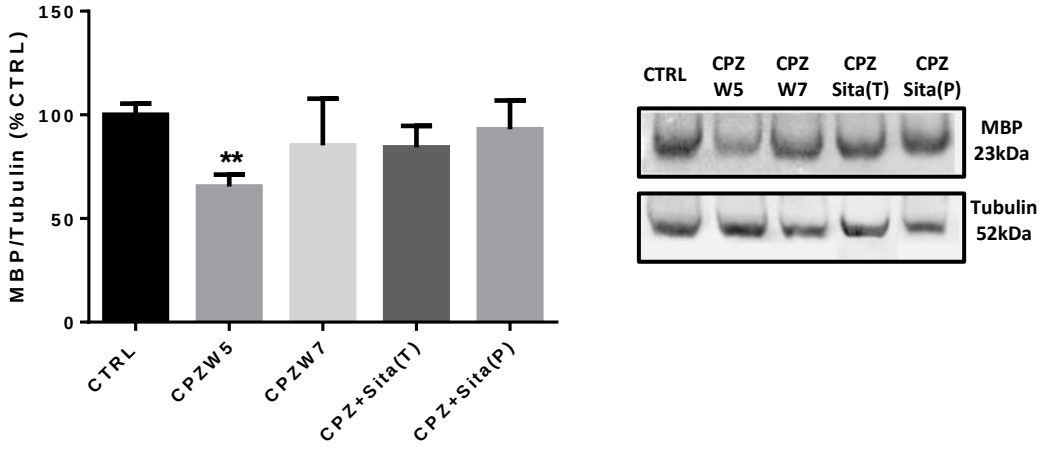

Figure 19: Evaluation of MBP levels in total ileum cell lysates by Western Blotting. Results are expressed as mean ± S.E.M. of 4 animals *per* group. Statistical differences were evaluated by ANOVA and Turkey's multiple comparison test. ** p <0.01 vs CTRL.

2 Evaluation and characterization of the contractile response of ileum segments isolated from the CPZ-induced MS animal model submitted to Sitagliptin treatment

2.1 Contractile response to acetylcholine

Firstly, to directly induce the maximum contraction of ileum smooth muscle, all segments were submitted to 100μM of exogenous ACh. Normally, ACh is used as an external standard to normalize data, namely if the contraction to this excitatory neurotransmitter is not altered.

Then, to understand if the results obtained for the different groups could be compared according to this, the ACh-induced contraction was analyzed for all groups. Nevertheless, the results showed statistically significant differences in ACh maximum contraction (Emax), with CPZ+Sita (T) group presenting a higher contractile response when compared to CTRL, CPZW5 and CPZW7 groups ($p < 0.01$ for all). The CPZ+Sita (P) group showed a tendency ($p > 0.01$) for increasing contraction in response to ACh (Figure 20). Considering these results, all data from functional studies will be presented from now on in absolute values of tension (mN) and not in percentage of ACh-induced contraction.

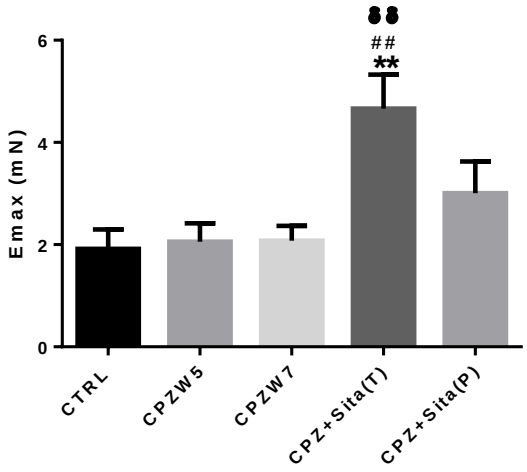

Figure 20: Maximum contractile response to ACh of mice isolated ileum with cuprizone-induced MS, expressed in mN of tension. Emax = maximum contraction in mN of tension. Results are expressed as mean ± S.E.M. of 13-16 segments of 4 animals *per* group. Statistical differences were evaluated by ANOVA and Turkey's multiple comparison test. ** $p < 0.01$ vs CTRL, ## $p < 0.01$ vs CPZW5 and δδ $p < 0.01$ vs CPZW7.

2.2 Contractile response to electrical field stimulation

Regarding functional studies performed on ileum segments isolated from all animal groups under evaluation, it was possible to observe a frequency-dependent contractile response to electrical field stimulation, within the range of frequencies used (1Hz-16Hz) (Figure 21-A).

Concerning the maximum contraction induced by electrical field stimulation, the CPZ+Sita (T) group presented a significant higher Emax compared to CPZW5 (Emax [CPZ+Sita (T)] = 1.19±0.24 mN, s/n = 15/4 vs Emax [CPZW5] = 0.46±0.08 mN, s/n = 13/4, $p < 0.05$). Furthermore, although there were no statistically significant differences, the CPZW5 group showed a trend towards a decrease in contraction in relation to the control group (Emax [CTRL] = 0.63±0.13 mN, s/n = 13/4, $p > 0.05$), which appears to indicate a possible

impairment of intestinal function in this group, not encountered in the CPZW7 group, in which the CPZ treatment was suspended after five weeks allowing remyelination. Additionally, CPZ+Sita (P) group also showed a trend to higher contraction than CPZW5 (Figure 21-B).

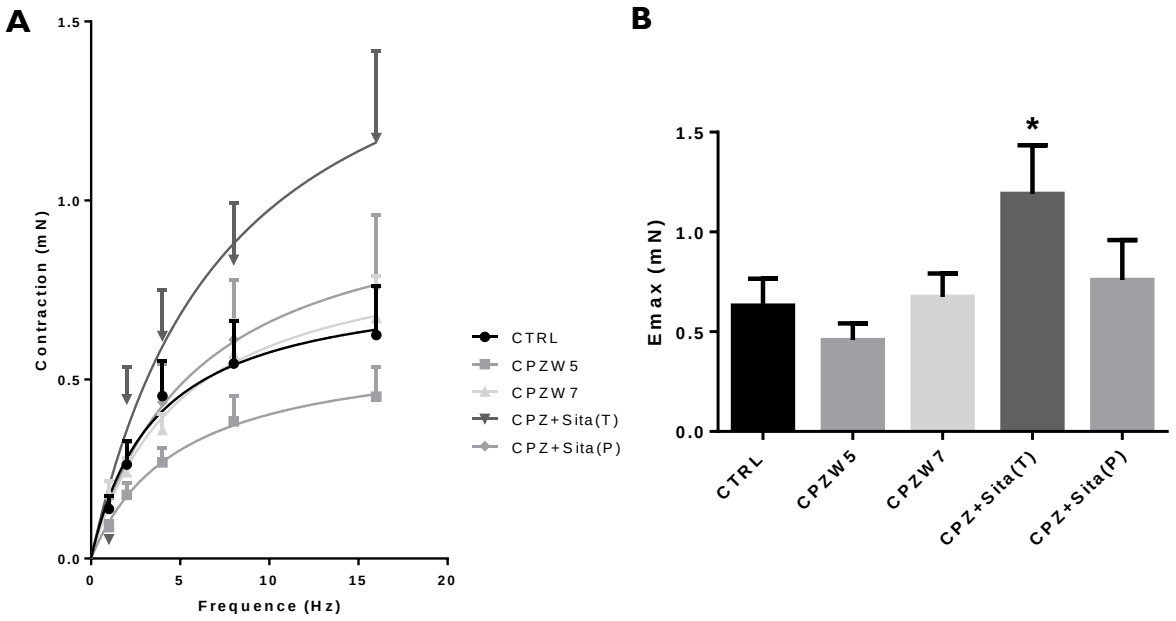

Figure 21: Frequency-response curves (A) and maximum responses (Emax) (B) to electrical field stimulation of isolated ileum of mice with cuprizone-induced MS. Emax = maximum contraction in mN of tension. Results are expressed as mean ± S.E.M. of 13-15 segments of 4 animals *per* group. Statistical differences were evaluated by ANOVA and Turkey's multiple comparison test. * p <0.05 vs CPZW5.

2.3 Effect of Exendin-3 on contractile response to electrical field stimulation

To determine the influence of the incretin GLP-1 on the contractile response to electrical field stimulation of the ileum isolated from both CPZ animal groups submitted to the treatment with Sitagliptin, FR curves were performed in the presence and in the absence of 100nM of Exendin-3, a selective antagonist of GLP-1R (K_D of 1.7nM at cloned human GLP-1R). As expected, since it has been described that GLP-1R mediates an inhibitory response on GI motility (Marathe *et al.*, 2011), the presence of the antagonist led to a significant increase (p <0.05) in the contractile response on both groups (Figure 22) (Emax [Sita(T)] = 100.0±0.00%, s/n=7/4 vs Emax [Sita(T)+Ex3] = 146.62±49.40%, s/n=8/4; Emax [Sita(P)] = 100.0±0.00%, s/n = 7/4 vs Emax [Sita(P)+Ex3] = 146.27±15.46%, s/n=8/4), strongly suggesting that this receptor has a major role on relaxation in conditions of DPP-IV inhibition.

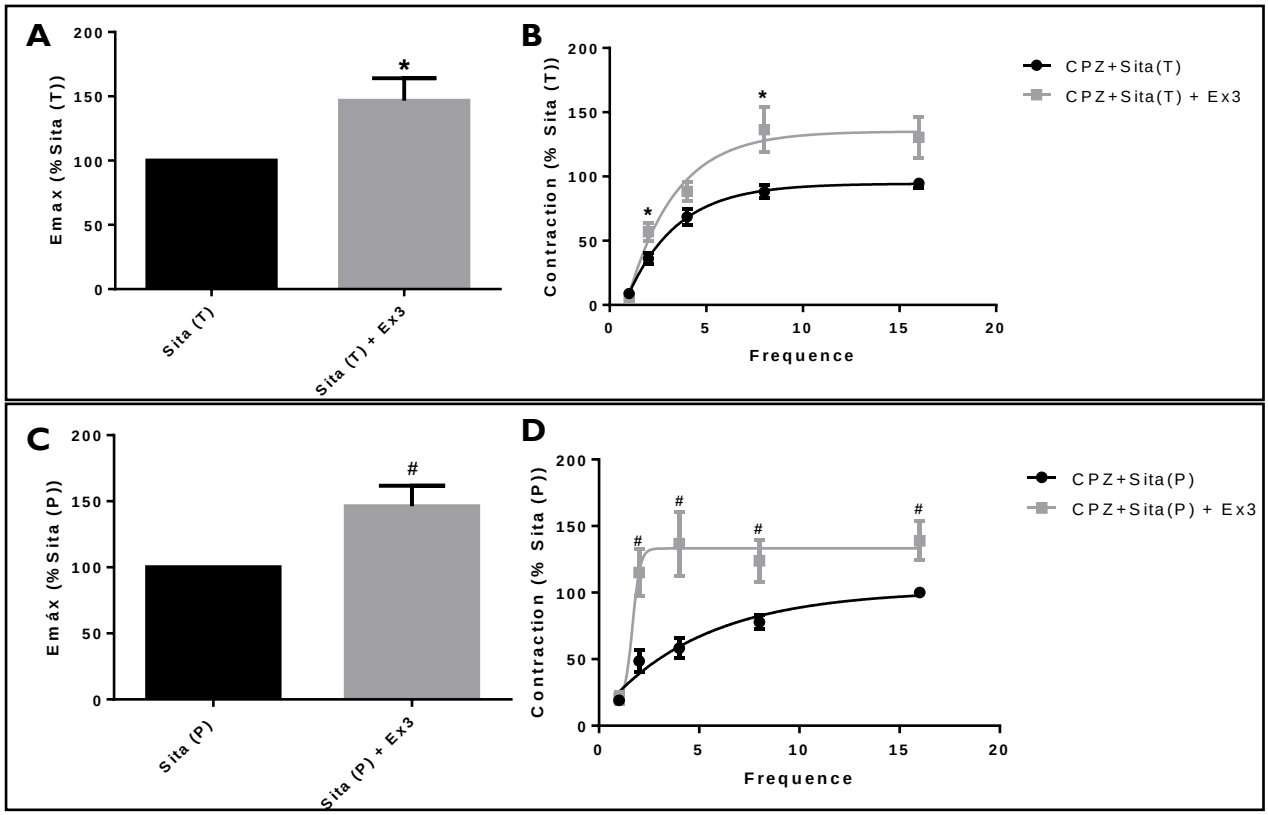

Figure 22: Maximum responses (Emax) (A and C) and frequency-contractile response curves (B and D) to electrical field stimulation of ileum isolated from both CPZ groups treated with Sitagliptin, in the presence and in the absence of 100nM of Exendin-3. Emax = maximum contraction in mN of tension. Results are expressed as mean ± S.E.M. of 13-15 segments of 4 animals *per* group. Statistical differences were evaluated by Student's *t* test. * p <0.05 vs Sita(T) # p <0.05 vs Sita(P).

3 Analysis of GLP-1R density in ileum segments isolated from the CPZ-induced MS animal model

Regarding the levels of GLP-1R in the isolated ileum from all groups of animals under study, the results showed, compared to CPZW5, a statistically significant increase of GLP-1R levels in the CPZ+Sita(P) group, that received Sitagliptin during the entire experiment as preventive therapy (CPZ+Sita (P) = 141.86±24.56% vs CPZW5 = 69.32±16.88%; p <0.05). Additionally, the CPZW5 also showed a trend to decreased levels of GLP-1R (p=0.059) when compared to the control group (Figure 23).

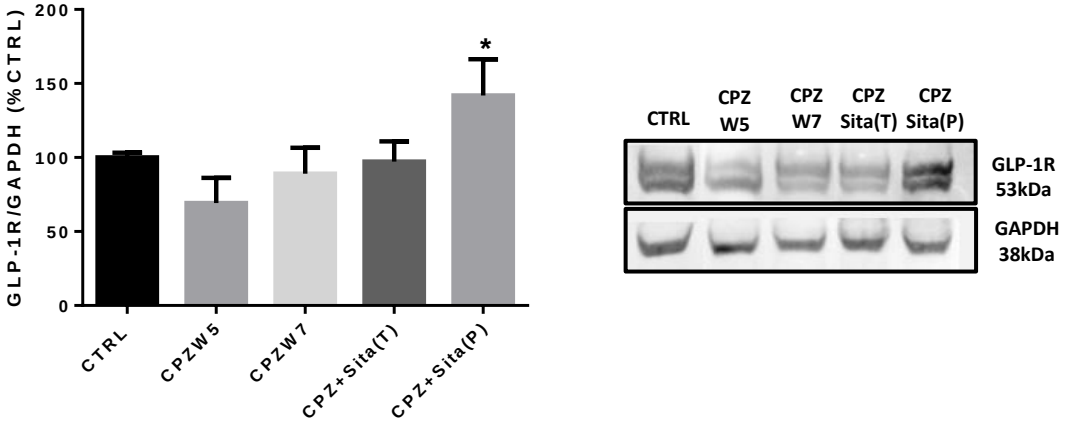

Figure 23: Evaluation of GLP-1R levels in total ileum cell lysates by Western Blotting. Results are expressed as mean ± S.E.M. of 4 animals *per* group. Statistical differences were evaluated by ANOVA and Turkey's multiple comparison test. * p <0.01 vs CPZW5.

4 Pharmacological characterization of the contractile response of ileum segments isolated from the CPZ-induced MS animal model

To evaluate the exact role of GLP-1 and NPY, another DPP-IV substrate, in intestinal motility in the presence of the disease, it is necessary to pharmacologically characterize the contractile response of the ileum isolated from the CPZ animal model by using selective antagonists of GLP-1R and NPY1R.

4.1 Demographic and biochemical profile

Concerning the weight gain (Figure 24, Graphics A and B), the results showed statistically significant differences between control and CPZ groups ($p < 0.05$), demonstrating a low percentage of weight gain in the CPZ group compared to the control one. In terms of water and food intake (Figure 24, Graphics C and F, respectively) during the 5-week experiment, only water intake presented a significant reduction in the CPZ group compare to the control group ($p < 0.001$). Regarding glycemia values (Figure 24, Graphic D), measure three times during treatment (at the beginning, at the 3rd week and at the 5th week), there were no significant differences between control and CPZ-treated animals. Lastly, in terms of thymus

weight (Figure 24, Graphic E), it is possible to verify a statistically significant difference between the two animal groups ($p < 0.001$), since CPZ-treated animals presented again an atrophy of thymus when compared to the control ones.

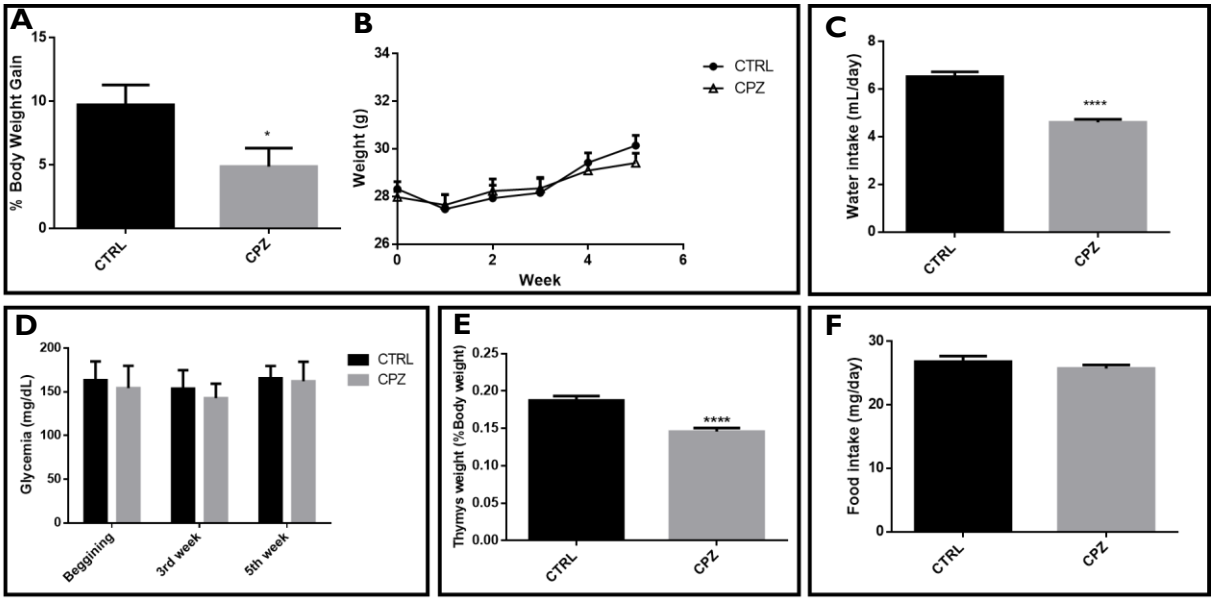

Figure 24: Demographic and biochemical profile data of the CPZ-induced MS animal model. A – Percentage of weight gain of the CPZ animal group and its control group (CTRL); B – Variation of weight, during a 5-week treatment; C – Water intake expressed by mL/day; D – Post prandial *ad libitum* glycemia values of both animal groups at the beginning of the treatment, at the 3rd week and at the 5th week (final of the treatment); E – Thymus weight expressed in percentage of body weight; F – Food intake expressed by mg/day. CTRL – control animals; CPZ – cuprizone-submitted animals. Results are expressed as mean ± S.E.M of 9-10 animals *per* group. Statistical differences were evaluated by Student's *t* test. *p <0.05 vs CTRL; ****p <0.001 vs CTRL.

4.2 Contractile response to electrical field stimulation

Regarding functional studies performed on the isolated ileum of both animal groups, it was possible to observe a frequency-dependent contractile response, within the range of frequencies used (Figure 25-A).

Concerning the maximum contraction (Emax), a significant low Emax was observed in the CPZ group when compare to the CTRL one (Emax [CPZ] = 0.553±0.0496 mN, s/n = 31/9 vs Emax [CTRL] = 0.782±0.105 mN, s/n = 30/9; $p < 0.05$) (Figure 24-B).

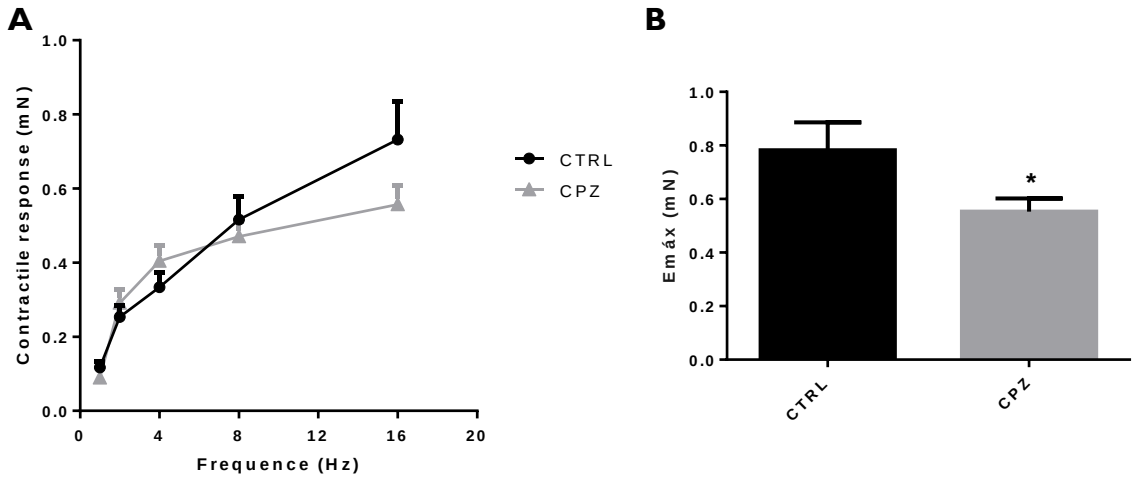

Figure 25: Frequency-contractile response curves (A) and maximum contraction values (B) to electrical field stimulation of the ileum isolated from control animals and CPZ-submitted animals. Emax = maximum contraction in mN of tension; CTRL – control animals; CPZ – cuprizone-submitted animals. Results are expressed as mean ± S.E.M. of 30-31 segments of 9 animals *per* group. Statistical differences were evaluated by Student's *t* test. * p <0.05 vs CTRL.

4.3 Effect of Exendin-3 and BVD-10 on the contractile response to electrical field stimulation

In order to pharmacologically characterize the receptor mediating the isolated ileum contractile response to electrical field stimulation in both control and CPZ-treated animals, FR curves were performed in the presence and in the absence of Exendin-3 (100nM), a selective antagonist of GLP-1R (K_D of 1.7nM at cloned human GLP-1R) or BVD-10 (100nM), a selective antagonist of NPY1R (pKi of 25.7nM at Y1R).

Concerning the Exendin-3, no significant effect was observed in the two groups (Emax [CTRL] = 100±0.00%, s/n = 7/4 vs Emax [CTRL+Ex3] = 93.08±7.219%, s/n = 8/4; Emax [CPZ] = 100±0.00%, s/n = 8/4 vs Emax [CPZ+Ex3] = 103.71±8.360%, s/n = 8/4) (Figure 26).

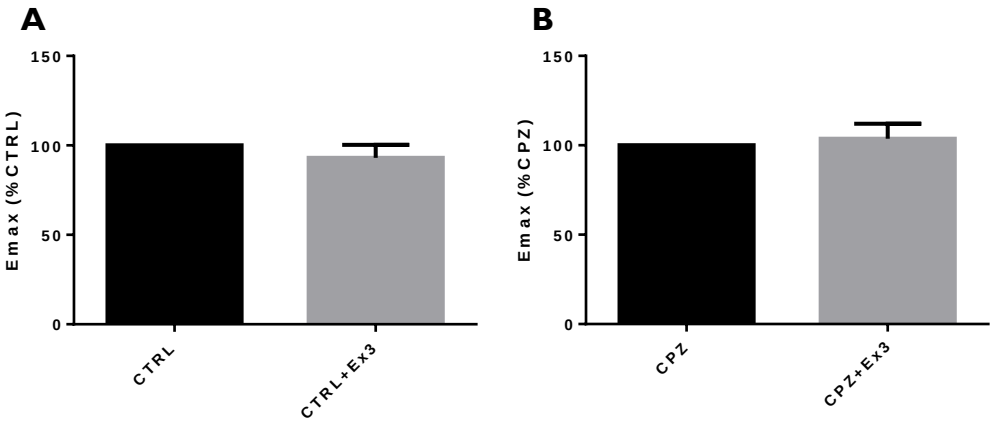

Figure 26. Maximum responses (Emax) of isolated ileum in the absence and presence of 100nM of Exendin-3, in both control and CPZ groups (A and B, respectively). Emax = maximum contraction in mN of tension; CTRL – control animals; CPZ – cuprizone-submitted animals. Results are expressed as mean ± S.E.M. of 7-8 segments of 4 animals *per* group.

Regarding the effect of BVD-10, it was possible to note a significant increase in the CPZ group maximum response to electrical field stimulation (Emax [CPZ+BVD10] = 148.8±15.29%, s/n = 10/5 vs Emax [CPZ] = 100±0.00%, s/n = 9/5; $p < 0.05$) (Figure 27-B). Notwithstanding, in the control group no alterations in the maximum response were observed in the presence of this antagonist (Figure 27-A), suggesting that YIR is able to mediate relaxation only in the presence of the disease.

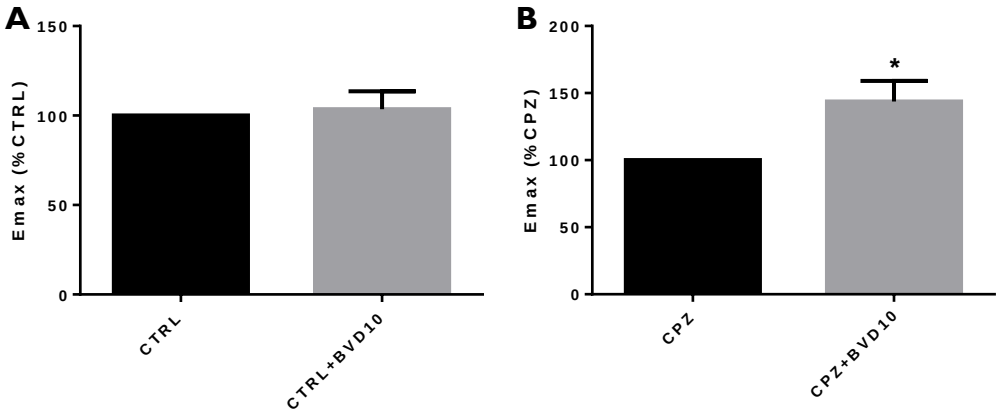

Figure 27. Maximum responses (Emax) of isolated ileum in the absence and presence of 100nM of BVD-10, in both control and CPZ groups (A and B, respectively). Emax = maximum contraction in mN of tension; CTRL – control animals; CPZ – cuprizone-submitted animals. Results are expressed as mean ± S.E.M. of 8-10 segments of 5 animals *per* group. Statistical differences were evaluated by Student's *t* test. * $p < 0.05$ vs CPZ.

CHAPTER V

DISCUSSION

Discussion

MS is the most prevalent chronic inflammatory disease of the CNS in which focal lymphocyte infiltration leads to damage of myelin and axons. Until today, the precise cause of MS is still unknown, but it appears to be triggered by a combination of genetic susceptibility and environmental factors. In the early stages of the disease, inflammation and neurologic disability are transient, lasting days or weeks, and remyelination occurs. In this stage, several patterns of lesions are observed, including immune-mediated inflammatory lesions (patterns I and II) and primary oligodendrogliopathy-associated lesions (patterns III and IV). However, after 10 to 20 years, many patients develop a progressive clinical course characterized by an extensive and chronic neurodegeneration, which eventually leads to impaired mobility and cognition (Compston and Coles, 2008; Popescu and Lucchinetti, 2012; Reich, Lucchinetti and Calabresi, 2018). In addition to central neurodegeneration, patients with MS also develop dysregulation of the ANS which results in various clinical symptoms, inducing GI dysfunction. In fact, this type of dysfunction affects approximately 70% of MS patients, with constipation being the most frequently reported GI symptom, followed by fecal incontinence (Pintér *et al.*, 2015; Wiesel *et al.*, 2001).

In terms of pharmacological treatment for MS, the several DMTs that have been developed in the past years have shown beneficial effects in patients with RRMS, including shortening the duration of acute exacerbations, decreasing their frequency and providing symptomatic relief. However, no curative therapies for MS are currently available and the most severe forms of the disease (progressive forms) are still practically untreatable (Goldenberg, 2012). Taking this into account and considering that human CNS can only be monitored by non-invasive techniques, the use of animal models have been essential to explore mechanisms of disease initiation and progression, as well as to test several novel therapeutical approaches for the disease (Procaccini *et al.*, 2015).

The most-studied animal model of MS is EAE that is characterized by an auto-immune reaction against the myelin proteins in the CNS. Despite its remarkably contribute to the knowledge of immune and inflammatory components of the disease allowing the development of novel therapeutic approaches, EAE model presents a great inflammatory background which complicates the study of other important components of the disease, including MS progression and neurodegeneration (Ransohoff, 2012). Nevertheless, toxic models are extensively used to study mechanisms of de- and re-myelination and neuronal repair which can help ameliorating or precluding progressive forms of MS. One of these models is the CPZ based model, a copper

chelating agent which, in addition to a normal rodent diet, causes mitochondrial dysfunction that leads to oligodendroglial cell death and subsequent demyelination, as well as profound activation of astrocytes and microglia (Procaccini *et al.*, 2015). This model provides insights into the determinants of oligodendrocytes cell death, since CPZ lesions are similar to pattern III lesions in human MS. Additionally, after the withdrawal of CPZ, spontaneous remyelination occurs, which makes this a good model to study mechanisms of damage and repair of myelin sheath (Ransohoff, 2012). Moreover, this is also a good model to support studies with new drugs that are capable of promoting neuroprotection, neurogenesis and neuronal development, and which also have anti-inflammatory effects. A good example of a drug class that fits these beneficial effects for the treatment of MS is the incretin-based therapy.

Incretin-based therapies, including GLP-1 agonists and DPP-IV inhibitors, are a drug class currently used for the treatment of diabetic patients. However, due to their proliferative, neuroprotective and anti-inflammatory properties, several studies have been performed using these drugs as a possible treatment of neurodegenerative diseases, namely MS (Al-Badri *et al.*, 2018). Incretin-based therapies, namely DPP-IV inhibitors, by increasing the bioavailability of their substrates GLP-1 and NPY, besides their neuroprotective effects at central level and metabolic effects at peripheral level, can have beneficial effects at other peripheral sites and on other symptoms presented by MS patients, namely on those related with the GI tract. In fact, GLP-1 and NPY have been described as having inhibitory effects in the GI motility being able to counteract some of the GI symptoms associated to the disease (Holzer, Reichmann and Farzi, 2012; Marathe *et al.*, 2011).

Based on this framework, the main aim of this study was to evaluate the effects of Sitagliptin treatment, a DPP-IV inhibitor, in the intestinal motility of an animal model of MS induced by CPZ intoxication, using the ileum as the target region, since this is one of the most pharmacologically active regions of the GI system. To achieve this objective, the contractile response to electrical field stimulation of ileum segments isolated from this CPZ-induced MS animal model was evaluated.

Firstly, demographic and biochemical profile data of the C57BL/6 mice groups under study was evaluate in order to confirm and characterize the animal model.

Concerning the body weight, both animal groups submitted to the five-week experiment were the ones who gained less weight, as expected. The control group and the group submitted to a preventive treatment with Sitagliptin [CPZ+Sita (P)], were the groups with a significant weight gain compared to CPZW5, among the seven-week experiment groups. Besides suggesting a body weight gain in the groups where CPZ was removed from the diet

in the last two weeks of the experiment, these data suggest an impairment on body weight gain in both animal groups submitted to CPZ intoxication without any additional treatment, which was already been described in the literature, since mice administered with CPZ tended to gain less weight (Torkildsen *et al.*, 2008).

Regarding water and food intake, both were evaluated as indicators of animal welfare. No statistical differences were observed in terms of water intake in the set of animals submitted to oral Sitagliptin administered in a jelly vehicle; however, in terms of food intake, statistically significant differences were observed. Indeed, CTRLW7 and CPZ group treated with Sitagliptin in the last two weeks of the experiments [CPZ+Sita(T)] ingested less food than the other two groups with the seven-week experiment. Despite of being significant, the ingestion of food did not change drastically in absolute values which allowed us to confirm, together with the water intake results, that the animal welfare was maintained.

Since Sitagliptin is an antidiabetic drug that interferes with glucose metabolism, glycemia values of the animals under experiment were also monitored. No differences were observed in the glycemia levels, which allowed us to prove that neither CPZ nor Sitagliptin altered glucose homeostasis. In fact, this was an expected result since Sitagliptin reduces blood glucose levels by a glucose-dependent mechanism which confers to this drug a very low risk of hypoglycemia (Scott, 2017). However, it was possible to observe a trend for higher values of glycemia in the final points of the treatment period (at week 5 for CTRLW5 and CPZW5 groups and at week 7 for the other four groups). This can be explained by the fact that these glycemia values were obtained before the animal sacrifice, right after the anesthetic administration. At this time point, the animals were subject to a high level of stress, which led to the increase of hormones such epinephrine and cortisol, both hyperglycemic (Marik and Bellomo, 2013).

In order to confirm the development of MS by CPZ intoxication, thymus was isolated from the animals and weighed just after sacrifice. Statistically significant differences were observed in the thymus weight of the CPZW7 group' animals, that presented a lighter thymus compared to both control groups. Although CPZ intoxication is not associated with great levels of inflammation (like the EAE animal model, for example), by mimicking the pathogenesis of MS due to cell death of oligodendrocytes, CPZ administration is also associated with increased levels of inflammatory cytokines, like IL-1β and TNF-α (Henriques, 2016), which in turn can promote thymus atrophy. Indeed, this was also described by Solti and collaborators, that observed a thymus atrophy in the CPZ mouse model (Solti *et al.*, 2015).

As previous discussed, DPP-IV enzyme is a serine protease widely distributed and enzymatically active in both membrane-bound and circulating forms that can be inactivated by

a specific class of drugs, the DPP-IV inhibitors (Mulvihill and Drucker, 2014). Belonging to this class of drugs, Sitagliptin was administrated to our animal model with the aim to increase the levels of GLP-1 and NPY, which are DPP-IV substrates that present anti-inflammatory, proliferative and neuroprotective properties. To confirm the effect of Sitagliptin, an assay was performed to evaluate serum DPP-IV activity in the animals under treatment. As expected, similar to what has been already described (Bhansali *et al.*, 2010), DPP-IV activity decreased approximately 80% on both animal groups submitted to Sitagliptin treatment , which confirms the inactivity of the enzyme and the anticipated consequent increased levels of GLP-1 and NPY.

In addition to the demographic and biochemical profile, there was also the need to molecularly confirm the CPZ-induced MS animal model, and evaluate if CPZ affected the ENS. Since this model induces demyelination, a good way to confirm this effect is to evaluate the levels of a myelin protein, such as MBP, what we have done in this study. Specifically, we evaluated this myelin protein because, according to previously described by Griffiths and collaborators, in PNS the most abundant myelin protein is the MBP, with PLP and MOG presenting lower levels in PNS than in CNS (Griffiths *et al.*, 1989). Analyzing the ileal levels of MBP by Western Blotting, it was possible to confirm in the ENS both the demyelination induced by CPZ administration and the demyelination. In fact, MBP ileal levels were significantly lower in the CPZW5 group, when compared to the control group. Additionally, MBP ileal levels partially recovered in the groups where CPZ was removed from the diet in the last two weeks of the experiment, confirming that in these groups spontaneous remyelination occurred, eventually potentiated by Sitagliptin treatment.

Taking into account that the main objective of this study was to evaluate the effect of Sitagliptin on the GI motility in this CPZ-induced MS animal model, functional studies were performed in the ileum segments isolated from all animal groups. Electrical field stimulation was used as a tool for the activation of the ileum segments intrinsic innervation, since it induces neuronal depolarization which, in turn, stimulates the release of neurotransmitters.

Firstly, in order to evaluate if ACh could be a good external standard to normalize data, all ileum segments were submitted to 100μM of exogenous ACh to directly induce the maximum contraction of ileum smooth muscle. However, statistically significant differences were observed among groups, with CPZ group treated with Sitagliptin in the last two weeks of the experiment presenting a significant higher response then the control group and both CPZ groups that did not receive any treatment. In the CPZ group submitted to a preventive treatment with Sitagliptin also presented a trend to higher contractile response to ACh,

although without statistically significant differences. This can be explained by the fact that Sitagliptin may alter the response to ACh, since in previous studies conducted in our research group the response to ACh improved in aorta isolated from an animal model of type 1 DM treated with Sitagliptin, being in agreement with an improvement of the endothelium (Franco, 2014). Thus, the results of functional studies could not be expressed in terms of percentage of ACh response as it could mask some important changes in the GI function.

Functional studies performed on ileum segments isolated from all animal groups under evaluation allowed to conclude that the contractile response of these segments was frequency-dependent to electrical field stimulation within the range of frequencies used. The maximum contraction induced by electrical field stimulation was analyzed in order to compare the contractile response between all experimental groups. In fact, the CPZ+Sita(T) group was the only one presenting a higher contractile response compared to the CPZW5 group. Indeed, this last group was the one who presented the lower contractile response to electrical field stimulation, suggesting an impairment on intestinal function in the group where the peak of demyelination occurred, according to MBP density levels. This result is in agreement with several reviews that reported constipation as the main GI dysfunction of MS patients (National Multiple Sclerosis Society, 2014).

Regarding the maximum contraction of the CPZ+Sita(T) group, in which Sitagliptin was administered in the last two weeks of the experiment, the elevated values can be explained by the fact that inhibition of DPP-IV leads to increased levels of their substrates. Several DPP-IV substrates are chemokines, like eotaxin, interferon-γ-inducible protein 10 (IP-10) and regulated on activation normal T-cell expressed and secreted (RANTES) (Drucker, 2007), that play an important role on inflammatory response, which in turn can increase GI motility. Additionally, substance P is also a DPP-IV substrate that is commonly co-expressed with ACh in enteric motor neurons, being these two excitatory neurotransmitters. In fact, substance P is capable of enhancing ACh contractile response, as already described by Li and collaborators (Li *et al.*, 2014), which can explain the significant higher contractile response to ACh in the same animal group.

Nevertheless, counteracting the results with Sitagliptin subacute treatment, in the preventive treatment with Sitagliptin [CPZ+Sita(P)], the contractile response to electrical field stimulation approached the control levels. This can be explained by the fact that several DPP-IV substrates, like GLP-1, NPY, PYY, among others, play an inhibitory effect of GI motility (Marathe *et al.*, 2011; Tough *et al.*, 2011). In fact, when functional studies were performed in the presence of Exendin-3, a selective antagonist of GLP-1R, it was possible to observe a statistically significant increase on the maximum contractile response of both groups submitted

to Sitagliptin, reflecting that this receptor is mediating relaxation in a significant manner under DPP-IV inhibition. As a matter of fact, PI3K/Akt/NOS signaling pathway associated to GLP-1R leads to NO production and to smooth muscle relaxation (Halim *et al.*, 2018).

Moreover, the results of functional studies are in conformity with GLP-1R density results obtained through Western Blotting. Indeed, GLP-1R density in ileum isolated from CPZ+Sita(P) group was significantly higher from the rest of the groups, including CPZ+Sita(T). Besides confirming the inhibitory effect of GLP-1R, these results reflect that this receptor adjusts its density in order to mediate relaxation and to approximate to the control situation during chronic treatment with this DPP-IV inhibitor. This compensatory effect was also described in other studies, including the one conducted by Tang and collaborators, where Sitagliptin improved aorta relaxation in an animal model of DM, which was associated with increased levels of GLP-1 (Tang *et al.*, 2016). This alteration of density in the preventive group and not in the treatment group, suggests that two weeks of treatment, during an already installed remyelination process, are not enough to induce alterations on the density of inhibitory receptors, in this case GLP-1R, thus enhancing the contractile response.

Finally, the density of GLP-1R in the CPZW5 group was slightly decreased, almost significantly ($p=0.059$), when compared to the control group. This suggests that the density of this receptor may be compromised in the demyelination phase in this animal model. This was also described by our research group in a previous study, where GLP-1R protein expression was significantly decreased at week 5 (peak of demyelination) in the cerebellum in the same animal model (Henriques, 2016). This downregulation of the receptor in the peak of demyelination and the upregulation of the receptor with the preventive treatment with Sitagliptin might be explained by compensatory mechanisms triggered by the disease and its treatment that take advantage of the anti-inflammatory and neuroprotective effects of GLP-1. In fact, this was recently described by Lee and collaborators who found that the downregulation of GLP-1R in the EAE animal model was reversed by the treatment with an GLP-1RA, which increased the anti-inflammatory and neuroprotective mechanisms linked to this receptor (Lee *et al.*, 2018).

Following this study, where it was evaluated the effect of Sitagliptin on the GI motility of the CPZ animal model, emerged the need to evaluate the exact role of GLP-1 in the intestinal motility, as well as NPY, another DPP-IV substrate, considering also the GI disorders associated with MS (particularly constipation like previously described in this discussion).

Once again, MS was induced by CPZ intoxication for five weeks in another set of animals and in order to pharmacologically characterize the contractile response to electrical field

stimulation of ileum segments isolated from the CPZ group and its control group, selective antagonist of the two peptides' receptors were used. Then, to characterize and confirm the induction of MS, demographic and biochemical profile of the two animal groups were assessed.

Through the body and thymus weight analyses it was possible to confirm the expected alterations on the metabolism and immune system of the CPZ-treated animals, compared to CTRL ones, since both parameters were significantly decreased in the CPZ group. Water and food intake were also evaluated as indicators of animal welfare. In opposition to what happened in the other set of animals, food intake didn't show any statistically significant differences between the two groups. However, regarding to water intake, CPZ-treated animals drank significantly less water than the controls, probably due to the unpleasant flavor in the water given by CPZ addition, not compensated by the ingestion of the jelly vehicle needed for Sitagliptin administration. Glycemia values in these two animal groups did not vary, similar to what happened in the other set of animals.

The performed functional studies on the isolated ileum of both animal groups of this set of experiments once again revealed a frequency-dependent contractile response and this time a significant low maximum contraction in the CPZW5 group compare to the CTRL one. Since the number of animals per group in this essentially *in vitro* study were higher than the animals per group in the one previously discussed conducive to the evaluation of Sitagliptin treatment, a statistically significant difference arose between CPZ-treated animals and controls. This difference can be explained by the demyelination induced by CPZ intoxication in the gut-brain axis neurons, or in the ENS, that lead to a failure in neuronal signaling and, consequently, to a decreased gut motility. Once more, this is in agreement with the reported constipation usually present in MS patients (National Multiple Sclerosis Society, 2014).

Concerning the pharmacological characterization of the contractile response to electrical field stimulation in this MS animal model, no significant differences were observed in FR curves performed in the presence of Exendin-3, the selective GLP-1R antagonist in any of the groups. Therefore, it is possible to conclude that GLP-1R does not have a significant contribution to ileum contractile response neither in control animals nor in animals with MS. So, this receptor, in the presence of the disease, is only significantly important as an inhibitor of GI motility when the peptide bioavailability increases after the administration of an DDP-IV inhibitor.

Since GLP-1 did not appear to interfere with the basal intestinal response to electrical field stimulation in our MS animal model, we decided to clarify the role of NPY in gut motility. For that, FR curves were performed in the presence of BVD-10, a selective Y1R antagonist. In CTRL animals, BVD-10 did not produce any effect; however, in the CPZ group, the contractile response in the presence of the antagonist was significantly higher, meaning that in the

presence of MS, NPY becomes relevant to intestinal function by increasing its contribution to the inhibitory effect on GI motility, probably contributing to the constipation reported in MS patients. Since in some studies, including the one conducted by Decressac and collaborators, it was possible to identify alterations in the levels of NPY-positive neurons in neurodegenerative diseases (Decressac and Barker, 2012), the results observed in our study suggest that, in the presence of the pathology, there may be also alterations on NPY levels or on Y1R expression, as a compensatory mechanism in terms of function, which pharmacological and molecular basis must be further investigated.

CHAPTER VI

CONCLUSION AND FUTURE PERSPECTIVES

Conclusion and future perspectives

With this study, we primarily intended to characterize the intestinal motility of an animal model of MS induced by CPZ and the effect of its treatment with Sitagliptin, a DPP-IV inhibitor, since that MS patients frequently complained about GI dysfunctions, namely constipation. We also tried to understand the pharmacological mechanisms underneath Sitagliptin effects on GI motility, evaluating GLP-1 and Y1 contributions, both DPP-IV substrates.

Firstly, all the demographic and biochemical parameters, together with MBP density of ileum segments isolated from the animal model, allowed us to characterize and confirm the MS induced by CPZ intoxication. DPP-IV activity measured in the serum also allowed us to confirm a successful administration of Sitagliptin and the consequent inhibition of DPP-IV enzyme.

Functional studies performed to evaluate the effects of demyelination induced by CPZ and remyelination promoted by Sitagliptin treatment indicated a frequency-dependent contractile response to electrical field stimulation of the ileum isolated from the different study groups. Moreover, it was possible to conclude that CPZ-submitted animals presented a decreased GI motility in the demyelination peak, which is in agreement with the elevated reports of constipation associated with MS. This impairment was more evident in the second set of animals, since the increased the number of animals per group, allowed us to attain statistical significance.

On the other hand, the treatment with Sitagliptin during remyelination significantly increased the contractile response. Moreover, the preventive treatment allowed to reach control levels. To this normalization, GLP-1 appears to be determinant, since GLP-1R levels measured by Western Blotting were increased in the preventive treatment group. These results were reinforced by the functional studies performed in the presence of the selective GLP-1R antagonist Exendin-3 confirming that this receptor is mediating an inhibitory effect on both groups treated with Sitagliptin. However, this receptor does not appear to contribute to the basal contractile response to electrical field stimulation in the control and CPZ animal groups not submitted to Sitagliptin. On the under hand, the presence of the selective YIR antagonist BVD-10 allowed us to conclude that NPY becomes a determinant contributor to the intestinal motility only in a disease state, exhibiting an inhibitory effect, which can be explained by a possible alteration on Y1R expression or on NPY levels that must be further investigated.

Considering all the results obtained so far, we can affirm that CPZ administration alters

the intestinal intrinsic innervation, similar to what happens in MS, being this a good animal model to study GI dysfunctions associated with the disease. Additionally, the treatment with Sitagliptin seems to reverse the detrimental effects of CPZ intoxication. So, Sitagliptin appears to be a good therapeutic option for MS not only because their anti-inflammatory and neuroprotective properties, but also by ameliorating the GI dysfunction of MS patients and, thus, contributing to improve the health-related quality of life of these patients.

In the future further investigation is needed to understand the inflammatory background associated with this animal model and if Sitagliptin treatment could ameliorate a possible increased in inflammation. Finally, in order to better understand the Sitagliptin effect on demyelination, an animal group with five weeks of CPZ intoxication and Sitagliptin treatment should be studied, also evaluating the effect on intestinal motility of other DPP-IV substrates, namely substance P, an excitatory peptide.

CHAPTER VII

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