Escola Superior
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Especialização em Farmacoterapia Aplicada

EFFECTS OF THE ORAL ANTIDIABETIC SITAGLIPTIN ON THYMIC T-CELL SUBSETS OF AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

Patrícia Isabel Figueiredo Couceiro

Coimbra, 29 de junho de 2022

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Trabalho de Projeto Original submetida à Escola Superior de Tecnologia da Saúde de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Farmácia – Especialização em Farmacoterapia Aplicada, realizado sob a orientação científica da Professora Doutora Sofia Andreia Domingues Viana e coorientação do Professor Doutor Manuel Amaro dos Santos Rosa.

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Resumo

Introdução: A Esclerose Múltipla (EM) é uma das principais causas de incapacidade neurológica em adultos jovens. A par com o contexto autoimune, os doentes com EM apresentam uma involução tímica precoce e alteração da homeostase das células T CD4 que parecem aumentar a suscetibilidade do doente à auto-reatividade. Os tratamentos disponíveis são apenas sintomáticos e não mitigam os processos centrais da doença: desmielinização e remielinização. Contudo, estudos recentes associam a inibição farmacológica do alvo CD26/DPP-IV com uma diminuição da incidência, início dos sintomas e gravidade geral da doença.

Objetivo: Dado o envolvimento do CD26/DPP-IV na maturação das células T no timo, no processo auto-reativo das células T e na inflamação autoimune, este trabalho visou avaliar o efeito de um inibidor da DPP-IV – o antidiabético oral sitagliptina – na modulação da involução tímica e respetivo benefício farmacológico num ensaio pré-clínico de EM.

Methods: Murganhos C57BL/6 adultos foram submetidos ao modelo de EM induzido por cuprizona. A sitagliptina foi administrada oralmente numa dose de 50mg/Kg de peso corporal. A caracterização da morte de células do timo (7-AAD) e subpopulações de células T (coloração extracelular CD4, CD8, CD3) foi realizada por citometria de fluxo. A expressão de genes que codificam proteínas associadas à mielina (MBB, PLP; cerebelo) foi analisada por RT-PCR. As experiências com animais receberam aprovação (# 12/2018) pelo organismo local (iCBR-FMUC) de Bem-Estar Animal (ORBEA).

Resultados: A sitagliptina promoveu a involução tímica durante a fase de desmielinização da doença, caracterizada por i) uma reversão do aumento global do ratio $CD3^{high/low}$ (sugerindo um bloqueio da maturação das células T) e ii) um fenótipo dominante de células T $CD8^{+}/CD3^{high}$. Surpreendentemente, a sitagliptina bloqueou a remielinização cerebelar espontânea induzida por suspensão da cuprizona.

Conclusões: Estes resultados permitem-nos concluir que a regeneração tímica e a remielinização central são processos que, embora simultâneos, são independentes. É improvável que uma intervenção com sitagliptina se traduza em ganhos terapêuticos neste modelo animal de EM. Estudos futuros serão necessários para avaliar o potencial dos inibidores da DPP-IV nas doenças autoimunes, permitindo revelar efeitos secundários plausíveis, que ainda carecem de esclarecimento.

Palavras-chave: Esclerose múltipla; Intoxicação por cuprizona; Involução tímica; Inibidores CD26/DPP-IV; Sitagliptina; Remielinização espontânea

Abstract

Introduction: MS is one of the leading causes of neurological disability in young adults. Besides autoimmune inflammation, MS patients display early thymic involution and perturbed naïve CD4 T-cell homeostasis that seem to increase patient's susceptibility to autoreactivity. Available treatments are only symptomatic and do not tackle the core of the disease: demyelination along with spontaneous remyelination. Notably, the pharmacological inhibition of CD26/DPP-IV was correlated with a decreased incidence, onset of symptoms and overall disease severity.

Aim: Given the involvement of CD26/DPP-IV on thymic T cell maturation, autoreactive T cells activation and autoimmune inflammation, the current work aimed to assess whether a currently approved DPP-IV inhibitor – sitagliptin (an antidiabetic drug) - would modulate thymic involution and provide pharmacological benefits in MS.

Methods: Adult C57BL/6 mice were assigned to the cuprizone-induced model of MS. Sitagliptin was orally given at a dose of 50mg/Kg BW. Flow cytometry allowed the characterization of thymocyte cell death (7-AAD) and T-cell subpopulations (CD4, CD8, CD3 extracellular staining). RT-PCR was employed to characterize the cerebellar myelin-related MBP/PLP gene expression. Animal experiments received approval (# 12/2018) by the local (iCBR-FMUC) Animal Welfare Body (ORBEA).

Results: Sitagliptin remodeled thymic involution during the demyelination phase of the disease, featured by i) an arrest in the overall increase in CD3 $^{high/low}$ ratio (suggestive of T cells maturation blockade) and ii) a dominant thymic $CD8^{+}/CD3^{high}$ T phenotype. Unexpectedly, sitagliptin blocked cuprizone-induced cerebellar remyelination.

Conclusion: From our observations it is possible to conclude that thymic regeneration and central remyelination are simultaneous, albeit independent processes. It is unlikely that sitagliptin affords protection in the cuprizone-animal model of MS. Future studies are encouraged to assess the potential of DPP-IV inhibitors repurposing in autoimmune diseases, allowing us to disclose plausible side-effects that are still lacking elucidation.

Keywords: Multiple Sclerosis; Cuprizone intoxication; Thymic involution; CD26/DPP-IV inhibitors; Sitagliptin; Spontaneous remyelination

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Abbreviations

7-AAD – 7- Aminoactinomycin D
aa – Amino Acid
ADA – Adenosine Deaminase
APCs – Antigen-Presenting Cells
ATP – Adenosine Triphosphate

BBB – Blood-Brain Barrier
BW – Body Weight

CNS – Central Nervous System
CPZ – Cuprizone
CSF – Cerebrospinal Fluid
cTECs – Cortical Thymic Epithelial Cells

DIS – Dissemination in Space
DIT – Dissemination in Time
DN – Double Negative
DP – Double Positive
DPP-IV – Dipeptidyl peptidase IV

EAE – Experimental Autoimmune Encephalomyelitis
EBNA1 – Epstein-Barr virus Nuclear Antigen 1
EBV – *Epstein-Barr* virus
ER – Endoplasmic Reticulum

FDA – Food and Drug Administration
GA – Glatiramer Acetate
GD – Gadolinium
GIP – Glucose-dependent Insulinotropic Peptide
GIRK – G-protein coupled inwardly rectifying potassium
GLP-1 – Glucagon-Like Peptide 1
GLP-1R – Glucagon-Like Peptide 1 receptor
GWAS – Genome Wide Association Studies

HLA – Human Leucocyte Antigen

IFN-γ – Interferon Gamma
IL – Interleukin

MBP – Myelin Basic Protein
MHC – Major Histocompatibility Complex

MHV – Mouse Hepatitis Virus
MOG – Myelin-Oligodendrocyte Glycoprotein
MRI – Magnetic Resonance Imaging
MS – Multiple Sclerosis
MSRV-Env – Multiple Sclerosis associated Retrovirus Envelope protein
MSSS – Multiple Sclerosis Severity Score

NA – Noradrenaline
NEDA – No Evidence of Disease Activity
NK – Natural Killer
NPY – Neuropeptide Y

OLGs – Oligodendrocytes
OPC – Oligodendrocyte Precursor Cell

PBS – Phosphate-Buffered Saline
PLP – Myelin Proteolipid Protein
PP – Pancreatic Polypeptide
PPMS – Primary-Progressive Multiple Sclerosis
PYY – Peptide YY

ROS – Reactive Oxygen Species
RNS – Reactive Nitrogen Species
RRMS – Relapsing-Remitting Multiple Sclerosis
RT-PCR – Real-time Polymerase Chain Reaction
RTEs – Naïve CD4 Recent Thymic Emigrants

SCFA – Short Chain Fatty Acid
SITA – Sitagliptin
SNPs – Single Nucleotide Polymorphisms
SPMS – Secondary-Progressive Multiple Sclerosis

T-reg – Regulatory Lymphocytes
T2DM – Type 2 Diabetes Mellitus
TCC – T Cells Clones
TCR – T cell receptor
TECs – Thymic Epithelial Cells
TH1 – T-helper 1
TMEV – Theiler's Murine Encephalomyelitis Virus
TNFα – Tumor Necrosis Factor alfa

UVR – Ultraviolet Radiation

Chapter I | **INTRODUCTION**

1. Multiple sclerosis – Epidemiology

Multiple sclerosis (MS) is the prototypic inflammatory disease of the central nervous system (CNS) that manifests progressively through brain and spinal cord dissemination in time and space, due mainly to autoimmune inflammation [1, 2]. 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 [3]. It is one of the main causes of neurological disability in young adults [1, 2]. 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 [4-7].

Although supportive treatment is available, including disability management, general symptom relief and psychiatric care, there is currently no cure for MS, and the number of MS patients has been significantly increasing over the past decades [1, 2]. The disease prevalence varies considerably, with high rates in Europe, North America, New Zealand and Australia, and low rates in Asia and Africa [8]. San Marino and Germany have the highest prevalence in the world (337 per 100,000 and 303 per 100,000, respectively), followed by the USA (288 per 100,00) (Figure 1) [8].

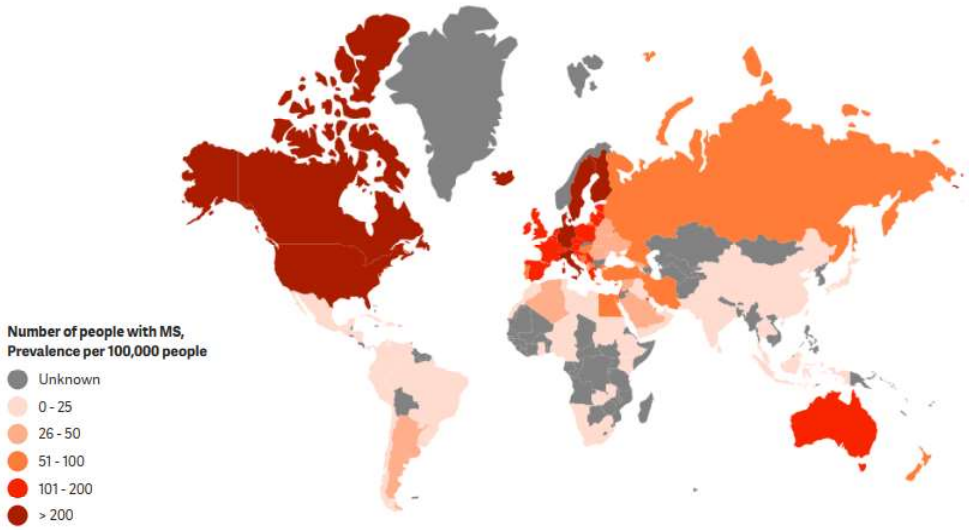


Figure 1. Number of people with MS - prevalence per 100,000 people. (Source: The Multiple Sclerosis International Federation, Atlas of MS, 3rd Edition (September 2020)).

According to the MS International Federation, the number of people with MS worldwide has increased from 2.3 million in 2013 to 2.8 million in 2020, which equates to 1 to 3,000 people in the world living with MS [8]. In fact, in countries with the highest prevalence, this numbers convert to 1 in every 300 individuals having the disease. Every five minutes, someone somewhere in the world is diagnosed with MS and its prevalence and incidence are globally increasing in a significant manner [8]. Several factors are likely to be involved in this increase, such as improved diagnosis as consequence of better access to neurologists and general healthcare, improved ability to count the number of MS patients, as well as an augmented median life expectancy for these individuals [2, 8].

Although MS incidence has been increasing generally [2, 9], this rise is more pronounced for women than for men, female patients being 2-3 times more frequent than male patients (Figure 2) [2, 9].

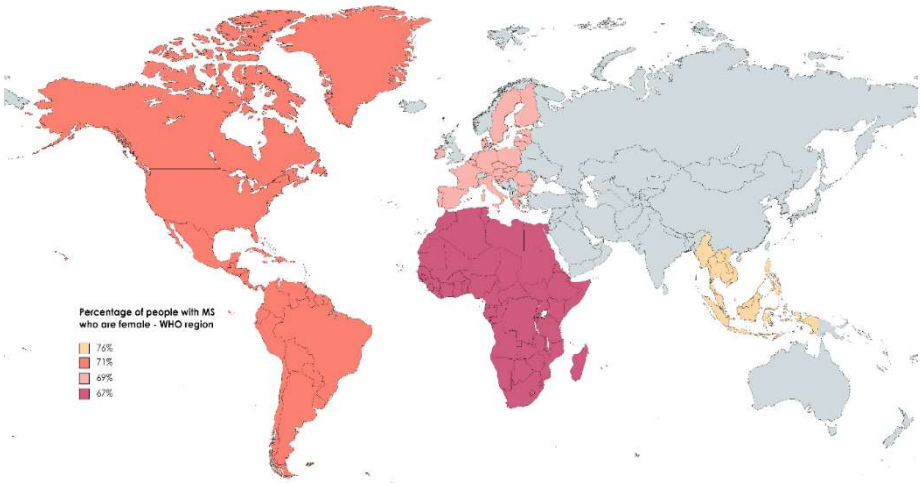


Figure 2. Percentage of people with MS who are female. (Source: The Multiple Sclerosis International Federation, Atlas of MS, 3rd Edition [8]).

The observed increased incidence of MS in women in the last decades might indicate change in women lifestyle for the past years. Factors such as late pregnancy, use of oral contraception, smoking, obesity, and stress have been listed as potential causes of the higher MS incidence in women nowadays [2]. MS incidence is also higher for individuals with Caucasian ancestry [1], but although the disease is less frequent for African Americans and Hispanics, it tends to have a more severe course when it occurs in these populations [1].

In the last decades, MS-associated mortality has been decreasing, potentially due to treatment development [10]. The current higher life expectancy as well as reduced mortality of MS patients are leading to a rise in disease prevalence and, therefore, to an increasing number of patients needing constant health care for this chronic condition.

1.1. Clinical definition

MS disease course is described by two key phenomena – relapses and progression – which might overlap in different stages of the disease [11, 12]. MS is considered to present three distinct phases, the first being the high-risk phase, the second the relapsing-remitting phase and, finally, the progressive phase, to which about 80% of patients ultimately evolve to [11, 12]. Each phase is further defined as active or inactive. Relapses are transient events of neurological disability that occur in MS patients, being either symptomatic or asymptomatic. A symptomatic relapse refers to a distinct, generally focal, acute or subacute event taking place in the CNS. In terms of pathology, both types of relapses reflect the occurrence of inflammatory demyelination, which might, or not, be associated with axonal damage and after symptomatic relapses, remission follows, which is a recovery phase characterized by some level of restoration of myelin. Most patients completely or almost completely recover after a relapse. However, in some cases there is only a partial recovery and, in rare situations, individuals never recover [12].

The disease is considered active when new symptomatic relapses or asymptomatic magnetic resonance imaging (MRI) activity exist, including enhancing hyperintense-T_1 lesions, new hyperintense-T_2 lesions and enlarged pre-existent hyperintense-T_2 lesions. On the other hand, the absence of activity for a year or more is considered "no evidence of disease activity" (NEDA), which also includes the absence of progression.

The progressive phase of MS is established one year after the clinical documentation, and can be considered active or inactive based on the presence or absence of relapses before the onset of the progressive disease course, respectively. Progressive phase onset is potentially age-dependent and might, or not, depend on disease duration.

Regarding MS clinical course, they are usually categorized in one of three types: Relapsing-Remitting MS (RRMS), Secondary Progressive MS (SPMS) and Primary Progressive MS (PPMS). Although it is considered that the different types of MS consist of a variable clinical expression of the same disease, the possibility that they reflect different diseases with dissimilar pathophysiologic mechanisms has not been ruled out [12].

RRMS is characterized by neurologic dysfunction attacks, also designated as episodes or flares, which develop acutely but could last for days and even weeks [1, 12]. After an episode, patients frequently regain their neurologic function completely in a period of time that varies from patient to patient, and between episodes neurologic function remains stable [1, 12]. RRMS patient's MRI data show evidence of acute and chronic inflammation [13].

The second type of MS – SPMS – initiates as a typical RRMS but, at a certain time point, the disease course is shifted, the acute episodes become less frequent and a steady deterioration of the neurological function occurs, independently of the existence of attacks [11, 13]. SPMS ultimately develops in most of RRMS patients and leads to high neurologic disability [14].

The third one, the PPMS composes only about 10% of MS cases and is characterized by a constant decline of the neurological function without acute episodes [15]. Similarly to what is observed in SPMS, PPMS patients display less evidence of active inflammation on MRI comparatively to those with RRMS [12]. PPMS patients also present a more balanced gender ratio, higher age of onset and worse prognosis when compared to those with RRMS [13].

MS diagnosis is based on two distinct neurological dysfunction episodes that occur, at least, 30 days apart from each other, in different locations of the CNS, in those with one relapse which show MRI evidence of dissemination in space (DIS) and dissemination in time (DIT) [1]. DIS is characterized by one or more T_2 lesions located in, at least, two of the four areas of the CNS – periventricular, juxta cortical, infra-tentorial and spinal cord [1, 16]. DIT is recognized by one of two criteria: (1) new T_2 lesions and/or gadolinium (GD)-enhancing on a follow-up MRI, with reference to a basal scan, regardless of its timing; (2) Presence of GD-enhancing asymptomatic lesions simultaneously with non-enhancing lesions at any moment [1].

In sum, all MS patients initiate the disease course with a high-risk period that is determined by environmental and genetic factors. The active pre-symptomatic phase follows, and this, in its turn, is followed by the relapsing-remitting phase. Ultimately, most patients evolve to the progressive phase. Therefore, MS is a dynamic pathology divided into different phenotypic phases, each phase being associated with disability variations due to uncomplete recoveries after relapses, progression, and extrinsic factors unrelated to the disease.

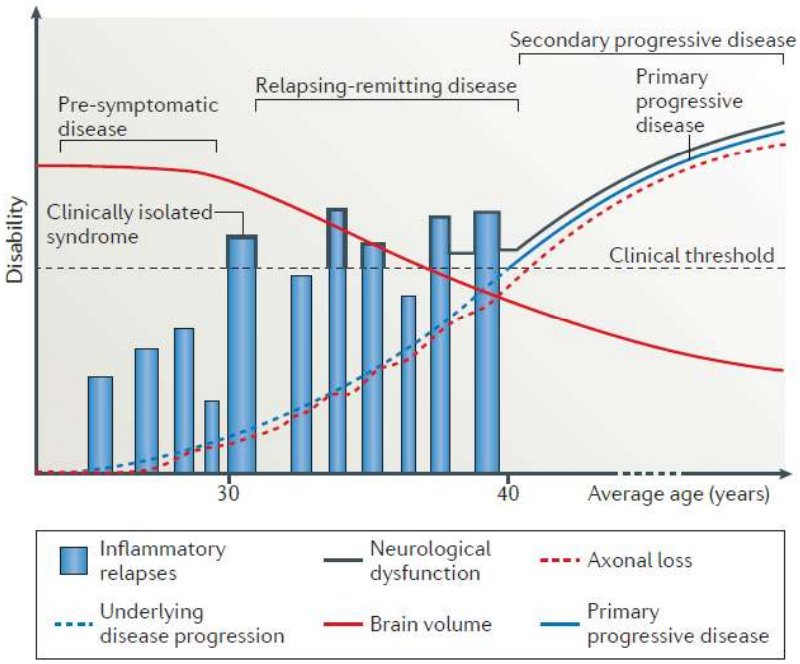


Figure 3. MS forms and clinical course. Approximately 85% of patients are affected by RRMS that is characterized by flare-ups (relapses) of symptoms followed by periods of remission (black line). After one or two decades, about 70% of RRMS patients present an irreversible progression form of clinical 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). (Taken from Dendrou *et al*., 2015 [4]).

1.2. Risk factors

The most impactful risk factor is the family history [17]. In fact, the risk for individuals related to MS patients' increases proportionally with the genetic similarity between them [13], particularly, the HLA classes I and II genes.

Several studies in particular, genome-wide association studies (GWAS) have identified approximately 110 non-HLA single nucleotide polymorphisms (SNPs) associated with the risk for MS development [18]. Virtually, all these SNPs are located near genes involved in the innate or adaptive immunity [1, 2, 18], which highlights the fact that MS is primarily an immune-mediated disease. Environmental factors and lifestyle are key aspects in disease development. Furthermore, unlike genetic factors, many of these features can be modified, having a tremendous potential for disease prevention.

1.2.1. Sunlight exposure and Vitamin D synthesis

Many authors in their studies have reported varied incidence and prevalence values for MS according to geographical latitude [1, 2, 13, 19]. This tendency has been partially explained by the differences in sun exposure and, consequently, vitamin D levels, at different latitudes [20]. This observation has been the basis for innumerous studies aiming to analyze the effects of sunlight exposure and vitamin D on the risk for MS, from whose results indicate that both ultraviolet radiation (UVR) as well as vitamin D are associated with a lower risk [1, 2, 9, 18]. Preclinical evidence suggest that sunlight exposure, even independently from vitamin D, have a protective effect in a MS experimental animal model which might be associated with UVR action on regulatory T cells and antigen-presenting dendritic cells [21]. However, it is still not acknowledged if it is a cause or a consequence of MS, since MS patients might potentially spend less time on the outside due to heat intolerance [22].

Despite the various referred observations suggest, it is still not fully understood whether vitamin D and/or sunlight exposure influence the risk for MS only in specific developmental stages or if this effect occurs throughout an individual's entire lifespan.

Several mendelian randomization studies have showed an association between genetic variants affecting serum vitamin D levels and susceptibility to MS, these studies being the most significate evidence of causal relationship between vitamin D and MS [23]. Therefore, it cannot be considered universally accepted.

1.2.2. *Epstein-Barr* virus

When thinking about the relationship between the Epstein-Barr virus (EBV) and MS, it is crucial to refer the "hygiene hypothesis", which states that multiple exposures to infectious agents during early childhood reduce the risk of MS development via modulation of the immune response towards regulatory and helper T cells (Th2) and attenuation of the proinflammatory activity of Th1 cells [9]. This hypothesis may explain in part MS

geographical distribution. An example of infectious disease that varies age-wise in different populations is the one caused by EBV [24]. In the case of developing countries, virtually all children are infected in the first years of their life, while in developed countries many children are not infected until adolescence [9]. EBV infection at an early age is typically asymptomatic, but when it occurs in adolescence or adulthood it frequently manifests itself as infectious mononucleosis, which has been associated with an increased risk for MS [2, 9]. Particularly, if the infection results in mononucleosis, the risk for MS double increases when compared to EBV-positive cases in which mononucleosis does not develop [9]. It is important to refer that both infectious mononucleosis and increased Epstein-Barr virus nuclear antigen 1 (EBNA1) antibody titers interact with HLA MS risk genetic variants [25, 26]. There is evidence of an interaction between infectious mononucleosis (EBV infection-resulting disease) and the HLA DRB1*15:01 allele that results in a higher risk for MS [27]. In addition, Sundström *et al.* [26] showed that less EBNA1 reactivity is required to increase the MS risk in individuals carrying the HLA DRB1*1501 allele when compared to the ones in which this allele was not present. Since the HLA risk alleles are involved in T cell adaptive immunity [28], these observations might point to common pathogenic pathways involved in MS pathogenesis.

1.2.3. Smoking, Alcohol and Caffeine consumption

Several evidences point to smoking as an important risk factor for MS, also affecting the disease course and progression [29]. Interestingly, the interaction between smoking and the risk for MS seems to be dose-dependent, as cumulative smoking is associated with a higher susceptibility to the disease [18]. In fact, a study employing a cohort of Swedish construction workers reported that ever-smokers presented an increased risk for MS [30] and regarding the effect of smoking in disease course and progression, O'Gorman *et al*. [31] showed that MS onset was approximately 4 years earlier in ever-smokers and Healy *et al*. [32] reported that when comparing patients who continued smoking after diagnosis, patients who quitted after diagnosis and patients that never smoked, the probability of disease worsening was higher for the smoking patients when compared to the other groups, suggesting smoking exacerbates MS pathology. Therefore, smoking has been associated with a faster rate of MS progression, as well as with an anticipated transition to the progressive phase of the disease [29]. Additionally, evidence show that ever smoker MS patients present a risk 3.6 times higher of transiting to the progressive phase of the disease than non-smoker patients [33]. Regarding relapses, another study reported that smoking a pack of cigarettes a day led to a 27% rise in relapse rate [34]. Regarding characteristic MS lesions, there is evidence of an effect of smoking in augmenting GD-enhancing lesions, as well as in increasing T1 and T2 lesion volume [35]. In addition, it was verified that smokers display a more severe neurodegenerative phenotype [35].

Besides smoking, passive exposure to cigarette smoke has also been associated with an increased risk for MS. Furthermore, the risk for developing MS is higher in children

carrying the HLA-DBR1*15 alleles when exposed to passive smoking [36]. In fact, passive smoking may explain the higher incidence of MS in women and children, since they are more prone to this kind of exposure.

Although the association between smoking and MS is well established, the basis of such relationship is difficult to define. One of the potential hypotheses is that smoking interacts with susceptibility genes, as it is seen for other autoimmune diseases such as rheumatoid arthritis [37].

Other interesting studies evaluating the role of alcohol and coffee consumption on MS have been somehow inconsistent. While some studies show no impact of these substances intake in MS [38], others suggest an inverse association between the two factors [39-41]. Furthermore, a cross-sectional analysis showed a positive effect of coffee consumption on disease course and progression for the relapsing form of MS [42]. However, the evidence currently available are not solid enough for substantiating any recommendations concerning coffee consumption in the context of MS.

Regarding alcohol intake, case-control studies evidence a dose-dependent inverse correlation between alcohol consumption and MS, this effect being more evident in women in comparison to men [41].

It is also important to take into consideration the psychological and societal roles of alcohol consumption, since it is associated to personal contact and social interactions, as well as reduced loneliness, by improving the mental health and general life quality of MS patients. However, there is an elevated MS associated-depression rate, which might lead to substance abuse [43].

1.3. Multiple sclerosis Pathophysiology

1.3.1. Basic aspects

Despite the physio pathological mechanisms underlying, MS presents a multifactorial nature, it has not been possible until now to determine and evaluate the exact cause of this disease. However, the available evidence suggests that MS pathophysiology is based on two major components: neuroinflammation and neurodegeneration. Nevertheless, several studies in the field of MS continues unknown due to the lack of knowledge on the initial events leading to disease emergence and an incomplete vision of the processes responsible for its development. The perception of MS as a disease with a metabolic impairment component in addition to the autoimmune and inflammatory ones, allows to further understand some of the aspects of the disease course, including the genetic susceptibility, environmental risk factors and the difference in incidence between males and females, simultaneously opening new avenues for therapy development. The blood-brain barrier (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 end feet of astrocytes. Normally 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. This peripheral tolerance breakdown is due to defects on regulatory mechanisms that are normally maintained by regulatory lymphocytes (Treg). Although the number of Treg 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 Treg 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. 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.

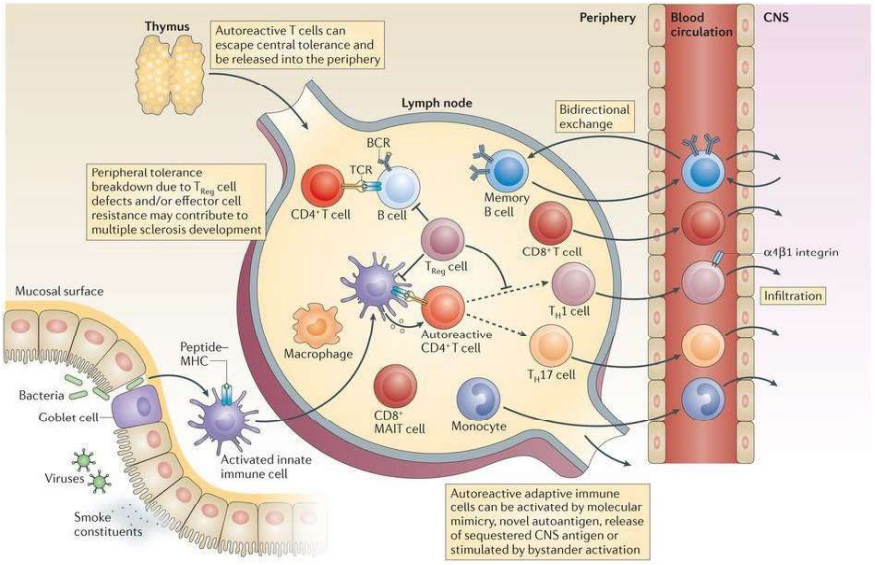


Figure 4. Immune system dysregulation outside CNS. BCR – B cell receptor; TCR – T cell receptor; CD8+ MAIT – CD8+ mucosa-associated invariant T cell. (Taken from Dendrou *et al*., 2015 [4]).

From one side 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. On the other side, T-helper 1 (TH1) and TH17 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 interferon gamma (IFN-γ) and interleukin (IL)-17 (cytokines produced by TH1 and TH17 cells, respectively) appear to effectively cross the BBB and accumulate within the brain and 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, it can be said that the axonal damage observed 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.

1.3.2. Autoimmunity

The idea that MS composes an autoimmune disorder is well defended by several lines of evidence. In fact, it can be assert that the immunological aspect is the most well-known physio pathological component of the disease, and it is directly associated with an underlying inflammatory state [44]. During the evolution, the immune system has developed several strategies to control in an appropriate way the immune responses. Central to this behavior is the positive selection of thymocytes in the thymic cortex and the removal of autoreactive T-cell clones (negative selection) in the medulla [45, 46], as well as the generation of the so-called T regulatory (Treg) cells [47]. The on the activation of autoreactive T lymphocytes through cross-reactivity by viral and/or bacterial antigens structurally similar to CNS proteins. The interaction between T cells and brain endothelial cells allows their migration to the CNS, leading to an exacerbated immune response and an extension of the number of molecules susceptible to become antigens (epitope spreading), which further fuels the inflammation [44]. Upon receptor activation, T lymphocytes differentiate into different subtypes of effector cells, including Th1, Th2 and Th17 cells [44]. Th1 cells display a pro-inflammatory phenotype and are responsible for the production of cytokines such as tumor necrosis factor alpha (TNF-α), IFN-γ, IL-12, IL-15 and IL-17 [44]. The potential involvement of deregulated Th1 cells in autoimmunity development in MS is highlighted by IFN-γ-enriched active lesions [48].

Together, the available observations highlight the deregulation of immune cells and their ability to reach the CNS as a key factor for MS development. However, it is imperative to define the source of such impairment and phenotypical skew in order to establish a causal relationship and potentially inhibit and/or prevent such events from occurring.

1.3.3. Demyelination

The complex process of demyelination and axonal degeneration are the main characteristics of the disease [49]. Sclerotic plaques are characterized by destruction of oligodendrocytes (OLGs) and myelin sheaths, followed by recognition of myelin epitopes by immune cells, giving rise to an autoimmune episode in which T cells attack myelin and the cells that produce it even further. Additionally, macrophages phagocyte and opsonize myelin, and B cells release antibodies against it [50].

However, many questions began to arise regarding such hypothesis, since there is evidence pointing to an initial neurodegenerative process occurring independently from

immune mediated inflammation [49, 51]. Thus, it has been suggested that besides immune cells, many other factors might be involved in the demyelination process. And additionally, multilayered structures of myelin are observed in the chronic phase of the disease [49], suggesting that such excessive myelin results from OLGs dysfunction in the spinal cord.
Similarly, TNF-α overexpression has been shown to associate with progressive demyelination in animals' models of CNS inflammation [52] but, it was established that such effect is dependent on the TNF-α receptor, since the receptor I displays a cytopathic action, but receptor II presents neuroprotective properties and might promote Treg cells function [52]. Furthermore, glutamate homeostasis is found to be altered in MS patients, leading to a central accumulation of this neurotransmitter that seems to affect oligodendrocytes, astrocytes, endothelial cells, and immune cells [53].

More recently, the hypothesis that demyelination occurs not only as a result of events beginning in the systemic compartment and reaching the CNS, but also as a consequence of OLGs damage, has arisen. In fact, type III MS lesions display nuclear condensation and fragmentation of OLGs [54], suggesting that such alterations are intrinsic to these cells.

Axonal degeneration occurs in parallel with the demyelinating events, contributing to disability progression of MS and the demyelination promotes a series of adaptive changes on axons, including an altered ionic channels distribution through the axolemma [55]. Although demyelination and axonal degeneration are usually paired as two simultaneous events which characterize MS, myelination does not seem to be completely necessary to maintain axonal integrity. Similarly to demyelination, axonal damage processes are multifactorial events, involving several different types of cells and mediators such as T lymphocytes, macrophages, antibodies, and free radicals, among others [50]. So, it is very important to consider the heterogeneity of the pathogenic mechanisms underlying axonal damage for the design of targeted therapies.

1.3.4. Remyelination

This process of remyelination consists on the formation of new myelin sheaths around axons residing in the CNS in response to the myelin loss characteristic of MS and it is considered a reparative process, allowing the restoration of axonal conductive functions, and conferring neurologic protection. Although myelin produced in these conditions shows to be morphologically and functionally different from the original myelin found before demyelinating events, the remyelination process displays significate similarities with the developmental myelin formation [56]. The remyelination can be divided in 2 main phases, the first being the recruitment phase, which consists of the colonization of the lesioned areas by oligodendrocyte precursor cells (OPCs); and the second being the differentiation phase, when these cells transform into myelin-producing OLGs and both are associated with distinct and specific molecular patterns, which are responsible for promoting a switch on OPCs from a proliferative environment to a differentiated one [56].

Giving the variety of mechanisms involved in remyelination events, remyelination failure probably results from dysfunctions in several pathways, and not from an isolated mechanism. One of the potential causes for remyelination failure in the context of MS is the incapability of OPCs to differentiate, possibly due to the lack of promoting molecules or, alternatively, to the presence of inhibitors, as is the case of hyaluronan which accumulates in MS lesions [57]. Taken together, the remyelination failure observed in MS probably results from a combined deregulation of several of the cellular and molecular mechanisms needed for it to occur, and unravelling such mechanisms in the context of MS may indorse the development of potential remyelination-promoting therapeutic agents.

The strategies for inducing remyelination can be divided in 2 main categories: (1) promoting endogenous remyelination and (2) myelinogenic cells transplantation [56]. Antibody therapy has been also revealing to be useful in promoting myelin regenerative events through modulation of OPCs recruitment and differentiation, by preventing OLGs death and via immunomodulatory effects [56].

1.3.5. The role of Thymus

Bone marrow derived progenitor cells undergo extensive differentiation in the thymus, a primary lymphoid organ, to originate the phenotypic markers and functional reactivities characteristics of mature T cells. The thymus has a central role for the control of organ-specific autoimmunity, both by generating regulatory T cells or by limiting the output of autoreactive T cells [58]. The surface expression of the co-receptor molecules CD4 and CD8 characterize the four main thymocyte populations. According to their specificity to T-cell receptors (e.g., $CD4^+$, $CD8^+$, and forkhead $P3^+$ regulatory T-cells), immature T-cells are selected in the thymus to develop functional and self-tolerant T-cell repertoires (positive selection) and induce a central tolerance to eliminate autoreactive T-cells (negative selection) [59].

The $CD4^-CD8^-$ double negative (DN) cells are the most immature population and firstly express CD8 before becoming $CD4^+CD8^+$ double positive (DP) cells. The maturation of DN thymocytes into the DP cells is heavily dependent on signaling through the CD3 complex [60]. Thus, CD3 positivity increases along thymocyte maturation process and the ratio of immature ($CD3^{low}$) and mature ($CD3^{high}$) thymocyte is often used to characterize T-cell maturation status. The maturation of thymocytes occurs in the thymic cortex, whereas the differentiation and negative selection of T-cells occurs in the thymic medulla [59]. Thus, DP cells relocate from the cortex to the thymic medulla and undergo positive and negative selection, eliminating most $CD4^+CD8^+$ DP cells before they maturate and acquire a $CD4^+$ or $CD8^+$ phenotype. Finally, non-autoreactive CD4 or CD8 single positive (SP) cells are permitted to leave the thymus and colonize the periphery [61].

T cells are mainly self-tolerant but defects in multiple mechanisms that control self-reactivity can break the tolerance phenomena, ultimately resulting in autoreactive T cells and T-cell mediated autoimmune diseases, as it is the case for MS. When the peripheral

tolerance mechanisms are 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. Thus, the thymus can have dual functions in MS: it can be the organ where potentially self-reactive T-cells mature and differentiate inducing or exacerbating the disease, but it is also the cradle of regulatory T-cells that can potentially suppress self-reactive immune responses, locally [62, 63]. Notably, RRMS patients display thymic involution and perturbed naïve CD4 T-cell homeostasis. It was observed that young patients have reduced generation of CD4 recent thymic emigrants (RTEs) and altered T-cell proliferative responses that fail to maintain naïve CD4 T-cell numbers with age. The early thymic involution and compensatory homeostatic peripheral T-cell proliferative responses are suspected to predispose patients to autoreactivity [64].

Immune thymic profile has been also explored in the experimental autoimmune encephalomyelitis (EAE) and the cuprizone mouse models of MS [58, 61]. In the later, Solti *et al.* do not indicate a functional relationship between cuprizone-induced thymus involution and the absence of inflammatory responses or the selective demyelination which was observed in the cuprizone model [61]. But instead of that, cuprizone-induced thymocyte and oligodendrocyte apoptosis seems to occur parallel to each other, and in both cases the toxin affects the most vulnerable cells in the given organ. It raises the possibility that similar selective elimination of the most vulnerable cell type in other organs is responsible for the absence of thriving that is characteristic of the cuprizone model. An important feature of the cuprizone model is that after termination of the toxin feeding, an accelerated thriving and regeneration occurs. Therefore, the cuprizone model could be valuable in studying thymus regeneration as well as the remyelination processes. This organ plays an essential role in the immune system by providing a unique environment to support cell development. The thymus is a primary lymphoid organ derived from the endoderm of the 3rd pharyngeal pouch implicated in the maturation of thymocytes and, therefore, key for establishing the immune surveillance. This organ does not contain self-renewing lymphoid precursor cells and therefore, is colonized by lymphoid precursors coming either from the fetal liver through mesenchyme or from the adult bone marrow via the blood vessels [65]. Within the thymus, the developing thymocytes move throughout a 3D thymic epithelial network, interacting with the thymic epithelial cells (TECs) of two histologically different compartments: the cortex and the medulla [66]. As such, the ability of the thymus to recruit, foster, and export αβT cells effectively determines how the peripheral immune system mounts effective responses. While early events in thymocyte development take place in the thymic cortex and are controlled by the cortical thymic epithelial cells (cTECs), the thymus medulla plays a pivotal role in events that ensure the correct formation of multiple αβT cell sub lineages [67-69].

1.4. Animals models of MS

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 [70-75]. 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.

1.4.1. 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 5). 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 [70, 75].

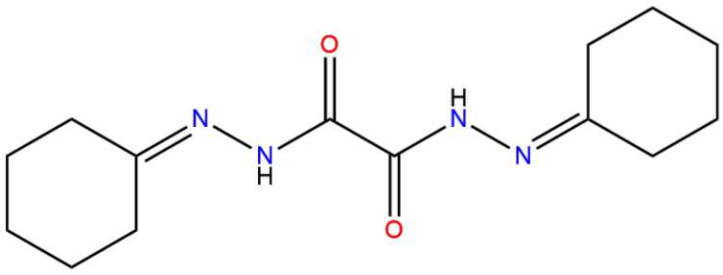


Figure 5. Cuprizone (CPZ) molecular structure. CPZ ($C_{14}H_{22}N_4O_2$) has a molecular weight (MW) of 278.356 g/mol, owns 2 H donors (N-H-), 4 H acceptors (=N-, =O), 2 routable bounds (N-N(H)) and 0 formal charge. CPZ is slightly soluble in water and extremely soluble in ethanol. (National Center for Biotechnology Information. PubChem Compound Database; CID=9723. Image taken from: http://en.chembase.cn/molecule-103437.html (accessed at March 10, 2022)).

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 [76]. 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^{2+} 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 mega mitochondria in the liver and in oligodendrocytes of mice treated with CPZ. The presence of this mega mitochondria is related to the increase of oxidative stress in oligodendrocytes, with consequent high levels of reactive oxygen species (ROS)/ reactive nitrogen species (RNS) and shortage of adenosine triphosphate (ATP), which can lead to a disruption of endoplasmic 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 [76, 77].

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 reproducible 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 [70, 77].

2. Dipeptidyl peptidase IV, Glucagon-like peptide 1 and NPY pathways

2.1. Dipeptidyl peptidase IV

Dipeptidyl peptidase IV (DPP-IV) is also known as T-cell activation antigen cluster of differentiation CD26 or adenosine deaminase (ADA)-binding protein. This 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 [78]. 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 [79]. As a transmembrane protein, the 110kD DPP-IV can be organized as a monomer, homodimer, or even as homotetramer on the surface of cells. However, only the dimerized forms present enzymatic activity, being this the predominant form of DPP-IV [80]. Additionally, to the transmembrane form, DPP-IV also presents a soluble circulating form (sDPP-IV) with 727 aa. The sDPP-IV 20 lacks the intracellular tails and transmembrane regions and represents a substantial proportion of DPP-IV activity in human serum (Figure 6) [79].

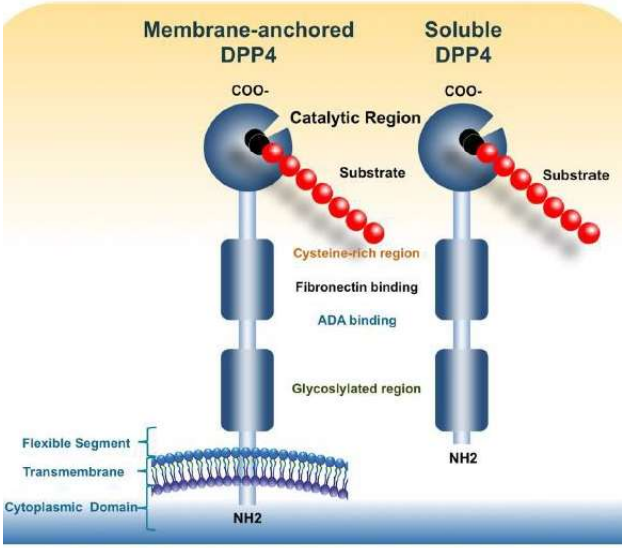


Figure 6. DPP-IV structure. Schematic representation of the membrane-bound monomer (left) and soluble DPP-IV (right). Both forms share many domains including glycosylated region, ADA binding domain, fibronectin bonding domain, cysteine-rich domain and catalytic domain. Only membrane-bound DPP-IV presents a cytoplasmic domain, a transmembrane domain and a flexible segment (Taken from Mulvihill *et al*, 2014 [79]).

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 [79]. However, its principal role is the enzymatic function, namely 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, chemokines, and others [78, 81]. 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 [78, 82].

2.2. Glucagon-like peptide 1

Glucagon-like peptide 1 (GLP-1) is an endogenous 30 aa incretin produced and secreted from enteroendocrine L cells, found in the jejunum, ileum and colon. This incretin is normally 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-17-37 and GLP-17-36 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-19-36 amide and/or GLP-19-37 [83-86].

Besides its insulinotropic effect, GLP-1 also promotes β-cell proliferation, differentiation and regeneration, insulin gene synthesis, islet cell mass increase and antiapoptotic pathways [85, 87]. Other authors' data suggested that GLP-1 might have additional roles other than glucose-lowering effects. In fact, several other studies have demonstrated that incretin-based therapies also present anti-inflammatory effect by reducing the production of inflammatory cytokines [88].

2.2.1. 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-1 receptor (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 [83, 89, 90]. 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 [91].

2.3. Neuropeptide Y

Neuropeptide Y (NPY) is a 36 aa peptide that was first isolated from porcine brain in 1982 [92]. 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 [93].

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 [94]. The NPY family consists not only of NPY, but also of peptide YY (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 [95]. Moreover, NPY exhibits a 70% homology with PYY and 50% homology with PP [96]. 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 [97]. NPY is one of the most abundant neuropeptides in the mammalian brain, being widely distribute within the CNS and peripheral nervous system. 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 [94], and in enteric neurons [98]. 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 [94].

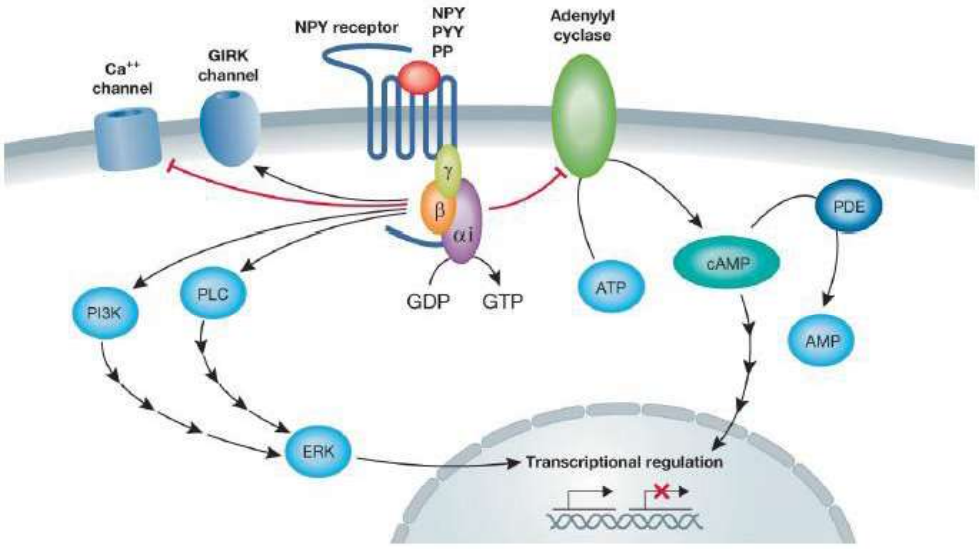


Figure 7. Overview of NPY receptors signal transduction. NPY receptors couple to the G-protein signaling cascade, leading to the inhibition of adenylyl cyclase. Furthermore, the activation of the G-protein complex can also lead to decreased Ca^{2+} channel activity and enhanced G-protein coupled inwardly rectifying potassium (GIRK) currents. (Taken from Brothers *et al*, 2010 [96]).

2.4. Dipeptidyl peptidase IV and T cell function

On T cells, CD26/DPP-IV interacts directly with antigen-presenting cells via Caveolin-1, resulting in an up-regulation of the CD86 co-stimulatory molecule and enhanced bonding of the immunological synapse. CD26 is able to trigger direct T cell activation and proliferation via CARMA1 mediated NF-κB activation in the T cell *in vitro.* In the periphery, findings are conflicting but the expression of CD26/DPP-IV is envisioned as a characteristic of memory T cells where CD26/DPP-IV bright cells respond maximally to recall antigens [99].

Moreover, CD26/DPP-IV is described as a mediator of lymphocyte migration and thymic maturation marker in both rodents and humans. It reaches the highest levels of expression in mature CD4 or CD8 single-positive cells within the thymus and is down-regulated in cells that undergo apoptosis [100].

As a matter of fact, in CD26/DPP-IV-deficient animals the overall number of leucocytes was found decreased and in humans, a reversible dose-dependent decrease in absolute lymphocyte numbers were observed in patients receiving DPP-IV-inhibitors [100]. Moreover, CD26-deficient animals also showed a reduced density of thymic medullar lymphocytes and most intriguingly, impairment of CD26 depleted memory T cells as well as RTE from the $CD4^+$ pool which was filled with naïve T cells instead [99].

2.5. Dipeptidyl peptidase IV and MS

MS patients display increased numbers of $CD26^+$ T cells which correlate with disease activity [101]. Moreover, human myelin basic protein (MBP)-specific $CD4^+$ T cell clones

(TCC) also showed higher expression levels of CD26/DPP-IV. Notably, DPP-IV inhibitors were found to suppress DNA synthesis and IFN-gamma, IL-4, and TNF-alpha production of the antigen-stimulated TCC, suggesting that CD26 plays a role in regulation of activation of autoreactive TCC [102]. In addition, IL-17-producing $CD4^+$ cells (Th17) - major contributors of autoimmune inflammation - were found to express high amounts of enzymatically active CD26/DPP-IV. Notably, pharmacological blockade of CD26 enzymatic center restores the CD26 negative regulation of the chemotactic $CD4^+$ T cell response to the inflammatory chemokines CXCL9-12. These results support the notion that CD26 may contribute to the orchestration of the immune response by Th17 cells in human inflammatory diseases, namely MS [103].

Notably, pharmacological inhibition of DPP-IV decreased incidence, onset of symptoms and overall disease severity in EAE significantly, increasing the secretion of the immunosuppressive cytokine TGF-beta1 in spinal cord tissue and plasma during acute EAE [104]. Moreover, a combined approach targeting both CD26/DPP-IV and APN/CD13 inhibition ameliorated the EAE mouse model of MS by a mechanism involving an active TGF-beta1-mediated anti-inflammatory effect at the site of pathology [105]. In addition, a decreased risk of incidence in autoimmune diseases such as systemic lupus erythematosus, psoriasis, multiple sclerosis, and inflammatory bowel disease was associated with the use of DPP-IV inhibitors in a large cohort of diabetic patients [106]. 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 [107], but also present an inflammatory effect mediated by the same receptor that is dependent on inhibition of DPP-IV [108]. 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 [109, 110].

Taken together, these results claim for further studies aimed to assess the pertinence of CD26/DPP-IV modulation as a pharmacological approach for prevention or treatment of MS.

3. 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 and DPP-IV inhibitors [85].

Nowadays, there are several DPP-IV inhibitors approved by FDA, including Sitagliptin, Vildagliptin 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 [111]. However, DPP-IV inhibitors have neurotrophic and immune regulating functions, as the catalytic core pf CD26/DPP-IV is part of the linking site required for co-stimulation, which has aroused interest in the application of these drugs for the management of neuroinflammatory/neurodegenerative diseases [99, 112].

3.1. Sitagliptin

Sitagliptin, whose chemical structure is represented on Figure 8, 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 [113, 114]. 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 HbA1C [115]. 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 [116, 117].

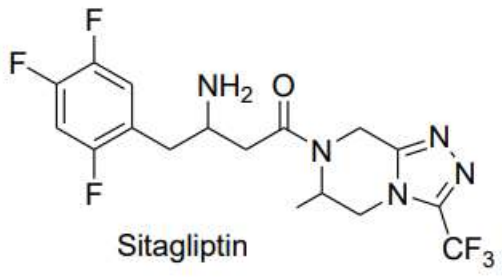


Figure 8. Chemical structure of Sitagliptin, a DPP-IV inhibitor. The chemical structure of Sitagliptin is (2*R*)-4-oxo-4[3-(trifluoromethyl)-5,6-dihydro[1,2,4]-triazolo-[4,3]-pyrazin-7-(8H)-yl]-1-(2,4,5-trifluorophenyl)-butan-2-amine. (Taken from Bhavya *et al,*2013 [113]).

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 [116, 117].

Chapter II | **MAIN OBJECTIVES**

Main objectives

MS is one of the leading causes of neurological disability in young adults, having an enormous personal, social, and economic impact. Besides autoimmune inflammation, MS patients display early thymic involution and perturbed naïve CD4 T-cell homeostasis that seem to increase patients' susceptibility to autoreactivity [64]. In MS animal models, immune thymic involution was also reported in both EAE and cuprizone intoxication paradigms [58, 61]. Yet, the functional significance of this pathological event in MS progression is still lacking clarification.

Nowadays, MS management is solely dependent on symptomatic treatments that do not tackle the core of the disease: demyelination along with spontaneous remyelination. As a matter of fact, there are still no effective pharmacological interventions available that target the remyelinating component of the disease and the most severe forms (progressive forms) are still practically untreatable [3]. Therefore, the discovery of innovative solutions that exert beneficial effects in the context of MS is a crucial step for relieving and managing the disease burden.

Given that drug discovery is a vast undertaking process, drug repurposing is an attractive approach as it holds the potential to provide fast access to therapeutics with demonstrated evidence of safety and efficacy. In this regard, currently approved antidiabetics (e.g. pioglitazone) or antidyslipidemics (e.g. simvastatin) have been studied for MS [118, 119]. Accordingly, the oral antidiabetics DPP-IV inhibitors are a drug class extensively studied given their neuroprotective and anti-inflammatory properties [112, 120, 121]. Notably, pharmacological inhibition of DPP-IV was correlated with a decreased incidence, onset of symptoms and overall disease severity in the EAE animal model [104]. In MS patients, recent evidences showcase the involvement of CD26/DPP-IV on autoreactive T cells activation and autoimmune inflammation [101, 102, 105, 106]. Collectively, these evidences support the assumption that the inhibition of the enzymatic activity of DPP-IV may be a useful tool for pharmacological interventions in MS.

In this sense, we conducted a preclinical MS *in vivo* assay to characterize:

1- The cuprizone-induced thymic involution dynamics that occurs in the demyelination and remyelination phases of the disease;

2- The sitagliptin therapeutic potential to modulate cuprizone-induced thymic involution and central demyelination/remyelination.

Chapter III | **MATERIAL & METHODS**

3.1. Animals and treatments

In this study, were used C57BL/6 male mice with 8 weeks age, obtained from Charles River Laboratories (Barcelona, Spain). These mice were housed in the iCBR bioterium facility (Coimbra Institute for Clinical and Biomedical Research), Faculty of Medicine, University of Coimbra, under controlled temperature (22±1ºC) and relative humidity (50-60%) and a 12-h light 12-h dark cycle. The animals were housed four *per* cage 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).

After 2 weeks of acclimatization, the animals were randomly divided into six groups (10 animals per experimental group): controls (CTR), which received vehicles only for 5 and 7 weeks, respectively; animals receiving CPZ only during 5 weeks and sacrificed at the end of week five (CPZ W5) (demyelination peak); animals receiving CPZ for 5 weeks followed by 2 weeks of withdrawal (CPZ W7) (early remyelination); animals receiving CPZ for 5 weeks and Sitagliptin (Januvia®, MSD, Portugal), orally from 2.5 week (CPZW5 SITA) and animals receiving CPZ for 5 weeks followed by 2 weeks of withdrawal and with Sitagliptin (Januvia®, MSD, Portugal), orally at W6 and W7 (CPZ W7 SITA) (Figure 9). CPZ 0.2% m/v was administered daily by oral gavage (dissolved in methylcellulose 1%, w/v), and Sitagliptin at a dose of 50 mg/kg was given through a semi-solid vehicle for voluntary consumption (Pill, Patent pending nº PCT/IB2021/053124).

All animals were monitored once a week for body weight and at W 0, 2, 5 and 7 for occasional glycemia. At the end, animals were anaesthetized by intraperitoneal injection of ketamine chloride (1 g/mL; Imalgene®) in chlorpromazine 2.5% (Largactil®). Blood was drawn by cardiac puncture for hematological and biochemical analyses. After cervical dislocation, the main organs were weighed for relative weight determination. Animal experiments were conducted according to the National and European Communities Council Directives of Animal Care and received approval (# 12/2018) by the local (iCBR) Animal Welfare Body (ORBEA).

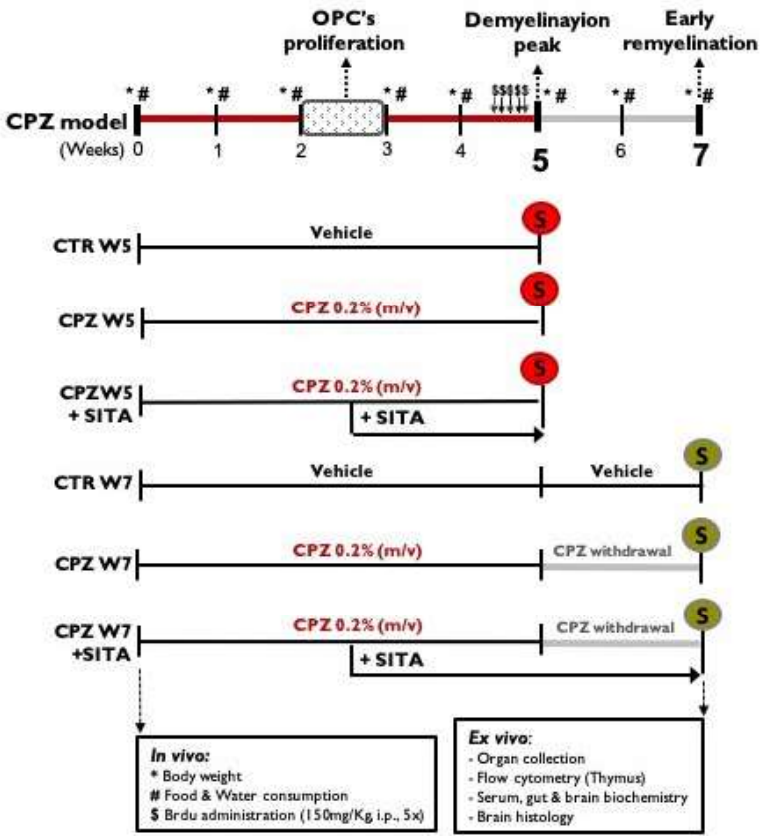


Figure 9. Experimental design. 60 C57BL/6J adult healthy mice were distributed among six groups (9-10 animals per experimental group). CTR W5/W7 groups were submitted to force-fed gavage of methylcellulose 1%, w/v), daily, for 5/7 weeks. CPZ W5 group was submitted to force-fed gavage of CPZ 0.2% (dissolved in methylcellulose 1%, w/v) for 5 weeks to induce demyelination. To study the early remyelination period, CPZ W7 group was submitted to force-fed gavage of CPZ 0.2% (dissolved in methylcellulose 1%, w/v) for 5 weeks followed by CPZ withdrawal for 2 weeks more. To assess sitagliptin effects in cuprizone-induced thymic atrophy, the drug was Pill-dosed daily (50mg/Kg), from week 2.5 (onset of OPC's proliferation) to the end of the experiments (W5 or W7). All experimental groups were daily dosed with the semi-solid vehicle (Empty Pill: CTR W5, CTR W7, CPZ W5, CPZ W7; SITA-PILL: CPZ W5+BB, CPZ W7+BB - Patent pending nº PCT/IB2021/053124). Abbreviations: CPZ, cuprizone; OPC's, oligodendrocytes progenitor cells; SITA, sitagliptin; S, sacrifice.

3.2. In vivo monitoring

3.2.1. Body weight

Body weight (BW) was carefully monitored before study commencement, once weekly during the study and the day before the sacrifices, using an analytical balance (CQT 2000 Core® Portable Compact Balance, Adam Equipment, USA).

3.2.2. Mortality and toxic signs

Classical signs of toxicity (e.g. changes in skin, hair, mucous membranes, eyes, and general behavioral manifestations) and any injury or illness were conducted once daily until the end of experiments.

3.2.3. Glycemic profile

Blood samples were collected from the tail vein and occasional measurements occurred at Week 0, 2, 5 and 7. The glucose levels were measured using a portable commercial glucometer kit (GlucoMen® aero 2K, A.MENARINI diagnostics).

3.3. Acquisition of thymus samples

The weight of the mice was measured at the beginning and at the end of the treatment period. After that, the animals were euthanized, and their chest was opened. The thymus was photographed, carefully dissected and their wet weight was measured in grams (absolute organ weight). The relative thymus weight of each animal was then calculated as follows:

$$\text{Relative Organ Weight} = \frac{\text{Absolute Organ Weight (g)}}{\text{Mouse Body Weight (g)}}$$

Thymus were freshly processed for other different techniques, frozen for histochemistry and immunohistochemistry, or homogenized in phosphate buffered saline (PBS) with a glass/glass homogenizer.

3.4. Sample preparation for flow cytometry

Thymus were excised from the mice and have been subjected to manual disruption in 1x PBS and filtered, by applying mild pressure through a 70-µm nylon cell strainer (Corning Cat. No. 352235), to obtain single-cell suspensions of thymocytes. Thymocytes were then counted using the hematological counter DxH500 (Beckman Coulter, Pasadena, CA, USA), and the concentration adjusted to $1x10^6$ cells/mL.

3.5. 7-AAD viability staining (flow cytometry)

The viability of thymocytes were evaluated through the 7- Aminoactinomycin D (7-AAD BD Pharmingen™, Nr. 559925) viability staining protocol. The fluorescent intercalator 7-AAD changes spectrally when it interacts with DNA. With emission maxima of 647 nm

and the ability to be stimulated by a 488 nm laser. The permeability of cell membrane are compromised during cell death allowing the formation of the 7-AAD/DNA complexes, which can be measured using flow cytometry. Therefore, 100uL of thymocytes suspensions were added to 12 x 75 mm cytometry tubes and stained with 5uL of 7-AAD compound for 30 min at 4ºC. In the end of incubation, 100uL of 1x PBS were added to cells and immediately acquired, or after wash with 2mL of 1x PBS and centrifuged at 500g for 5 minutes, in an 8-color flow cytometer BD FACSCanto II (BD Biosciences, San Jose, CA, USA) with BD FACSDiva software (Becton Dickinson, San Jose, CA, USA).

3.6. Extracellular staining (flow cytometry)

The identification of CD3, CD4 and CD8 positive cells within the thymocytes were assessed by flow cytometry. The extracellular antibodies used for staining were anti-CD45 (APC-Cy7 Rat Anti-Mouse CD45, Nr. 557659), anti-CD3 (FITC Hamster Anti-Mouse CD3e, Nr. 553062), anti-CD4 (PE-Cy7 Rat Anti-Mouse CD4, Nr. 561099) and anti-CD8 (PE Rat Anti-Mouse CD8a, Nr. 553033). The anti-CD45 antibody was used to discriminate thymocytes and the remaining to identify the subpopulations of T cells from other cell types. All antibodies were provided by BD Pharmingen^{TM}. Briefly, 100uL of thymocytes was added to 12 x 75 mm cytometry tubes and stained with the extracellular antibodies for 15 min, in the dark at RT. After incubation, red blood cells were lysed with BD Lysing Solution (Becton Dickinson, San Jose, CA, USA), for 10 min and centrifugated at 450g for 5 min. The supernatant was discarded and cell suspensions were washed one time with 1x PBS and centrifuged in the same conditions. In the end, samples were resuspended in 200uL of 1x PBS and acquired in an 8-color flow cytometer BD FACSCanto II (BD Biosciences, San Jose, CA, USA) with BD FACSDiva software (Becton Dickinson, San Jose, CA, USA).

All the cytometry raw data were analyzed with FlowJo™ Software v.10.7 (BD Life Sciences, Ashland, OR, USA). The gating strategy (Figure 10) for thymocytes started with the exclusion of dead cells, positive for 7-AAD dye, and doublets. Then, single cell thymocytes were identified by positivity for CD45 molecule. Within thymocytes, it was evaluated the expression of CD3 molecule to identify the CD3dim and CD3bright populations, and according to the expression of CD4 and CD8 molecules it was identified the $CD4^{-}$ $CD8^{-}$, $CD4^{+}$ $CD8^{-}$, $CD4^{+}$ $CD8^{+}$, and $CD4^{-}$ $CD8^{+}$ populations. Whose were evaluated within total thymocytes, and particularly within CD3dim and CD3bright populations.

Additionally, gated on CD3dim and CD3bright it was also assessed the CD4 and/or CD8 subpopulations.

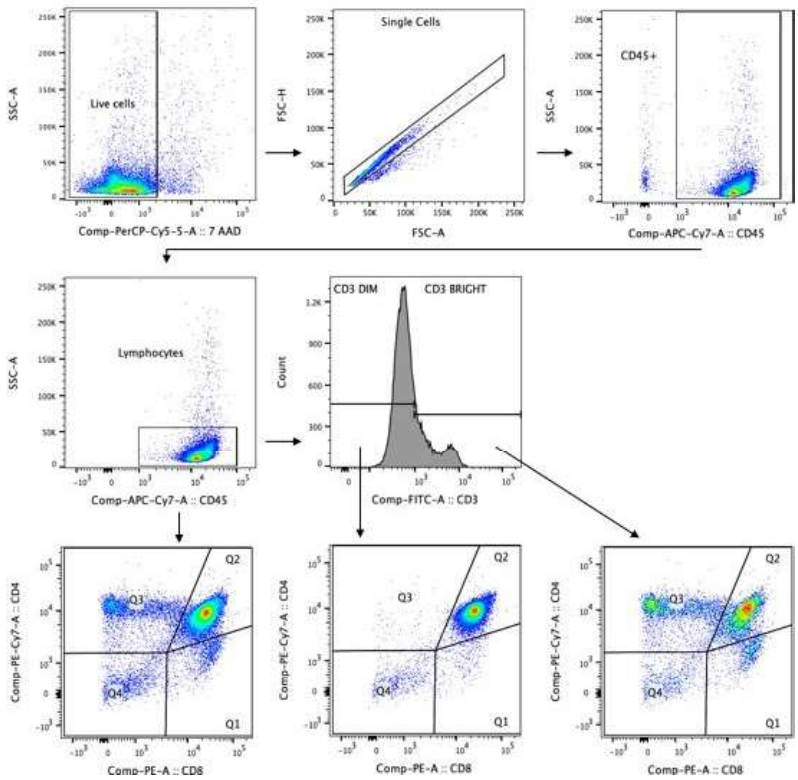


Figure 10. Representative image of the gating strategy applied to identify the thymocytes and the expression of the CD3, CD4 and CD8 molecules. First, exclusion of dead cells, positive for 7-AAD dye, and doublets. Then, single cell thymocytes were identified by positivity for CD45 molecule. Within thymocytes, it was evaluated the expression of CD3 molecule to identify the CD3dim and CD3brigh populations, and according to the expression of CD4 and CD8 molecules it was identified the $CD4^-$ $CD8^-$, $CD4^+$ $CD8^-$, $CD4^+$ $CD8^+$, and $CD4^-$ $CD8^+$ populations. Whose were evaluated within total thymocytes, and particularly within CD3dim and CD3bright populations. Cytometry raw data were analyzed with FlowJo™ Software v.10.7 (BD Life Sciences, Ashland, OR, USA). Q1 – $CD8^+$ $CD4^-$; Q2 – $CD8^+$ $CD4^+$; Q3 – $CD8^-$ $CD4^+$; Q4 – $CD8^-$ $CD4^-$.

3.7. Gene expression analysis

3.7.1. RNA extraction

For cerebellar samples, RNA was extracted from 30-50 mg of frozen tissue (preserved in RNA later Stabilization Solution, R-0901, Sigma Aldrich) using the the NZY Total RNA Isolation Kit (nzytech, MB13402), according to the manufacturer's instructions. RNA concentrations were determined (NanoDrop® ND-1000 Spectrophotometer). Samples were stored at -80ºC until subsequent analysis.

3.7.2. cDNA synthesis

Synthesis of complementary Deoxyribonucleic acid (cDNA) was performed using a Xpert cDNA Synthesis Mastermix (GK81.0100, Lot. 7E2709A, GRISP). For each tube, it was pipetted the volume corresponding to 2 µg RNA, 10 µL of Mastermix and water (to a final volume of 19 µL. Then, in the thermocycler (1861096, T100TM Thermal Cycler, Bio-Rad)

cDNA was synthesized following the Xpert cDNA Synthesis Mastermix protocol. Samples were stored at -20 ºC.

3.7.3. RT-PCR

A mixture was prepared containing 10 µL of Sybr Green (iTaq Universal SYBR Green Supermix 1725124, Bio-Rad), 0.4 µL of mix primers (Table 1) and 7.6 µL of autoclaved water. 18 µL of this mixture and 2 µL of the sample were transferred into each well. Realtime polymerase chain reaction (RT-PCR) protocol consisted of 1 cycle for initial denaturation (10 min at 95ºC), followed by 40 cycles comprising the following steps: 15s, 95 ºC; 45s, 58 or 60 ºC; 30 s at 72 ºC. Standardization was achieved with GeNorm algorithm, where gene stability was attained with Hypoxanthine Phosphoribosyltransferase (HPRT) and Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH). The relative expression ratio of each of the target gene was computed on the basis of ΔΔCt (2-ΔΔCp) values. Results are expressed as percentage of control.

Table 1. Primer sequences and real-time PCR conditions

Gene	Primer sequence		Temp. (ºC)
	Forward	Reverse	
GAPDH	CGA CTT CAA CAG CAA CTC	TGT AGC CGT ATT CAT TGT	58
HPRT	TCC ATT CCT ATG ACT GTA	CAT CTC CAC CAA TAA CTT	58
MBP	GCC TGT CCC TCA GCA GAT TT	GTC GTA GGC CCC CTT GAA TC	58
PLP	CAG GCA GAT CTT TGG CGA	TGA TGC CCA CAA ACG TTG	60

3.8. Statistical analysis

Results were expressed as means ± standard errors of the mean (S.E.M.) using GraphPad Prism® software, version 8.2.1 (GraphPad Software, Inc., La Jolla, CA, USA). The distribution of continuous variables was analyzed using the Kolmogorov-Smirnov test to assess significant deviations from normality. One-way analysis of variance (ANOVA, followed by Bonferroni's test for multiple comparisons) or the nonparametric Kruskal-Wallis test (followed by the Dunn's test for multiple comparisons) were used for normally or non-normally distributed data, respectively. Repeated measures ANOVA, followed by Bonferroni post-hoc test, were used to compare parameters evolution during the experimental period. A p value <0.05 was considered statistically significant.

Chapter IV | **RESULTS**

4.1. The impact of sitagliptin in cuprizone-induced thymic atrophy – Demyelination phase (Week 5)

Body weight (BW) was monitored weekly during the entire study period. On the first two weeks there were no differences in BW across all experimental groups. From W3 onwards, a significant difference in BW was observed between CPZ W5 animals and controls ($p<0.05$). SITA-treated animals also showed a similar growth curve pattern to the CPZ-intoxicated ones even though it only reached statistical significance at W5 (Figure 11).

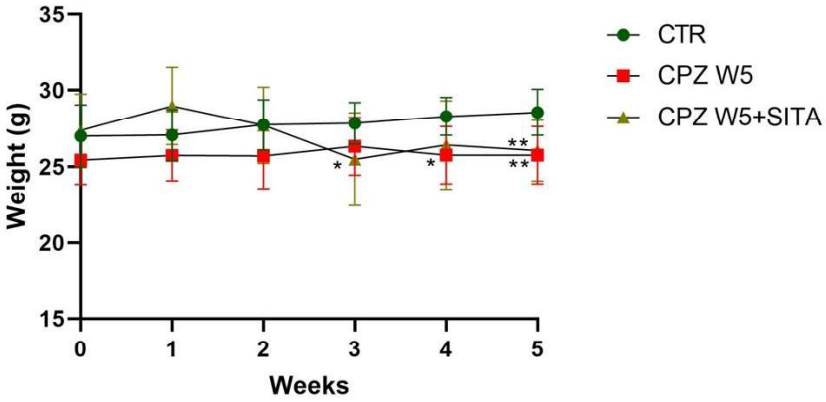


Figure 11. Body Weight evolution for the three experimental groups from Week 0 to Week5. Results are expressed as mean ± S.E.M of 8-10 animals per group. Repeated measures ANOVA * $p<0.05$, ** $p<0.01$ vs CTR. *Untreated (dark green); CPZW5 – cuprizone-treated at week 5 (red); CPZSITAW5 – cuprizone-Sitagliptin at week 5 (light green)*.

Glycemia was monitored at Week 0, 2 and 5. CPZ-intoxicated animals showed a trend to decrease glycemia values, but no statistical significance was observed. Sitagliptin didn't evoke any significant change in occasional glycemia (Figure 12).

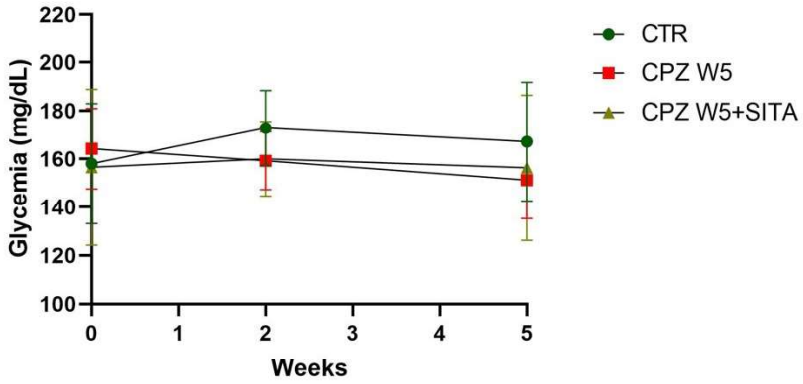


Figure 12. Occasional glycemia for the three experimental groups at Week 0, 2 and 5. Results are expressed as mean ± S.E.M of 8-10 animals per group. *Untreated (dark green); CPZW5 – cuprizone-treated at week 5 (red); CPZSITAW5 – cuprizone-Sitagliptin at week 5 (light green).*

Thymus wet weight was measured at the day of euthanasia (W5). Both absolute and relative thymus weight display a marked reduction in the CPZ-intoxicated and SITA-treated animals ($p<0.05$, $p<0.01$) (Figure 13).

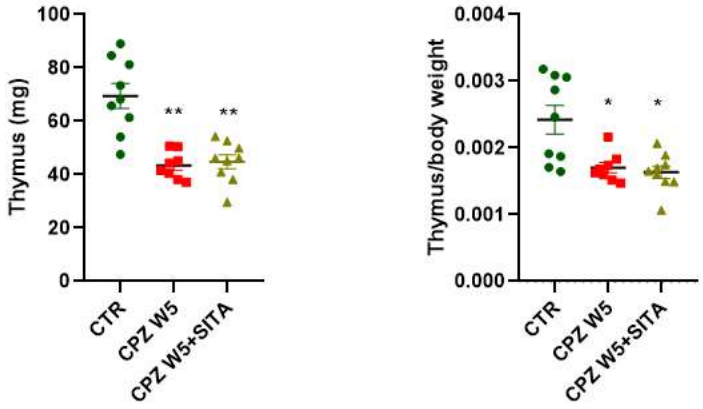
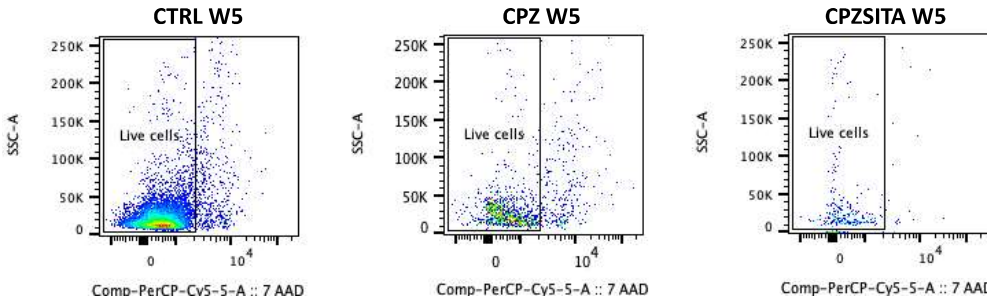


Figure 13. Absolute thymus weight (left) and relative thymus weight (right) for the three experimental groups. Results are expressed as mean ± S.E.M of 8-10 animals per group. One way ANOVA * p<0.05, ** p<0.01 vs CTR. Untreated (dark green); CPZW5 – cuprizone-treated at week 5 (red); CPZSITAW5 – cuprizone-Sitagliptin at week 5 (light green).

Then, we investigated whether cuprizone-induced thymic atrophy paralleled thymocytes death. In the CPZW5 condition (12288 ± 11134 events, n = 5) there are far fewer total events which indicates a lower number of viable cells which is borderline in the statistical significance (p=0.0537) (Figure 14). There is a significant decrease in total events in the CPZ W5 SITA (2753 ± 2296 events, n = 5) condition compared to CTRL (53408 ± 5601 events, n = 7, p < 0.0001). Similarly, one sees a trend towards lower viability in the CPZ and CPZW5 SITA groups which indicates that CPZ induced death (Figure 14).

A.



B.

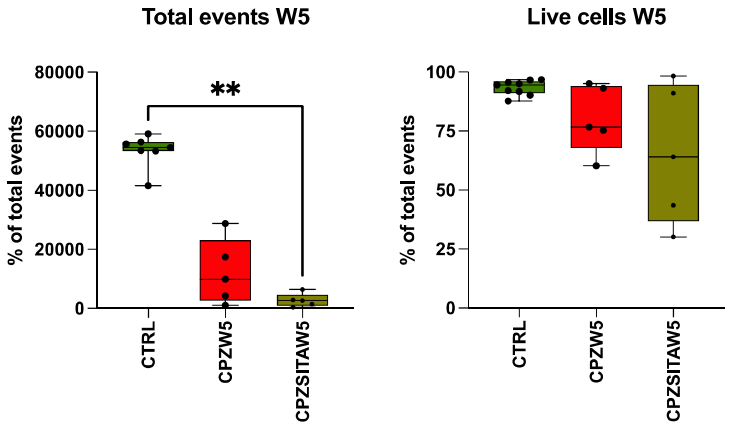
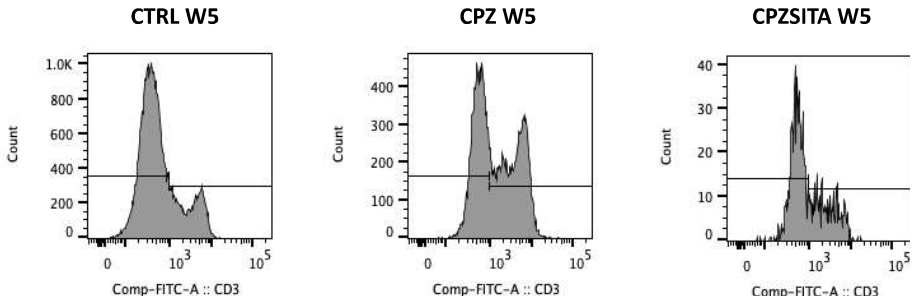


Figure 14. Characterization of CPZ-induced cell death (Week 5). Thymus suspensions cell death was determined using multiparametric flow cytometry with 7-AAD staining. A) Representative dot-plots of the cut-off for positive 7-AAD staining of the 3 experimental groups. B) From left to right total events and live cells of mice treated with CPZ for 5 weeks. Results are presented as mean ± standard deviation. CTRL – Untreated (dark green); CPZW5 – cuprizone-treated at week 5 (red); CPZSITAW5 – cuprizone-Sitagliptin at week 5 (light green); **p < 0.01.

To further analyze cuprizone's effect on the thymus, we performed flow-cytometry and access the MFI of CD3 in the thymus where it can be seen that the fluorescence intensity of CD3 is different in the CTRL, CPZ W5 and CPZ W5 SITA groups. Total CD3 MFI shows a significant increase in CPZW5 compared to CTRL ($p<0.01$) and this translates into a significant decrease in $CD3^{low}$ ($p<0.01$) and a significant increase $CD3^{high}$ ($p<0.01$). The $CD3^{high}/CD3^{low}$ ratio is also significantly increased ($p<0.01$) in the CPZW5 group. SITA-treated animals displayed a CD3 phenotype similar to control animals (Figure 15).

A.



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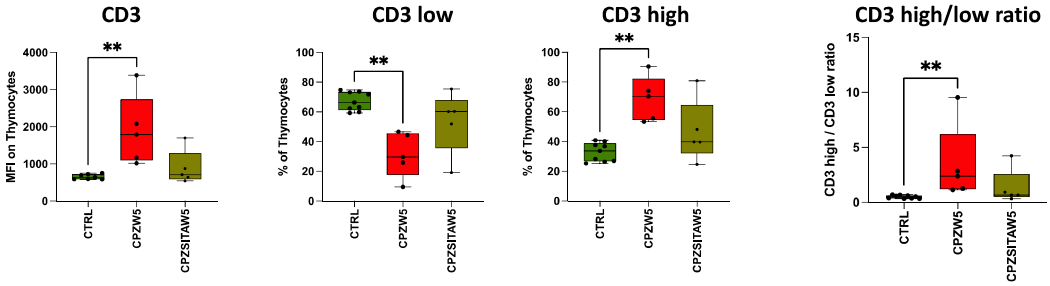
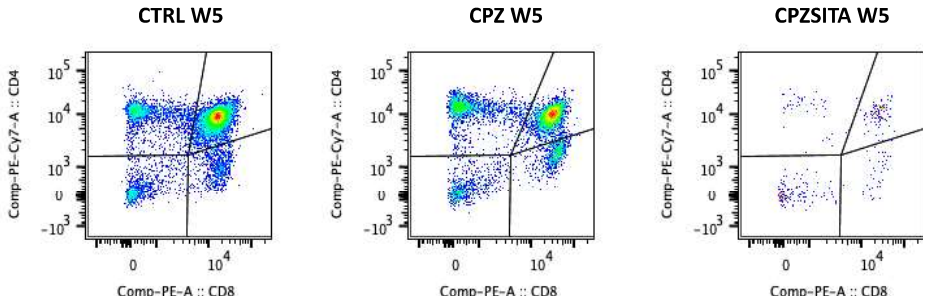


Figure 15. Characterization of thymocytes CD3 positivity. Flow cytometry was performed on thymus suspensions of untreated (CTRL, dark green) cuprizone-treated (CPZW5, red) and cuprizone-Sitagliptin (CPZSITAW5 light green) mice. Results are presented as representative line-charts (A) and points diagrams (B) as mean ± standard deviation. **p < 0.01.

Then, we intended to identify the T-cell subpopulation that was most sensitive to CPZ intoxication. Flow-cytometry analysis revealed that CPZ decreases substantially $CD4^+CD8^+$ DP T cells ($p<0.05$) while elevating the $CD4^-CD8^-$ DN T cells ($p<0.05$) and $CD4^+CD8^-$ T cells ($p<0.05$). Sitagliptin-treatment animals also showed a decrease in the percentage of $CD4^+CD8^+$ DP T cells ($p<0.05$) but an elevated percentage of $CD4^-CD8^-$ DN T cells ($p<0.05$). Notably, a significant increase of $CD4^-CD8^+$ T cells was observed ($p<0.05$) without any significant alterations in $CD4^+CD8^-$ T cells subpopulation (Figure 16).

A.



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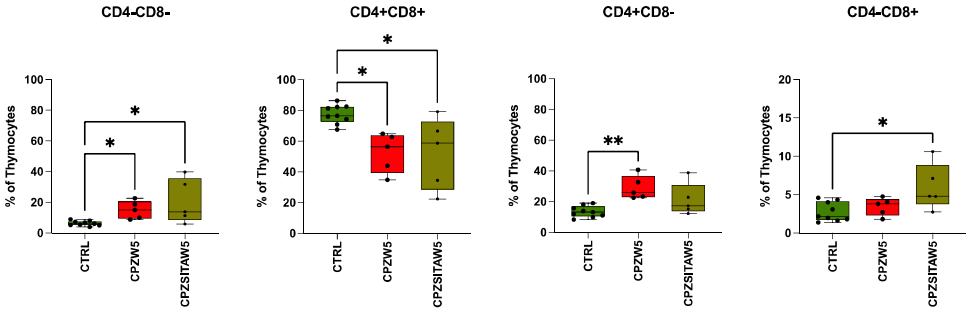


Figure 16. Characterization of thymocytes subpopulations. Flow cytometry was performed on thymus suspensions of untreated (CTRL, dark green) cuprizone-treated (CPZW5, red) and cuprizone-Sitagliptin (CPZSITAW5 light green) mice. Results are presented as dot-plots (A) and points diagrams (B) as mean ± standard deviation. DN: $CD4^-/CD8^-$ cells (lower left quadrant); CD4: CD4+ cells (lower right quadrant); CD8: CD8+ cells (upper left quadrant); DP: CD4+/CD8+ cells (upper right quadrant). ***p < 0.01.*

Given the i) profound alterations on CD3 positivity and thymocyte subpopulations induced by cuprizone and ii) the fact that sitagliptin abrogated the elevation of CD3 positivity, $CD3^{high}/CD3^{low}$ ratio and elicited a shift towards increased $CD4^-CD8^+$ T cells, we then filtered the results for the $CD3^{low}$ and $CD3^{high}$ subpopulations and characterize T cell subset frequencies. We observed that most $CD4^-CD8^-$ DN T cells and $CD4^+CD8^+$ DP T cells were characterized by a $CD3^{low}$ positivity ($p<0.01$) upon cuprizone intoxication even though the DP T cells showed a slight decrease in CD3 positivity. Consistently, the cuprizone induced increase in $CD4^+CD8^-$ T cells were mostly $CD3^{low}$ ($p>0.05$), denoting an immature phenotype. Remarkably, sitagliptin induced major changes in CD3 positivity in T cell subsets regardless the lack of alterations on the MFI of total CD3. Particularly, sitagliptin increased the percentage of $CD3^{bright}$ $CD4^-CD8^-$ DN T cells ($p<0.01$) while decreasing the percentage of $CD3^{bright}$ $CD4^+CD8^+$ DN T cells ($p<0.05$). Notably, we found that the increase in $CD4^-CD8^+$ T cells upon sitagliptin treatment corresponds mainly to $CD3^{bright}$ cells ($p<0.05$), a marker of lymphocyte maturation (Figure 17).

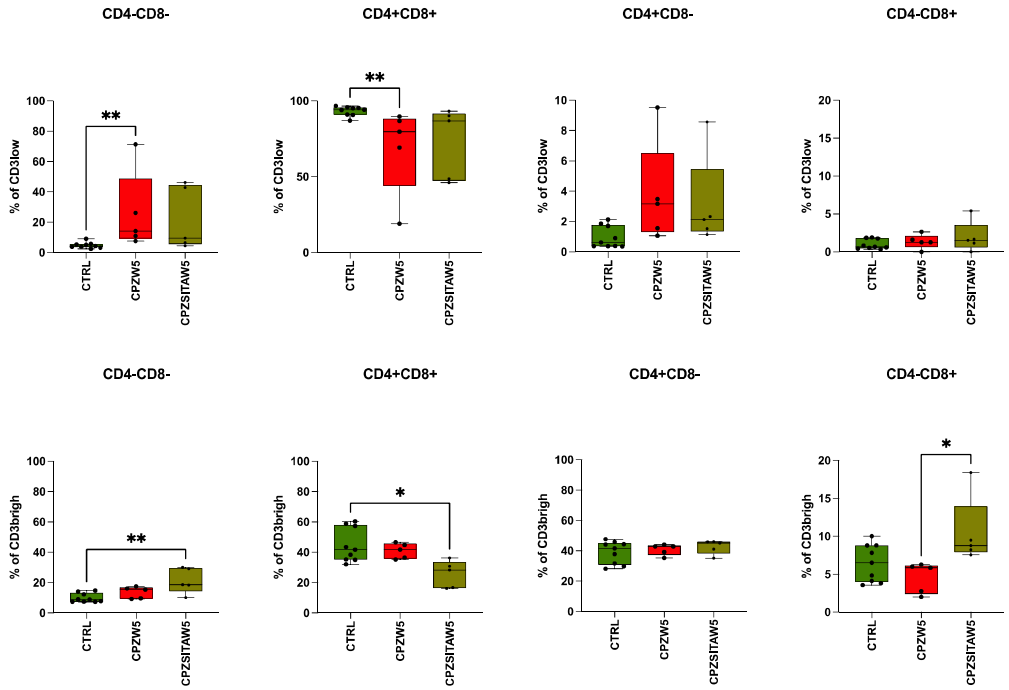


Figure 17. Effect of cuprizone treatment on CD3 positivity of thymocyte subpopulations. Flow cytometry was performed on thymus suspensions of untreated (CTRL, dark green) cuprizone-treated (CPZ W5, red) and cuprizone-Sitagliptin (CPZ SITA W5 light green) mice. Results are presented as points diagrams as mean ± standard deviation.

Finally, we intended to understand the central effects of cuprizone and sitagliptin treatments in order to understand the clinical relevance of cuprizone-induced thymic involution as well as sitagliptin thymocyte remodeling. Cerebellum was chosen given the group previous work showing that it is a target brain region of cuprizone intoxication [122]. Consistently, we found that intoxicated animals show decreased MBP and PLP gene expression levels, denoting a demyelinated pattern ($p<0.0001$). Surprisingly, sitagliptin aggravated the demyelination process ($p<0.05$ CPZ W5 vs CPZ W5 SITA) (Figure 18).

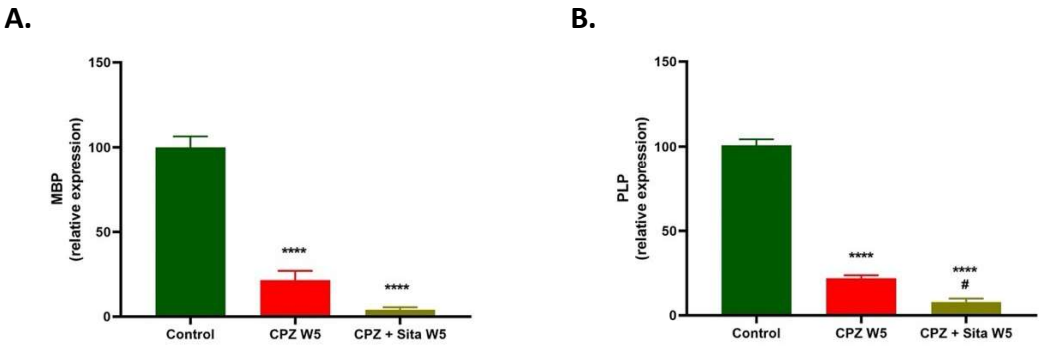


Figure 18. Cerebellar gene expression of myelin basic protein (MBP, A) and proteolipid protein (PLP, B) for the three experimental groups at Week 5. Results are expressed as mean ± S.E.M of 4-6 animals per group. One-way ANOVA **** $p<0.0001$ vs CTR; # $p<0.05$ vs CPZ W5. Untreated (dark green); CPZW5 – cuprizone-treated at week 5 (red); CPZSITAW5 – Cuprizone-Sitagliptin at week 5 (light green).

4.2. The impact of sitagliptin in cuprizone-induced thymic atrophy – Remyelination phase (Week 7)

Body weight (BW) was monitored weekly during the entire study period. Similarly to what we found in the previous protocol (W5), animals intoxicated with cuprizone showed a compromised body weight evolution in the first 5 weeks, reaching statistical significance at W4 ($p<0.05$) and W5 ($p<0.01$). Upon cuprizone withdrawal, animal reacquired a trend to normalize the body weight gain. SITA-treated animals also showed a similar growth curve pattern to the CPZ-intoxicated ones but only reaching statistical significance at W5 ($p<0.01$). It was also found that sitagliptin treatment did not altered body weight gain from W5 to W7 (Figure 19).

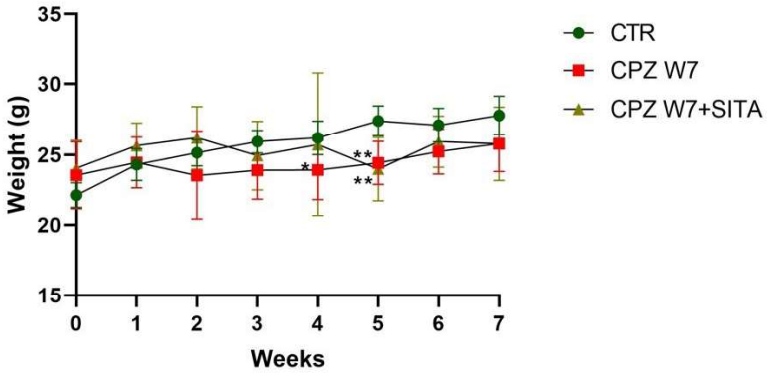


Figure 19. Body Weight evolution for the three experimental groups from Week 0 to Week7. Results are expressed as mean ± S.E.M of 8-10 animals per group. Repeated measures ANOVA * $p<0.05$, ** $p<0.01$ vs CTR. *Untreated (dark green); CPZW7 – cuprizone-treated at week 7 (red); CPZSITAW7 – cuprizone-Sitagliptin at week 7 (light green).*

Glycemia was monitored at Week 0, 5 and 7. No statistical significance was observed in any time-point within the three experimental groups. (Figure 20).

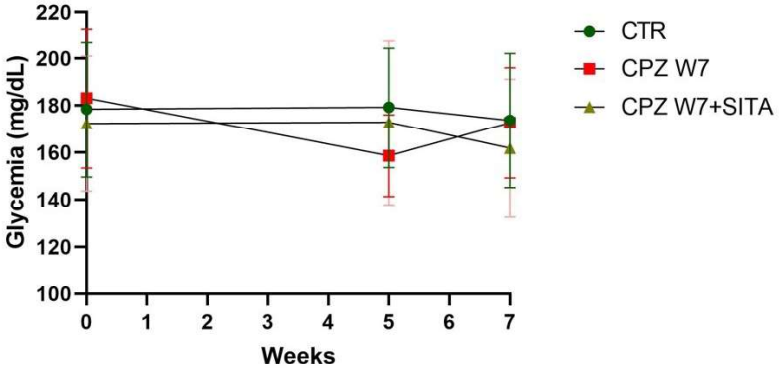


Figure 20. Occasional glycemia for the three experimental groups at Week 0, 5 and 7. Results are expressed as mean ± S.E.M of 8-10 animals per group. *CTRL – Untreated (dark green); CPZW7 – cuprizone-treated at week 7 (red); CPZSITAW5 – cuprizone-Sitagliptin at week 7 (light green).*

Thymus wet weight was measured at the day of euthanasia (W7). Both absolute and relative thymus weight display values similar to the control animals in the CPZ-intoxicated and SITA-treated animals (Figure 21).

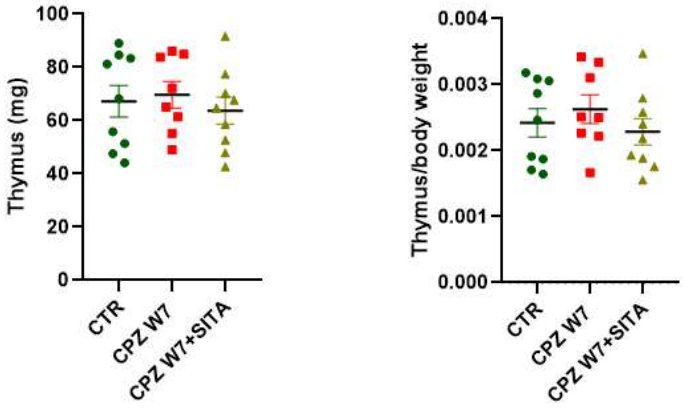
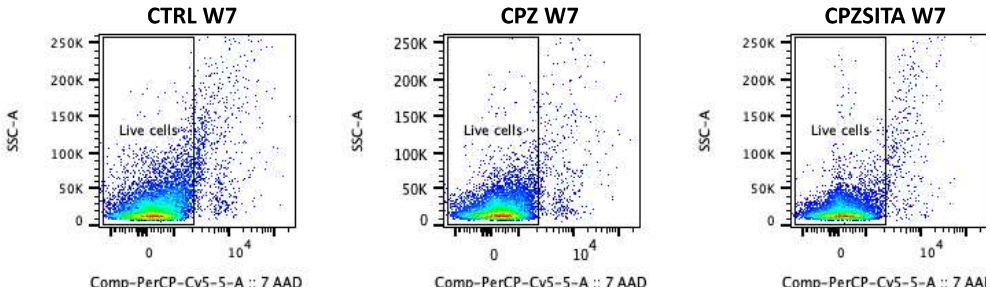


Figure 21. Absolute thymus weight (left) and relative thymus weight (right) for the three experimental groups. Results are expressed as mean ± S.E.M of 8-10 animals per group. CTRL – Untreated (dark green); CPZW7 – cuprizone-treated at week 7 (red); CPZSITAW5 – cuprizone-Sitagliptin at week 7 (light green).

Next, we investigated whether cuprizone-induced thymic atrophy was still present upon CPZ withdrawal for 2 weeks – Week 7 (the remyelination period). No significant changes were observed in any experimental group, denoting that thymus regenerate upon toxin suspension (Figure 22). Sitagliptin did not induced significant alterations in the thymic regeneration process.

A



B

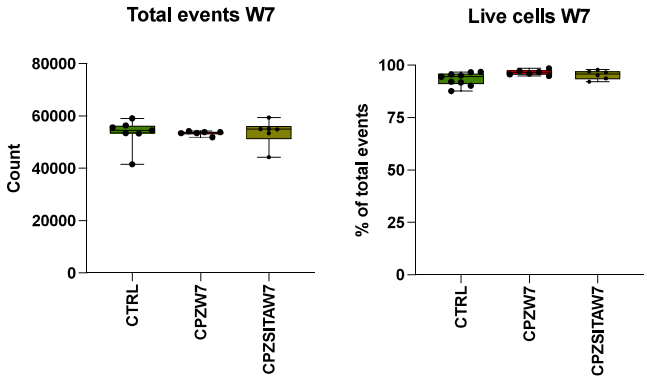


Figure 22. Characterization of CPZ-induced cell death (Week 7). Thymus suspensions cell death was determined using multiparametric flow cytometry with 7-AAD staining. A) Representative dot-plots of the cut-off for positive 7-AAD staining of the 3 experimental groups. B) From left to right total events and live cells of mice treated with CPZ at Week 7. Results are presented as mean ± standard deviation. *CTRL – Untreated (dark green); CPZW7 – cuprizone-treated at week 7 (red); CPZSITAW5 – cuprizone-Sitagliptin at week 7 (light green).*

To further analyze cuprizone's effect on the thymus upon 2 weeks of toxin withdrawal, we performed flow-cytometry and access the MFI of CD3. Total CD3 MFI, $CD3^{low}$, $CD3^{high}$ and $CD3^{high}/CD3^{low}$ ratio were found restored to control levels. SITA-treated animals displayed a CD3 phenotype similar to control animals (Figure 23).

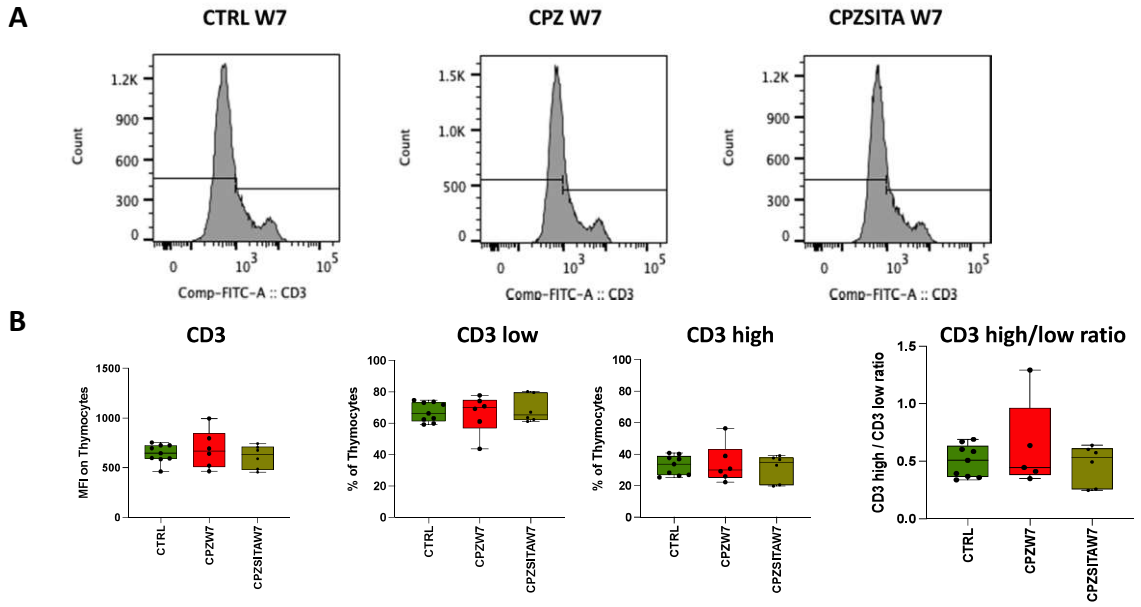


Figure 23. Characterization of thymocytes CD3 positivity. Flow cytometry was performed on thymus suspensions of untreated (CTRL, dark green) cuprizone-treated (CPZ W7, red) and cuprizone-Sitagliptin (CPZ SITAW7 light green) mice. Results are presented as representative line-charts (A) and points diagrams (B) as mean ± standard deviation.

Then, we intended to characterize the reversible nature of CPZ-induced altered T cells subpopulations 2 weeks following CPZ withdrawal. In addition, we also aimed to understand whether SITA-treatment could affect T cells subpopulations remodeling at the remyelination phase (W7). Flow-cytometry analysis revealed unaltered T cells subpopulations in all experimental groups, implying that 2 weeks of toxin withdrawal is sufficient to reestablish thymocytes dynamics. Again, SITA-treatment didn't perturb thymic T cells recovery (Figure 24).

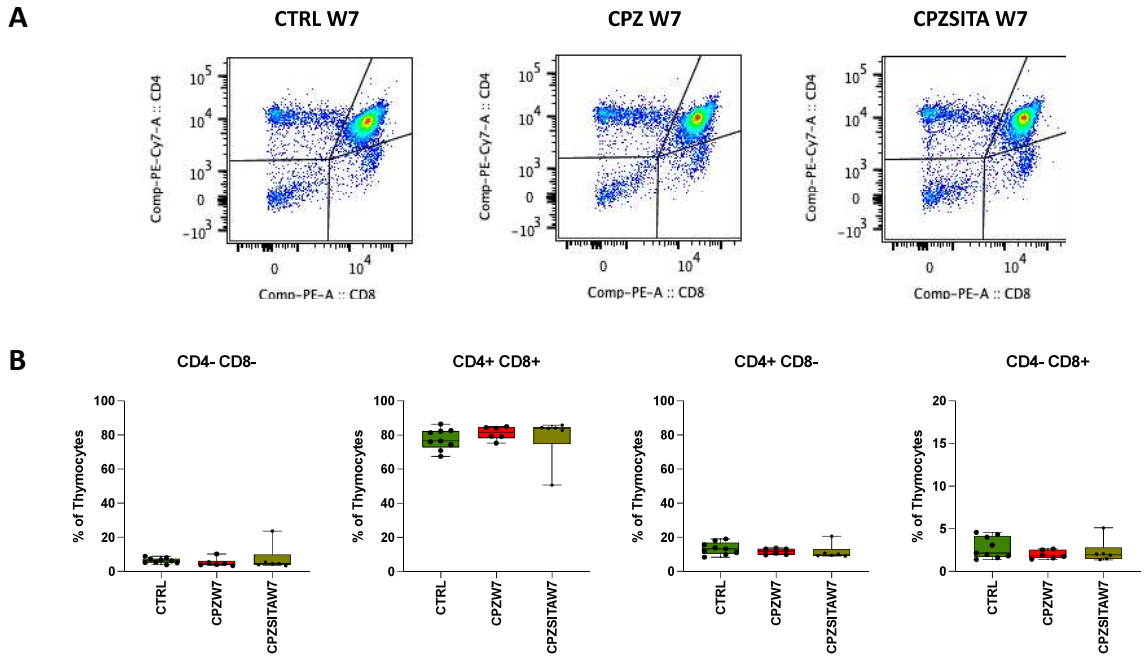


Figure 24. Effect of cuprizone treatment on thymocyte subpopulations. Flow cytometry was performed on thymus suspensions of untreated (CTRL, dark green) cuprizone-treated (CPZW7, red) and cuprizone-Sitagliptin (CPZ SITAW7 light green) mice. Results are presented as dot-plots (A) and points diagrams (B) as mean ± standard deviation. DN: CD4-/CD8- cells (lower left quadrant); CD4: CD4+ cells (lower right quadrant); CD8: CD8+ cells (upper left quadrant); DP: CD4+/CD8+ cells (upper right quadrant).

Next, we characterized the CD3 positivity of thymocyte subpopulations in all experimental groups. In opposition to what was observed at W5, we found no significant changes on CD3 positivity of any T cell subset in any experimental group (Figure 25).

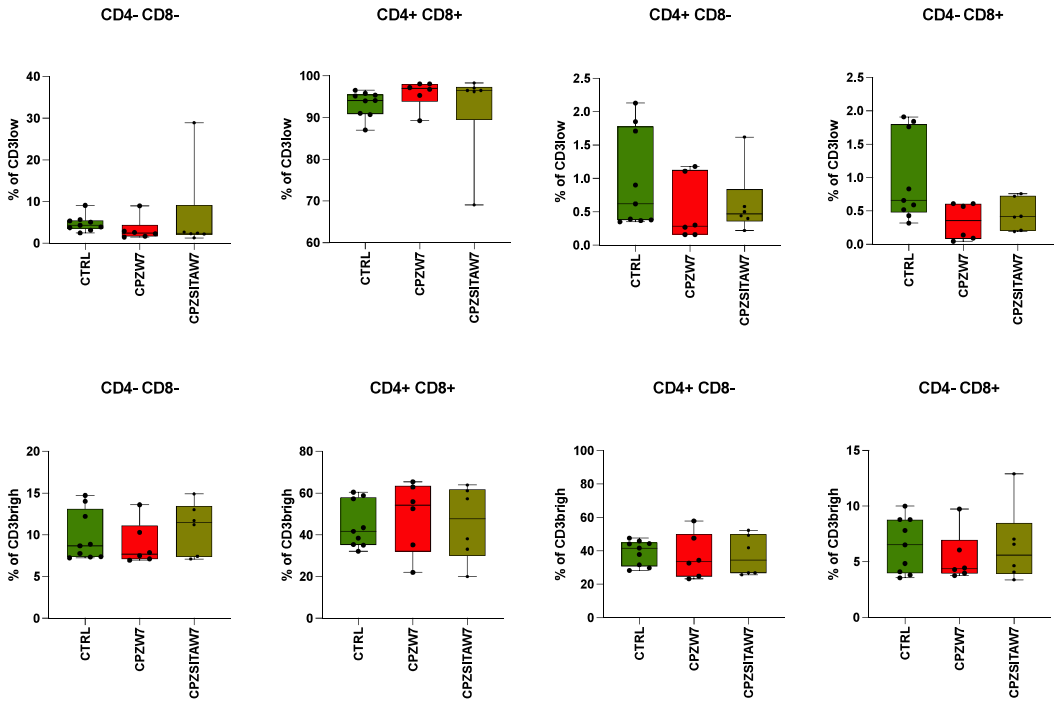


Figure 25. Effect of cuprizone treatment on CD3 positivity of thymocyte subpopulations. Flow cytometry was performed on thymus suspensions of untreated (CTRL, dark green) cuprizone-treated (CPZW5, red) and cuprizone-Sitagliptin (CPZ SITA W7 light green) mice. Results are presented as points diagrams as mean ± standard deviation.

Finally, we intended to understand the central effects of cuprizone and sitagliptin treatments in all experimental groups in the remyelination phase. Consistently, we found that intoxicated animals show MBP and PLP gene expression levels similar to control animals, denoting a remyelinated pattern. Unexpectedly, sitagliptin arrested the remyelination recovery typically observed at W7 (MBP, $p<0.001$ vs CTL /CPZ W7; PLP, $p<0.05$ vs CTL and $p<0.01$ vs CPZ W7) (Figure 26).

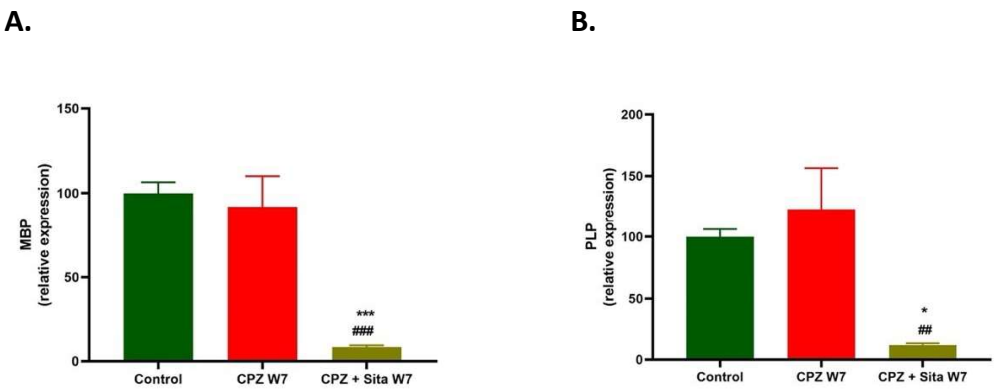


Figure 26. Cerebellar gene expression of myelin basic protein (MBP, A) and proteolipid protein (PLP, B) for the three experimental groups at Week 7. Results are expressed as mean ± S.E.M of 4-6 animals per group. One-way ANOVA * $p<0.05$ vs CTR; ## $p<0.01$ vs CPZ W5; *** $p<0.001$ vs CTR; ### $p<0.001$ vs CPZ W5. *Untreated (dark green); CPZW5 – cuprizone-treated at week 5 (red); CPZSITAW5 – cuprizone-Sitagliptin at week 5 (light green).*

Chapter V | **DISCUSSION & CONCLUSIONS**

Discussion and conclusions

MS is the most prevalent chronic inflammatory disease of the CNS. In the early stages of the disease, autoimmune inflammation and neurologic disability are transient, lasting days or weeks, and remyelination occurs. However, after 10 to 20 years, many patients develop a progressive clinical course characterized by an extensive and chronic neurodegeneration, eventually leading to impaired mobility and cognition [123]. Besides, MS patients also display other systemic perturbations, namely early thymic involution and perturbed naïve CD4 T-cell homeostasis that are suspected to predispose patients to autoreactivity [64].

MS patients benefit from symptomatic treatments that shorten the duration of acute exacerbations, decreasing their frequency and providing symptomatic relief. Most of these approved pharmacological agents, such as interferon β-1b, interferon β-1a, glatiramer acetate (GA), fingolimod or teriflunomide (to name just a few) tackle the patient's immune system. For instance, fingolimod blocks the migration of lymphocytes from lymph nodes, thereby reducing the number of lymphocytes in the periphery that, in turn, may reduce the lymphocyte migration into the CNS. GA also modifies the immune system, inducing and activating suppressor T cells in the periphery. Teriflunomide displays a cytostatic effect on proliferating B and T cells as well [3, 124]. Collectively, the currently approved disease modifying treatments mostly rely on T cells as a key pharmacological target. Yet, no curative therapies for MS are currently available.

Considering that human CNS can only be monitored by non-invasive techniques, the use of animal models has been essential to explore mechanisms of disease initiation and progression, as well as to test several novel therapeutic approaches for the disease [73]. The EAE and the Cuprizone intoxication models can mimic some clinical and pathological aspects of the disease and are the most widely used in preclinical research to broaden our understanding of MS. Remarkably, immune thymic profile has been also observed in both EAE and Cuprizone animal models regardless their divergent pathophysiological mechanisms [58, 61]. The consistent pattern of thymic involution reported in MS patients and animal models, along with its putative relationship with increased predisposition to autoimmunity, justifies the need of additional research aimed to explore the functional significance of thymic involution in MS progression in order to develop new pharmacological approaches able to effectively halt or slow down MS.

Given that i) CD26/DPP-IV holds an important role in T cell activation and proliferation and is a thymic maturation marker in both rodents and humans [100] and that ii) CD26/DPP-IV appears to be a key player on autoreactive T cells activation and autoimmune inflammation in MS [101, 102, 105, 106], we asked whether a currently approved DPP-IV inhibitor – sitagliptin – would modulate thymic involution and provide pharmacological benefits in MS. To this end, the cuprizone (CPZ) intoxication animal model was established and characterized.

The standard CPZ animal model of MS consists in the administration of 0.2% (w/w) CPZ for 5 weeks to induce acute demyelinating lesions followed by the CPZ withdrawal for additional 2 weeks to enable for spontaneous remyelination. In the present work, we found that CPZ administration impaired the animals' grow curves during the intoxication period as animals tended to gain less weight when compared to control ones (W5). This observation was not surprisingly since previous reports also show that mice administered with CPZ tended to gain less weight [75]. Still, the animals from the CPZ W7 group showed a trend to gain more weight than the ones composing the CPZ W5, an expected outcome since the seven-week protocol includes the toxin withdrawal to accomplish a recovery period. Since food and water consumption was similar between all experimental groups (data not shown), we assume that the observed body weight alterations are due to cuprizone intoxication itself. Moreover, sitagliptin didn't elicited any significant alteration in food consumption nor reversed cuprizone-induced body weight loss despite its anorexigenic properties [125].

Given that sitagliptin is an antidiabetic drug, occasional glycemia values were also monitored. Neither cuprizone nor sitagliptin altered circulating glycemic values. Again, this was an expected observation since sitagliptin reduces blood glucose levels by a glucose-dependent mechanism, which is the main reason for the very low risk of hypoglycemia presented by sitagliptin [126]. Then, we looked for cuprizone and sitagliptin effects on thymus, a primary lymphoid organ and the site of T-cell maturation and selection. After 5 weeks of cuprizone administration, a significant thymic tissue mass loss was observed. Since cuprizone treatment resulted in a significant weight loss for the animals as well, we normalized their thymic mass to their body mass, and found this relative thymus mass still reduced in the CPZ W5 mice, indicative of a disproportional thymus involution, as previously reported [61]. What was not knew was if thymus was able to regenerate upon 2 weeks of cuprizone withdrawal, analogous to what is described for CNS remyelination. Herein, we were able to disclose the reversible nature of cuprizone's deleterious effects on the thymus since no alterations were observed in thymus weight at W7. Thus, we highlight this cuprizone paradigm as a useful and valuable model to study thymus regeneration.

Besides thymus wet weight, we also followed the cuprizone and sitagliptin effects in the total number of live thymocytes by the 7-AAD staining protocol, a fluorescent intercalator that undergoes spectral shift upon binding to GC regions of DNA and therefore allows us to distinguish between viable/dead cells [127]. Surprisingly, we found that sitagliptin aggravated CPZ-induced thymocytes death at the peak of demyelination (W5). Consequently, we move forward and aimed to assess thymocyte subpopulations and the corresponding maturation process.

Cortical thymic DN $CD4^{-}CD8^{-}$ T cells are the most immature thymocyte population and relocate to the thymic medulla for maturation and differentiation. Since signaling through the CD3 complex is core for T cell maturation, we applied the ratio of immature ($CD3^{low}$) and mature ($CD3^{high}$) thymocyte to characterize T-cell maturation status upon cuprizone and sitagliptin treatments. We found that cuprizone intoxication increased total

CD3 MFI and the CD3 $^{high/low}$ ratio at W5, hinting for a boosted rate of T cell maturation. Then, we intended to identify the T cell subpopulation that was most sensitive to cuprizone effects. We found a decreased number of DP $CD4^+CD8^+$ T cells and increased $CD4^+$ T cell frequency with CD3 positivity similar to control levels. Others have previously shown that one week of cuprizone treatment resulted in a significant lower proportion of immature DP $CD4^+CD8^+$ T cells. The same also holds true for the EAE onset [58]. Herein, we corroborate those observations with an extended protocol of cuprizone intoxication (5 weeks) and confirm that cuprizone eliminates immature thymocytes preferentially. However, while one week of cuprizone intoxication elicits an increased frequency of both $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells, 5 weeks of CPZ exposition consolidates the mature $CD4^+$ helper T cell lineage as the most prevalent one [61]. Curiously, immature $CD4^+CD8^+$ DP thymocytes have been found to display the highest susceptibility to increased corticoid levels and experience a marked stress-induced reduction [128]. Thus, future studies are needed to help us to elucidate if the thymic alterations are solely a consequence of increased corticosterone and/or increased production of pro-inflammatory cytokines, or if there is a cause-consequence relation between what happens in the CNS and in the thymus.

Unexpectedly, sitagliptin induced profound changes in thymic remodeling during the demyelination phase of cuprizone intoxication. Firstly, it arrested the overall increase in CD3 $^{high/low}$ ratio induced by CPZ, suggestive of T cells maturation blockade. Moreover, sitagliptin reshaped T cells phenotype upon cuprizone treatment into a dominant $CD4^-CD8^+$ T cell. Surprisingly, those cells were mostly $CD3^{high}$, hinting for a prevalent (and selective) mature CD8 phenotype. CD8 T cells are essential players of the adaptive immune system and may display both effector and regulatory roles. Notably, CD8 T cells are emerging as important effector and regulatory cells in MS. For instance, cytotoxic effector T cells can be detected within perivascular cuffs and parenchymal lesions with their cytolytic granules polarized towards demyelinated axons, indicative of imminent T cell mediated killing [129]. By the other hand, $CD8^+$ Treg cells render suppression of recurrent relapsing episodes in the EAE mice and resistance to EAE induction [130]. Indeed, a significant reduction of the number of $CD8^+$ Treg cells in the circulation of multiple sclerosis patients has been observed [131].

Analogous to our findings, the inhibition of DPP-IV/CD26 was associated with changes in $CD8^+$ T effector memory subset in the NOD mice with increased protection against autoimmunity to β pancreatic islets. Actually, DPP-IV inhibition increased the expression of CD26 on $CD8^+$ effector memory T cells from both the spleen and pancreatic lymph nodes [132]. Accordingly, sitagliptin-induced DPP-IV inhibition did not arrest the maturation of CD8 T cell subset in our experimental setting. CD26/DPP-IV is expressed in CD8 T cells and $CD8^+CD26^{high}$ T cell subsets have been characterized as early effector memory T cells that exert a cytotoxic effect [133]. Collectively, we propose future studies aimed to assess CD26/DPP-IV expression/activity in thymic T cell subsets and brain samples. Moreover, it will be of utmost importance to further disclose the presence of circulating/central CD8 T

cells as well as the cytotoxic or regulatory nature of sitagliptin-induced CD8 T cell differentiation and maturation in the cuprizone-intoxicated thymus.

Remarkably, the effects of sitagliptin on thymic T cell subsets in the demyelination period actually paralleled a robust downregulation of myelin-related genes expression. Moreover, sitagliptin did not perturbed the spontaneous thymic regeneration observed 2 weeks following cuprizone withdrawal. However, in this remyelination period, we observed a very expressive negative impact of sitagliptin treatment in the CNS taking into account the severe blockade of myelin-related gene expression in cerebellar samples, in opposition to the spontaneous remyelination pattern observed in the cuprizone intoxicated animals (W7). First, it seems reasonable to infer that in the cuprizone-intoxicated model, thymic regeneration and central remyelination are independent, albeit simultaneous processes, as sitagliptin treated animals show thymic regeneration but lack central remyelination. Secondly, it is unlikely that this drug actually affords protection in the cuprizone-animal model of MS regardless the effector or regulatory nature of CD8 T cell subsets induced by sitagliptin given the robust negative impact observed in the expression of myelin-related proteins in cerebella.

DPP-IV has a range of additional functions besides being a protease for substrates relevant to energy homeostasis. In this regard, CD26 is considered to be an important regulator of T-cell function. Such pleiotropic effects of DPP-IV encourage the potential uses of its inhibitors other than type 2 diabetes, including autoimmune diseases. However, some of these effects may manifest as adverse events and may not yet have been reported in the currently literature [134]. Given the results reported herein, it appears to be prudent to keep an eye on DPP-IV inhibitors and autoimmune diseases and future preclinical studies should be conducted to further disclose not only the potential therapeutic effect of DPP-IV inhibitors in autoimmunity but also the plausible side-effects in aforesaid disorders.

Chapter VI | **REFERENCES**

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