Universidade de Lisboa

Faculdade de Farmácia

TARGET S100B TO REDUCE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS PATHOGENESIS

Catarina da Silva Barros

Dissertation supervised by Professor Doctor Adelaide Maria Afonso Fernandes Borralho and co-supervised by Doctor Andreia Pereira Barateiro Macedo

Master course in Biopharmaceutical Sciences

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The studies presented in this thesis were performed in the Neuron-Glia in Health and Biology group, at the Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, under the supervision of Adelaide Fernandes, PhD, and the co-supervision of Andreia Barateiro, PhD.

The studies included in this thesis were selected as poster communications:

Barros C., Pascoal P., Santos G., Barateiro A., Freitas R., Brites D., Graça L., Fernandes A. Inhibition of S100B prevents ex vivo demyelination and improves EAE clinical score. 19th Biennial Meeting for the Society for Free Radical Research International (SFRRI). June 4th-7th, 2018.

Barros C., Pascoal P., Santos G., Barateiro A., Freitas R., Brites D., Graça L., Fernandes A. S100B targeting prevents *ex vivo* demyelination and reduce EAE severity. 10th iMed.ULisboa Postgraduate Students' Meeting & 3rd i3DU Meeting. July 24th-25th, 2018.

Barros C., Pascoal P., Santos G., Barateiro A., Freitas R., Brites D., Graça L., **Fernandes A**. Targeting S100B reduces demyelination-associated pathogenesis in *ex vivo* and *in vivo* models mimicking Multiple Sclerosis. 34th Congress of the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS). October 10th-12th, 2018.

The studies included in this thesis were selected as oral communications:

Barros C., Pascoal P., Santos G., Barateiro A., Freitas R., Brites D., Graça L., Fernandes A. (2018). S100B blockage prevents *ex vivo* demyelination and ameliorates EAE clinical score. Reunião de Primavera do Grupo de Estudos de Esclerose Múltipla: *"A caminho da personalização? Novas perspetivas no tratamento e monitorização na esclerose múltipla centradas no doente"*. May 18th-19th, 2018.

Work presented in this master thesis was supported by Medal of Honor L'Oréal for Women in Science, Innovation grant Ordem dos Farmacêuticos and GMSI–Merck to AF, and by Fundação para a Ciência e Tecnologia UID/DTP/04138/2013 to iMed.ULisboa, and post-doctoral grant SFRH/BPD/96794/2013 to AB.

To my family.

"It always seems impossible until it's done."
-Nelson Mandela

Resumo

A Esclerose Múltipla (EM) é uma doença autoimune, crónica e inflamatória que afeta o sistema nervoso central (SNC) e é considerada a doença neurológica não traumática e incapacitante mais comum em adultos jovens. Estima-se que em todo o mundo existam, aproximadamente, 2.3 milhões de pessoas com EM. A EM enquadra-se nas doenças associadas ao sistema imunitário, uma vez que a sua ativação resulta na formação de lesões focais no SNC e na neuroinflamação que, consequentemente, conduz à desmielinização e lesão axonal. Estudos recentes demonstraram que a molécula inflamatória S100B, quando libertada em quantidades excessivas, está relacionada com processos inflamatórios e desmielinizantes. Curiosamente, usando o modelo *ex vivo* de desmielização, a inibição do S100B através de um fármaco específico, a pentamidina, mostrou benefícios a nível dos processos inflamatórios, apontando o S100B como possível alvo terapêutico.

Este trabalho focou-se na modulação do S100B, pela utilização da pentamidina no modelo animal de EM, a Encefalopatia Autoimune Experimental (EAE), onde previamente demonstrámos o envolvimento do eixo S100B-RAGE. O nosso principal objetivo baseou-se no estudo do papel da pentamidina no modelo *in vivo* da EM, a fim de melhorar o curso da doença e prevenir a desmielinização e a neuroinflamação.

Inicialmente, observámos que os animais tratados com pentamidina desenvolveram uma menor disfunção motora e tiveram uma recuperação mais rápida. Com este tratamento conseguimos ainda prevenir a perda de mielina. Verificámos, também, que o tratamento com a pentamidina levou a um aumento do marcador de oligodendrócitos (OL) maturos (MBP) e uma diminuição do número de células progenitoras $NG2^+$. Neste modelo demonstrámos ainda que os animais induzidos com EAE têm uma significativa ativação de astrócitos e microglia, confirmada pela elevada densidade de células $GFAP^+$ e $Iba1^+$, respetivamente. O tratamento com pentamidina preveniu ambas as ativações, diminuindo a reatividade glial, assim como a expressão de S100B e consequente resposta pro-inflamatória.

Em suma, os nossos resultados permitem-nos concluir que a proteína inflamatória S100B está envolvida na patologia associada à EM e que, usando um tratamento profilático com pentamidina, conseguimos prevenir os efeitos tóxicos associados à patogénese da doença. Assim, a inibição do S100B pode ser uma nova estratégia terapêutica não só para reduzir o dano associado à patologia da EM, mas também melhorar a recuperação.

Palavras-chave: EAE, reatividade glial, neuroinflamação, esclerose múltipla, S100B

Abstract

Multiple Sclerosis (MS) is a complex chronic immune disease in the central nervous system (CNS), causing neurological disability in young people aged between 20 to 40 years. It is considered one of the world's most common neurological disorder that affect approximately 2.3 million people worldwide. The disease symptoms result from attacks that damaged the nerve fibers in the CNS, due to the overactivation of the immune system. Furthermore, the inappropriate activation of the immune system results in focal lesions on CNS, inflammation and destruction of myelin sheaths of neurons, main features of MS pathology. Recently, the inflammatory molecule S100B, in toxic levels, was directly correlated with demyelination and inflammatory processes using an *ex vivo* demyelinating model. The beneficial outcome of its inhibition with pentamidine, an S100B-binding drug, in our demyelinating model indicates S100B as an emerging therapeutic target in MS.

In here, we aimed to target S100B in the animal model of MS, the experimental autoimmune encephalomyelitis (EAE), once we already demonstrated the involvement of S100B-RAGE axis. Indeed, we will use EAE to study whether by targeting S100B we may ameliorate the disease course and MS-like pathogenesis.

Firstly, we observed that the animals treated with pentamidine reach a lower disease clinical score and have a faster recovery. Moreover, histological analysis showed that EAE-induced animals have an increased percentage of demyelination, which was reduced upon pentamidine treatment. These results were corroborated by the loss of MBP marker, as well as protein expression when compared with pentamidine treated mice. Regarding gliosis, pentamidine decreased glia reactivity (both astrocytes and microglia) and the density of S100B-expressing astrocytes in EAE-induced animals. Furthermore, the inflammatory response was attenuated in pentamidine treated animals preventing the exacerbated expression of pro-inflammatory cytokines and even increasing the anti-inflammatory response.

Overall, our results indicate that S100B is involved in MS pathology and that, by using pentamidine, we could prevent the detrimental effects of this protein when in pathological levels. So, its inhibition may be a new therapeutic strategy not only to reduce damage but, hopefully, to improve recovery.

Keywords: EAE, glia reactivity, neuroinflammation, multiple sclerosis, S100B

AGRADECIMENTOS

Em primeiro lugar, gostaria de agradecer à Investigadora Coordenadora Dora Brites, investigadora principal do grupo *Neuron Glia Biology in Health and Disease*, por me ter dado a oportunidade de desenvolver a minha dissertação neste grupo. Obrigada por nos fazer pensar sempre mais à frente, pela partilha de experiências e conhecimento, e por nos incentivar a ser mais e melhor como pessoas e profissionais nesta área.

À minha orientadora, Professora Doutora Adelaide Fernandes, agradeço de coração! Para mim é difícil falar de alguém, que tão naturalmente se tornou tão próxima de mim. Ainda me lembro da primeira vez que fui falar contigo. Mal eu imaginava o que estava para vir! Adelaide, estou grata pela tua partilha diária, confiança e, acima de tudo, pela amizade que conseguimos construir nestes dois anos. Foi um privilégio ter-me cruzado contigo e ter tido a oportunidade de participar ativamente neste projeto. Nunca vou esquecer o abraço que me deste quando o meu coração estava apertado, nem das conversas profissionais e pessoais que tivemos ao longo deste ano, as quais me fizeram crescer, amadurecer e acreditar em mim. Contigo aprendi que com persistência e dedicação conseguimos sempre alcançar os nossos objetivos. Diariamente, eu agarro-me a isso e nos dias menos bons, não desisto. Tenho que te agradecer pela tua disponibilidade em todos os momentos do meu percurso, por acreditares sempre em mim, por ouvires todas as minha ideias após leituras e reflexões, pela tua boa disposição e por seres a melhor que podia ter escolhido. Ficar-te-ei grata para sempre!

À minha coorientadora, Doutora Andreia Barateiro. Nem sei por onde começar... Ao fim de um ano, criámos uma profunda cumplicidade. Entendes os meus discursos complicados e aquelas expressões tipicamente minhas. Desabafo contigo facilmente, rimos juntas, ouves-me com atenção, ajudas-me a lidar com as minhas frustrações quando ando meia perdida, aconselhas-me como ninguém e foste a pessoa que nunca duvidou das minhas capacidades. Eu sei que não gostas que te agradeça, mas obrigada. Por tudo, mesmo! No meio do caos, tu vias sempre a luz ao fundo do túnel a brilhar e à espera que eu lá chegasse. Foste incansável e dedicada comigo desde o início. Perante momentos menos bons lembraste-me que desistir não era opção. Serás sempre um exemplo para mim, de esforço e dedicação em tudo o que fazes. Obrigada pela partilha de experiências e diálogos construtivos nas nossas idas diárias ao IMM, pelos conselhos e por toda confiança que depositaste em mim. No início tive uma coorientadora, no final acabei com uma amiga. Obrigada, Andreia, do fundo do coração.

Gostaria de agradecer à Professora Doutora Alexandra Brito e ao Professor Doutor Rui Silva pela disponibilidade e pelas questões levantadas que me deixaram sempre a pensar mais além e que contribuíram para o meu conhecimento. Gostaria também de

agradecer à Professora Doutora Ana Rita Vaz, pela simpatia e paciência que sempre demonstrou ter ao longo deste ano.

Gostaria de expressar o meu agradecimento ao Professor Doutor Luís Graça e à Raquel, por ter colaborado connosco e nos ter transmitido o conhecimento acerca do modelo animal associado à Esclerose Múltipla.

Agora vem a parte difícil. Um especial obrigada a todos os meus colegas de trabalho que fizeram parte desta fase da minha vida! Era impensável para mim neste ano conhecer pessoas que se tornaram tão amigos e que eu desejo que nunca mais saiam da minha vida. Voltaria a escrever outra tese sobre tudo o que aprendi, aqui, só para voltar a passar momentos com cada um de vocês. Sempre dissemos que este ano daria para escrever um livro! Um obrigada a ti, Catarina, por seres a típica Catarina. Tens a capacidade de me fazer sentir bem e à vontade. Quando menos esperava estávamos a partilhar a nossa vida e momentos, sem timidez ou receio. Quero que continues a fazer parte desta caminhada. Duvido que acabe por aqui. À Sofs, por seres as duas faces da mesma moeda. És a serena e a extrovertida. Não foi à primeira, mas de pouco e pouco, fomos confiando uma na outra até que havia coisas não precisavam de ser ditas, bastava um olhar. Já te conheço o suficiente para saber quando precisas de ser ouvida e quando precisas de um abraço de amiga. Mesmo que não queiras, eu vou lá estar, sempre. À Pipa! Sem ti, este ano não tinha sido a mesma coisa. Apesar das nossas diferentes opiniões, tu foste sempre uma pessoa coerente e correta com todos. Nunca desistas do que realmente queres fazer e nunca, mas nunca, duvides do que és capaz. Espero, de coração, que sejas muito feliz. À Dani (eu sei que não gostas), no ano passado tínhamos uma mão cheia de histórias, este ano rebentámos a escala. Engraçado como rapidamente fizemos o clique e como as nossas parvoíces se juntaram e nos ajudaram a descontrair e provocaram momentos e risos inevitáveis e incontroláveis. Obrigada por toda a tua má disposição matinal que nós conseguimos reverter. E, se é isto que queres, não desistas! À Martinha, obrigada pelas horas intermináveis de partilha. Os nossos almoços tiveram outro valor contigo lá. Vou estar aqui sempre que precisares. A todas, um grande obrigada por fazerem parte do meu dia-a-dia em todos os momentos, por partilharem as minhas angústias mas também todos os meus momentos de felicidade e vitórias. Vocês são as maiores. Não teria sido igual sem vocês. A cave das cusquices, dos sorrisos, do choro, das frustrações. Obrigada por todas as memórias que levo deste ano.

Cátia, começo por ti, e não é por seres a mais velha, é mesmo porque és a maior. Obrigada por toda a paciência que tiveste comigo, nesta última fase. Obrigada pelos desabafos e conselhos que me deste enquanto descongelávamos arcas e me ajudavas no ChemiDoc. Nunca vou esquecer certas coisas que me disseste e vou sempre lembrar-me de ti como o exemplo que eu quero seguir. Não tenho dúvidas nenhumas do futuro brilhante

que vais ter. Gonçalo, chegaste connosco e, rapidamente, te tornaste um de nós. Obrigada pelas discussões, espírito critico e opiniões que me faziam sempre pensar de outra forma. Obrigada pela partilha de experiências e conhecimento. Por alinhares nas nossas brincadeiras e por estares sempre disposto a ajudar no que fosse preciso (menos para encher as caixas de pontas). Um mega obrigada. À Sara, foste a pessoa que mais me mostrou que se queremos muito uma coisa, não devemos desistir. Eu sei que tu não vais desistir e mostraste-me como ver o lado bom quando me sentia perdida. Obrigada pelo teu profissionalismo, pela tua boa disposição diária e pela amizade. Por fim, à Marta, por toda a ajuda que me deste desde o primeiro dia aqui no laboratório.

Às meninas novas, Maria e Beatriz, espero que aproveitem este ano, que aprendam muito e que aceitem de braços abertos todos os desafios. Muito bom trabalho e sorte às duas.

A todos os meus colegas do CBF, obrigada por todo o companheirismo e amizade que demonstraram ao longo desta curta aventura. À minha alentejana, Catarina, um especial obrigada. Nunca vou esquecer a força que me deste ao longo deste ano. Foste tu a primeira pessoa com quem partilhei o meu principal objetivo aqui, o que iria fazer e sem saber o impacto que teria em ti. Também por ti, dediquei-me ainda mais a este projeto, não só pensando no que nos move enquanto investigadores, mas também no impacto que o nosso trabalho tem nos outros. Não falamos todos os dias, nem sentimos essa necessidade. Quando precisamos, estamos lá uma para a outra. Eu sei que também sentes os mesmo.

Não posso esquecer de agradecer ao início de tudo, à FCT! Onde tudo começou e por tudo o que me deste de bom, mas também pelas cabeçadas dadas, que me obrigaram a pensar muitas vezes sobre as melhores decisões a tomar durante o meu percurso por lá. Sem isso, não teria a persistência e a força de vontade. Provavelmente, não tinha chegado onde cheguei. Obrigada às pessoas que apareceram no meu caminho e ficaram. Ao Pedro, por seres a pessoa que está lá para tudo. Um amigo que se cruzou comigo logo no primeiro ano e que ficou e aos poucos, fomos conquistando tudo o que queríamos. Cada objetivo individual alcançado era uma vitória para os dois! Ainda hoje continuamos assim. Mesmo com todos os nossos desentendimentos conseguimos chegar ao final dos dias cheios de emoções. Rimos, choramos, partilhamos, refilamos e somos honestos: dizemos a verdade um ao outro mesmo que custe. Estamos sempre lá um para o outro e eu sei que isso vai continuar, independentemente do que o futuro nos reserva. A todo o grupo que se criou no primeiro, apesar dos diferentes caminhos, permanecem no meu coração até hoje. Às pessoas que entraram casualmente na minha vida: Wendy, Nuno, à banda do momento e a todos os outros. Obrigada pelo carinho e por estarem sempre de braços abertos. Aos meus padrinhos, por serem o exemplo a seguir! Vou sempre reconhecer os vossos sapatos. Aos meus afilhados, por terem feito parte da minha vida académica e não só.

Ao meu Algarvio, Cláudio. Ao meu informático, por me transmitir calma e serenidade, quando eu me sentia a pessoa mais stressada do mundo. Aceitas-me como sou: a workaholic que não deixa nada para trás. Felizmente, ensinaste-me a organizar melhor o meu tempo e a aproveitar o pouco tempo que temos para os dois. Também fazes parte deste ano, deste trabalho e do meu crescimento pessoal e relacional. Mesmo sem eu estar presente, tu fazias questão que eu soubesse o orgulho que sentes em mim e que estás do meu lado a apoiar as minhas decisões. Obrigado por seguires o meu trabalho e por mostrares vontade em saber tudo o que envolve. Obrigada pela tua paciência, pela pura amizade, pelo teu amor e carinho. Este ano é a tua vez e eu vou estar aqui pronta como tu estiveste para mim. Sempre a sorrir para ti e contigo

À minha família. Mami, tu és a maior. Obrigada por todo o teu apoio, desde sempre. Estiveste ao meu lado e sempre me incentivaste a seguir o que mais gosto, sem receios. Apanhei a tua garra e com isso vou conquistando o que nunca imaginei. Ao João, por ser o oposto de mim. Somos o cão e o gato, mas parte do que eu sou agora deve-se a ti. Obrigada Johnny, por seres mais mimoso que eu. Ao meu avô, por ser o meu herói. Obrigada por acompanhares e estares presente em todos os meus grandes momentos. Apoias-me em tudo, e obrigada por isso. À minha avó, por se manter presente. É uma felicidade ver-te e é sempre um aperto no coração deixar-te. Obrigada avó, nunca vou esquecer as coisas bonitas que me dizes sempre que me vês. Ao meu pai, obrigada pelo apoio que me deste e o orgulho que demonstraste ter durante estes 5 anos. Ao resto da minha família que está longe, mas sempre perto. Madrinha, padrinho, tios e primos, um bem-haja por acreditarem em mim e me incentivarem a lutar pelo que quero e pelo que me faz feliz. Quem sabe se o meu futuro não será junto de vocês!

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I- INTRODUCTION

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Abbreviations

APC	Antigen-presenting cells
BBB	Blood-brain barrier
Ca^{2+}	Calcium
cdc-42	Cell division cycle-42
CD	Cluster differentiation
CFA	Complete Freund's Adjuvant
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
DMTs	Disease-modifying therapies
DMF	Dimethyl fumarate
EAE	Experimental autoimmune encephalomyelitis
Iba-1	Ionized calcium binding adaptor molecule-1
IL	Interleukin
Ig	Immunoglobulin
I.P.	Intraperitoneal
iNOS	Inducible nitric oxide synthetase
LFB	Luxol fast blue
FDA	Food and Drug Administration
GFAP	Glial fibrillary acid protein
GM	Grey matter
IRT	Immune reconstitution therapy
LPC	Lysophosphatidylcholine
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
NG2	Neuron-glia antigen 2
nM	Nanomolar
OL	Oligodendrocytes
OPCs	Oligodendrocytes precursor cells
PBS	Phosphate buffer
PFA	Paraformaldehyde
PLP	Proteolipid protein
PP MS	Primary Progressive MS

PR MS	Progressive relapsing MS
PSD-95	Postsynaptic protein density-95
PTx	Pertussis toxin
RAGE	Receptor for advanced glycation end products
RhoA	Ras homologous member A
ROS	Reactive oxygen species
RR MS	Relapsing-remitting MS
RT	Room temperature
SC	Spinal cord
SEM	Standard error of the mean
SP MS	Secondary progressive MS
S.C.	Subcutaneous
Th cells	T helper cells
TMEV	Theiler's Murine Encephalomyelitis Virus
TNF	Tumor necrosis factor
Treg cells	Regularory T cells
WM	White matter
μM	Micromolar

I - INTRODUCTION

1. Multiple Sclerosis: an overview

Multiple Sclerosis (MS) is an autoimmune, inflammatory and neurodegenerative demyelinating disorder of the central nervous system (CNS) white and grey matter (Hafler, 2004). MS is the most common cause of non-traumatic disability among young adults between 20 to 40 years, besides traumatic injury (Raffel et al. 2016). According to World Health Organization (WHO), in Europe, the estimated prevalence is about 80 per 100 000 people and, worldwide, over 2.5 million people suffer with MS (WHO, 2008). Particularly in Portugal, recent studies estimated a prevalence around 56.2 per 100 000 people (De Sá et al., 2012; Kobelt et al., 2017). Despite its prevalence, MS has also a high social, labor and economic impact. Once it is a non-traumatic neurological disability among young adults, the early loss of work ability and the increase incapacity overtime has an average cost of 13 € billion per year (Sobocki et al., 2007).

MS is a complex disease and it is well-characterized by 1) focal lesions on CNS; 2) neuroinflammation; and 3) destruction of myelin sheaths of neurons (Costantino et al., 2008). Generally, the pathophysiology is correlated with the inappropriate activation of the immune system that leads to the disruption of the blood-brain barrier (BBB) becoming more permeable and allowing the infiltration of immune cells within the CNS parenchyma (Howard and Weiner, 2009; Ransohoff, 2012). The activation of microglia, oxidative stress, mitochondrial dysfunction, and the release of cytotoxic mediators are considered the main consequences of immune cell invasion (Compston and Coles, 2008; Obermeier et al., 2013). Furthermore, all of these processes promote more inflammation, formation of demyelinated plaques, axonal degeneration, followed by loss of motor function, namely paralysis (Compston and Coles, 2008).

Demyelination has been described as a hallmark of autoimmune and inflammatory responses (Merrill and Scolding, 1999). The loss of myelin sheaths of neurons has serious consequences at both cognitive and motor levels. Symptomatically, depending on the nerve

attacked in the CNS, the beginning of MS starts with common features such as muscle weakness, fatigue, cognitive impairment, ataxia, visual disturbances and vertigo (Koriem, 2016; Raffel et al., 2016). Besides these common well-known signs of the disease, patients can also develop neuropsychiatric symptoms that are highly prevalent and frequently disregarded in clinical settings (Haussleiter et al., 2009). Diaz-Olavarrieta et al. shown that 95% of the relapsing-remitting MS (RRMS) patients develop neuropsychiatric symptoms, highlighting for depression (79%), anxiety (37%) and irritability (35%) (Diaz-Olavarrieta et al., 1999).

Regarding all research, the etiology of MS is still not fully understood. Although, several investigators believe that the cause of developing MS disease involves a combination of environmental exposure and genetic susceptibility (Compston and Coles, 2008). Infections with Epstein-Barr virus, low sunlight exposure, smoking and vitamin D deficiency are some of the main environmental factors associated with the disease (Compston and Coles, 2008; Raffel et al., 2016). Meta-analysis studies have shown a direct association between smoking and MS susceptibility but, further studies are needed to completely understand its association with disease progression (Handel et al., 2011). Recently, it was also reported that human MS-derived microbiota contains factors that can contribute to the pathogenesis of the disease (Berer et al., 2017). Concerning genetic susceptibility, the Human Leukocyte Antigen gene was the one identified and strongest related with the risk of developing MS (Didonna and Oksenberg, 2015).

1.1. Disease Course of Multiple Sclerosis

The disease course of MS shows high heterogeneity among patients and within the same patient, remaining an unpredictable disease and the main cause of irreversible neurologic disability (Compston and Coles, 2008). In 1996, the US National Multiple Sclerosis Society Advisory Committee on Clinical Trials in Multiple Sclerosis defined four clinical subtypes: 1) relapsing-remitting (RR); 2) primary progressive (PP); 3) secondary progressive (SP); and 4) progressive relapsing (PR) **(Figure I.1)** (Lublin et al., 2014). More recently, the Committee decided that the category PRMS should be eliminated and the patients are now categorized as PP patients (Lublin, 2014; Lublin et al., 2014).

RRMS is the most common form of MS, affecting approximately 80-90% of the patients. It is characterized by complete or partial recovery between relapses (or “attacks”) (Hafler, 2004). The periods between relapses are characterized by lack of disease progression. However, due to the failure of the CNS to remyelinate and regenerate axons, focal neurological deficits and persistent symptoms start to accumulate (Miller and Leary, 2007).

SPMS appears after an initial RR disease course and it is characterized by relapses with apparent recovery, although the symptoms become progressive and remission is no longer obtained. In this MS subtype, the disease pathology is described by chronic CNS inflammation, incapacity to remyelinate, gliosis and, neuronal and axonal degeneration (Hampton et al., 2008).

Around 10-15% of MS patients develop PPMS without passing through RR phase. At this stage, it comes the worst prognosis because patients experience an increase attack frequency with poor or without recovery (Nylander and Hafler, 2012). At least, a small percentage (≈10%) of patients develop PRMS with clear acute relapses with or without full recovery, where the periods between relapses are characterized by continuous progression (Lublin et al., 2014).

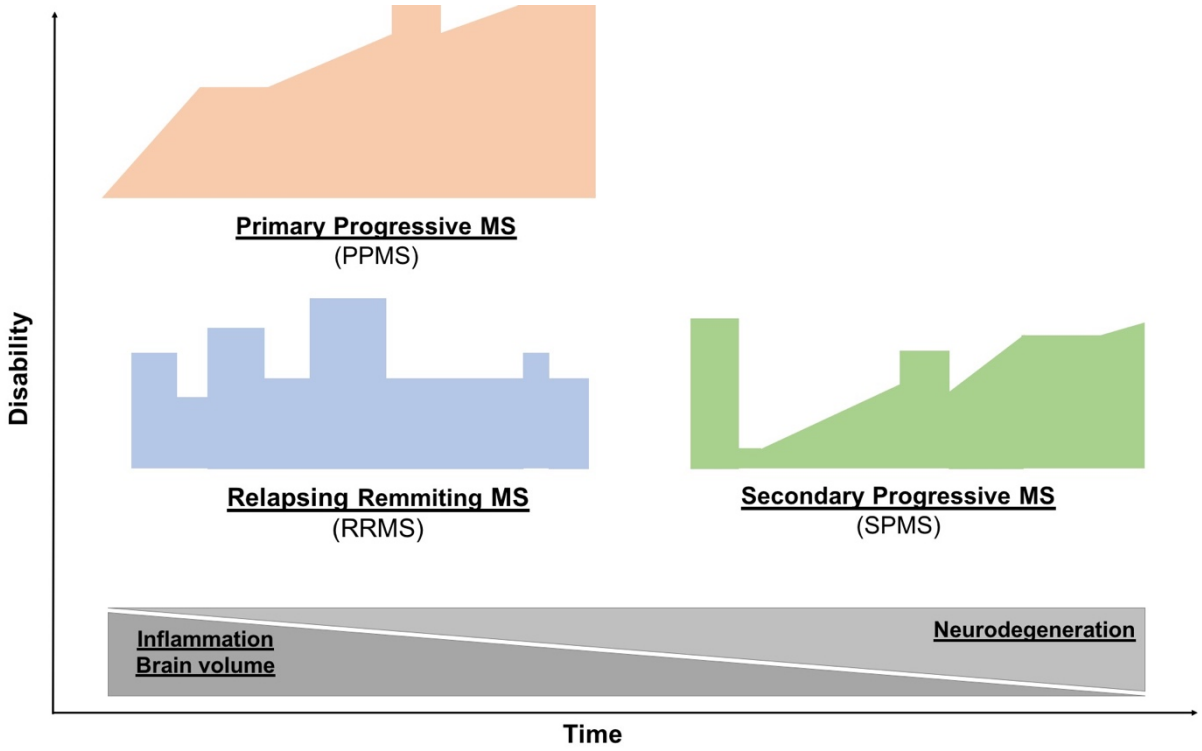

Figure I. 1: Schematic representation of Multiple Sclerosis disease course over time (Lublin, 2014). The relapsing-remitting (RR) disease course is characterized with regular clinical attacks. With time, the relapses become less frequent, the recovery is incomplete, and the patients' disability is accompanied by a decrease of brain volume or brain atrophy.

The development of MS pathogenesis is highly connected with inflammatory and neurodegenerative phases. In progressive phenotypes, the neurological decline is accompanied by CNS atrophy, decreasing brain volume **(Figure I.1)**. Disease-modifying therapies (DMTs), such as interferon and glatiramer acetate, are first-line treatments that are able to reduce the frequency of relapses, slowing down the accumulation of physical disability (Compston and Coles, 2008). Furthermore, the newest DMTs, namely Natalizumab and Fingolimod, are proven to help in both prevention of the formation of inflammatory lesions, decrease the number and severity of clinical attacks, and slow the accumulation of

disability **(Section 1.3.)** (Axisa and Hafler, 2016). Unfortunately, there are no effective therapeutic options that completely help in patients' disability and promote remyelination. So, it is essential to find safe and efficient therapeutic options in order to combat physical and cognitive symptoms and improve life quality for MS patients.

1.2. Features of cellular and molecular mechanisms involved in Multiple Sclerosis immuno-pathophysiology

The physiological pattern of MS comprise two main steps: 1) myelin sheath destruction and formation of lesions in the CNS; and 2) inflammation (Koriem, 2016). In addition to the autoreactive immune cells, the major pathological mechanisms associated with brain atrophy and related with a permanent clinical disability are axonal degeneration, neuronal loss, gliosis and oligodendrocyte (OL) dysfunction (Compston and Coles, 2008; Howard and Weiner, 2009).

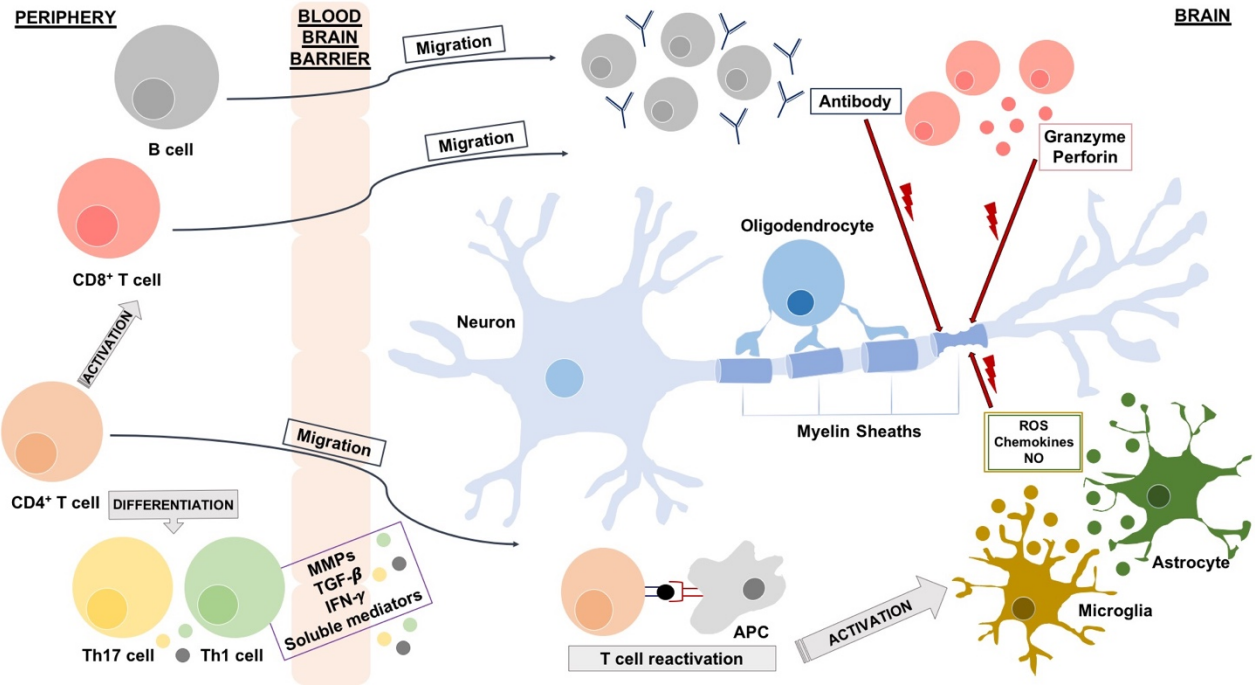

Figure I. 2: Schematic representation of CNS inflammatory cascade in Multiple Sclerosis. T cells must be activated in the periphery and gain access to the CNS where they are reactivated by antigen presenting cells (APCs). Cytokines secretion and metalloproteinases (MMPs) are responsible for the disruption of the blood-brain barrier (BBB) allowing the migration of immune cells ($CD4^+$ and $CD8^+$ T cells, and B cells) to CNS parenchyma. Within CNS, activated $CD4^+$ T cells secrete pro-inflammatory cytokines, $CD8^+$ T cells produce granzymes and perforins and B cells secret autoantibodies consequently creating an inflammatory environment, leading to myelin, oligodendrocyte and axonal damage. The reactivation of T cells also triggers astrocytes and microglia reactivity that release cytokines, chemokines and reactive oxygen species (ROS) contributing for the demyelination process. IFN – Interferon; NO – Nitric oxide; Th – T helper cell; TGF – Transforming Growth Factor

1.2.1. Immunopathology underlying immune cell infiltration

Generally, autoimmune disorders such as MS, arise when the immune system loses its tolerance ability and become unresponsiveness to antigens or over activated against self-molecules attacking its own tissues (Goverman, 2009). The pathology of MS is highly related to the increase migration of peripheral immune cells (T and B cells) through the brain physical barrier, the BBB, due to its disruption. The increased secretion of pro-inflammatory molecules leads to an increase permeability of the barrier expanding the recruitment of immune cells and, furthermore promoting more inflammation and tissue damage **(Figure I.2)** (Dendrou et al., 2015).

The existence of T cells that are reactive against self-myelin proteins, namely myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), have a crucial role in the development of autoimmunity and CNS disorders (Goverman, 2009). Thus, the dysregulation of T cell responses and the failure to suppress effector T cells causes excessive immune activation resulting in pathology (Joller et al., 2012).

$CD4^+$ T cells are the central mediators of the adaptive immune response, being responsible for cytokine secretion and cellular defenses against a pathogen. When triggered by antigen-presenting cells (APCs), naïve T cells can differentiate into regulatory T (Treg) cells and T helper (Th) cells, namely Th1, Th2, and Th17, that differ depending on the cytokine production and function (Fletcher et al., 2010; Zhu et al., 2010). Both Th1 and Th17 cells are described to be pathogenic, once they are responsible for the secretion of high amounts of pro-inflammatory cytokines, promote inflammatory responses and contribute to autoimmunity. Additionally, Treg cells exert an important role in the maintenance of immunosuppression, keeping pathogenic Th1 and Th17 cells in check (Leung et al., 2010). Treg cells and its transcription factor, FoxP3, have a critical role in autoimmunity acting as main regulators of cytokine production and in the inhibition of T cell activation (Fontenot et al., 2003).

Overall, in MS-related immunopathology, the Th cells are increased, while Treg cells are reduced not being able to suppress Th1 and Th17 pro-inflammatory activity (Zhu et al., 2010). Once, $FoxP3^+$ Treg cells can suppress the activation, proliferation and effector functions (cytokine production) of immune cells, it makes it a powerful target in the prevention of disease (Sakaguchi et al., 2010). Infiltrating $CD4^+$ T cells are also able to promote OL damage following demyelination **(Section 1.2.2.1.)** and glial activation **(Section 1.2.2.2.)**, through the action of soluble inflammatory and cytotoxic mediators (Dendrou et al., 2015).

In addition to $CD4^+$ T cells, $CD8^+$ T cells can also have both suppressive and cytotoxic functions (Wootla et al., 2012). Their interaction with the major histocompatibility complex

(MHC) class I antigens is the way to induce their response and react against autoantigens, for example, MBP. $CD8^+$ T cells also secrete great amounts of molecules, such as granzymes and perforins which will induce cellular damage (Friese and Fugger, 2009; Wootla et al., 2012). Recent evidences suggested a correlation between the release of perforin and the axonal injury present in MS plaques (Howe et al., 2007; Wootla et al., 2012). Curiously, histopathological studies also indicate that these cells are most common in MS patients' brain being present in higher number than $CD4^+$ T cells (Dendrou et al., 2015).

B cells are also important supporters of the immune system. They are multifunctional cells being involved in natural, adaptive and autoantibody production (Hampe, 2012). During an immune response, B cells can acquire APCs phenotype and can interact with T cells either by activation or inhibition, producing inflammatory cytokines (Hampe, 2012). Curiously, the number of infiltrating B cells in CNS when compared to T cells, is higher and it is thought to be related with MS progression (Dendrou et al., 2015). The cytokines produced by B cells have an impact on mechanisms of immunity and autoimmunity, modulating the migration of dendritic cells, activating the migration of macrophages, exerting regulatory functions and providing stimulatory signals (Hampe, 2012). Studies also revealed an increase release of pro-inflammatory cytokines and a decrease of anti-inflammatory cytokines in MS patients, corroborating that B cells are associated with MS (Bar-Or et al., 2010). Nowadays, several studies also revealed a relation between the presence of B cells and the immunopathology of MS. For instance, the production of autoantibodies by B cells is a way of diagnosis that can be detectable in the cerebral spinal fluid (CSF) and more than 50% of demyelinating lesions in MS patients are characterized by the deposition of pathogenic antibodies that target the OL (Yanaba et al., 2008; Dendrou et al., 2015). Moreover, in progressive phenotypes of MS, it was found the presence of meningeal B-cell follicles containing germinal centers, which can be considered a hallmark of chronic inflammation (Magliozzi et al., 2004; Dendrou et al., 2015).

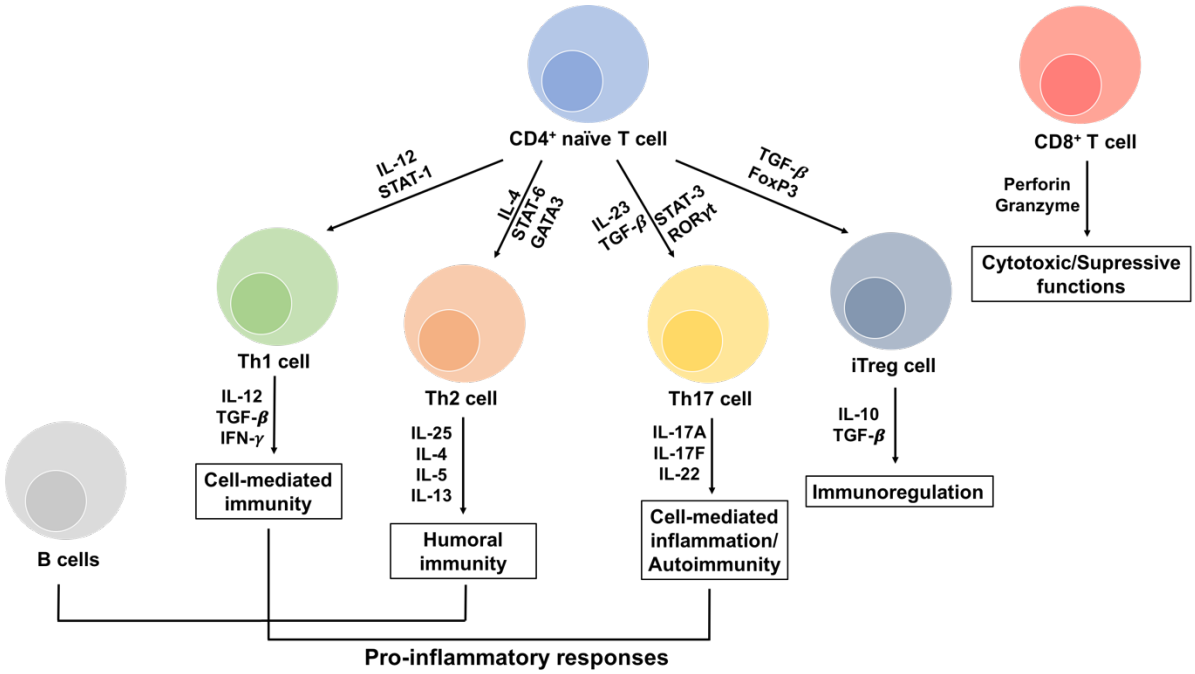

Figure I. 3: Adaptive immune system. CD4⁺ T cells can differentiate into three subtypes, T helper cell (Th)1, Th2, Th17, and iTreg cells, based on the cytokine microenvironments. B cells and Th2 cells are correlated with humoral immunity and anti-inflammatory responses. In opposite, the Th1 and Th17 cells are mainly related with pro-inflammatory responses. The CD8⁺ T cells exert both cytotoxic and suppressive functions. FoxP3 - Forkhead box protein 3; IL – Interleukin; IFN – Interferon; ROR – Retinoid-related Orphan Receptor; STAT – Signal Transducer and Activator of Transcription; TGF – Transforming Growth Factor; Treg – Regulatory T cell

1.2.1. Pathophysiological characteristics of the CNS

MS refers to “multiple scars” that accumulate in regions of the brain and spinal cord (SC). As it was referred previously, the pathogenesis of MS is highly heterogeneous and it is mainly associated with the disruption of the BBB, leading to immune cells invasion within the CNS (Howard and Weiner, 2009; Macchi et al., 2015). Consequently, the activation of T cells and CNS glia, as well as the large secretion of pro-inflammatory cytokines and chemokines will have an impact in the immunologic and neurologic forms of the disease, leading to myelin damage **(Figure I.2)** (Macchi et al., 2015).

Demyelinated areas, known as focal plaques, in the white and grey matter, SC and cortex, are the main pathologic hallmarks of MS patients **(Section 1.2.2.3.)** (Koriem, 2016). In RRMS, focal inflammation and demyelination are responsible for the white matter (WM) lesions. In addition to inflammatory lesions, CNS atrophy and increased axonal loss are also associated with the progressive phenotypes of MS and, therefore with the development of SPMS (Dendrou et al., 2015).

1.2.1.1. Neurons and oligodendrocytes as main victims in demyelination

Neurons are the main cells of the CNS with cell bodies in the grey matter (GM). Although the morphology can differ in some features, they all contain three common principal regions. Each neuron is composed by a soma that is extended into multiple branched processes called dendrites, and then, an axon that sends outgoing signals to the other cells (Saladin, 2014). The neurons are the main cells responsible for the undergoing communication of the nervous system with the other parts of the organism, through three main processes: 1) excitability, and the ability to respond and detect stimuli; 2) conductivity, and the capacity to react quickly reaching other cells; and 3) secretion, that is the transmission and release of neurotransmitters. Importantly, the propagation of signals is dependent of either the formation of myelin by OLs and the nodes of Ranvier, that are unmyelinated areas, where sodium channels regulate electrical impulse conduction (Bradl et al. 2010, Mitew et al. 2014).

Furthermore, neuroglia are the supportive cells of the brain, SC, and peripheral nerves. OLs are specialized glial cells that provide support and insulation to neurons in the CNS by enwrapping the axons with myelin sheaths, providing them protection and nutritional support (Merrill and Scolding, 1999). They are known as the myelinated cells of the CNS comprising about 5 to 10% of the total glia population. Myelin formation occurs when oligodendrocyte precursor cells (OPCs), migrate through the CNS and differentiate into mature OL. During OL maturation, there are four transitional stages that can be identified according to the migratory capacity, morphology and expression of specific markers (Barateiro and Fernandes, 2014). Firstly, it occurs the generation of OPCs, followed by the differentiation into preoligodendrocytes (or late OPCs) that, overtime, become immature OL and, finally, mature OL. OPCs are highly proliferative cells with increased migratory capacity being the main source for OL replacement and myelin repair. They also express high levels of platelet-derived growth factor-receptor α and proteoglycan neuro-glia antigen (NG)-2 and they are characterized by their short multipolar ramifications (Barateiro and Fernandes, 2014; Armada-Moreira et al., 2015). Since late OPCs express the same markers as OPCs, they can also be identified by NG2 staining. In immature (or pre-myelinating) OL, the cell morphology starts to change becoming cells with longer ramified branches. Moreover, mature OLs starts to form compact enwrapping sheaths around the axons, being responsible for the expression of myelin proteins (Podbielska et al., 2013; Barateiro and Fernandes, 2014). Overall, the proliferation and migratory capacity decrease, the cell morphology changes and different markers can be identified during OL development (Barateiro and Fernandes, 2014).

As it was mentioned above, the myelin sheaths provide insulation around nerve fibers. Concerning its synthesis, OL and Schwann cells are the responsible entities within the CNS and the peripheral nervous system, respectively (Chamberlain et al. 2016). Full myelination of axons is only completed in adolescence, being accompanied with a progressively increase of the WM volume in the brain. The composition of myelin comprises about 20% proteins, mainly MBP and proteolipid protein (PLP), and 80% lipids, such cholesterol, phospholipids and glycolipids (Saladin, 2014). Besides MBP and PLP, MOG is also present with less abundance, although it is the one that induces strongest responses in MS patients (Grau-López et al., 2009; Podbielska et al., 2013).

Regarding myelin repair, transcription factors and Rho-family of small guanosine triphosphatases (Rho GTPases) are some of the molecules that are involved in the processes. Olig1 and Olig2 are two transcription factors important in the regulation of oligodendrogenesis (Armada-Moreira et al., 2015). Indeed, experiments using knock-out strains of these genes have shown a direct relation with the generation of OL complex morphologies that fail to myelinate (*Olig1*) and a failure in the synthesis of OPC (*Olig2*) (Lu et al. 2002, Maire et al. 2010). Overall, these experiments revealed the importance of these genes in the regeneration of OL and, possibly, in remyelination (Chamberlain et al., 2016). Relatively to Rho GTPases, cell division cycle (cdc)-42, Ras-related C3 botulinum toxin substrate (Rac)-1, and Ras homologous member (Rho) A are the main subfamilies described to regulate the changes in cellular morphology during OL differentiation (Laura Feltri et al., 2008; Barateiro and Fernandes, 2014). Indeed, Rho-GTPases are implicated in a variety of cellular processes, such as in cell polarization, transcriptional and membrane transport regulation. Particularly in the CNS, cdc42 and Rac1 act as positive regulators of morphological changes being increased during OL differentiation, while RhoA protein act as negative regulator inhibiting the process of elongation (Liang, 2004).

MS is the most common chronic demyelinating disease, once it is incapable of spontaneous remyelination in progressive and later stages of MS, which are known to be more neurodegenerative than inflammatory. Demyelination is associated with a failure to regenerate OL and replace myelin, exacerbating axonal loss and leading to neuroinflammation (Chamberlain et al., 2016). Indeed, the myelin sheaths play a vital role in the maintenance of axonal homeostasis, stability, and integrity (Merrill and Scolding, 1999; Mitew et al., 2014).

1.2.1.2. Neuroinflammation and glial activation

Besides gradual neuroaxonal loss and brain atrophy, inflammation is also present during the clinical course of MS. Briefly, the development of inflammation in MS is mainly mediated by T cells and activated macrophages/microglia (Lassmann et al., 2007). This

process starts with the transmigration of autoreactive $CD4^+$ T cells through the BBB into the CNS where they are reactivated by microglia that act as APCs. This is accompanied by the release of pro-inflammatory cytokines which activate and recruit immune cells. Within the pro-inflammatory environment, occur the mechanisms that mediate myelin, OL and axon damage, leading to neurologic dysfunction **(Figure I.2)** (Naegele and Martin, 2014).

Neuroinflammation act as a consequence of neuronal degeneration eliciting the production and release of pro-inflammatory cytokines (Hirsch and Hunot, 2009; Xanthos and Sandkühler, 2013). Besides the presence of cytokines, neuroinflammation is also mediated by chemokines, secondary messengers, nitrogen species and reactive oxygen species (ROS). CNS glia, including microglia and astrocytes, are also responsible for the production of these mediators (Xanthos and Sandkühler, 2013; DiSabaro J. et al., 2016). Their accumulation can amplify both inflammatory and immune responses contributing to an exacerbation of demyelination and neurodegeneration (Lassmann et al., 2007). In active and chronic lesions of MS patients, $CD4^+$ and $CD8^+$ T cells, and activated microglia are the main infiltrated components (Herz et al., 2010).

Microglia are the central mediators of neuroinflammation being 10% of the CNS population and are present in the parenchyma of a healthy brain (DiSabaro J. et al., 2016). They are known as the resident macrophages of the CNS and act as the first line of defense against brain injury, responding to dangerous signals and initiating inflammatory responses (Domingues et al., 2016). Microglial reactivity to external stimuli can be reparative, however, when uncontrolled can lead to an exacerbate release of neurotoxic and pro-inflammatory factors (Fernandes, Miller-Fleming, & Pais, 2014). The neurotoxic substances that are produced, promote and support axonal damage, leading to a progressive neurodegeneration (Dendrou et al., 2015). Consequently, when CNS homeostasis start to change, microglia becomes overactivated leading to morphologic alterations, microgliosis, increasing the expression of mediators and become phagocytic cells (Caldeira et al., 2014; Fernandes et al., 2014).

Regarding morphological changes, as a consequence of brain injury, microglia change from a highly branched vigilant state (healthy conditions) to a more amoeboid or rounded reactive form, reducing the complexity of their shape (Petersen and Dailey, 2004; Koriem, 2016). Importantly, these morphological changes can be observed by staining for ionized calcium binding adaptor molecule (Iba)-1, a specific marker of microglia. Moreover, depending on the stimuli, the duration and the environment they encounter, microglia can acquire different phenotypes: 1) a classic neurotoxic phenotype (M1); and 2) an alternate anti-inflammatory phenotype (M2) (Fernandes, Miller-Fleming, & Pais, 2014). These changes allow them to participate in the cytotoxic response, immune regulation and injury resolution.

M1 microglia phenotype is associated with acute infection being essential for the defense against microorganisms. Principally, M1 consists of a pro-inflammatory phenotype associated with cytotoxic responses (Goldmann and Prinz, 2013). It is well-characterized by the release of pro-inflammatory cytokines and can be influenced by T cells, mainly by its subtype, Th1 cells. Interferon-γ, which is released from Th1 cells, and lipopolysaccharide are the main inducers of M1 proliferation. When induced, microglia release inflammatory mediators, including interleukin (IL)-1β, IL-6, IL-12, and inducible nitric oxide synthase (iNOS) **(Table I.1)** (Goldmann and Prinz, 2013; Salter and Stevens, 2017).

Contrariwise, M2 microglia phenotype plays a crucial role in tissue repair, in the secretion of extracellular matrix proteins and growth factors, promoting phagocytosis and contributing to remyelination processes (Correale, 2014). Microglia can also acquire this phenotype by interplay with Th2 cells, once they release some anti-inflammatory cytokines, such as IL-4 and IL-10. This phenotype is characterized by the expression of high levels of arginase and transforming growth factor-β, which is related to the protective role of Th2 cells and the induction of Treg cells (Kettenmann et al., 2011; Goldmann and Prinz, 2013). In addition, M2 microglia phenotype can be subdivided into three main classes, M2a, M2b, and M2c that have different functional properties and specific markers. Specifically, M2a microglia phenotype is responsible for phagocytosis and tissue repair, regeneration and remodeling; M2b or immunoregulatory phenotype is stimulated by immune complexes and is related with the modulation of different conditions of inflammation; and the M2c, also known as an acquired-deactivating phenotype, is associated with an increased neuronal loss **(Table I.1)** (Brites and Vaz, 2014).

Microglia have been associated with the development of cortical GM lesions in progressive MS. Also, M1 phenotype promote even more neuroinflammation and demyelination with an increase clinical disability (Correale, 2014). Studies have also shown a protective role of microglia during active phases of demyelination. Anti-inflammatory M2 microglia containing ingested-derived lipids are prone to contribute for the resolution of inflammation, producing anti-inflammatory cytokines and growth factors that can inhibit further lesion development and promoting regeneration (Boven et al., 2006). Indeed, the switch from M1 to M2 polarization play an important role in remyelination processes, once there is a secretion of neurotrophic and regenerative factors, promoting OL differentiation and recruitment (Miron et al., 2013; Miron and Franklin, 2014). Also, in the peak of the disease, there is an increased expression of the cell surface markers cluster differentiation (CD)-45, CD86 and MHC-II, which is a consequence of the response to demyelination (Goldmann and Prinz, 2013). Although, CNS myelination depends not only on microglia but also on the crosstalk between astrocytes and oligodendrocytes

Table I. 1: Microglia subtypes and the respective inflammatory mediators and phenotypic markers.

Phenotype	M1 (classical)	M2 (alternative-activated)		
Subtype	M1	M2a	M2b	M2c
Functions	Pro-inflammatory	Anti-inflammatory	Immunoregulatory	Immunosuppressive
Released inflammatory mediators	IL-1β; IL-6; TNF-α; iNOS	IL-10; IL-4; TNF-β	IL-10; TNF-α; IL-1β; IL-6	IL-10; TNF-β
Phenotypic markers	MHC-II; CD45; CD86	FIZZ1; Arg1; SOCS-1	SOCS-3	Arg1; SOCS-3
Function	- Antigen presentation; - Secretion of toxic molecules	- Myelin phagocytosis; - Secretion of regenerative factor; - Promotion of OL differentiation and remyelination		

Arg – Arginase; FIZZ1 – Protein found in inflammatory zone; IL – Interleukin; iNOS – Inducible Nitric Oxide Synthase; MHC – Major Histocompatibility Complex; OL – Oligodendrocytes; TNF – Tumor Necrosis Factor; SOCS – Suppressor Cytokine Signaling

Additionally to microglia, astrocytes are also great supporters of the CNS, providing a physical and metabolic environment for neuronal activities. They exert roles as immunocompetent cells by secreting neurotrophic and/or neurotoxic factors, namely S100B protein, or augmenting the immune response (Miljković et al., 2011). They respond to all sorts of CNS insults, undergoing a transformation called "reactive astrogliosis", and therefore altering their morphology and expression. In several neurodegenerative disorders or after brain injury, it is observed the glial scar formation which is known to be the outcome of this reactivity (Sofroniew, 2009; Hamby and Sofroniew, 2010). Recently, it was revealed that neuroinflammation is also responsible for the induction of two different phenotypes of astrocytes, A1 and A2, as well as in microglia. Indeed, the A1 phenotype represent a gain of neurotoxic functions, destroying neurons and mature OLs; and the A2 phenotype mostly upregulate neurotrophic factors developing a protective environment (Liddelow et al., 2017). Relatively to specific molecular markers, the glial fibrillary acid protein (GFAP) expression is essential for the identification and characterization of astrocytes in healthy and pathological tissues (Sofroniew and Vinters, 2010).

Concerning the role of astrocytes in health and disease, the activation and loss of vessels coverage represent early lesions and it is accompanied with BBB dysfunction, which is a hallmark of MS progression. They are also responsible for cytokines production, namely IL-12, IL-23 and IL-25, being capable of regulate the differentiation of $CD4^+$ T cells into a more pro-inflammatory phenotype and promoting a cytotoxic activity of $CD8^+$ T cells. Moreover, B cells activation can also be controlled by astrocytes through the B cell activating factor, which is known to be increased in MS lesions (Domingues et al., 2016).

1.2.1.3. Neuropathology: White matter and grey matter lesions

Besides all immunopathology and neuroinflammation present in MS immune-pathogenesis, this disease is characterized by focal demyelinated lesions in the brain and SC WM and also in GM. This so called “plaques” can be distinguished accordingly to their immunological activity or by the infiltration of immune cells, in WM or GM, respectively **(Table I.2)** (Lassmann et al., 2007; Prins et al., 2015). Pathologically, both white and grey matter lesions are highly heterogeneous, varying among patients in their location, shape and size.

Concerning WM lesions, anatomical localization explain some neurological symptoms diagnosed in MS patients, namely visual dysfunction, auditory impairment, and motor and sensory deficits (Prins et al., 2015). Again, the early lesions are quite heterogeneous and it is thought to arise from T-cell mediated inflammation alongside with antibody deposition, activation of complement, loss of myelin proteins and dysfunction of OL unit (Popescu and Lucchinetti, 2015).

Although MS is known as a WM disease, GM demyelination has also been described. GM cortical lesions are also present in the pathogenesis of human MS, specifically in the cerebral cortex, deep GM structures (thalamus and hippocampus), cerebellum and SC. It is known that the presence of this lesions can explain certain cognitive impairments, present in almost 50% of patients carrying MS disease (Bö et al., 2007; Prins et al., 2015). Briefly, cortical demyelinated lesions can be classified either by leukocortical, intracortical or subpial, based on their location (Popescu and Lucchinetti, 2015). Cortical pathology is also associated with meningeal aggregates, an increase neuronal loss and neurite injury, which is reflected in both progression and irreversible clinical disability (Lucchinetti et al., 2011).

Inflammation, demyelination and axonal loss are the pathological hallmarks present in all MS stages. Overall, in WM lesions, OLs are known to be the initiators of lesion formation, together with a prominent astrogliosis and morphological changes of microglia (Bö et al., 2013). Therefore, chronic demyelinated GM lesions have lack of inflammatory infiltration and, when compared to WM lesions, it is observed less activation of both microglia and astrocytes. Regarding demyelination, it is more extensive in GM than WM, however there is no correlation between them (Bö et al., 2013; Prins et al., 2015). Overtime, MS neuropathology changes, being mainly influenced by demyelinating activity, disease severity and clinical course, as well as by the use of therapeutic interventions.

Table I. 2: Main characteristics of white and grey matter lesions, in multiple sclerosis patients.

	White matter lesion	Grey matter lesion
CNS regions	Optic nerves and frontal lobe tracts; Brainstem and cerebellum; Periventricular WM; Spinal cord	Motor cortex; Cerebellum; Spinal cord
Cell influx	Infiltrating T cells and macrophages	Little or no inflammatory cells; Ectopic B-cell follicles
T cell site of entry	Blood-brain barrier	Meninges, choroid plexus, or subarachnoid space.
Glial cell characteristics	Higher number than in GM; Microglial activation; Astrogliosis; Oligodendrocyte loss	Less number than in WM; Less microglia activation and astrogliosis; Oligodendrocytes damage
Pathological pattern of lesions	Pattern I – 15% of MS patients; T cell and macrophage infiltration, active demyelination and activated microglia; Pattern II – 58% of MS patients; same as above, but with IgG deposition and activation of complement; Pattern III – 26% of MS patients; infiltrating inflammatory cells and microglia, no IgG deposition, and loss of OLs; Pattern IV – rare; nonapoptic death of OL, absence of remyelination	Cortical demyelinating lesions: Type I – leukocortical lesions that include both WM and deeper cortex; highly inflammatory; Type II – intracortical lesions; located within the cortex, centered on blood vessels, and do not extend to the surface of the brain or subcortical WM; Type III – subpial lesions; extend from the pia into the deeper cortical layers
Clinical implications	Visual, auditory and organ dysfunction, such bladder and bowel; Motor and sensory deficits	Increase motor and sensory symptoms; Cognitive impairment

CNS – central nervous system; Ig – immunoglobulin; GM – grey matter; WM – white matter

1.3. Diagnosis, Biomarkers and Current Treatments of Multiple Sclerosis

In initial phases of the disease, the number of relapses and the development of different symptoms is used as a primary diagnosis. An early and precise diagnosis of MS is very important once there are no effective treatments, and overtime becomes more difficult to reduce neurodegeneration. The primary assessment is supported by magnetic resonance imaging (MRI) (Hafler, 2004). Although MRI is an ambiguous technique, give us information about WM lesions via evaluation of T2-hyperintense lesions and gadolinium-enhancing T1 lesion (Nylander and Hafler, 2012). Using the contrast enhancing agent, gadolinium, it is possible to detect damages in the BBB, indicating active lesion areas (Polman et al., 2011). Recently, the Magnetic Resonance Imaging in Multiple Sclerosis (MAGNIMS) society has proposed a standardized MRI protocol to assist in the diagnosis of MS, excluding other similar pathologies (Filippi et al., 2016). The analysis of CSF also provides supportive

evidence. This test includes the count of white blood cells, which is usually seven times higher in patients than in healthy people; and the presence of oligoclonal bands by electrophoresis, which is also higher in MS patients once the immune system is over activated producing great amounts of auto-antibodies (Link and Huang, 2006; Brownlee et al., 2016). Neurophysiological testing of evoked potentials in the visual, sensory and auditory pathway is also used as a supportive evidence of MS, being less invasive than a lumbar puncture. These diagnoses allows the identification of silent lesions and the analysis of prolonged latency reflects demyelination zones (Brownlee et al., 2016).

During disease course, a variety of events occur, and the use of biomarkers give us a precise, sensitive and reliable form of control, detecting disease activity and assessing therapeutic efficacy. Biomarkers are related to the measurement of proteins, lipids and nucleic acids in the blood or CSF reflecting disease-related or drug-related processes (Harris and Sadiq, 2014). These molecules are part of MS diagnosis, for instance through the analysis of oligobands in CSF and white matter T2-T1 lesions by MRI (Housley et al., 2015). The release of soluble markers associated with neurodegeneration and glial activation are also considered biomarkers. Several CSF biomarkers, such as CXCL13 and S100B, have been related to certain pathologic processes underlying disease activity and disease progression (Axisa and Hafler, 2016; Barateiro et al., 2016). Once B cells are involved in MS disease, CXCL13 is a biomarker that indicates the humoral immune response and serve as a measure of the therapeutic efficacy of B-cell targeting therapies (Förster et al., 1996). S100B, an astrocyte proliferation marker, is also one potential biomarker of neuronal and glial cell damage **(Section 2)** (Axisa and Hafler, 2016). Besides its importance as a biomarker for MS diagnosis, previous research from ongoing work of our group have shown that S100B is augmented in CSF, serum and *post-mortem* plaques of MS patients, being considered a potential therapeutic target to reduce MS pathogenesis **(Section 2.2.)** (Barateiro et al., 2016).

Therapeutic approaches to MS are based on altering the function of the immune system, either by using immunosuppressive drugs or by modulating molecules. However, these strategies are only successful in early stages of MS disease, not having effective therapeutic options for progressive phenotypes. A complex disease, such MS, require treatments that influence both neuro-restoration, remyelination, neuro-protection and inflammation (Howard and Weiner, 2009). Currently, there are 13 DMTs that have been approved for the treatment of MS by Food and Drug Administration (FDA) (Vargas and Tyor, 2017). Dimethyl fumarate (DMF) is one of the latest first-line approved drugs to treat relapsing forms of MS. The mechanism underlying this drug is not fully understood, although it is known that DMF can downregulate T cell proinflammatory responses and can also be implicated in the balance between pro- and anti-inflammatory B cell responses (Li et al.,

2017). When first-line therapies fail to work, the second-generation therapies, such as Natalizumab and Fingolimod, provide more effective disease control, although with significant adverse effects (Raffel et al., 2016). Natalizumab, a monoclonal antibody, binds to a cell adhesion molecule (anti-$\alpha 4\beta 1$ integrin) expressed on the surface of lymphocytes and monocytes, preventing inflammatory cells from entering the brain through the BBB contributing to inflammation and causing WM lesions (Yednock et al., 1992; Miller et al., 2003). Fingolimod, an immunomodulatory drug, is the first approved DMT for relapsing forms of MS. Indeed, this drug sequester autoreactive lymphocytes in lymph nodes reducing cell infiltration in the CNS (Groves et al., 2013). Fingolimod, as well as Natalizumab, decrease disability and reduces relapses frequency by approximately 50% of clinical exacerbations in RR phases. In the last few years, clinical trials in MS establish the importance of B cells as therapeutic targets in this disease (Raffel et al., 2016). Furthermore, immune reconstitution therapy (IRT) has been the new investment for MS treatment, once it provides the ability to induce long-term remission, and thus, a possibility of a cure. This kind of therapy has high efficacy, however it is only an option in RR disease course, once we observe a recurrence of inflammatory activity. Recently, alemtuzumab, a monoclonal antibody, was the first IRT licensed by the European Medicines Agency and FDA and has shown promising results as a rescue therapy or as first line drug in severe-onset MS (Gallo et al., 2017). Although, the future goal in MS therapy should pass through combined therapy either by limiting the immune cell infiltration but also preventing neuroinflammation and neurodegeneration, which correlates directly with clinical symptoms and disease progression.

2. S100B

S100 proteins are a family of calcium (Ca^{2+})-binding to EF-hand domains that trigger conformational changes and allow interactions with other proteins. These proteins are thought to be Ca^{2+} sensors, modulating biological activity via Ca^{2+} binding (Michetti et al., 2012). Curiously, this family of proteins was termed S100 once it is soluble in 100% saturated ammonium sulfate solution (Rothermundt et al., 2003). Currently, it is known that S100 proteins can act either through intracellular regulators and extracellular signaling. Intracellularly, they are involved in the regulation of protein phosphorylation; cell growth and motility; cell cycle; transcription, differentiation and cell survival; and can promote inflammation and migration through interaction with target proteins including enzymes, receptors, transcription factors and nucleic acids. On the other hand, extracellular S100 proteins can act in both autocrine and paracrine manner via activation of surface receptors, namely through the receptor for advanced glycation end products (RAGE) (Michetti et al., 2012; Donato et al., 2013). Both trophic and toxic effects of extracellular S100B are mediated

in the brain by RAGE. In the human brain, S100B occurs in a multimeric state in human brain, and the most prominent form is a tetramer. Indeed, binding studies revealed that tetrameric S100B binds to RAGE with higher affinity than dimeric S100B (Ostendorp et al., 2007a).

RAGE is responsible for the interaction with a large number of ligands belonging to the S100 family. It is a member of the immunoglobulin (Ig) superfamily being expressed at low levels in normal tissues and it is involved in inflammatory disorders. Its gene is located within the MHC class II locus, which is associated with the activation of innate immunity, acting as a pattern recognition receptor (Xie et al., 2008). Structurally, RAGE consists of: 1) three extracellular Ig-like domains (one N-terminal and two C-type Ig domains); 2) a transmembrane helix; and 3) a intracellular negatively charged C-terminal tail (approximately, 42 amino acids) (Ostendorp et al., 2007a; Xie et al., 2008). RAGE can bind to products of non-enzymatic glycoxidation, β-sheet fibrils characteristic of amyloid, proinflammatory-like mediators such as S100 family, and amphoterin. It is important to highlight that RAGE binding to receptors does not accelerate degradation but can begin a period of cellular activation leading to inflammation (Schmidt et al., 2001). RAGE and its high expression is also related to a variety of diseases, namely Alzheimer's disease, diabetes and inflammatory diseases (Ostendorp et al., 2007a).

During pathological conditions, S100 proteins are mainly found in biological fluids, and can be used as biomarkers. Specifically, S100B, a small Ca^{2+}-binding protein, is one of the S100 proteins that can be used as biomarker of disease diagnosis being detected in CSF or in peripheral blood, including for MS disease **(Section 2.2.)** (Villarreal et al., 2014; Barateiro et al., 2016). Upon S100B binding, there is an activation of RAGE expression, expanding S100B levels that is found to be increased in several tumors, aging diseases, chronic epilepsy or HIV infection, and in acute and chronic inflammatory diseases (Ostendorp et al., 2007a; Donato et al., 2009; Villarreal et al., 2014).

2.1. Intracellular and Extracellular role of S100B

As it was mentioned above, S100B is a member of the S100 family of Ca^{2+}-binding protein that can exert both intracellular and extracellular functions (Brozzi et al., 2009). S100B is a 10,5 kDa protein located on chromosome 21q22.3 and it is abundant in the nervous system, where it is mainly expressed by astrocytes, mature OLs and Schwann cells (Huttunen et al., 2000; Adami et al., 2001).

Within cells, S100B is located in the cytoplasm and it is associated with membranes and certain cytoskeleton elements. Moreover, S100B act as Ca^{2+} sensors and as a stimulator of cell proliferation and migration; an inhibitor of apoptosis; and it also contributes to the

regulation of the microtubules assembly/disassembly and type III intermediate filaments (Brozzi et al., 2009; Donato et al., 2013). Additionally, it is also involved in the regulation of energy metabolism, cell proliferation and differentiation, and regulation of cell morphology in neurons and glial cells **(Figure I.4)** (Rothermundt et al., 2003; Deloulme et al., 2004; Kleindienst et al., 2007). Overall, it is quite evident that S100B plays a protective role if it acts within the cells, as an intracellular regulator, at physiological levels

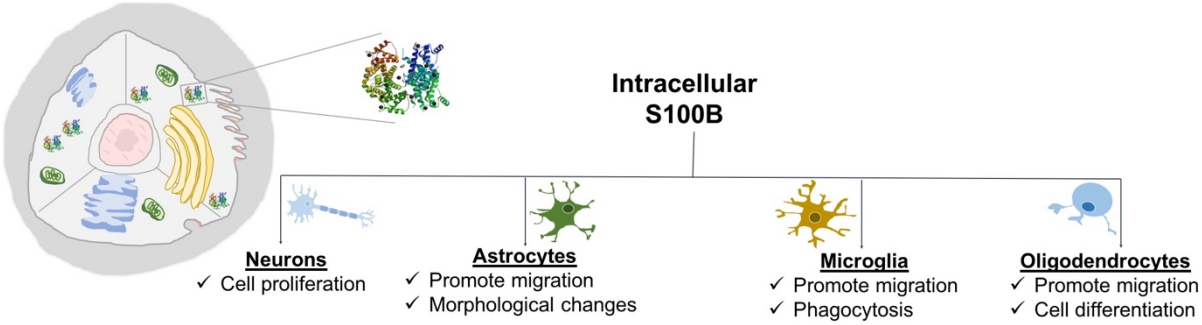

Figure I. 4: Schematic representation of S100B intracellular effects in CNS neurons and glial cells, including astrocytes, microglia and oligodendrocytes. Intracellularly**,** S100B acts as a signaling molecule promoting neuronal proliferation, oligodendrocyte differentiation, astrocyte morphology and helps in microglia/astrocyte migration. X-ray structure of human Ca^{2+}-S100B, in Protein Data Bank.

In the CNS, S100B is constitutively secreted by astrocytes. The secretion is mediated and regulated by metabolic stress conditions or upon exposure to agents, namely, serotonin agonists, low levels of glutamate, lysophosphatidic acid or pro-inflammatory cytokines (Brozzi et al., 2009; Donato et al., 2009). Actually, extracellular S100B might be dependent on the activation of RAGE, that is present on the cell surface (Donato et al., 2009).

Extracellularly, S100B also acts in neurons, astrocytes, and microglia where it has different effects, depending on its concentration **(Figure I.5)**. Indeed, S100B protein is also known to have double-life, acting either as a neurotrophic or neurotoxic molecule. Physiological conditions in the brain are shown to be around nanomolar (nM) concentrations. At this concentration, S100B exert trophic effects mainly protecting hippocampal neurons, stimulating neuronal outgrowth mediated by RAGE-dependent activation, and preventing motor neurons degeneration (Huttunen et al., 2000; Gerlach et al., 2006). Besides neurons, it also affects microglia and astrocytes in different manners. In an autocrine way, S100B promote astrocyte proliferation and survival (Rothermundt et al., 2003; Donato et al., 2013). However, in microglia, it attenuate the activation being implicated in the regulation of its activity (Adami et al., 2001; Donato et al., 2013).

Contrariwise, upon injury S100B can achieve micromolar (μM) concentrations, which is associated with neurodegenerative or apoptosis-inducing effects (Rothermundt et al., 2003). Astrogliosis is one of the consequences, in parallel with iNOS expression, nitric oxide (NO)

and pro-inflammatory cytokines (IL-1β and tumor necrosis factor (TNF)-α) secretion (Huttunen et al., 2000). Astrocyte exposure to S100B induces several alterations in its morphology toward a reactive stellate phenotype. Neuronal apoptosis also occurs through S100B interaction with RAGE causing high levels of ROS accompanied by its release, followed by activation of caspase cascade (Rothermundt et al., 2003; Gerlach et al., 2006). In addition, S100B has also the ability to induce microglia activation and, therefore contribute to NO release and to create a pro-inflammatory cytokine environment (Donato et al., 2009). Evidences suggest that extracellular S100B effects can promote gliosis via RAGE/Rac-1-Cdc42 pathways and activate nuclear factor kappa B (Villarreal et al., 2014).

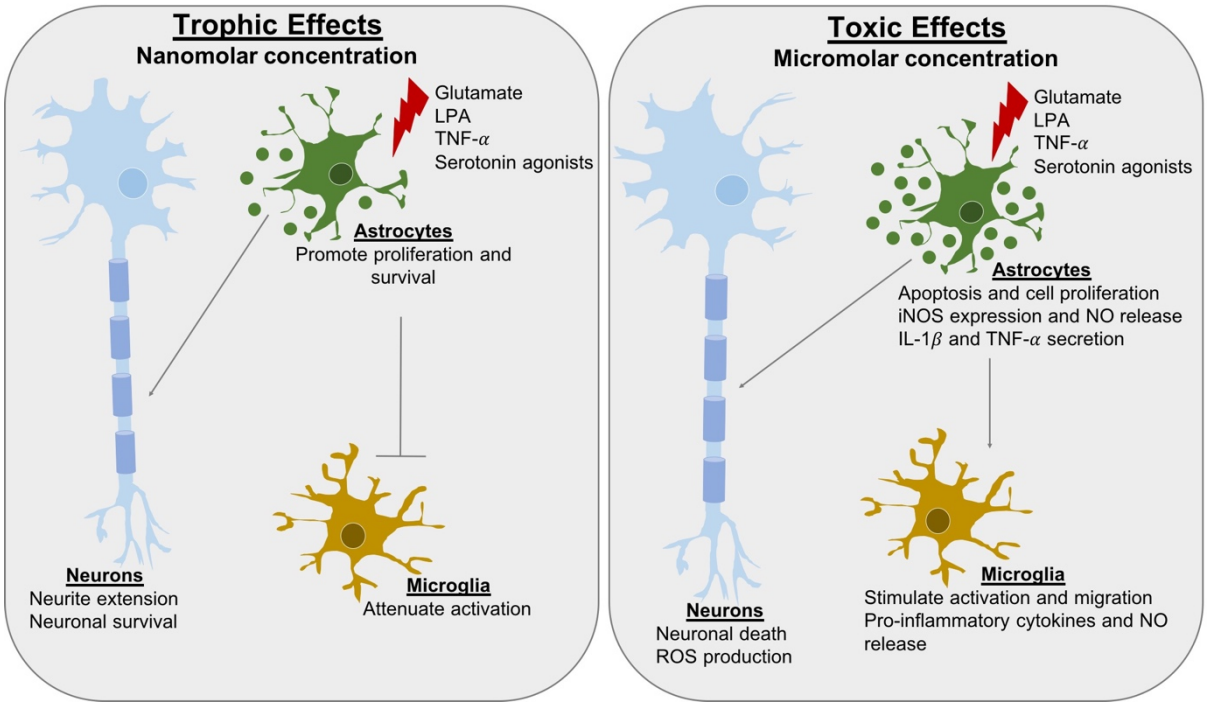

Figure I. 5: Schematic representation of S100B extracellular effects in CNS neurons and glial cells, namely astrocytes and microglia. Upon exposure to glutamate, LPA, serotonin agonists and TNF- α, S100B is released from astrocytes exerting paracrine and autocrine effects. Depending on its concentration, nanomolar (left) or micromolar (right), S100B can have both trophic or toxic effects, respectively. IL – Interleukin; LPA – Lysophosphatidic acid; NO – Nitric Oxide; ROS – Reactive Oxygen Species; TNF – Tumor Necrosis Factor

S100B protein has been extensively studied over the past years, and there is no doubt between the intracellular and extracellular effects in the nervous system cells. The relationship between S100B, neurodegeneration, and neuroprotection comes from data obtained through the analysis of extracellular effects. The release of S100B from astrocytes can be reduced by augmenting the levels of glucose following by inhibition of Src kinase, or just upon treatment with high doses of glutamate (Donato et al., 2009). Furthermore, the S100B therapeutic neutralization through inhibitors, namely antagonists, drugs and specific

antibodies, could be a good strategy to overcome neurodegeneration and inflammation associated with the toxic effects of this molecule **(Section 2.3.)**.

2.2. S100B and Multiple Sclerosis

MS is a heterogeneous disease involving either neuroinflammation, glial activation and oligodendrocytes pathology. The presence of S100B within the CNS and its elevated levels in biological fluids of several pathological conditions, made S100B a protein candidate for a potential biomarker.

Moreover, Michetti and his collaborators were the first group to detect S100B in extracellular biological fluids, particularly in the CSF of MS patients, in the acute phase of exacerbation, however, almost undetectable during remission (Michetti et al., 1979). Another study showed that, there is a significant trend of the CSF S100B levels during disease progression, more elevated in RRMS decreasing in SPMS and even lower in PRMS, in accordance to the decrease of an inflammatory status. It was also shown the presence of S100B in acute lesions of *post-mortem* brain tissues of patients with RRMS (Petzold et al., 2002). Furthermore, studies also described that patients with increased S100B levels reflected both axonal and glial pathology, while after immunosuppressive therapies the levels decreased (Bartosik-Psujek et al., 2011). Indeed, after these findings showing the importance of S100B in different phases of disease progression, this protein gained some status in MS research once can serve as diagnostic and prognostic biomarker and can be used as a tool to monitor immunosuppressive treatments.

Some ongoing work in our group increased the interest in this protein. In human CSF and serum samples from MS patients, it was confirmed a significant increase of S100B levels at the time of diagnosis and that it is highly upregulated in active and chronic MS lesions of post-mortem samples, mainly in astrocytes. Moreover, RAGE expression was predominantly observed in macrophages/microglia in active lesions. Using an *ex vivo* demyelinating model, it was demonstrated that S100B is overexpressed and released upon demyelination, alongside with the activation of astrocytes and microglia (Barateiro et al., 2016). Once it was revealed the relevance of this protein in MS, it is of our interest to find therapeutic approaches to neutralize and diminish the levels of pathological S100B.

2.3. Therapeutic approaches to neutralize S100B

Taking in advantage the presence of S100B in several events within CNS and its direct implication in the MS disease, further studies were done to see its significance as a therapeutic target.

Therapeutic antibody-mediated neutralization of S100B is one of the promising

approaches that shown interesting results in reducing the effects of extracellular S100B at μM concentration. Recent work of our group showed that incubation of brain slices with anti-S100B antibody partially prevented the demyelination caused by lysophosphatidylcholine (LPC), reduced astrogliosis and prevented cytokine and inflammasome expression (Barateiro et al., 2016).

Additionally, S100B can be neutralized through a RAGE-specific antagonist, FPS-ZM1. Results from the co-incubation of S100B with RAGE antagonist showed the reduction of S100B toxic effects, namely by the prevention of S100B-induced OPC differentiation and OL morphological maturation arrest, loss of axonal integrity and, consequently myelination impairment. This strategy also supported the role of S100B-RAGE engagement on the creation of an inflammatory environment. Indeed, this study also described that, by using an *ex vivo de novo* demyelinating model, co-incubation with μM levels of S100B and RAGE antagonist, decreased both myelination and inflammation (Santos et al., 2018).

Furthermore, studies were also performed to elucidate whether S100B-RAGE axis were indeed involved in mechanisms underlying MS pathology. Using organotypic cerebellar slice cultures incubated with RAGE antagonist, FPS-ZM1, have shown to promote OL differentiation and maturation and, actually, be protective against both demyelination and axonal injury. Additionally, the inhibition of S100B-RAGE axis was able to prevent gliosis and, subsequently the inflammatory milieu. Given these results, we were able to clarify the role of these axis and that, therefore, its blockage could be a potential new therapeutic strategy (unpublished data, Santos, G., 2018. PhD thesis).

Once previous results have shown the role of S100B-RAGE axis, studies were performed using an *ex vivo* demyelinating model treated with pentamidine, an antiprotozoal drug, with a specific affinity to the S100B molecule (Markowitz et al., 2004; McKnight et al., 2012)**.** This drug was first identified in 1930 for the treatment of *Pneumocystic carinii* and it is already a FDA approved drug (Sands et al., 1985). These studies have shown that pentamidine not only prevented demyelination by blocking toxic S100B, but also prevented the exacerbated expression of pro-inflammatory cytokines, and therefore supported remyelination (unpublished data, Pascoal, P. 2017. Master thesis). These preliminary results showed that this drug is able to neutralize toxic S100B preventing its destructive effects in MS-like pathogenesis.

Collecting all data, further studies should be done using an animal model of MS, to further clarify the potential pharmacological targeting of S100B in MS disorder **(Section 3)**. So, it is of great interest to explore whether these strategies may ameliorate MS-like pathogenesis and immune response, becoming a new way of therapeutic intervention, improving patients' life.

3. Experimental models to study Multiple Sclerosis

The unsatisfactory and ineffective therapy for the treatment of MS as well as the complete knowledge of its pathophysiology, led to the development of experimental models that mimic some hallmarks of disease. Mice and rats are the more used animal models, once they are easy to manipulate and genetically closeness to humans, being the preferential target to study and develop human diseases.

In MS research, it has been used a variety of animal models to study either inflammation, autoimmunity, demyelination and remyelination, considering each of the pathogenic process for both relapsing MS (inflammatory) and for chronic progressive MS (neurodegenerative). Among the different experimental animal models used to study MS, the experimental autoimmune encephalomyelitis (EAE) is the most frequently used in research. Besides EAE, there are two major categories of animal models that can be used: 1) the virus-induced chronic demyelinating disease model; and 2) the toxic-induced model of demyelination **(Section 3.1.)** (Denic et al., 2011)**.** Besides all good things that the *in vivo* models bring to MS research, they represent an extensive cost, either economically or ethically (Mix et al., 2010). To overcome this issue, cerebellar organotypic slice cultures have also been used to mimic demyelination and inflammatory processes when exposed to toxins, namely LPC, in the absence of the immune system response (Doussau et al., 2017).

Due to the heterogeneity of MS, it is not possible for a single animal model to capture and incorporate the spectrum of human MS and its variability in clinical, radiological, immunological, pathological and genetic features (Mix et al., 2010; Procaccini et al., 2015). However, over time, there is an increased interest in discovering useful and relevant animal models that allow the study of either immune- and CNS-pathological mechanisms of MS concerning its therapeutic and reparative treatments.

3.1. *In vivo* Animal Models

Regarding *in vivo* models, the three most studied animal models of MS summarized in **Table I.3** are (Denic et al., 2011):

1) The EAE, that is useful to study CNS inflammation, immune surveillance and immune-mediated tissue injury **(Section 3.1.1.)**;

2) The virus-induced model of demyelination, using Theiler's Murine Encephalomyelitis Virus (TMEV), to reproduce chronic disease course and to study the role of axonal injury and repair **(Section 3.1.2.)**;

3) The toxic-induced model of demyelination, using cuprizone and LPC, to study the mechanisms of demyelination and remyelination **(Section 3.1.3.)**.

Table I. 3: Characteristics of the different in vivo models of multiple sclerosis.

Model of MS	Mechanism	Application	Involved cells
Experimental Autoimmune Encephalomyelitis	Immunization with a specific myelin protein ($PLP_{139-151}$ or MOG_{35-55})	Myelin destruction; neuroinflammation, and immune system activation	T cells, B cells, and microglia
Virus-induced model	Intracerebral infection with picornavirus, such as TMEV	Study of axonal damage, and inflammatory-induced demyelination	Glial cells, and T cells
Toxic-induced model	Feeding 0.2% cuprizone, or LPC injection	Study of de- and re-myelination processes	Glial cells

LPC- Lysophosphatidylcholine; PLP – Proteolipid Protein; MOG – Myelin Oligodendrocyte Glycoprotein; TMEV – Theiler's Murine Encephalomyelitis Virus

3.1.1. Experimental Autoimmune Encephalomyelitis: immunity and inflammation

As it was mentioned above, EAE is the most extensively and widely studied animal model of autoimmune and inflammatory disorders. It provides a flexible, rapid and potent platform for research, once there are many features that resemble for clinical MS (Ransohoff, 2012). EAE is the animal model more versatile of autoimmune nature, without the involvement of viruses and toxins (Batoulis et al., 2011). Besides the positive things, recent criticism of EAE has been made since there were promising results that have shown to be inefficient in human MS, suggesting a more careful use of the model (Denic et al., 2011).

The induction of inflammation and demyelination can be observed through active immunization with auto-antigens derived from CNS proteins, such as PLP, MOG, and MBP, or by passive transfer of encephalitogenic T cells between animals of the same species (Teixeira et al., 2005; Rangachari and Kuchroo, 2013). Briefly, immunization is performed using adjuvants and by subcutaneous (s.c.) injections containing synthetic peptides derived from myelin protein. Consequently, there is the activation of peripheral antigen-specific T cells that enter in the CNS confronting myelin antigens and therefore, inducing disease (Denic et al., 2011). Although complete Freund's adjuvant (CFA) alone is able to induce strong inflammatory responses, exert immunomodulatory functions and increase the permeability of BBB, when added to pertussis toxin (PTx) result in a more severe and reliable form of disease (Teixeira et al., 2005; Procaccini et al., 2015).

The oscillatory symptoms and clinical courses can be variable depending on the genetic background, the source and dose of antigenic material and, even the immunization protocol itself. Emphasizing the different genetic background, currently, there are two mice strains more frequently used, namely the SJL/J mice and C57BL/6 mice (Batoulis et al., 2011; Ransohoff, 2012). Briefly, the SJL/J mice can be immunized by synthetic $PLP_{139-151}$,

showing patterns of relapses following recovery and so, reproducing the RR-MS disease course. This model provided proof of the central role of $CD4^+$ T cells in the immune pathogenesis (McRae et al., 1992; Whitham et al., 1991). EAE is mostly induced in C57BL/6 mice using MOG_{35-55} peptide. In here, it is important the dosage under specific conditions, since the type of disease that is reproduced can change depending on it. Indeed, a high and low concentration of peptide reproduce chronic-progressive EAE and RR-EAE, respectively (Berard et al., 2010). This model contributed to the study on the immune pathogenesis of MS, revealing the contribution of B cells, $CD8^+$ and $CD4^+$ T cells and CNS glia cells (Rangachari and Kuchroo, 2013). In addition to this mice models, it can also be used knockout strains to elucidate the role of some involved cells and targets, namely S100B protein. It is also important to highlight that, in classic EAE model clinical changes are evaluated by a standardized 5-points scale, further explained in detail (**II – Methods**) (Berard et al., 2010; Batoulis et al., 2011).

Although EAE is commonly used in research as a model for MS, it has some limitations and some differences relatively to lesions in MS **(Table I.4)**. For instances, in EAE lesions, $CD4^+$ T cells are predominantly in the perivascular infiltrates, while in human MS lesions the predominant immune cells are $CD8^+$ T cells (Hauser et al., 1986). Both Th1 and Th17 cells can induce classic EAE, however Th17 cells are responsible to induce a more severe form of EAE; indeed, both cells have been identified in the CNS and CSF of MS patients (Jager et al., 2009; Batoulis et al., 2011; Procaccini et al., 2015). Moreover, EAE does not reproduce relapses which difficult the study of remyelination. Histologically, this model is mainly described by inflammatory infiltration in the brain and SC WM accompanied with focal demyelination; while MS is mainly a brain disease characterized by focal demyelinated plaques in the cerebral, cerebellar cortex and also, in the SC (Day, 2005; Ransohoff, 2012). Besides its limitations, EAE model changed the course of MS understanding and research, contributing to the knowledge of autoimmunity and neuroinflammation with the final aim of developing novel therapeutic approaches.

3.1.2. Virus-induced demyelination model: virus as a critical environmental susceptibility factor

Molecular mimicry mechanisms have been linked with the pathogenesis of many autoimmune disorders, including MS. It was reported that Epsein-Barr Virus, Measles Virus, and Human Herpesvirus 6 organisms are capable of molecular mimicry mechanisms using myelin as target (Oldstone, 2005). Viruses have been linked to MS as a critical environmental susceptibility factor and to study its contribution the virus-induced demyelination model was developed (Miller et al., 1996).

TMEV is a mouse enteric pathogen with a single stranded RNA picornavirus. TMEV have two main strains, 1) a highly virulent that cause fatal encephalitis, and 2) a less virulent that is used as a murine model of MS. The last model can develop both monophasic and persistent neurologic disease, and a biphasic that reproduce chronic demyelinating lesions (Denic et al., 2011).

Briefly, TMEV infects macrophages and CNS glial cells during chronic phases, being directly involved in demyelination. This model provides us the origin of some behavioral signs following by demyelination of murine CNS; although, the pathogenesis of TMEV-induced myelination differs from what it is seen in MS (Ransohoff, 2012). Unlike what happens in EAE model, the disease is always chronic-progressive in susceptible mice and can only be induced in mice, and not in other different species such as other rodents and primates **(Table I.4)** (Procaccini et al., 2015).

3.1.3. Toxic-induced models of demyelination: processes of focal de- and re-myelination

To fulfill the limitations in the EAE model, namely the absence of remyelination, the toxic-induced model of demyelination was developed. This is an alternative *in vivo* model that use toxins not to mimic MS disease, but to study the processes of focal de- and re-myelination **(Table I.4)** (Denic et al., 2011).

One of the most used is cuprizone known as a copper chelator that is able to promote copper deficiency and, therefore myelin damage. This toxin has as main target mature OLs inducing demyelination without affecting other cell types present in the CNS. Concisely, animals are fed with 0.2% cuprizone during 4 to 6 weeks causing dysfunction of mitochondrial complex and toxicity of OLs. Then, cuprizone administration is suppressed, which allows the generation of new OLs from the pool of OPCs and the formation of myelin sheaths, promoting remyelination (Matsushima and Morell, 2001).

Moreover, we can also study focal demyelination through the injection of a toxin LPC, an activator of phospholipase A2. This toxin induces high focal areas of demyelination due to toxic effects on myelin sheaths, without affecting adjacent cells or axons. Like cuprizone, after a few weeks, complete remyelination occurs (Jeffery and Blakemore, 1995).

These models brought to research more knowledge about important processes in MS disease, namely the understanding of de- and re-myelination. Indeed, these models also brought the capability of isolate lesions as discrete events with spatiotemporal predictability, providing insights into the cellular and molecular mechanisms (Miller and Fyffe-Maricich, 2010; Ransohoff, 2012). However, they fail in the absence of ongoing immune activity, an important contribute to MS disease (Batoulis et al., 2011).

Table I. 4: Advantages and limitations of the different in vivo models used to study multiple sclerosis disease.

Animal Model	Advantages	Limitations
EAE	Flexible, potent and rapid platform for research; Histopathology in accordance with MS lesions; Immunization induce autoreactive inflammatory T cells infiltration of the CNS; Good model to study autoimmunity, neuroinflammation, cytokine biology and immunogenetics	Remyelination cannot be well studied; EAE is a disease of subpial spinal cord white matter, whereas MS is a brain disease with prominent demyelination of the cerebral and cerebellar cortex
Virus-induced model	Demyelination is immune-mediated; Some viruses are proved to be related to MS susceptibility; The clinical manifestation is very similar to chronic-progressive MS	The pathogenesis (demyelination) differs from that in MS; TMEV can only be induced in mice and does not cause pathology in humans
Toxic-induced models	Study of cellular and molecular determinants of remyelination; Provides insights into the determinants of OL cell death, without affecting other cells	Absence of ongoing immune activity, as seen in MS

CNS – central nervous system; EAE – Experimental Autoimmune Encephalomyelitis; MS – Multiple Sclerosis; OL – oligodendrocyte; TMEV – Theiler's Murine Encephalomyelitis Virus

All together and taking advantages of recent findings **(Section 2.3.)**, we decided to test one way of neutralizing S100B through a specific S100B-binding drug, pentamidine. To accomplish this, we will use as an *in vivo* model of MS, the EAE, once it is the one that mimics in more detail the entrance of immune cells in the CNS leading to demyelination and neuroinflammation. Indeed, initial studies using the EAE model were done to demonstrate/validate the role of S100B and RAGE axis. Furthermore, results show a remarkable increase in S100B and its receptor expression, as well as gene expression of pro-inflammatory cytokines (unpublished data, Pascoal, P. 2017. Master thesis).

4. Aims

The main purpose of this project is **to understand the role of the inflammatory molecule S100B in MS-like pathogenesis and, hopefully reduce or even prevent disease severity by using a drug-modifying therapy (pentamidine)**. To accomplish this objective, we will use the MS *in vivo* autoimmune and inflammatory model, EAE, and evaluate animal clinical score and CNS pathogenesis. The specific aims are:

1) **To understand whether the binding of the drug pentamidine to S100B may reduce EAE clinical score.** For this we will use, a cohort of animals that will be divided in two groups: 1) the vehicle group, EAE-induced; and 2) the treated group, EAE-induced and treated with pentamidine since the day of induction. The clinical score will be evaluated daily following a 5-scale, until the end of the treatment. Body weight will be also measured.
2) **To evaluate if S100B targeting decrease CNS pathogenesis in the inflammatory EAE model.** We will measure the areas of demyelination and myelin-related molecules by histopathological staining and immunostaining, respectively. In this phase, glial cells activation and inflammatory status will be also assessed.

II – MATERIAL AND METHODS

1. Animals

To perform EAE studies, we used female C57BL/6 mice acquired from Instituto de Medicina Molecular (IMM), with 8-10 weeks of age. The animals were kept in a controlled environment at 21ºC and 55-67% humidity, on 12- hours light/dark cycles and fed with food and water in *ad libitum* diet. Furthermore, before starting the experiments, the animals were in acclimatization conditions for one week to adapt to the new conditions and avoid animal stress.

Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional Animal Care and Use Committee and the national animal affairs regulatory office (Direção Geral de Alimentação e Veterinária). The best efforts were made to minimize the number of animals used and their suffering.

2. Experimental Autoimmune Encephalomyelitis

C57BL/6 mice were divided into two different groups (total number per group = 5): 1) vehicle group, the EAE-induced group receiving daily saline; and 2) treated group, the EAE-induced group receiving daily pentamidine (4 mg/kg body weight, 2.4 mg/ml daily, via i.p.; Sigma-Aldrich, Merck). EAE was induced in female C57BL/6 mice with 8 to 10 weeks of age. On day 0, mice were immunized by a s.c. injection with 125 μg of MOG_{35-55} (1.25 mg/mL) peptide emulsified in CFA supplemented with 4 mg/mL of heat-inactivated *Mycobacterium tuberculosis*. The MOG_{35-55}/CFA emulsion was injected in both sides of the groin of the mouse with 100 μL. Additionally, to achieve full immunization, the mice were immediately injected intravenously with 100 μL of PTx (200 ng/100 μL) in phosphate buffer (PBS) on the

day of induction and 48 hours later, as described in **Figure II.1**. All animals were injected with the MOG_{35-55}/CFA emulsion and PTx, in the same conditions. During the induction procedure, the mice were anesthetized to reduce stressful situations. Immediately after the induction, the vehicle and treated group were injected via intraperitoneal (i.p.) with PBS and pentamidine, respectively.

For 30 days after induction, all mice were weighted and monitored daily. Clinical signs of EAE were also assessed using a scale ranging from 0 to 5 grades that is characterized by an ascending paralysis beginning at the tail (score 1), followed by limb and forelimb (score 2 and 3, respectively), leading to quadriplegia (score 4) and death (score 5). A more detailed description is given in **Table II.1**. Importantly, the EAE model was generally considered a success if its score exceeded the score 2.

At day 31, the animals were sacrificed, and tissues (specifically, brain and SC) were collected to perform histochemical, immunofluorescence and biochemical analyses, as it will be described below.

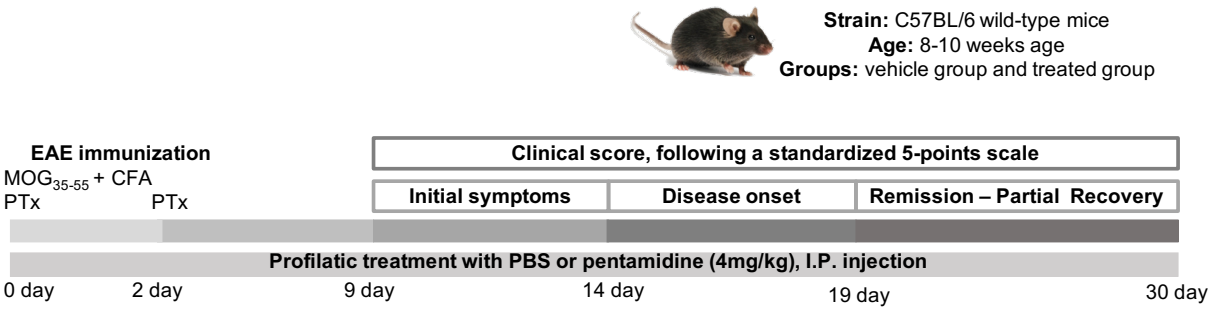

Figure II. 1: Global schematic EAE induction timeline, in C57BL/6 mice with 8-10 weeks age. To achieve full EAE immunization, an emulsion composed by MOG_{35-55} peptide and CFA was injected via subcutaneous at day 0. In addition, PTx toxin was also injected intravenously, at day 0 and 48 hours after. Alongside with mice immunization, PBS and pentamidine were injected in the respective animals' group (e.g. Vehicle group and Treated group). Weight and clinical score were also daily assessed during the experiment. 31 days post-induction, the mice were sacrificed, and blood, spinal cord and brain were collected. CFA – Complete Freund's Adjuvant; EAE – Experimental Autoimmune Encephalomyelitis; I.P. – intraperitoneal injection; MOG – Myelin Oligodendrocyte Glycoprotein; PBS – phosphate buffer; PTx – Pertussis Toxin

Table II. 1: Detailed clinical score with respective clinical observations. Euthanasia is recommended after the mouse score 4.0 after 2 days. When the mouse is euthanized because of severe paralysis, the last clinical evaluation score is given for the respective mouse for the rest of the experiment.

EAE Clinical Score	Clinical Observations
0.0	No obvious changes in motor function compared to non-immunized mice. When picked up by base of tail, the tail has tension and is erect. Hind legs are usually spread apart. When the mouse is walking, there is no gait or head tilting.
0.5	Tip of tail is limp. When picked up by base of tail, the tail has tension except for the tip. Muscle staining is felt in the tail, while the tail continues to move.
1.0	Limp tail. When picked up by base of tail, instead of being erect, the whole tail drapes over finger. Hind legs are usually spread apart. No signs of tail movement are observed.
1.5	Limp tail and hid leg inhibition. When picked up by base of tail, the whole tail drapes over finger. When the mouse is dropped on a wire rack, at least one hind leg falls through consistently. Walking is very slightly wobbly.
2.0	Limp tail and weakness of hind legs. When picked up by base of tail, the legs are not spread apart, but held closer together. Walking has a clearly apparent wobbly. –OR– Mouse appears to be at score 0.0, but there are no obvious signs of head tilting when the walk is observed. The balance is poor.
2.5	Limp tail and dragging of hind legs. Both hind legs have the same movement, but both are dragging at the feet. –OR– No movement in one leg and it is completely dragging, but movement in the other leg.
3.0	Limp tail and complete paralysis of hind legs. –OR– Limp tail and almost complete paralysis of hind legs. One or both hind legs are able to paddle, but neither is able to move forward. –OR– Limp tail with paralysis of one front and one hind leg.
3.5	Limp tail and complete paralysis of hind legs. In addition to: Mouse is moving around the cage, but when placed on its side, is unable to right itself. Hind legs are together on one side of the body. –OR– Mouse is moving around the cage, but the hind quarters are flat.
4.0	Limp tail, complete hind legs and partial front leg paralysis. Mouse is minimally moving around the cage but appears alert and feeding.
4.5	Limp tail, complete hind legs and partial front leg paralysis, no movement around the cage. Mouse is not alert and has minimal movement. The mouse barely responds to contact.
5.0	Mouse is complete paralyzed.

EAE – Experimental Autoimmune Encephalomyelitis

3. Histological analysis

After performing the *in vivo* procedures, animals of both groups were weighted and then, sacrificed. Mice were anaesthetized with a non-lethal dose of isoflurane, a commonly used anesthetic, and intracardially perfused through the left heart ventricle with 4% of

paraformaldehyde (PFA) using a peristaltic pump. Mice brain was removed and fixed/preserved in PFA at 4ºC. Therefore, the samples were cryoprotected with 40% sucrose in PBS and then, snap-frozen in TissueTek® OCT™ compound (Sakura Finetek Europe, Netherlands). Finally, the samples were cross-sectioned with serial coronal cryostat sections with 20 μm thickness, at -20º C (Cryostat Leica CM S3050) and collected in both glass slides and in 24-well plates as free-floating.

The slices were stained for Luxol Fast Blue (LFB) and hematoxylin staining and visualized by light microscopy to assess the degree of demyelination and cells infiltration, respectively. The slices were allowed to dry overnight (approximately, 16 hours) at room temperature (RT). Then, they were incubated with 0.1% LFB solution in 96% ethanol and 10% acetic acid, overnight, at 56.ºC. To clean the excess stain, the slices were washed with distilled H_2O. In addition, tissue differentiation was performed using 0.05% lithium carbonate solution followed by 70% ethanol, and these steps were repeated until the white and gray matter were distinguishable. The slices were then rinsed with distilled H_2O and counterstaining with hematoxylin, for 10 minutes, at RT, and rinsed with tap water for 5 minutes. Hydrochloric acid was also used to differentiate the slices followed by water one last time, for 5 minutes. Finally, dried samples were mounted with Entellan (Merck, K12572938). The images were taken on an optical microscope, Leica DC 100 camera (Leica, Wetzlar, Germany) with x10 magnification under a bright field. Then, the level of myelination was quantitatively evaluated by determining the percentage of brain area that was stained with LFB staining, using the ImageJ (Fiji Is Just) software.

4. Immunohistochemistry procedure

After brain sectioning, slices in glass slides were post-fixed in 4% PFA for 10 minutes following by washing three times with PBS 10 minutes each. Then, to permeabilize the tissue, the slices were incubated with 0.25% Triton X-100 in PBS for 10 minutes and then, incubated with blocking solution containing 5% bovine serum albumin, 5% fetal bovine serum and 0.1% Triton X-100 in PBS solution for one hour, at RT. After blocking, the slices were probed with the primary antibody **(Table II.2)** diluted in the blocking solution, for approximately 48 hours, at 4ºC. Following incubation, the slices were washed three times for 10 minutes each with PBS, and therefore incubated with the appropriate secondary fluorescence antibodies for approximately 2 hours, at RT. The secondary antibodies were also diluted in blocking solution **(Table II.3)**. Afterwards, the slices were washed three times for 10 minutes each with PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:1000, approximately 5 minutes) to stain the cell nuclei. Finally, the slices were washed three times for 5 minutes each with PBS and then, mounted using Fluoromount-G (Southern

Biotech, Birmingham, AL) for fluorescence/confocal microscopy. Fluorescent images were obtained by confocal microscopy using Leica DMi8-CS inverted microscope with Leica LAS X software. Furthermore, the different z-stacks were merged and analyzed with ImageJ (Fiji Is Just) software.

Table II. 2: List of primary antibodies used in immunohistochemistry procedures. Marker and its respective target. The table also highlights for the dilution used and primary Ab host and brand.

Marker	Target	Primary Antibody: dilution, host and brand		
NG2 Neuron-glia 2	Immature oligodendrocytes	1:100	Rabbit	Millipore AB5320
MBP Myelin Binding Protein	Mature oligodendrocytes	1:200	Rat	BioRad MCA409S
Iba-1 Ionized calcium-binding adapter molecule 1	Microglia	1:250	Rabbit	Wako 019-19741
GFAP Glial fibrillary acidic protein	Astrocytes	1:100	Mouse	NovoCastra 6035278
S100B	S100B	1:250	Rabbit	DAKO Z0311

Table II. 3: Secondary antibodies used in immunohistochemistry procedures.

Secondary Antibody	Host and brand	Dilution
Alexa 488, green anti-rabbit	Invitrogen Goat, A11008	1:500
Alexa 594, red anti-mouse	Invitrogen Goat, A11005	
Alexa 594, red anti-rat	Invitrogen Donkey, A21209	

Using Image J software, it was possible to evaluate immature and mature OLs, astrogliosis and microglia activation. The number of positive cells for each type was counted from merged z-stacks. Approximately, 6-7 z-stacks were taken per slice per condition, reducing variation in image acquisition. Furthermore, three specific regions were analyzed that are usually related to demyelination and inflammation: region I – fimbria, region II – internal capsule, and region III – perivascular space **(Figure II.2)**. Indeed, NG2, S100B, GFAP and Iba-1 positive cells were counted in the determined regions, and the results were given by the mean cell number for each region. Regarding oligodendrogenesis, the percentage of area marked with MBP staining was measure and normalized to the area of each region.

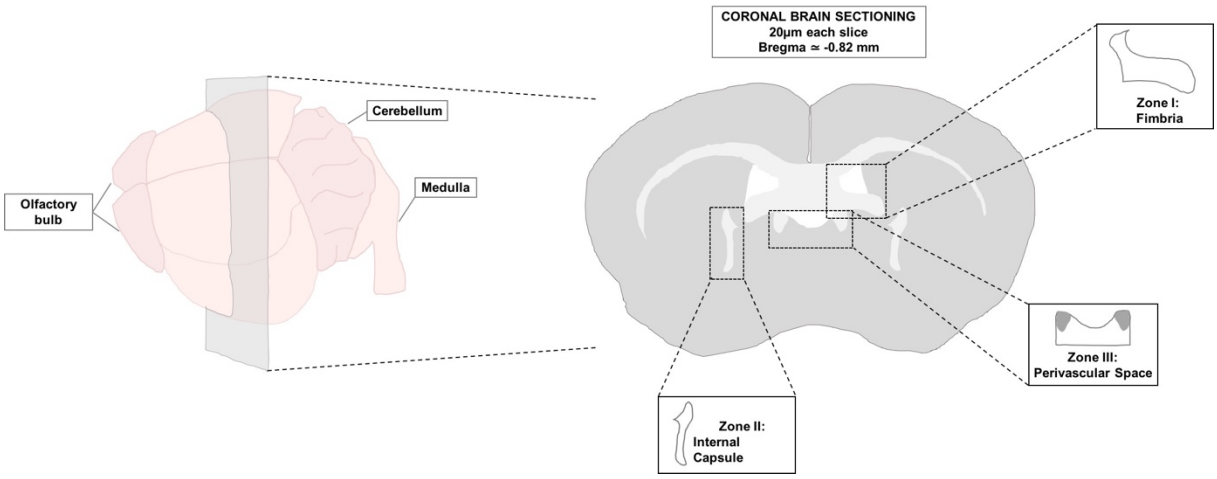

Figure II. 2: Representative brain coronal slice indicating the evaluated regions: fimbria (I), internal capsule (II), and perivascular space (III).

5. Gene expression levels

To determine the expression levels of genes, isolation of total RNA was done from EAE slices using RiboZol^{TM} reagent method, according to the manufacture's guidelines (VWR Life Science, USA). Following RNA extraction, the RNA concentration and purity was checked using NanoDrop ND-100 Spectrophotometer (NanoDrop Techonologies, Wilmington, DE, USA).

5.1. Semi-quantitative RT-PCR (qRT-PCR)

After RNA isolation and quantification, the samples were reversibly transcribed into complementary DNA (cDNA) using Xpert cDNA Synthesis Mastermix kit (GRiSP), under manufacturer's instructions. Furthermore, the cDNA was amplified by semi-quantitative RT-PCR (qRT-PCR) on a 7300 Real-Time PCR System (Applied Biosystem, Madrid, Spain) by the excitation and emission of Xpert Fast SYBR Mastermix (GRiSP). The cycle conditions were previously optimized: 50ºC for 2 minutes, 95.ºC for 10 minutes followed by 40 cycles at 95ºC for 15 seconds and 62ºC for 1 minute. The PCR was performed in 384-well plates, in which each sample was performed in duplicate. The sequences used as primers are listed in the **Table II.4**. β-actin was used as an endogenous control to normalize the expression levels.

Semi-quantification of the transcription level of the genes was measured using ΔΔCt comparative method. This value shows the cycle number extrapolated from the intersection between the amplification curve and the threshold line, known as the Ct value. The results were normalized to the endogenous gene, β-actin, and were obtained by the formula, $2^{-\Delta\Delta Ct}$. For each sample, ΔCt was calculated by the difference between the Ct value of the gene of interest and the mean Ct value of β-actin. Then, ΔΔCt value of one sample was achieved by

the difference between its ΔCt value and the ΔCt value of the sample chosen as reference, in our case the vehicle group (EAE induced+saline).

Table II. 4: List of the primers used for each sample.

Gene	Primer	
	Forward	Reverse
PSD-95	cgaggatgccgtggcagcc	catggctgtggggtagtcagtgcc
SYP	tcaggactcaacacctcagtgg	aacacgaaccataagttgccaa
TNF-α	tactgaacttcggggtgattggtcc	cagccttgtcccttgaagagaacc
IL-1β	caggctccgagatgaacaac	ggtggagagctttcagctcata
IL-10	atgctgcctgctcttactga	gcagctctaggagcatgtgg
β-actin	gctccggcatgtgcaa	aggatcttcatgaggtagt

IL – interleukin; PSD-95 – Postsynaptic protein density 95; SYP – Synaptophysin; TNF – Tumor Necrosis Factor

6. Protein expression analysis

To characterize protein expression, total protein extraction from slice homogenates was performed. Slice tissues were dissociated using RIPA (Radio-Immunoprecipitation Assay) buffer followed by sonication and centrifugation at 12,000 g for 10 minutes, at 4ºC. After the extraction of proteins, total protein concentrations were measured using the BCA protein assay kit following manufacture's guidelines (Pierce™ BCA Protein Assay, ThermoFisher Scientific), and stored at -80ºC.

6.1. Western Blot

Protein samples were prepared with buffer containing 0.25 M Tris-base (pH 6.8), 4% (w/v) sodium dodecyl sulphate, 40% (v/v) glycerol, 0.2% (w/v) bromophenol blue and 1% (v/v) β-mercaptoethanol following by heating during 5 minutes, at 100ºC. The samples were loaded in equal amounts, separated on a Tris-Tricine gel and then, transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were incubated in blocking solution with 1% Tween 20-Tris buffered saline (T-TBS) in 5% (w/v) non-fat milk, for 1 hour at RT. Therefore, the membranes were incubated overnight at 4ºC with the primary antibody mentioned in **Table II.5**. In the following day, membranes were washed and incubated in the same blocking buffer as above with the secondary antibody **(Table II.6)**, for 1 hour, at RT. After washing, the membranes were incubated using WesternBright Sirius reagent (Advansta, Menlo Park, CA, USA) for 1 minute. The bands were detected and visualized in ChemiDoc™ (Bio-Rad Laboratories, Hercules, CA, USA) equipment. The relative intensities of the protein bands were analyzed using ImageLab™ analysis software (Bio-Rad Laboratories). Results were normalized to the expression of β-actin.

The membranes were reutilized, following incubation with the stripping buffer containing Tris-base pH 6.8, SDS, β-mercaptoethanol and HCl for 30 minutes, at 50ºC, in order to start a new immunoblotting protocol.

Table II. 5: List of primary antibodies used in western blot procedures. Marker and its respective target. The table also highlights for the dilution used and primary Ab host and brand.

Marker	Target	Primary Antibody: dilution, host and brand		
NG2 Neuron-glia 2	Immature oligodendrocytes	1:250	Rabbit	Millipore AB5320
MBP Myelin Binding Protein	Mature oligodendrocytes	1:250	Rat	BioRad MCA409S
S100B	S100B	1:750	Rabbit	DAKO Z0311
β-actin	β-actin	1:10000	Mouse	Sigma A5441

Table II. 6: Secondary antibodies used in western blot procedures.

Secondary Antibody	Host and brand	Dilution
Anti-rabbit HRP	Santa Cruz Biotechnology sc-2004	1:5000
Anti-mouse HRP	Santa Cruz Biotechnology sc-2004	
Anti-rat HRP	Santa Cruz Biotechnology sc-2032	

7. Statistical Analysis

In the present project, all results are presented as mean ± standard error of the mean (SEM). The differences between treatment conditions were established by two-tailed unpaired Student's t t-test with Welch's correction, using GraphPad Prism 6.0 for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com), as appropriate.

III - RESULTS

1. Pentamidine reduce the severity and improves recovery of chronic-EAE in C57BL/6 mice

S100B-RAGE axis has been described as an important target in neuroinflammatory disorders, including MS. *In vivo* and *ex vivo* studies demonstrated that, at micromolar concentrations, this axis is involved in either neuronal impairment and oligodendrogenesis processes, but also in glia activation and in the creation of a pro-inflammatory environment (Santos et al., 2018). As it was mentioned above, recent studies using the *ex vivo* demyelinating model demonstrated the protective role of pentamidine, a S100B-binding drug, being able to reduce demyelination and neuroinflammation (unpublished data, Pascoal, P. 2017. Master thesis). So, to clarify the role of this drug in the *in vivo* model, we used the EAE model, where we also previously verified increased levels of S100B expression upon EAE induction.

For this, we used a cohort of animals that were divided in 2 groups: EAE-induced group receiving daily saline (EAE), and EAE-induced receiving daily doses of pentamidine (EAE+Pnt; 4 mg/kg body weigh; according to previously used in another mice study Esposito et al., 2012) initiated following mice immunization with MOG_{35-55}. Once the EAE model display motor symptoms (as described in **Table II.1**), we also monitored the animals clinical score until the end of the experiment, as well as mice body weight **(Figure III.1)**.

EAE induction was performed as diagramed in **Figure II.1**. The mice, from both groups, were observed for a period of 30 days after immunization. The EAE animals developed an acute form of EAE equivalent to score 3 and 4, which peaked around days 17-18 post-immunization. The disease onset was followed by a slight spontaneous recovery around day 21-23 post-immunization without full motor recovery. Actually, 70-80% of the vehicle group showed clinical symptoms superior to 2, meaning that EAE induction was successful. In comparison to EAE-induced animals, the EAE+Pnt developed the disease onset later,

reaching score 2 approximately in day 20, and showed a less severity in motor symptoms. Curiously, in the end of the experiment, 70% of the EAE mice treated with pentamidine, EAE+Pnt, presented a clinical score equal to 0 or 1, correspondent to normal motor function and initial loss of tail movement. Furthermore, as can be observed, the treatment started in the same day as EAE immunization, which seems to indicate a delay of the appearance of first clinical signs in EAE+Pnt mice **(Figure III.1 A).**

The first few days post-EAE induction animals suffered an overall decrease of mice body weight, that can be due to the stressful situation during EAE induction procedure. Upon the emergence of first clinical signs (around day 10), the EAE group continue to lose body weight in a more marked way than the EAE+Pnt group, difference that was maintained until the end of the experiment **(Figure III.1 B)**. However, the variability between experiments was elevated, and so further studies should be done to find a pattern in body weight loss and disease severity.

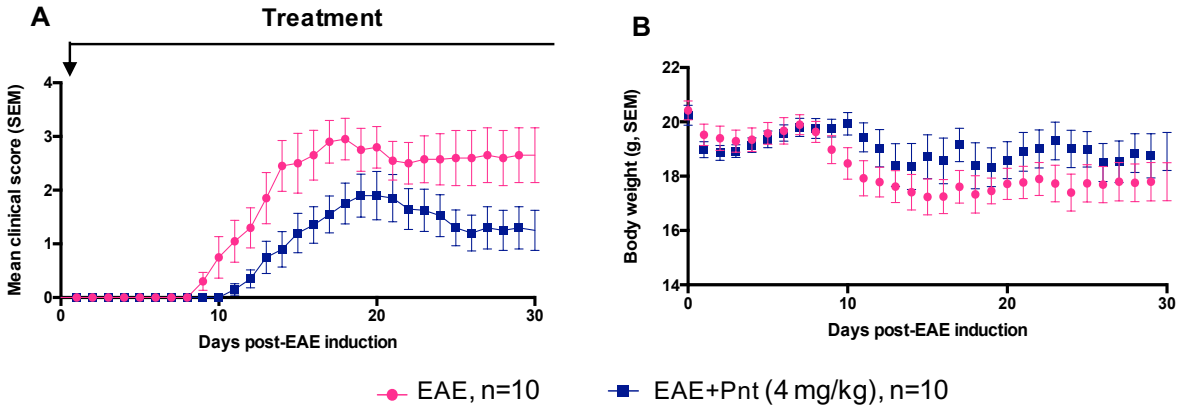

Figure III. 1: Pentamidine treatment ameliorates EAE clinical score and animal weight loss. (A) Clinical observations of vehicle (EAE) and treated (EAE+Pnt) group during 30 days after mice immunization. Treatment with pentamidine started in the day of immunization, and these animals showed not only a delayed of first clinical signs but also in disease onset. The clinical score is a numeric value related to disease severity that was given during each day of the experiment following a 5-point standardized scale. (B) For 30 days, body weight was also measure in both EAE and EAE+Pnt animals. Results are expressed as mean ±SEM of n=5 per group, in two independent experiments. Pnt – Pentamidine; SEM - standard error of the mean

Indeed, we can conclude that prophylactic treatment with the S100B-binding drug, pentamidine, did 1) affect the disease onset, 2) ameliorate EAE disease course, and 3) improve recovery.

2. Pentamidine leads to a significant prevention of myelin loss in EAE-induced animals but no changes in synaptic markers

Commonly, the EAE model is one of the animal models used to study and understand autoimmune and inflammatory features and, furthermore, it is a powerful resource for the development of new therapeutic approaches. One of the main characteristics of this model is the destruction of normal and healthy myelin sheaths resulting in demyelination and, consequently, axonal damage (Day, 2005). Indeed, to assess the effect of pentamidine on CNS damage, we next performed histopathological analyzes of brain sections, using LFB and hematoxylin staining **(Figure III.2)**.

In EAE group, as can be observed in **Figure III.2 A**, there is a loss of LFB-associated blue coloration when compared to the EAE+Pnt one that is directly related to increased demyelination levels. Furthermore, to confirm the obvious loss of myelin, quantification of the area occupied by LFB staining was performed. As expected, EAE animals showed an augmented damage of myelin sheaths, which was significantly prevented upon treatment with pentamidine. Actually, we observed an almost 70% increase in myelin content in the EAE+Pnt animals when compared to EAE group **Figure III.1 B**.

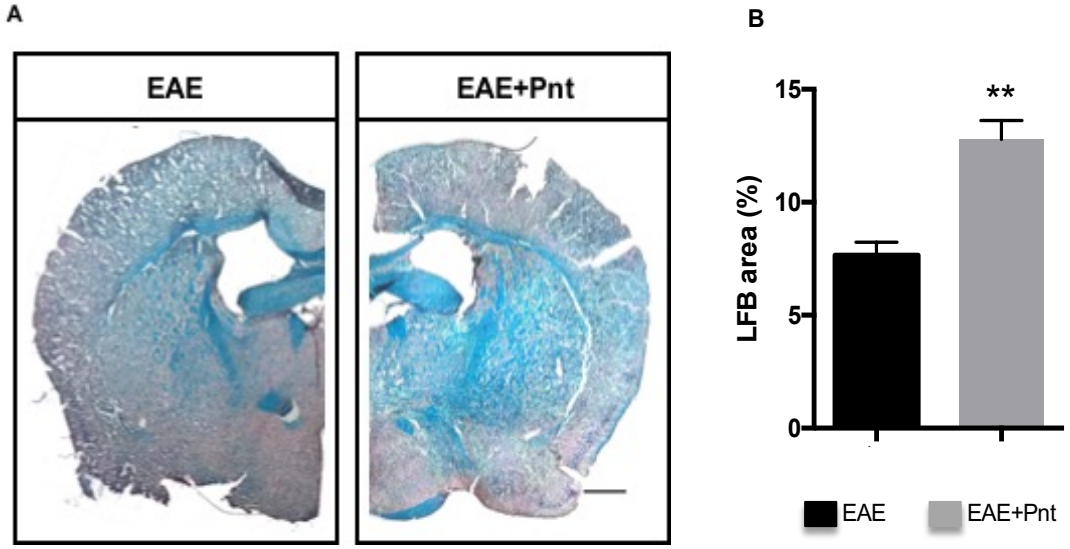

Figure III. 2: Pentamidine attenuates EAE-induced demyelination in brain slices. (A) Representative images of demyelinated regions in both EAE and EAE+Pnt groups. Luxol fast blue stain (LFB, blue) for myelin show a loss of myelin in EAE-induced group, which it is not observed in animals treated with pentamidine. Bregma: 0.82 mm. Scale bar: 400 µm. (B) Graph bars represent quantitative analyzes of the percentage of area occupied by LFB staining. Results are expressed as mean ±SEM of n=5 per group. **p<0.01 vs EAE. Pnt – pentamidine; SEM – standard error of the mean

Next, given our recent data in the *ex vivo* model of demyelination showing a neuroprotective effect of pentamidine (unpublished data, Pascoal, P. 2017. Master thesis), we decided to evaluate if in EAE-induced animals we could also detect neuronal dysfunction

and if pentamidine treatment could prevent it. For these, we evaluated gene expression of two commonly used markers for pre- and post-synaptic neuronal compartments, synaptophysin for presynaptic vesicles and postsynaptic density protein (PSD)-95 for dendritic spine formation, respectively, by qRT-PCR **(Figure III.3)**.

Although no significant changes were observed, animals treated with pentamidine (EAE+Pnt) showed a slight decrease in synaptophysin mRNA expression (0.79-fold). In accordance, preliminary data following western blot analysis, also demonstrated that synaptophysin protein expression was decreased (data not shown). On the other hand, the PSD-95 mRNA expression levels showed a tendency to be augmented in animals treated with pentamidine (1.3-fold), possibly indicating a potential neuroprotective effect

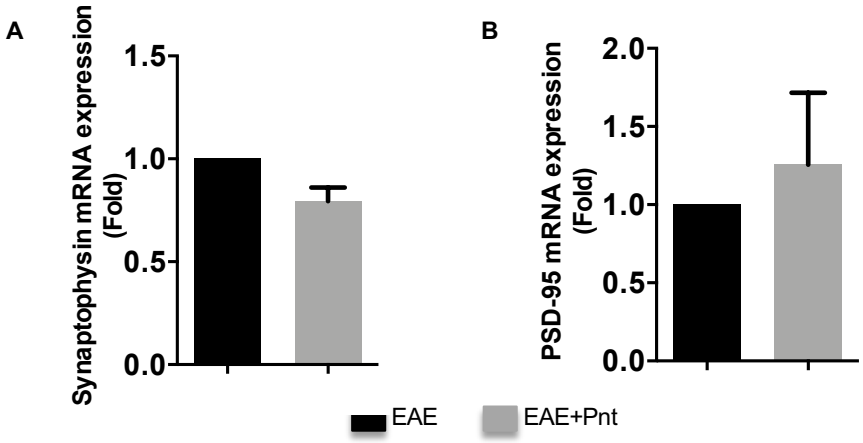

Figure III. 3: Pentamidine treatment slightly alters the expression of synaptic genes. Relative (A) synaptophysin and (B) PSD-95 (postsynaptic protein density 95) mRNA levels were determined by qRT-PCR with the ΔΔCt method. The results were normalized to β-actin. Results are expressed as mean ±SEM of n=5 per group. Pnt – pentamidine; SEM – standard error of the mean

Together, these results suggest that the inhibition of S100B through pentamidine may prevent demyelination and have a partial effect on axonal injury.

3. Pentamidine prevents oligodendrogenesis impairment caused by EAE insult

OLs are known as the myelinating cells of the CNS, resulting from the activation, proliferation, migration and differentiation of OPCs. Pathological processes of MS lead to dysfunction and apoptosis of OL following demyelination and neurodegeneration. Given the importance of OLs, one of the new therapeutic treatments that is arising is related to the promotion of remyelination, through oligodendrogenesis processes (Chamberlain et al., 2016). Knowing that, we decided to address the role of pentamidine as a preventing drug for oligodendrogenesis impairment. For these, immunostaining for immature and mature OL (NG2 and MBP marker, respectively) was performed followed by quantification of total area,

in the case of MBP, or positive cells, for NG2, in three specific demyelinating regions, as mentioned in **Figure II.2**. The results were normalized to the total area of each region. Additionally, to corroborate immunostaining results, protein expression analysis of the same markers was performed by western blot **(Figure III.4)**.

Confirming our previous results, EAE insult caused a loss of mature OL in demyelinated areas, which was significantly prevented in EAE+Pnt treated animals. As shown in **Figure III.4 A**, the area occupied by MBP marker, augmented in all analyzed regions in animals treated with pentamidine (1.87-fold, $P<0.01$ (region I), 1.33-fold, $P<0.05$ (region II), and 1.29-fold, $P<0.05$ (region III)). Moreover, the loss of mature OL was accompanied with an increased number of $NG2^+$ cells, indicative of their proliferation and accumulation in response to the pathological damage, or delay in the differentiation ability into mature OL. Prophylactic treatment with pentamidine helped in the maintenance of healthy myelin, preventing the occurrence of "normal" demyelination due to EAE insult. As we prevented the loss of mature OL, EAE+Pnt mice showed a decrease in number of $NG2^+$ cells in the fimbrias (0.74-fold, region I) and internal capsule (0.74-fold, region II) of treated animals. These results were corroborated by protein expression of OL markers as shown in **Figure III.4 D and E**. Indeed, when compared to the vehicle group, we observed an increased expression of MBP (1.36-fold) and a decreased expression of NG2 (0.43-fold) in EAE+Pnt mice, although not significant **(Figure III.4 F and G)**.

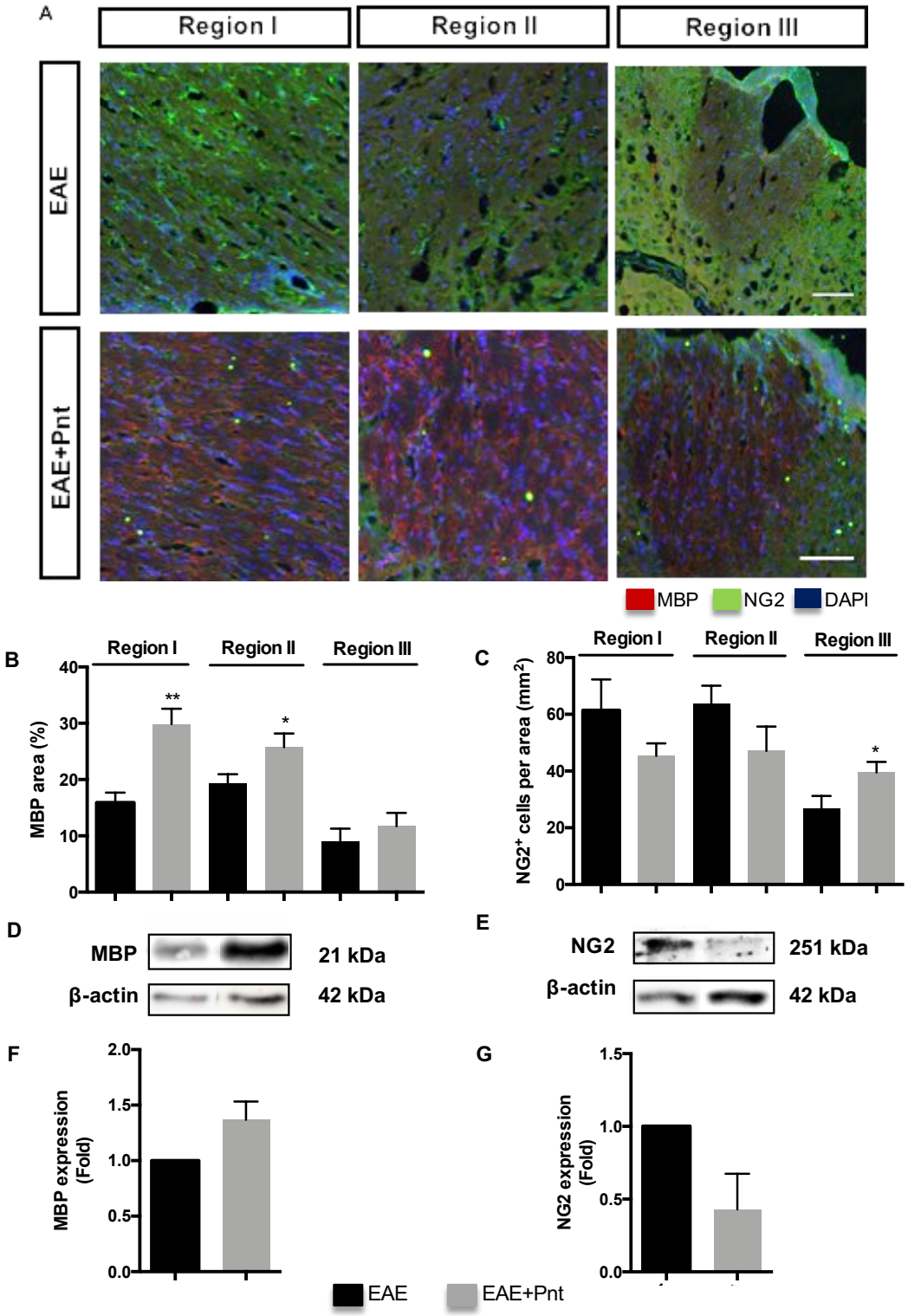

Figure III. 4: EAE induced *de novo* oligodendrogenesis impairment is prevented by pentamidine treatment. (A) Brain sections were immunostained for mature oligodendrocytes (myelin basic protein, MBP, red) and immature oligodendrocytes (neuron-glia antigen 2, NG2, green) and evaluated in three different regions, fimbria (I), internal capsule (II) and perivascular zone (III). Nuclei were counterstained with DAPI (blue). Magnification: 20x. Scale bar: 90 µm. Graph bars represent the area occupied by (B) MBP and (C) NG2 staining in three specific areas. Representative results of (D) MBP and (E) NG2 in total brain homogenates, analyzed by western blot. Graph bars represent the fold change for (F) MBP and (G) NG2 using scanning densitometry normalized to β-actin. Results are expressed as mean ± SEM of n=5 per group, in two independent experiments. *p<0.05, and **p<0.01 vs. EAE. Pnt – pentamidine; SEM – standard error of the mean

EAE induction reduced the percentage of MBP, in favor of an increase of immature $NG2^+$ cells indicating a possible replacement of the lost cells. Curiously, this scenario was reverted in animals submitted to pentamidine treatment, EAE+Pnt. Indeed, these results suggest that pentamidine can also act in the prevention of mature OLs loss and in the early recruitment of progenitor's cells.

4. Pentamidine prevents astroglial reactivity and increased S100B expression in EAE-induced animals

Alongside with myelin degeneration, MS and EAE model are characterized by an activation of both astrocytes and microglia. Regarding astrocytes, they are known as the CNS cell type that highly express and secrete S100B (Sofroniew and Vinters, 2010). Indeed, previous results from our lab showed increase astrogliosis and S100B expression levels followed EAE-insult (unpublished data, Pascoal, P. 2017. Master thesis). Knowing this, we aimed to evaluate whether pentamidine could prevent astrogliosis and the increased levels of S100B. So, we decided to double stain both EAE and EAE+Pnt slices for a commonly used astrocytic marker (GFAP) and S100B. As it was mentioned in the previous section, positive cells were counted and quantified in three particular regions **(Figure II.2)** that are highly related to demyelination. The results were normalized to the area of each region.

EAE insult caused a marked astrocytic activation, seen by an elevated number of cells expressing GFAP, S100B and expressing both $S100B^+/GFAP^+$, namely at the fimbria and internal capsule. Interestingly, pentamidine treatment significantly reduced the number of $GFAP^+$ cells (0.67-fold, $P<0.01$ (I); 0.64-fold, $P<0.01$ (region II); and 0.35-fold, $P<0.001$ (region III)), together with a decrease of $S100B^+$ cells (0.71-fold, $P<0.05$ (region I), 0.74-fold, $P<0.05$ (region II), and 0.40-fold, $P<0.001$ (region III)), namely in perivascular zone **(Figure III.5 B and C).** In accordance, EAE+Pnt animals also showed reduced levels of $S100B^+/GFAP^+$ cells (0.69-fold (region I); 0.72-fold, $P<0.05$ (II); and 0.44-fold, $P<0.01$ (III)), essentially in the perivascular area **(Figure III.5 D)**. Moreover, exposure to pentamidine prevented the elevated levels of cells expressing S100B, meaning that this drug might be inhibiting S100B binding to RAGE, preventing its own expression. In accordance, protein expression of S100B (0.85-fold) was also exhibiting the same pattern **(Figure III.5 E and F)**.

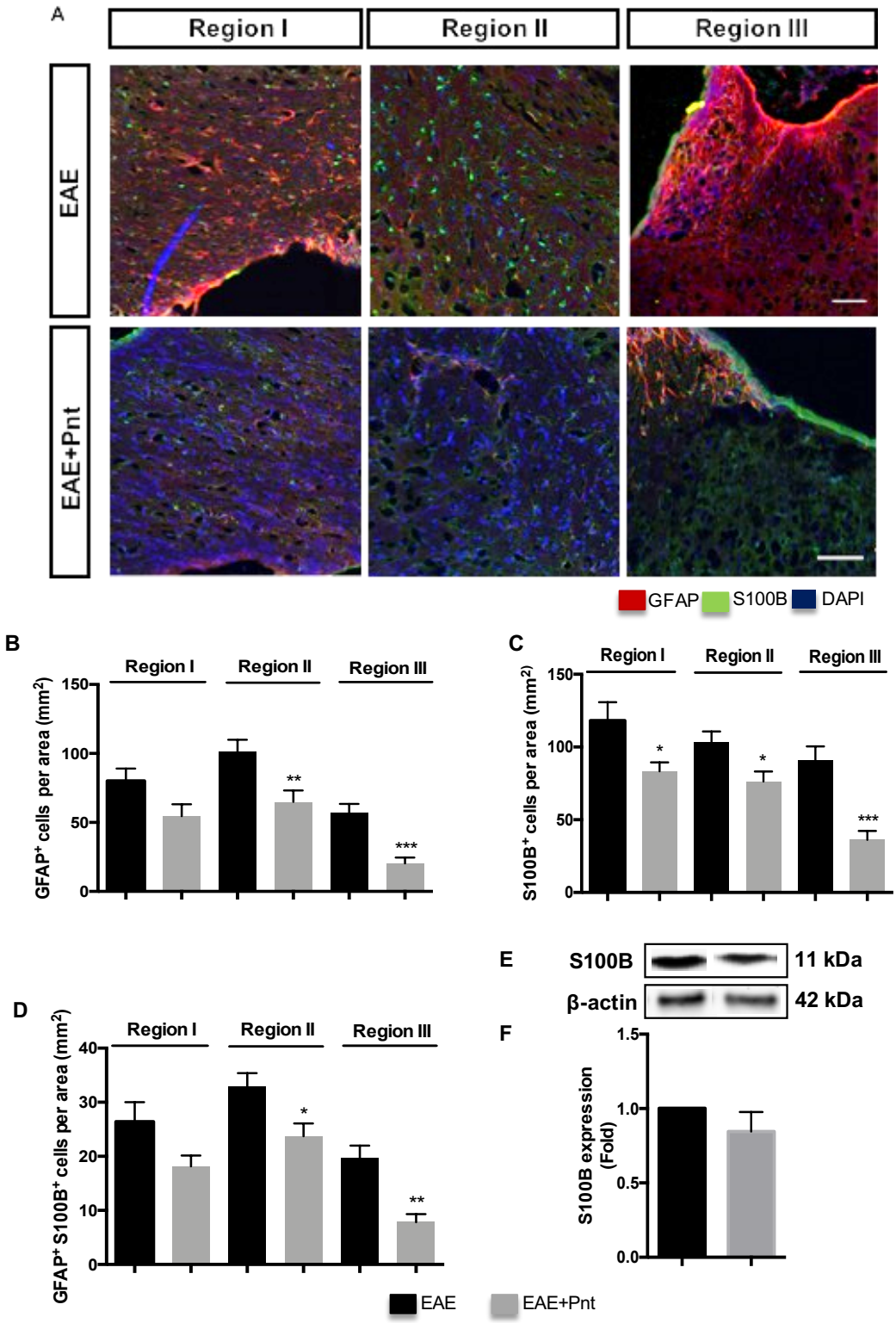

Figure III. 5: Pentamidine significantly prevents astrogliosis and the high expression of S100B elicited by EAE induction. (A) Brain sections were immunostained for astrocytes (glial fibrillary acidic protein, GFAP, red) and S100B (green), and evaluated in three different regions: fimbria (I), internal capsule (II) and perivascular zone (III). Nuclei were counterstained with DAPI (blue). Magnification: 20x. Scale bar: 90 µm. Graph bars represent the quantification of the relative number of (B) $GFAP^+$, (C) $S100B^+$ and (D) $GFAP^+/S100B^+$ cells per area (mm^2), in three specific areas. (E) Representative results of S100B in total brain homogenates, analyzed by western blot. (G) Graph bars represent the fold change obtained for S100B using scanning densitometry normalized to β-actin. Results are expressed as mean ±SEM of n=5 per group, in two independent experiments. *p<0.05, **p<0.01, and ***p<0.001 vs. EAE. Pnt – pentamidine; SEM – standard error of the mean

As already known, EAE induction enhances the levels of cells expressing S100B and GFAP. These results suggest that pentamidine treatment reduced the number of cells expressing S100B, as well as the presence of reactive astrogliosis, known to be a S100B-related inflammatory response.

5. EAE-induced microglia activation is prevented by pentamidine

As mentioned above, S100B is involved in both astrocytes and microglia reactivity. Regarding microglia, recent studies have shown that, after demyelination insult, there is an increase microglial number and a change in their morphology to a more amoeboid state, so called pro-inflammatory phenotype (Barateiro et al., 2016). Knowing that, we decided to evaluate the degree of reactive microgliosis resulting from EAE insult and upon pentamidine treatment. Indeed, we performed double immunostaining of both EAE and EAE+Pnt slices with antibodies for both MBP and Iba-1, a commonly used marker for microgliosis **(Figure III.6)**.

As expected, EAE group revealed a marked microgliosis, showing increased numbers of Iba1^{+} cells. This consequence of EAE induction appear to be significantly diminish in animals treated with pentamidine (0.56-fold, P<0.05 (region I);0.71-fold (region II); 0.67-fold, P<0.05 (region III)), mainly in the fimbrias and the perivascular zones **(Figure III.6 B)**.

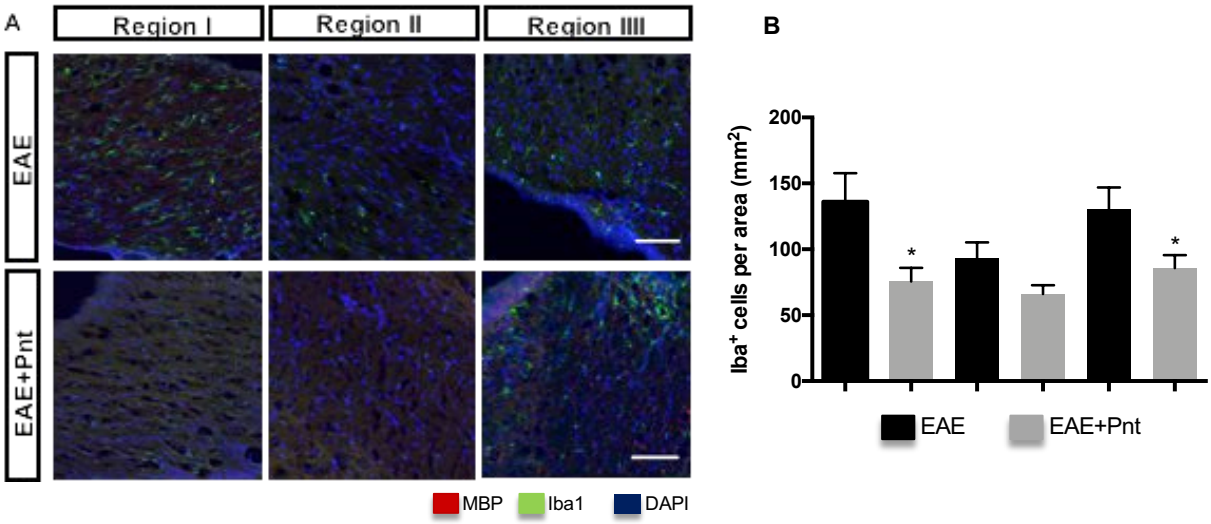

Figure III. 6: EAE insult induces a reactive microgliosis, which is prevented by pentamidine treatment. (A) Brain sections were immunostained for mature oligodendrocytes (myelin basic protein, MBP, red) and microglia (ionized calcium-binding adapter molecule 1, Iba1, green) and evaluated in three different regions, fimbria (I), internal capsule (II) and perivascular zone (III). Nuclei were counterstained with DAPI (blue). Magnification: 20x. Scale bar: 90 µm. (B) Graph bars represent the quantification of the relative number of Iba1^{+} cells per area (mm^{2}). Results are expressed as mean ±SEM of n=5 per group, in two independent experiments. *p<0.05 vs. EAE. Pnt – Pentamidine; SEM – standard error of the mean

Regarding microglia, EAE induction promoted microglia activation. However, this effect was prevented in EAE+Pnt animals, suggesting that pentamidine is an effective drug in the prevention of both microglial and astroglial activation.

6. Pentamidine prevents the inflammatory response induced by EAE insult

Alongside with demyelination and neurodegeneration, micromolar concentrations of S100B, can activate CNS glia, mainly microglia and astrocytes, leading to the production and release of pro-inflammatory cytokines (Donato et al., 2009). Knowing these, we wanted to assess the effect of pentamidine on the inflammatory response. So, we next extracted total RNA from both animals slices and analyzed gene expression of pro-inflammatory cytokines, namely TNF-α, IL-1β, and anti-inflammatory cytokine, as IL-10, by qRT-PCR.

As depicted in **Figure III.7 A**, gene expression of both pro-inflammatory TNF-α and IL-1β cytokines were significantly decreased in the EAE+Pnt group when compared to the EAE-induced group (0.16-fold, P<0.001; and 0.62-fold, P<0.05, respectively). Additionally, we also observed a vast increase in IL-10 mRNA expression levels (6.80-fold, p<0.05) in animals treated with pentamidine, suggesting an induction of a strong anti-inflammatory response **(Figure III.7 B)**.

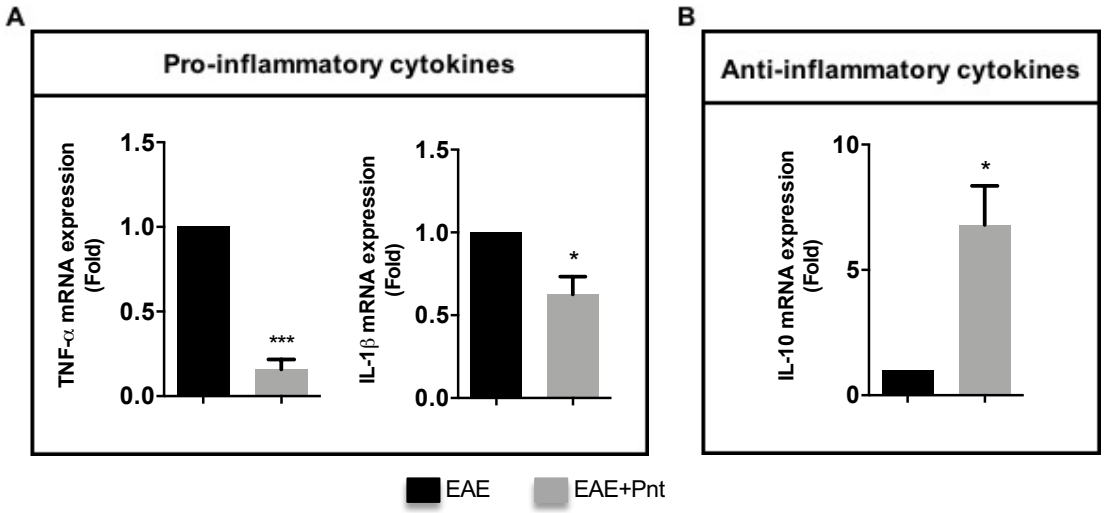

Figure III. 7: Pentamidine alters the inflammatory profile resulted from EAE insult, reducing the expression of pro-inflammatory cytokines, and increasing the expression of anti-inflammatory cytokine. Relative (A) TNF-α and IL-1β, and (B) IL-10 mRNA levels were determined by qRT-PCR with the $\Delta\Delta$Ct method. The results were normalized to β-actin. Results are expressed as mean ±SEM of n=5 per group. *p<0.05, and ***p<0.001 vs. EAE. IL – interleukin; Pnt – pentamidine; SEM – standard error of the mean; TNF – tumor necrosis factor

Together, these data show that EAE induction promote an exacerbated pro-inflammatory response. Moreover, pentamidine treatment was able to prevent the pro-inflammatory environment, suggesting a possible role as an anti-inflammatory drug.

Overall, our results showed that pentamidine, a S100B-binding drug, can act either in the prevention of demyelination and mature OL loss, but also in both astrocytes and microglia reactivity. As we reverted the glial reactivity scenario, we were able to prevent the exacerbated expression inflammatory factors (as TNF-α and IL-1β), suggesting a shift from the pro-inflammatory environment to a more anti-inflammatory one. Moreover, animals induced with EAE and treated with pentamidine reached a lower clinical score and had a faster recovery, suggesting that this drug may be used as a new therapeutic strategy for MS with a broaden efficacy.

IV - DISCUSSION

MS is known as a complex inflammatory demyelinating autoimmune disorder of the CNS. Regarding disease pathogenesis, glial reactivity, inflammation, OL toxicity and axonal degeneration culminate in the formation of demyelinated plaques, a major hallmark of MS (Compston and Coles, 2008). In the past few years, S100B has been linked to a range of neuroinflammatory and neurodegenerative disorders (Ostendorp et al., 2007b). In the case of MS, we showed that S100B is elevated in both CSF and serum of MS patients, being upregulated in active and chronic lesions. Additionally, we recently showed a correlation between abnormal levels of S100B and its impact in demyelination and inflammatory processes, using an *ex vivo* demyelinating model (Barateiro et al., 2016). Besides all research involved to find new drugs, the complexity of the disease difficult the emergence of efficient and safe treatments.

The presence of S100B in the brain and its implications in MS pathogenesis developed a great interest in our research as a potential therapeutic target. Through an *ex vivo* demyelinating model, we demonstrated three different ways of neutralizing S100B, through antibody-mediated therapy directed to S100B (Barateiro et al., 2016), use of S100B receptor RAGE-specific antagonist FPS-ZM1 (Santos et al., 2018) and drug therapy, using the S100B-binding drug pentamidine (unpublished data, Pascoal, P. 2017. Master thesis). Although all therapies demonstrated to be efficient in the reduction of S100B toxic effects, we were very interest in the preventive/protective effect of pentamidine. Indeed, S100B inhibition with this drug have showed promising results in the *ex vivo* demyelinating model, once we were able to prevent both demyelination and inflammation, but also support remyelination (unpublished data, Pascoal, P. 2017. Master thesis). So, here we provided preliminary evidence for the preventive effect of pentamidine *in vivo*, using the EAE model, a common animal model to study MS.

As previously mentioned, the EAE model is extensively used to study neuroinflammation and neurodegeneration related to MS disease. Firstly, we wanted to

understand whether prophylactic treatment with pentamidine could prevent EAE clinical disability. EAE-induced animals showed a chronic disease course that was maintained along the experiment. The same was not observed in EAE+Pnt animals, where we see an attenuation of the clinical score together with an increase of myelin preservation. Overall, we demonstrated that pentamidine improves clinical outcome and recovery, which led us to hypothesized that S100B inhibition may have a direct CNS-specific effect.

The EAE model is characterized by severe demyelination, inflammation and neurodegeneration. Indeed, using LFB staining, we observed a marked decrease in myelin content in the EAE group. The almost absence of pathology was detected in mice treated with pentamidine, thus preventing the disease and subsequent histological damage. Thus, our data is in line with previous results using *ex vivo* models demonstrating that augmented S100B levels produced by a demyelinating insult can interfere in this process, once its inhibition prevented the loss of myelin. Alongside with demyelination, the EAE insult is known to have an effect in both axonal damage and neuronal disability. Studies showed synaptic impairment caused by MOG immunization, either by the reduction of pre-synaptic proteins (e.g. synaptophysin), but also through the reduction of post-synaptic proteins (e.g. PSD-95) (Zhu et al., 2003). In addition, overexpression of S100B in several neurological disorders has been related to chronic neuronal damage, and moreover to its involvement in synaptic plasticity processes (Nishiyama et al., 2002). Indeed, we previously observed that EAE-induced animals, when compared to non-induced ones, have reduced levels of both synaptophysin and PSD-95 (unpublished data, Pascoal, P. 2017. Master thesis). Surprisingly, comparatively to EAE-induced mice, animals treated with pentamidine showed an apparent pre-synaptic degeneration as revealed by synaptophysin reduction, while post-synaptic expression levels were upregulated. The PSD-95 increased expression levels in later disease onset may represent a compensatory mechanism, as already described in other studies (Savioz et al., 2015). Nevertheless, further studies should be performed, for instance the use of electrophysiology, to demonstrate whether pentamidine is able to prevent synaptic loss and neuronal degeneration, and improve brain network communication.

The failure in the formation of new myelin sheaths onto demyelinated axons leads to the formation of multifocal sclerotic plaques. The progressive development of MS and EAE disease is accompanied by OL destruction and further oligodendrogenesis impairment. Due to inflammation, the mature OLs, which are the main source of myelination, are destroyed in the demyelinating regions and the repair depends on the recruitment of OPCs to the injured areas (Aharoni et al., 2008). Concordantly, in a previous study we confirmed that EAE-induced mice showed an overall decrease of mature OL seen by the loss of MBP staining, that was accompanied with an increase number of $NG2^{+}$ precursor cells (unpublished data, Pascoal, P. 2017. Master thesis). Upon the inhibition of S100B through pentamidine

treatment, the EAE-induced animals showed a reverted scenario, with a prevention of mature OL loss and an overall decrease in cells expressing NG2, although not significant. Moreover, recent studies revealed that, besides the use of NG2 as a marker for OPCs in studies of myelin repair, it is also known that NG2 is expressed by macrophages and pericytes in demyelinated lesions (Moransard et al. 2011; Cejudo-Martin et al. 2016). Indeed, NG2 is prominently accumulated within and around areas of leukocyte infiltration (Moransard et al., 2011). Curiously, we observed an accumulation of $NG2^+$ cells in the perivascular regions, known to be the place where exists abundant cell infiltration. Nevertheless, toxic levels of S100B have detrimental effects in oligodendrogenesis processes inhibiting OL differentiation and maturation, as previously reported by us (Santos et al., 2018) and so, may directly affect remyelination delaying myelin formation (Barateiro et al., 2015). Therefore, through pentamidine specific binding, we were able to prevent oligodendrogenesis dysfunction and consequently, demyelination. Additional studies should be performed to understand the dual properties of this drug in the prevention of myelin loss or, even more attractively, in the remyelinating processes.

As pointed out already, in parallel with OL damage and synaptic injury, the EAE model is characterized by concomitant microglia activation, followed by severe astrogliosis. As already described by Donato et.al, S100B can affect neurons, astrocytes and microglia via engagement of RAGE and, curiously, micromolar concentrations of S100B can lead to astrocyte proliferation and migration (Brozzi et al., 2009). In agreement, EAE-induced mice showed severe astrocytosis in specific demyelinated regions, which was significantly prevented in EAE+Pnt mice. These results go in line with previous *ex vivo* demyelinating studies that demonstrated that pentamidine was capable of inducing a significant decrease in GFAP expression and so, preventing astrocyte activation (unpublished data, Pascoal, P. 2017. Master thesis.). Concordantly, in previous studies we also observed an elevated number of $S100B^+$ cells in the EAE-induced animals, in parallel with high number of $S100B^+$ astrocytes, known as the main producers, when compared to non-induced animals (unpublished data, Pascoal, P. 2017. Master thesis). Interestingly, using pentamidine we were also able to prevent the expression of increased S100B levels known to occur upon EAE insult. In fact, S100B targeting by pentamidine was already tested in other neurodegenerative disease models where S100B is increased, such as the Alzheimer's disease model, where pentamidine showed to reduce both S100B expression as well as its receptor, RAGE (Cirillo et al., 2015).

S100B exerts extracellular functions that are mediated by RAGE, transducing different effects on a variety of cell types with different outcomes (Donato et al., 2009). Microglia, as well as S100B, can exert both trophic and toxic effects depending on the external stimuli. Moreover, upon demyelination, microglia are activated and rapidly migrate to the site of injury

for clearing out myelin debris to further help in remyelination (Lampron et al., 2015). Indeed, even after 30 days post-induction, we observed an elevated and acute number of cells expressing Iba1, a marker for microglia, that was significantly higher than in non-induced animals (unpublished data, Pascoal, P. 2017. Master thesis). Curiously, microglia were mostly seen in demyelinated areas, suggesting a possible recruitment of microglia, known as CNS resident cells, in an attempt to clear damaged myelin. In fact, several studies reported the importance of microglia as phagocytic cells, showing that its clearance properties goes with a pro-regenerative contribution to the events occurring upon CNS damage (Neumann et al., 2009). Interestingly, pentamidine prevented microglia activation which may account for a preventive role in disease initiation and subsequent myelin damage. With these results, we may speculate that pentamidine by acting on S100B, may indirectly prevent both astrocytes and microglia activation.

Regarding inflammation, S100B triggers gliosis promoting the release of pro-inflammatory cytokines which are deleterious for OL and may exacerbate demyelination (Barateiro et al., 2015). Typically, microglia M1 phenotype tend to release pro-inflammatory cytokines, as IL-1β and TNF-α, while microglia M2 phenotype release anti-inflammatory factors, namely IL-10. In accordance, our previous results showed that EAE-induced animals have an increased expression of both pro-inflammatory cytokines (IL-1β and TNF-α), and a reduction in anti-inflammatory cytokines expression when compared to non-induced animals (unpublished data, Pascoal, P. 2017. Master thesis). Here, we observed that pentamidine treatment was able to ameliorate the inflammatory environment produced by EAE induction, since it significantly prevented the release of pro-inflammatory cytokines, namely IL-1β and TNF-α. In fact, this pentamidine effect was already observed in our *ex vivo* demyelinating studies (unpublished data, Pascoal, P. 2017. Master thesis.) and also in experimental models of Alzheimer's disease (Cirillo et al. 2015). Furthermore, pentamidine treatment also increased the expression of the anti-inflammatory cytokine IL-10. Interestingly, this interleukin, that is expressed by regulatory T cells, play an important role in preventing uncontrolled T cell-mediated tissue destruction and its mRNA expression in the CNS is increased during recovery phases of EAE, indeed exerting a role in EAE disease course (Samoilova et al., 1998; Cua et al., 2001). Overall, pentamidine was able to modulate the inflammatory environments preventing the production of inflammatory cytokines as seen upon disease induction and, more importantly, having anti-inflammatory properties, favoring a less pro-inflammatory milieu which may improve remyelination.

Collectively, these results suggest that pentamidine, given as a prophylactic treatment, change the disease outcome reducing the clinical score and preventing severe demyelination. Together with the prevention of astrocyte and microglia reactivity, this

treatment avoided the exacerbated release of pro-inflammatory factors and, inversely, induced the expression of anti-inflammatory ones. Alongside with the *ex vivo* studies, these results using the *in vivo* model of MS reinforces the concept that S100B is involved in MS/EAE pathology and that its inhibition, using pentamidine, can be a good therapeutic strategy to reduce CNS damage and, hopefully, improve recovery.

Concluding Remarks

In previous *ex vivo* demyelinated studies, we demonstrated that pentamidine have beneficial effects in the prevention of demyelinating and inflammatory pathogenesis. Then, with EAE studies, we ensured that S100B-RAGE axis was active, as well as inflammation and demyelination, two main characteristics of MS disease. Further curiosity emerged, and, with this thesis, we aimed to understand the role of S100B in EAE-pathogenesis and, hopefully, prevent disease severity by drug-modifying therapy using pentamidine.

In the first part of our EAE study, we day-by-day followed the mice and accompanied an increased loss of motor function in EAE-induced animals, which was not that severe in the group treated animals with pentamidine. Indeed, prophylactic treatment with pentamidine did ameliorate EAE clinical score and improved a faster recovery. Regarding CNS pathogenesis, S100B targeting prevented demyelination and, subsequently, OLs impairment. Along with a decrease in astrocytes and microglia activation, we also prevented exacerbated expression of pro-inflammatory cytokines, favoring an anti-inflammatory environment. Taking all together, our data suggest that pentamidine confers neuroprotection upon EAE insult and may be used as a modulator of inflammation. More attractively, pentamidine is already an FDA approved drug, which allow a potential drug repositioning for MS and, optimistically, a new hope for MS patients.

However, further studies with EAE and pentamidine administration should be performed to understand possible immunomodulatory and remyelinating properties of the drug. As a part of the CNS, it would also be interesting to investigate the role of pentamidine in SC pathogenesis, once it is one of the main areas affected in the EAE model. Recently, it was reported that transgenic overexpressing S100B animals, have impaired learning and electrophysiological disturbances, suggesting that S100B may also affect cognition. Indeed, behavior and cognitive studies should also be done, as well as electrophysiology, to assess if pentamidine may also prevent EAE-associated psychopathology. Until now, we can conclude

that pentamidine is a good preventive, and hopefully a therapeutic strategy as well, to reduce EAE-severity and CNS damage.

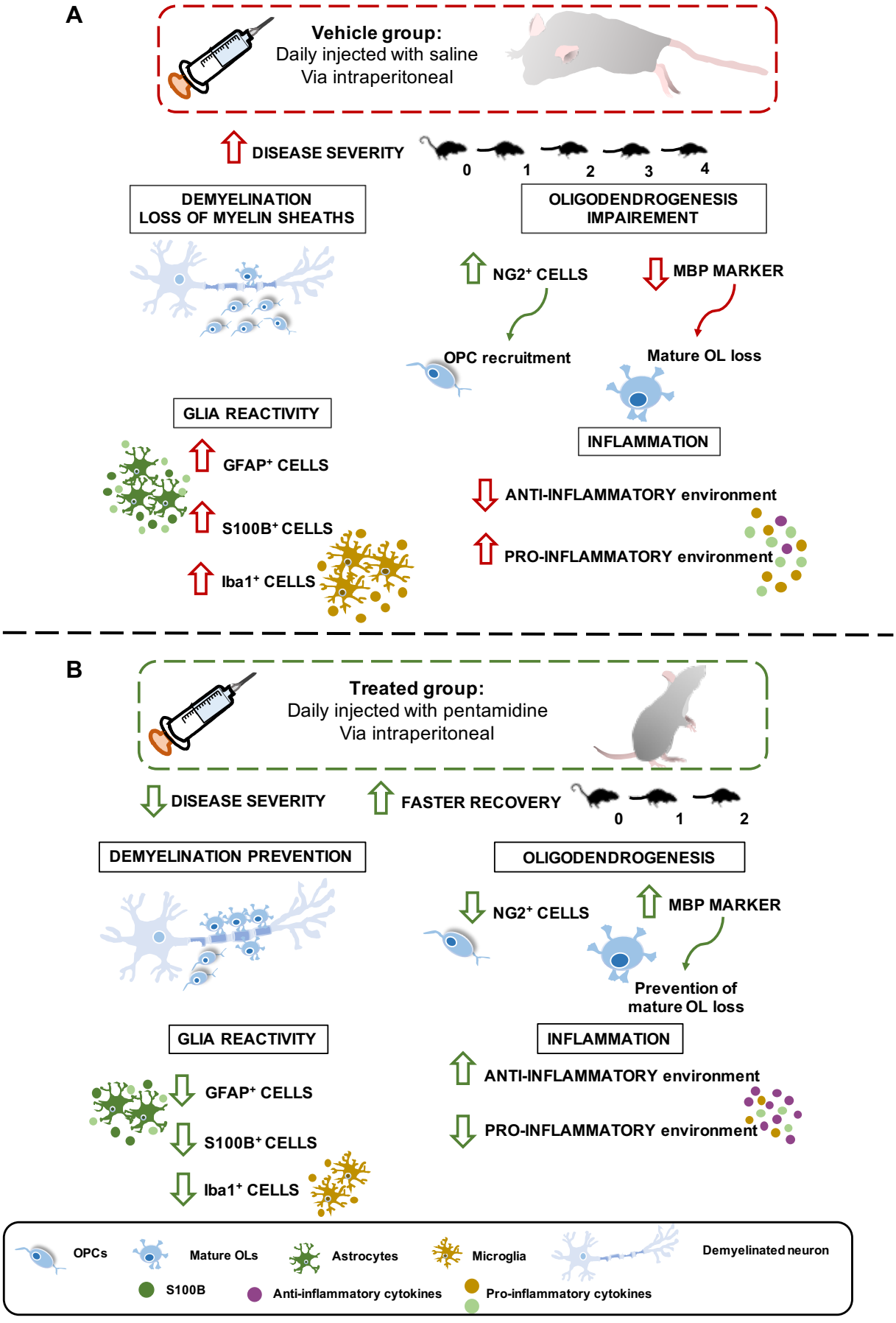

Figure IV. 1. Schematic representation of the major findings in this thesis. *In vivo* studies were performed using an animal model of multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE), where the cohort of animals were divided in two groups: EAE-induced group receiving daily saline Vehicle group (A) and EAE-induced receiving daily doses of pentamidine Treated group (B). EAE induction lead to an increase disease severity, known to be associated with an increase clinical disability. The EAE pathology showed a severe loss of myelin sheaths (demyelination), accompanied with a loss of mature oligodendrocytes (OL, myelin basic protein, MBP) and a recruitment of immature OL (neuron-glia 2, NG2), known as oligodendrocytes precursor cells (OPCs). Moreover, we also observed an increased in glial reactivity confirmed by the increased density of GFAP and Iba1 positive cells, as well as an exacerbated expression of pro-inflammatory cytokines favoring a more inflammatory environment. These results go in line with preliminary data comparing non-induced and EAE-induced animals (unpublished data. Pascoal, P. 2017. Master thesis.). Interestingly, we found that inhibition of S100B, through an S100B-binding drug, pentamidine, could prevent disease severity (maximum clinical score observed was 2) and improve a faster recovery. Modulating S100B expression, we prevented the loss of myelin sheaths and oligodendrogenesis impairment, decreased gliosis and shifted the inflammatory environment to a more anti-inflammatory one. In opposite to the vehicle group, pentamidine treatment inhibited the overall detrimental effects of increased S100B levels, known to contribute to MS-like pathology.

IV – REFERENCES

Adami C, Sorci G, Blasi E, Agneletti AL, Bistoni F, Donato R. 2001. S100B expression in and effects on microglia. Glia 33:131–142.

Aharoni R, Herschkovitz A, Eilam R, Blumberg-Hazan M, Sela M, Bruck W, Arnon R. 2008. Demyelination arrest and remyelination induced by glatiramer acetate treatment of experimental autoimmune encephalomyelitis. Proc Natl Acad Sci.

Armada-Moreira A, F. Ribeiro F, M. Sebastião A, Xapelli S. 2015. Neuroinflammatory modulators of oligodendrogenesis. Neuroimmunol Neuroinflammation:263–273.

Axisa P-P, Hafler DA. 2016. Multiple sclerosis: genetics, biomarkers, treatments. Curr Opin Neurol [Internet] 29:345–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27058221

Bar-Or A, Farwaz L, Fan B, Darlington P, Rieger A, Ghorayeb C, Calabresi P, Waubant E, Hauser S, Zhang J, Smith C. 2010. Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? Ann Neurol 67:452–61.

Barateiro A, Afonso V, Santos G, Cerqueira JJ, Brites D, van Horssen J, Fernandes A. 2016. S100B as a Potential Biomarker and Therapeutic Target in Multiple Sclerosis. Mol Neurobiol 53:3976–3991.

Barateiro A, Fernandes A. 2014. Temporal oligodendrocyte lineage progression: In vitro models of proliferation, differentiation and myelination. Biochim Biophys Acta - Mol Cell Res 1843:1917–1929.

Barateiro A, Santos G, Afonso V, Brites D, Fernandes A. 2015. Role of S100B on oligodendrocyte physiology and pathology. In: Oligodendrocytes: Biology, Functions and Role in the Pathology of Diseases.

Bartosik-Psujek H, Psujek M, Jaworski J, Stelmasiak Z. 2011. Total tau and S100b proteins in different types of multiple sclerosis and during immunosuppressive treatment with mitoxantrone. Acta Neurol Scand 123:252–256.

Batoulis H, Recks MS, Addicks K, Kuerten S. 2011. Experimental autoimmune encephalomyelitis - achievements and prospective advances. APMIS 119:819–830.

Berard JL, Wolak K, Fournier S, David S. 2010. Characterization of relapsing-remitting and chronic forms of experimental autoimmune encephalomyelitis in C57BL/6 mice. Glia 58:434–445.

Berer K, Gerer A. L, Cekanaviciute E, Jia X, Xiao L, Xia Z, Liu C, Klotz L, Stauffer U, Baranzini E. S, Kumpfel T, Hohlfeld R, Krishnamoorthy G, Wekerle H. 2017. Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. Proc Natl Acad Sci U S A:1–6.

Bö L, Esiri M, Evangelou N, Kuhlmann T. 2013. Demyelination and Remyelination in Multiple Sclerosis. In: Myelin Repair and Neuroprotection in Multiple Sclerosis. . p 23–45.

Bö L, Geurts JJG, Van Der Valk P, Polman C, Barkhof F. 2007. Lack of correlation between cortical demyelination and white matter pathologic changes in multiple sclerosis. Arch Neurol 64:76–80.

Boven LA, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen RQ, Boot RG, Aerts JM, Amor S,

Nieuwenhuis EE, Laman JD. 2006. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain 129:517–526.

Bradl M, Lassmann H, Schreiner B, Palle P, Waisman A, Becher B, Buch T, Bradl M, Lassmann H, Funfschilling U, Supplie M, Mahad D, Boretius S, Saab A, Edgar J, Lee Y, Morrison B, Li Y, Lengacher S, Farah M, Hoffman P, Watkins T, Emery B, Mulinyawe S, Barres B, Snaidero N, Möbius W, Czopka T, Hekking L, Mathisen C, Verkleij D, Pfeiffer S, Warrington A, Bansal R, Haber M, Vautrin S, Fry E, Murai K, Boggs J, Rangaraj G, Heng Y, Liu Y, Harauz G, McTigue D, Tripathi R, Nave K, Locatelli G, Wörtge S, Buch T, Ingold B, Frommer F, Sobottka B, Gensert J, Goldman J, Keirstead H, Blakemore W, Nunes M, Roy N, Keyoung H, Goodman R, McKhann G, Jiang L, Fancy S, Zhao C, Franklin R, Franklin R, Ffrench-Constant C, Sim F, Zhao C, Franklin R, Arenella L, Herndon R, Ludwin S, Ludwin S, Bakker D, Scolding N, Morgan B, Houston W, Linington C, Campbell A, Compston D, Fernandez-Gamba A, Leal M, Maarouf C, Richter-Landsberg C, Wu T, Morelli L, Makinodan M, Okuda-Yamamoto A, Ikawa D, Toritsuka M, Takeda T, Kimoto S, Knapp P, Wolswijk G, Chang A, Tourtellotte W, Rudick R, et al. 2010. Oligodendrocytes: biology and pathology. Acta Neuropathol [Internet] 119:37–53. Available from: http://link.springer.com/10.1007/s00401-009-0601-5

Brites D, Vaz AR. 2014. Microglia centered pathogenesis in ALS: insights in cell interconnectivity. Front Cell Neurosci [Internet] 8. Available from: http://journal.frontiersin.org/article/10.3389/fncel.2014.00117/abstract

Brownlee WJ, Hardy TA, Fazekas F, Miller DH. 2016. Diagnosis of multiple sclerosis: progress and challenges. Lancet [Internet] 6736:292–302. Available from: http://linkinghub.elsevier.com/retrieve/pii/S014067361630959X

Brozzi F, Arcuri C, Giambanco I, Donato R. 2009. S100B protein regulates astrocyte shape and migration via interaction with Src kinase: Implications for astrocyte development, activation, and tumor growth. J Biol Chem 284:8797–8811.

Caldeira C, Oliveira AF, Cunha C, Vaz AR, FalcÃ£o AS, Fernandes A, Brites D. 2014. Microglia change from a reactive to an age-like phenotype with the time in culture. Front Cell Neurosci [Internet] 8. Available from: http://journal.frontiersin.org/article/10.3389/fncel.2014.00152/abstract

Cejudo-Martin P, Kucharova K, Stallcup WB. 2016. Role of NG2 proteoglycan in macrophage recruitment to brain tumors and sites of CNS demyelination. Trends cell Mol Biol.

Chamberlain KA, Nanescu SE, Psachoulia K, Huang JK. 2016. Oligodendrocyte regeneration: Its significance in myelin replacement and neuroprotection in multiple sclerosis. Neuropharmacology 110:633–643.

Cirillo C, Capoccia E, Iuvone T, Cuomo R, Sarnelli G, Steardo L, Esposito G. 2015. S100B inhibitor pentamidine attenuates reactive gliosis and reduces neuronal loss in a mouse model of Alzheimer's disease. Biomed Res Int 2015.

Compston A, Coles A. 2008. Multiple sclerosis. Lancet [Internet] 372:1502–17. Available from: http://www.sciencedirect.com/science/article/pii/S0140673608616207

Correale J. 2014. The role of microglial activation in disease progression. Mult Scler J [Internet] 20:1288–1295. Available from: http://journals.sagepub.com/doi/10.1177/1352458514533230

Costantino CM, Baecher-Allan C, Hafler DA. 2008. Multiple sclerosis and regulatory T cells. J Clin Immunol 28:697–706.

Cua DJ, Hutchins B, LaFace DM, Stohlman SA, Coffman RL. 2001. Central Nervous System Expression of IL-10 Inhibits Autoimmune Encephalomyelitis. J Immunol.

Day MJ. 2005. Histopathology of EAE. In: Experimental Models of Multiple Sclerosis. . p 25–43.

Deloulme JC, Raponi E, Gentil BJ, Bertacchi N, Marks A, Labourdette G, Baudier J. 2004. Nuclear expression of S100B in oligodendrocyte progenitor cells correlates with differentiation toward the oligodendroglial lineage and modulates oligodendrocytes maturation. Mol Cell Neurosci 27:453–465.

Dendrou CA, Fugger L, Friese MA. 2015. Immunopathology of multiple sclerosis. Nat Rev Immunol [Internet] 15:545–558. Available from: http://www.nature.com/doifinder/10.1038/nri3871

Denic A, Johnson AJ, Bieber AJ, Warrington AE, Rodriguez M, Pirko I. 2011. The relevance of animal models in multiple sclerosis research. Pathophysiology 18:21–29.

Diaz-Olavarrieta C, Cummings JL, Velazquez J, Garcia de al Cadena C. 1999. Neuropsychiatric Manifestations of Multiple Sclerosis. J Neuropsychiatry Clin NeurosciThe J Neuropsychiatry Clin Neurosci 11:5151–57.

Didonna A, Oksenberg JR. 2015. Genetic determinants of risk and progression in multiple sclerosis. Clin Chim Acta 449:16–22.

DiSabaro J. D, Quan N, Godbout J. 2016. Neuroinflammation: The devil is in the details. J Neurochem 139:136–153.

Domingues HS, Portugal CC, Socodato R, Relvas JB. 2016. Oligodendrocyte, Astrocyte, and Microglia Crosstalk in Myelin Development, Damage, and Repair. Front Cell Dev Biol [Internet] 4. Available from: http://journal.frontiersin.org/Article/10.3389/fcell.2016.00071/abstract

Donato R, Cannon BR, Sorci G, Riuzzi F, Hsu K, Weber DJ, Geczy CL. 2013. Functions of S100 proteins. Curr Mol Med 13:24–57.

Donato R, Sorci G, Riuzzi F, Arcuri C, Bianchi R, Brozzi F, Tubaro C, Giambanco I. 2009. S100B's double life: Intracellular regulator and extracellular signal. Biochim Biophys Acta - Mol Cell Res 1793:1008–1022.

Doussau F, Dupont JL, Neel D, Schneider A, Poulain B, Bossu JL. 2017. Organotypic cultures of cerebellar slices as a model to investigate demyelinating disorders. Expert Opin Drug Discov 12:1011–1022.

Esposito G, Capoccia E, Sarnelli G, Scuderi C, Cirillo C, Cuomo R, Steardo L. 2012. The antiprotozoal drug pentamidine ameliorates experimentally induced acute colitis in mice. J Neuroinflammation [Internet] 9:774. Available from: http://jneuroinflammation.biomedcentral.com/articles/10.1186/1742-2094-9-277

Fernandes A, Miller-Fleming L, Pais TF. 2014. Microglia and inflammation: conspiracy, controversy or control? Cell Mol Life Sci 71:3969–3985.

Filippi M, Rocca MA, Ciccarelli O, De Stefano N, Evangelou N, Kappos L, Rovira A, Sastre-Garriga J, Tintor?? M, Frederiksen JL, Gasperini C, Palace J, Reich DS, Banwell B, Montalban X, Barkhof F. 2016. MRI criteria for the diagnosis of multiple sclerosis: MAGNIMS consensus guidelines. Lancet Neurol 15:292–303.

Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KHG. 2010. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. Clin Exp Immunol 162:1–11.

Fontenot JD, Gavin MA, Rudensky AY. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol [Internet] 4:330–336. Available from: http://www.nature.com/doifinder/10.1038/ni904

Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. Cell 87:1037–1047.

Friese MA, Fugger L. 2009. Pathogenic CD8 + T cells in multiple sclerosis. Ann Neurol 66:132–141.

Gallo P, Centonze D, Marrosu MG. 2017. Alemtuzumab for multiple sclerosis: the new concept of immunomodulation. Mult Scler Demyelinating Disord.

Gerlach R, Demel G, König HG, Gross U, Prehn JHM, Raabe A, Seifert V, Kögel D. 2006. Active secretion of S100B from astrocytes during metabolic stress. Neuroscience 141:1697–1701.

Goldmann T, Prinz M. 2013. Role of microglia in CNS autoimmunity. Clin Dev Immunol 2013.

Goverman J. 2009. Autoimmune T cell responses in the central nervous system. Nat Rev Immunol [Internet] 9:393–407. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19444307

Grau-López L, Raïch D, Ramo-Tello C, Naranjo-Gómez M, Dàvalos A, Pujol-Borrell R, Borràs FE, Martínez-Cáceres E. 2009. Myelin peptides in multiple sclerosis. Autoimmun Rev 8:650–653.

Groves A, Kihara Y, Chun J. 2013. Fingolimod: Direct CNS effects of sphingosine 1-phosphate (S1P) receptor modulation and implications in multiple sclerosis therapy. J Neurol Sci.

Hafler D. 2004. Multiple Sclerosis. J Clin Invest 113:788–794.

Hamby ME, Sofroniew M V. 2010. Reactive astrocytes as therapeutic targets for CNS disorders. Neurotherapeutics [Internet] 7:494–506. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2952540&tool=pmcentrez&rendertype=abstract

Hampe CS. 2012. B Cells in Autoimmune Diseases. Scientifica (Cairo) [Internet] 2012:1–18. Available from: http://www.hindawi.com/journals/scientifica/2012/215308/abs/

Hampton DW, Anderson J, Pryce G, Irvine KA, Giovannoni G, Fawcett JW, Compston A, Franklin RJM, Baker D, Chandran S. 2008. An experimental model of secondary progressive multiple sclerosis that shows regional variation in gliosis, remyelination, axonal and neuronal loss. J Neuroimmunol 201–202:200–211.

Handel AE, Williamson AJ, Disanto G, Dobson R, Giovannoni G, Ramagopalan S V. 2011. Smoking and multiple sclerosis: an updated meta-analysis. PLoS One 6:e16149.

Harris VK, Sadiq SA. 2014. Biomarkers of therapeutic response in multiple sclerosis: current status. Mol Diagn Ther [Internet] 18:605–617. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4245485/pdf/40291_2014_Article_117.pdf

Hauser SL, Bhan a K, Gilles F, Kemp M, Kerr C, Weiner HL. 1986. Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. Ann Neurol 19:578–587.

Haussleiter IS, Brüne M, Juckel G. 2009. Psychopathology in multiple sclerosis: Diagnosis, prevalence and treatment. Ther Adv Neurol Disord 2:13–29.

Herz J, Zipp F, Siffrin V. 2010. Neurodegeneration in autoimmune CNS inflammation. Exp Neurol 225:9–17.

Hirsch EC, Hunot S. 2009. Neuroinflammation in Parkinson's disease: a target for neuroprotection? Lancet Neurol 8:382–397.

Housley W, Pitt D, Hafler DA. 2015. Biomarkers in Multiple Sclerosis. Clin Immunol:51–58.

Howard L, Weiner M. 2009. The Challenge of Multiple Sclerosis: How Do We Cure A Chronic Heterogeneous Disease? Ann Neurol 65:239–248.

Howe CL, Adelson JD, Rodriguez M. 2007. Absence of perforin expression confers axonal protection despite demyelination. Neurobiol Dis 25:354–359.

Huttunen HJ, Kuja-Panula J, Sorci G, Agneletti AL, Donato R, Rauvala H. 2000. Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. J Biol Chem 275:40096–40105.

Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. 2009. Th1, Th17, and Th9 Effector Cells Induce Experimental Autoimmune Encephalomyelitis with Different Pathological Phenotypes. J Immunol [Internet] 183:7169–7177. Available from: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901906

Jeffery ND, Blakemore WF. 1995. Remyelination of mouse spinal cord axons demyelinated by local injection of lysolecithin. J Neurocytol 24:775–781.

Joller N, Peters A, Anderson AC, Kuchroo VK. 2012. Immune checkpoints in central nervous system autoimmunity. Immunol Rev 248:122–139.

Kettenmann H, Hanisch U-K, Noda M, Verkhratsky A. 2011. Physiology of Microglia. Physiol Rev [Internet] 91:461–553. Available from: http://physrev.physiology.org/cgi/doi/10.1152/physrev.00011.2010

Kleindienst A, Hesse F, Bullock MR, Buchfelder M. 2007. The neurotrophic protein S100B: value as a marker of brain damage and possible therapeutic implications. Prog Brain Res 161:317–325.

Kobelt G, Thompson A, Berg J, Gannedahl M, Eriksson J. 2017. New insights into the burden and costs of multiple sclerosis in Europe. Mult Scler 23:1123–1136.

Koriem KMM. 2016. Multiple sclerosis: New insights and trends. Asian Pac J Trop Biomed 6:429–440.

Lampron A, Larochelle A, Laflamme N, Préfontaine P, Plante M-M, Sánchez MG, Yong VW, Stys PK, Tremblay M-È, Rivest S. 2015. Inefficient clearance of myelin debris by microglia impairs remyelinating processes. J Exp Med.

Lassmann H, Brück W, Lucchinetti CF. 2007. The immunopathology of multiple sclerosis: An

overview. In: Brain Pathology. Vol. 17. . p 210–218.

Laura Feltri M, Suter U, Relvas JB. 2008. The function of rhogtpases in axon ensheathment and myelination. Glia 56:1508–1517.

Leung S, Liu X, Fang L, Chen X, Guo T, Zhang J. 2010. The cytokine milieu in the interplay of pathogenic Th1/Th17 cells and regulatory T cells in autoimmune disease. Cell Mol Immunol [Internet] 7:182–189. Available from: http://www.nature.com/doifinder/10.1038/cmi.2010.22

Li R, Rezk A, Ghadiri M, Luessi F, Zipp F, Li H, Giacomini PS, Antel J, Bar-Or A. 2017. Dimethyl Fumarate Treatment Mediates an Anti-Inflammatory Shift in B Cell Subsets of Patients with Multiple Sclerosis. J Immunol.

Liang X. 2004. Signaling from Integrins to Fyn to Rho Family GTPases Regulates Morphologic Differentiation of Oligodendrocytes. J Neurosci [Internet] 24:7140–7149. Available from: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.5319-03.2004

Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, Bennett ML, Münch AE, Chung WS, Peterson TC, Wilton DK, Frouin A, Napier BA, Panicker N, Kumar M, Buckwalter MS, Rowitch DH, Dawson VL, Dawson TM, Stevens B, Barres BA. 2017. Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541:481–487.

Link H, Huang YM. 2006. Oligoclonal bands in multiple sclerosis cerebrospinal fluid: An update on methodology and clinical usefulness. J Neuroimmunol 180:17–28.

Lu QR, Sun T, Zhu Z, Ma N, Garcia M, Stiles CD, Rowitch DH. 2002. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell 109:75–86.

Lublin FD. 2014. New multiple sclerosis phenotypic classification. Eur Neurol 72:1–5.

Lublin FD, Reingold SC, Cohen JA, Cutter GR, S??rensen PS, Thompson AJ, Wolinsky JS, Balcer LJ, Banwell B, Barkhof F, Bebo B, Calabresi PA, Clanet M, Comi G, Fox RJ, Freedman MS, Goodman AD, Inglese M, Kappos L, Kieseier BC, Lincoln JA, Lubetzki C, Miller AE, Montalban X, O'Connor PW, Petkau J, Pozzilli C, Rudick RA, Sormani MP, St??ve O, Waubant E, Polman CH. 2014. Defining the clinical course of multiple sclerosis: The 2013 revisions. Neurology 83:278–286.

Lucchinetti CF, Popescu BFG, Bunyan RF, Moll NM, Roemer SF, Lassmann H, Brück W, Parisi JE, Scheithauer BW, Giannini C, Weigand SD, Mandrekar J, Ransohoff RM. 2011. Inflammatory Cortical Demyelination in Early Multiple Sclerosis. N Engl J Med [Internet] 365:2188–2197. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa1100648

Macchi B, Marino-Merlo F, Nocentini U, Pisani V, Cuzzocrea S, Grelli S, Mastino A. 2015. Role of inflammation and apoptosis in multiple sclerosis: Comparative analysis between the periphery and the central nervous system. J Neuroimmunol 287:80–87.

Magliozzi R, Columba-Cabezas S, Serafini B, Aloisi F. 2004. Intracerebral expression of CXCL13 and BAFF is accompanied by formation of lymphoid follicle-like structures in the meninges of mice with relapsing experimental autoimmune encephalomyelitis. J Neuroimmunol:11–23.

Maire CL, Wegener A, Kerninon C, Oumesmar BN. 2010. Gain-of-function of olig transcription factors enhances oligodendrogenesis and myelination. Stem Cells 28:1611–1622.

Markowitz J, Chen I, Gitti R, Baldisseri DM, Pan Y, Udan R, Carrier F, MacKerell AD, Weber DJ. 2004. Identification and characterization of small molecule inhibitors of the calcium-dependent S100B-p53 tumor suppressor interaction. J Med Chem.

Matsushima GK, Morell P. 2001. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. Brain Pathol 11:107–116.

McKnight LE, Raman EP, Bezawada P, Kudrimoti S, Wilder PT, Hartman KG, Godoy-Ruiz R, Toth EA, Coop A, MacKerell AD, Weber DJ. 2012. Structure-based discovery of a novel pentamidine-related inhibitor of the calcium-binding protein S100B. ACS Med Chem Lett.

McRae BL, Kennedy MK, Tan LJ, Dal Canto MC, Picha KS, Miller SD. 1992. Induction of active and adoptive relapsing experimental autoimmune encephalomyelitis (EAE) using an encephalitogenic epitope of proteolipid protein. J Neuroimmunol 38:229–240.

Merrill JE, Scolding NJ. 1999. Mechanisms of damage to myelin and oligodendrocytes and their relevance to disease. Neuropathol Appl Neurobiol 25:435–458.

Michetti F, Corvino V, Geloso MC, Lattanzi W, Bernardini C, Serpero L, Gazzolo D. 2012. The S100B protein in biological fluids: More than a lifelong biomarker of brain distress. J Neurochem 120:644–659.

Michetti F, Massaro A, Murazio M. 1979. The nervous system-specific S-100 antigen in cerebrospinal fluid of multiple sclerosis patients. Neurosci Lett 11:171–175.

Miljković D, Timotijević G, Stojković MM. 2011. Astrocytes in the tempest of multiple sclerosis. FEBS Lett 585:3781–3788.

Miller DH, Khan OA, Sheremata WA, Blumhardt LD, Rice GPA, Libonati M ichele A, Willmer-Hulme AJ, Dalton CM, Miszkiel KA, O'Connor PW. 2003. A controlled trial of natalizumab for relapsing multiple sclerosis. N Engl J Med.

Miller DH, Leary SM. 2007. Primary-progressive multiple sclerosis. Lancet Neurol 6:903–912.

Miller RH, Fyffe-Maricich SL. 2010. Restoring the balance between disease and repair in multiple sclerosis: insights from mouse models. Dis Model Mech [Internet] 3:535–539. Available from: http://dmm.biologists.org/cgi/doi/10.1242/dmm.001958

Miller SD, Dal Canto MC, Kim BS, Melvold RW. 1996. Theiler's Murine Encephalomyelitis Virus (TMEV)-Induced Demyelination: A Model for Human Multiple Sclerosis. Methods 3:453–461.

Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, Van Wijngaarden P, Wagers AJ, Williams A, Franklin RJM, Ffrench-Constant C. 2013. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat Neurosci.

Miron VE, Franklin RJM. 2014. Macrophages and CNS remyelination. J Neurochem.

Mitew S, Hay CM, Peckham H, Xiao J, Koenning M, Emery B. 2014. Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. Neuroscience 276:29–47.

Mix E, Meyer-Rienecker H, Hartung HP, Zettl UK. 2010. Animal models of multiple sclerosis-Potentials and limitations. Prog Neurobiol 92:386–404.

Moransard M, Dann A, Staszewski O, Fontana A, Prinz M, Suter T. 2011. NG2 expressed by macrophages and oligodendrocyte precursor cells is dispensable in experimental autoimmune encephalomyelitis. Brain.

Naegele M, Martin R. 2014. Chapter 03 – The good and the bad of neuroinflammation in multiple sclerosis. In: Handbook of Clinical Neurology. Vol. 122. . p 59–87.

Neumann H, Kotter MR, Franklin RJM. 2009. Debris clearance by microglia: An essential link between degeneration and regeneration. Brain.

Nishiyama H, Knöpfel T, Endo S, Itohara S. 2002. Glial protein S100B modulates long-term neuronal synaptic plasticity. Proc Natl Acad Sci.

Nylander A, Hafler D a. 2012. Multiple sclerosis. J Clin Invest 122:1180–1188.

Obermeier B, Daneman R, Ransohoff RM. 2013. Development, maintenance and disruption of the blood-brain barrier. Nat Med [Internet] 19:1584–1596. Available from: http://www.nature.com/doifinder/10.1038/nm.3407

Oldstone MBA. 2005. Molecular mimicry, microbial infection, and autoimmune disease: evolution of the concept. Curr Top Microbiol Immunol [Internet] 296:1–17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16329189

Ostendorp T, Leclerc E, Galichet A, Koch M, Demling N, Weigle B, Heizmann CW, Kroneck PMH, Fritz G. 2007a. Structural and functional insights into RAGE activation by multimeric S100B. EMBO J [Internet] 26:3868–78. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1952220&tool=pmcentrez&rendertype=abstract

Ostendorp T, Leclerc E, Galichet A, Koch M, Demling N, Weigle B, Heizmann CW, Kroneck PMH, Fritz G. 2007b. Structural and functional insights into RAGE activation by multimeric S100B. EMBO J [Internet] 26:3868–3878. Available from: http://emboj.embopress.org/cgi/doi/10.1038/sj.emboj.7601805

Petersen MA, Dailey ME. 2004. Diverse Microglial Motility Behaviors during Clearance of Dead Cells in Hippocampal Slices. Glia 46:195–206.

Petzold a, Eikelenboom MJ, Gveric D, Keir G, Chapman M, Lazeron RHC, Cuzner ML, Polman CH, Uitdehaag BMJ, Thompson EJ, Giovannoni G. 2002. Markers for different glial cell responses in multiple sclerosis: clinical and pathological correlations. Brain 125:1462–1473.

Podbielska M, Banik N, Kurowska E, Hogan E. 2013. Myelin Recovery in Multiple Sclerosis: The Challenge of Remyelination. Brain Sci [Internet] 3:1282–1324. Available from: http://www.mdpi.com/2076-3425/3/3/1282/

Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, Fujihara K, Havrdova E, Hutchinson M, Kappos L, Lublin FD, Montalban X, O'Connor P, Sandberg-Wollheim M, Thompson AJ, Waubant E, Weinshenker B, Wolinsky JS. 2011. Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. Ann Neurol 69:292–302.

Popescu BFG, Lucchinetti CF. 2015. Neuropathology of multiple sclerosis. In: Multiple Sclerosis: A Mechanistic View. . p 181–200.

Prins M, Schul E, Geurts J, van der Valk P, Drukarch B, van Dam AM. 2015. Pathological differences between white and grey matter multiple sclerosis lesions. Ann N Y Acad Sci 1351:99–113.

Procaccini C, De Rosa V, Pucino V, Formisano L, Matarese G. 2015. Animal models of Multiple Sclerosis. Eur J Pharmacol 759:182–191.

Raffel J, Wakerley B, Nicholas R. 2016. Multiple Sclerosis. Medicine (Baltimore) 44:537–541.

Rangachari M, Kuchroo VK. 2013. Using EAE to better understand principles of immune function and autoimmune pathology. J Autoimmun 45:31–39.

Ransohoff RM. 2012. Animal models of multiple sclerosis: the good, the bad and the bottom line. Nat Neurosci 15:1074–1077.

Rothermundt M, Peters M, Prehn JHM, Arolt V. 2003. S100B in brain damage and neurodegeneration. Microsc Res Tech [Internet] 60:614–632. Available from: http://doi.wiley.com/10.1002/jemt.10303

De Sá J, Alcalde-Cabero E, Almazán-Isla J, Sempere A, De Pedro-Cuesta J. 2012. Capture-recapture as a potentially useful procedure for assessing prevalence of multiple sclerosis: Methodologic exercise using portuguese data. Neuroepidemiology 38:209–216.

Sakaguchi S, Miyara M, Costantino CM, Hafler DA. 2010. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol [Internet] 10:490–500. Available from: http://www.nature.com/doifinder/10.1038/nri2785

Saladin K. 2014. Anatomy & Physiology: The Unity of Form and Function. Anat Physiol Unity Form Funct [Internet]:1248. Available from: https://books.google.com/books?id=lmr8nQEACAAJ&pgis=1

Salter MW, Stevens B. 2017. Microglia emerge as central players in brain disease. Nat Med [Internet] 23:1018–1027. Available from: http://www.nature.com/doifinder/10.1038/nm.4397

Samoilova EB, Horton JL, Chen Y. 1998. Acceleration of experimental autoimmune encephalomyelitis in interleukin-10-deficient mice: Roles of interleukin-10 in disease progression and recovery. Cell Immunol.

Sands M, Kron MA, Brown RB. 1985. Pentamidine: a review. Rev Infect Dis [Internet] 7:625–634. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3903942

Santos G, Barateiro A, Gomes CM, Brites D, Fernandes A. 2018. Impaired oligodendrogenesis and myelination by elevated S100B levels during neurodevelopment. Neuropharmacology.

Savioz A, Leuba G, Vallet PG. 2015. A framework to understand the variations of PSD-95 expression in brain aging and in Alzheimer's disease. Ageing Res Rev.

Schmidt AM, Yan S Du, Yan SF, Stern DM. 2001. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. J Clin Invest 108:949–955.

Sobocki P, Pugliatti M, Lauer K, Kobelt G. 2007. Estimation of the cost of MS in Europe: Extrapolations from a multinational cost study. Mult Scler 13:1054–1064.

Sofroniew M V. 2009. Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci 32:638–647.

Sofroniew M V., Vinters H V. 2010. Astrocytes: Biology and pathology. Acta Neuropathol 119:7–35.

Teixeira SA, Varriano AA, Bolonheis SM, Muscará MN. 2005. Experimental autoimmune

encephalomyelitis: A heterogenous group of animal models to study human multiple sclerosis. Drug Discov Today Dis Model 2:127–134.

Vargas DL, Tyor WR. 2017. Update on disease-modifying therapies for multiple sclerosis. J Investig Med.

Villarreal A, Seoane R, Torres AG, Rosciszewski G, Angelo MF, Rossi A, Barkert PA, Ramos AJ. 2014. S100B protein activates a RAGE-dependent autocrine loop in astrocytes: Implications for its role in the propagation of reactive gliosis. J Neurochem 131:190–205.

Whitham R, Bourdette D, Hashim G, Herndon R, Ilg R, Vanderbark A, Offner H. 1991. Lymphocytes from SJL/J mice immunized with spinal cord respond selectively to a peptide of proteolipid protein and transfer relapsing demyelinating experimental autoimmune encephalomyelitis. J Immunol 1:101–107.

WHO. 2008. Atlas Multiple Sclerosis resources In The World 2008. WHO Press:56.

Wootla B, Eriguchi M, Rodriguez M. 2012. Is multiple sclerosis an autoimmune disease? Autoimmune Dis 1.

Xanthos DN, Sandkühler J. 2013. Neurogenic neuroinflammation: inflammatory CNS reactions in response to neuronal activity. Nat Rev Neurosci [Internet] 15:43–53. Available from: http://www.nature.com/doifinder/10.1038/nrn3617

Xie J, Reverdatto S, Frolov A, Hoffmann R, Burz DS, Shekhtman A. 2008. Structural basis for pattern recognition by the receptor for advanced glycation end products (RAGE). J Biol Chem [Internet] 283:27255–69. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18667420

Yanaba K, Bouaziz JD, Matsushita T, Magro CM, St.Clair EW, Tedder TF. 2008. B-lymphocyte contributions to human autoimmune disease. Immunol Rev 223:284–299.

Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L, Karin N. 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. Nature.

Zhu B, Luo L, Moore GRW, Paty DW, Cynader MS. 2003. Dendritic and synaptic pathology in experimental autoimmune encephalomyelitis. Am J Pathol.

Zhu J, Yamane H, Paul WE. 2010. Differentiation of effector CD4 T cell populations. Annu Rev Immunol [Internet] 28:445–489. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3502616&tool=pmcentrez&rendertype=abstract