Escola Superior
de Tecnologia
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Politécnico de Coimbra

Beatriz Antunes Rodrigues

PRECLINICAL EVALUATION OF THE NUTRACEUTICAL POTENTIAL OF BLUEBERRY-BASED ANTIOXIDANT FIBER IN EXPERIMENTAL MULTIPLE SCLEROSIS

Dissertação no âmbito do Mestrado em Farmácia - Especialização em Farmacoterapia Aplicada orientada pela Professora Doutora Sofia Andreia Domingues Viana e coorientada pelo Doutor Flávio Nelson Fernandes Reis e pela Mestre Ana Carolina Marques Ferreira e apresentada à Escola Superior de Tecnologia da Saúde do Politécnico de Coimbra

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Abreviations

5-HT – Serotonin

Aβ - Amyloid-beta

BB – Blueberry Leaves Biomass

BBB – Blood-Brain Barrier

BDNF – Brain-Derived Neurotrophic Factor

BMI – Body Mass Index

CCR9 - C-C Chemokine Receptor Type 9

CGA - Chlorogenic Acid

CIS – Clinical Isolated Syndrome

CNS – Central Nervous System

COX - Cyclooxygenase

CPZ – Cuprizone

CSF – Cerebrospinal Fluid

CYP – Cytochrome P

DIS – Dissemination in Space

DIT – Dissemination in Time

DMTs – Disease-Modifying Treatments

EAE – Experimental Autoimmune Encephalomyelitis

EBNA1 – Epstein-Barr Nuclear Antigen 1

EBV – Epstein-Barr virus

EDSS – Expanded Disability Status Scale ´

EMA – European Medicines Agency

FDA – Food Drug Administration

FoxP3 – Forkhead Box P3

GABA – Gamma-Aminobutyric Acid

GBA – Gut-Brain-Axis

GI – Gastrointestinal

GIS – Gut Immune System

GM – Gut Microbiota

GWAS – Genome-Wide Association Studies

HFD – High-fat diet

HLA – Human Leukocyte antigen

IFN-β – Interferon beta

IFN-γ – Interferon gamma

IL– Interleukin

IM – Infectious Mononucleosis

LDL – Low-Density Lipoproteins

MHC – Major Histocompatibility Complex

MRI – Magnetic Resonance Imaging

MS – Multiple Sclerosis

NF-κB – Nuclear Factor Kappa B

NO – Nitric Oxide

NOS – Nitric Oxide Species

OLG – Oligodendrocyte

OPC – Oligodendrocyte Progenitor Cell

PACs – Proanthocyanidins

PCs – Polyphenolic Compounds

PPMS – Primary-Progressive Multiple Sclerosis

PPP – Purchasing Power Parity

PRMS – Progressive-Relapsing Multiple Sclerosis

PUFAs – Polyunsaturated Fatty Acids

RAS – Renin-Angiotensin System

RIS – Radiographically Isolated Syndrome

ROS - Reactive Oxygen Species

RRMS – Relapsing-Remitting Multiple Sclerosis

S1P – Sphingosine 1-Phospase

SCFAs – Short-Chain Fatty Acids

SNPs – Single-Nucleotide Polymorphisms

SPF – Specific Pathogen-Free

SPMS – Secondary-Progressive Multiple Sclerosis

TAC – Total Antioxidant Capacity

T2DM – Type 2 Diabetes Mellitus

Th1 - Type 1 T-Helper

Th17 - Type 17 T-Helper

Tm – Memory T

TNF- α – Tumor Necrosis Factor alpha

TNFR1- Tumor Necrosis Factor Receptor 1

TNFR2 - Tumor Necrosis Factor Receptor 2

TPC – Total Phenolic Contents

Treg – T Regulatory

UVR – Ultraviolet Radiation

Ω – Omega

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Resumo

A Esclerose Múltipla (EM) é uma doença autoimune do Sistema Nervoso Central (SNC), que se manifesta através do cérebro e da medula espinal, desencadeando perturbações motoras e não motoras na fase prodrómica, inclusive gastrointestinais. A hiper-reactivade das células T relativamente aos auto-peptídeos da mielina ocorre após a ativação das mesmas nos tecidos periféricos, nomeadamente a nível intestinal.

Os linfócitos T e B periféricos originam um padrão inflamatório robusto no SNC e, consequentemente, mecanismos de autorreparação, proliferação e diferenciação das células progenitoras dos oligodendrócitos, tornando-se ineficazes para ultrapassar a desmielinização. Cerca de 70% dos fatores de risco da doença possuem origem não genética: dieta e a disbiose da microbiota intestinal, reconhecida como um dos principais fatores de risco ambiental, para a suscetibilidade e progressão da doença.

Os polifenóis, devido às suas propriedades antioxidantes, anti-inflamatórias e pré-bióticas, apresentam efeitos promotores de saúde. Os dados clínicos demonstram os efeitos positivos das intervenções dietéticas no controlo da sintomatologia e da recidiva da EM. Por exemplo, as dietas à base de plantas com um teor enriquecido em polifenóis e/ou pré-bióticos/probióticos atuam em compartimentos do eixo intestino-cérebro, mantendo uma microbiota simbiótica saudável, aumentando a tolerância à autoimunidade, reduzindo o stress oxidativo, a inflamação e, por último, melhorando o bem-estar do doente. A reversão da disbiose da microbiota através de antioxidantes e de pré-bióticos é uma estratégia terapêutica emergente para o controlo da EM. Em particular, os constituintes das plantas do mirtilo demonstram reduzir a severidade da doença em diferentes contextos patológicos e exercer efeitos pleiotrópicos benéficos. O objetivo desta dissertação centra-se na avaliação do potencial nutracêutico de uma intervenção com fibras antioxidantes à base de mirtilo, denominada biomassa BB, nas perturbações do eixo intestino-cérebro observadas num modelo experimental de EM: murganhos adultos C57BL/6 induzido por cuprizona. BB foi administrada diariamente por via oral (500 mg / kg) da semana 2.5 à 5 (CpzW5 + BB) ou à 7 (CpzW7 + BB). Estas experiências receberam aprovação (# 12/2018) do Órgão de Bem-Estar Animal (ORBEA) local (iCBR-FMUC).
Relativamente à inflamação do cólon, a BB não pareceu proteger contra a perda de células caliciformes, nem prevenir a hiperplasia (coloração de Hematoxilina e Eosina), mas reduziu

significativamente a inflamação intestinal nas fases de desmielinização e remielinização. Nas caraterísticas fisiopatológicas da EM, observou-se que a biomassa não protege contra a desmielinização (coloração de Kluver Barrera), demonstrando não ser benéfica num episódio de surto. Contudo, na fase de remielinização, parece melhorar a recuperação, tendência esta corroborada por técnicas experimentais complementares. Além disso, observou-se ainda que a biomassa promove substancialmente a reatividade da microglia, refletida em termos de aumento do número e comprimento dos processos, e do número de extremidades (microscopia confocal). Enquanto na fase de desmielinização não foram observadas diferenças. Este padrão manteve-se consistente em todas as regiões cerebrais analisadas.

Em resumo, este estudo realça o potencial nutracêutico da BB na modulação do eixo intestino-cérebro, com o objetivo de aumentar a eficiência da remielinização. Serão necessários mais estudos para aprofundar estas hipóteses e para melhor elucidar os mecanismos subjacentes aos efeitos observados.

Palavras-chave: Folhas de Mirtilo; Esclerose Múltipla; Microbiota Intestinal; Remielinização; Células da Microglia

Abstract

Multiple Sclerosis (MS) is a central nervous system (CNS) autoimmune disease that manifests progressively through the brain and spinal cord converging into motor/psychiatric disabilities and extra non-motor symptoms such as gastrointestinal disturbances as soon as the prodromal phase. Notably, aberrant T-cells hyperreactivity to myelin self-peptides occurs upon T cells activation in peripheric tissues, namely the gut. Peripheric T and B cells as well as other resident immune cells give rise to a robust inflammatory/oxidative pattern in the CNS and self-repair mechanisms - oligodendrocyte progenitor cells proliferation and differentiation - become ineffective to outstep demyelination. Seventy percent of disease's risk factors stem from nongenetic components such as the diet, and gut microbiota (GM) dysbiosis has been recently acknowledged as a chief environmental risk factor to both susceptibility and progression of MS.

Polyphenols (PCs) display widely acknowledged health-promoting effects that arise from their antioxidant, anti-inflammatory and prebiotic properties. Available clinical evidence show that targeted dietary interventions exert positive effects on controlling MS symptomatology and relapse. For instance, plant-based diets with an enriched PCs content and/or prebiotics/probiotics can act on multiple gut-brain axis' compartments, allowing the maintenance of a healthy symbiotic GM, increasing autoimmunity tolerance while refraining oxidative stress and inflammation, ultimately improving patient wellness. Reversal of GM dysbiosis through antioxidants and prebiotics is therefore an emerging therapeutic strategy for MS management. In particular, components of blueberry (BB) plants have been showing to exert beneficial pleiotropic effects and to reduce disease severity in different pathological contexts, including MS. Thus, the aim of this thesis relies on assessing the nutraceutical potential of a blueberry-based antioxidant fiber intervention, called BB biomass, on gut-brain axis perturbances observed in experimental MS. To this end, adult C57BL/6 mice were assigned to the cuprizone-induced model of MS. BB biomass was orally administered (500 mg/kg) daily from week 2.5 to 5 (CpzW5+BB) or 7 (CpzW7+BB). Animal experiments received approval (# 12/2018) by the local (iCBR-FMUC) Animal Welfare Body (ORBEA). Regarding colonic inflammation, BB did not seem to protect against the loss of Goblet cells nor prevent hyperplasia (Hematoxylin and Eosin staining), but significantly reduced intestinal inflammation in both demyelination and

remyelination phases. Regarding the pathophysiological hallmarks of MS in the CNS, it was observed that BB biomass does not protect against demyelination (Kluver Barrera staining), so it's not beneficial in the episode of outbreak. However, in the remyelination phase, BB seems to enhance recovery, a trend that has been corroborated by complementary experimental techniques. Moreover, BB was found to significantly promote microglia reactivity as reflected in terms of increased branch number and length and number of endpoints (Confocal microscopy). Conversely, no differences were observed in the demyelination phase. This pattern remained consistent across all analyzed cerebral regions.

In summary, this study highlights the nutraceutical potential of BB biomass in modulating the gut-brain axis affected by MS, with the aim of enhancing remyelination efficiency. Further research will be required to explore these hypotheses in greater depth and to better elucidate the mechanisms underlying the observed effects.

Key words: Blueberry Leafs; Multiple Sclerosis; Gut Microbiota; Remyelination; Microglia Cells

Chapter I | INTRODUCTION

1.1. Multiple Sclerosis: An Overview

Multiple Sclerosis (MS) is a chronic inflammatory and heterogeneous disease. It's an acquired neurodegenerative immuno-mediated disorder of the Central Nervous System (CNS), characterized by demyelination, neuronal loss, gliosis and primary/secondary axonal degeneration, that occurs from the very early stages of the disease. Myelinated axons are the principal target of MS attacks, which can cause varying degrees if damage both myelin and axons. Briefly, it's a neurological condition that is prejudicial for the brain and spinal cord, which control all body functions, causing a diverse array of symptoms and signs due to differential involvement of motor, sensory, visual and autonomic systems (Dighriri et al., 2023; Doshi & Chataway, 2016; Haki et al., 2024; Vidal-Jordana & Montalban, 2017).

Although significant progressive advances in preventive and supportive treatments have been made in recent years, MS is considered the leading cause of nontraumatic neurological impairment among young adults, with long-term implications in their professional and personal lives, having a huge impact functionally, financially and on quality of life (Ascherio & Munger, 2016; Dighriri et al., 2023; Dobson & Giovannoni, 2019; Hassan-Smith & Douglas, 2011; Oh et al., 2018; Pugliatti et al., 2006; Thompson et al., 2018; Yamout & Alroughani, 2018).

The discovery of MS origin continues to be a challenge, but with the ongoing studies, a multifactorial theory is accepted. The current understanding of the natural history of the disease, its immunopathogenesis points and the most recent investigations infer that it derives from a hypothetic fusion of hereditary predisposition and a potential non-genetic trigger such as environmental agents including Epstein-Barr virus (EBV), vitamin D or ultraviolet light exposure, obesity/increased Body Mass Index (BMI) during adolescence, and cigarette smoking, that results in a sustaining autoimmune disease with constant immunological attacks to the CNS (Dighriri et al., 2023; Filippi et al., 2018; Haki et al., 2024; Pugliatti et al., 2006; Thompson et al., 2018; Vidal-Jordana & Montalban, 2017; Yamout & Alroughani, 2018).

1.1.1. Epidemiology

The prevalence of MS is rising all over the world since 1950s, with several factors likely contributing to this increase, including a better access to neurologists and general healthcare, prolonged survival due to medical advances, new therapies and better counting methods nationally. Over the past decades, diagnostic criteria and methods have suffered changes as new evidence and expert guidelines arise. This improvement has been allowing an increasingly early, accurate and quick diagnostic (Dighriri et al., 2023; Dobson & Giovannoni, 2019; Filippi et al., 2018; Thompson et al., 2018; Yamout & Alroughani, 2018).

The disease is associated with a high societal and economic burden: around 85.4 billion dollars in the United States in 2019 (Bebo et al., 2022; Filippi et al., 2018). While in Europe, a cross-sectional study in 16 countries demonstrated the mean costs: 22,800€ PPP in mild, 37,100€ PPP in moderate and 57,500€ PPP in severe disease (Kobelt et al., 2017). The costs are higher than those for stroke and Alzheimer's disease because of its extensive duration, the subsequent loss of productivity, the need for assistance in activities of daily living and the cost of immunomodulatory treatments (Pugliatti et al., 2006).

According to the latest atlas published by the Multiple Sclerosis International Federation, over 2 million people worldwide, that equates to 1 in 3000 people in the world, are living with this disorder (Dighriri et al., 2023; Filippi et al., 2018; Yamout & Alroughani, 2018).

In terms of geographic location, the prevalence of MS varies greatly, having the highest rates in North America and Europe (140 and 108 cases per 100.000 respectively), and is also common in Canada, New Zealand, and Southern Australia. While in East Asia and Sub-Saharan Africa, the disease is less prevalent (2.2 and 2.1 cases per 100.000) (Ascherio & Munger, 2016; Doshi & Chataway, 2016; Vidal-Jordana & Montalban, 2017; Yamout & Alroughani, 2018). Therefore, on the one hand those of Northern European descent and Caucasians have a higher risk of developing the disease, with a decrease in prevalence as one moves away from the equator (Haki et al., 2024). On the other hand, this neurodegenerative disease is rare in Asian, black, Native Americans and Māori individuals. Prevalence estimates range from 2 per 100 000 individuals in Asia to ~1 per 1000 individuals in Western countries, although a prevalence of 1 per 400 individuals has been reported in some countries with a high latitude (Filippi et al., 2018). The geographical differences are

predominantly a marker of ethnic origin and cultural habits (wearing protective clothing/duration of sun exposure) underlining the importance of the population genetic and lifestyle makeup(Ascherio & Munger, 2016; Dobson & Giovannoni, 2019; Hassan-Smith & Douglas, 2011; Yamout & Alroughani, 2018).

As described above, the disease is more common in northern Europe, however, there are exceptions, because this effect varies according to where the individuals live in early life. Migration studies indicate that populations who relocate from low-risk to high-risk locals during infancy present a higher chance of developing MS (Dighriri et al., 2023). Minorities in the United States of America, such as Hispanic Americans and black Americans, experience faster disease progression than White Americans (Thompson et al., 2018).

Although the average age of onset ranges between 20 and 40 years, the disease can manifest at any age. Almost 10% of cases are diagnosed before the age of 18 and in some rare cases, MS is diagnosed after 50, which are known as “late-onset multiple sclerosis” (LOMS) (Dighriri et al., 2023). The age of MS onset follows a similar pattern across different regions, with low incidence in childhood, rapidly increasing after adolescence, reaching a peak between 25 and 35 years, and then slowly declining (Ascherio & Munger, 2016; Yamout & Alroughani, 2018). In most representative series, the distribution of patients by age at onset of the disease is essentially well-shaped, with onset before the age of 20 years in around 10%, from ages 20 to 40 years in 70%, and after the age of 40 years in 20% of the cases (Confavreux & Vukusic, 2008). The highest prevalence rates have been estimated for the age group 35 – 64 years old for both sexes and for all countries (Pugliatti et al., 2006).

In the early 1900s, the MS sex ratio was about equal, but since then has progressively increased to 3:1, being more prevalent among women (Dighriri et al., 2023; Haki et al., 2024). Currently, women are two to three times more affected than men, with a trend toward higher values in the most recent studies and this difference seems to be increasing in some areas of the world (Ascherio & Munger, 2016; Confavreux & Vukusic, 2008; Oh et al., 2018; Thompson et al., 2018; Yamout & Alroughani, 2018).

This increased female preponderance of MS can be linked to a possible role of environmental risk/ lifestyle factors that mainly affect women, for example occupation, increased cigarette smoking, obesity, birth control and childbirth (Filippi et al., 2018). Moreover, women not only are generally more susceptible to autoimmune conditions, that

may be hormonally related, as also are more inclined to seek medical assistance for subtle symptoms and a benign course is more common in women, which is diagnosed more accurately (Hassan-Smith & Douglas, 2011; Oh et al., 2018; Yamout & Alroughani, 2018).

A longitudinal study in Canada over the past 50 years suggests that different smoking habits could explain the increase MS frequency in females (Hassan-Smith & Douglas, 2011; Oh et al., 2018; Yamout & Alroughani, 2018). This practice enhances MS risk by approximately 50% and can explain up to 40% of the raised incidence of MS in women, through the hypothesis that the organic solvents and smoked tobacco cause post-translational modifications via antigen presentation in the lungs (Dobson & Giovannoni, 2019).

Compared with the general population, MS patients have a higher mortality rate and a lifetime expectancy approximately 10 years shorter, especially with comorbidities (psychiatric disorders, cerebrovascular and cardiovascular disease, diabetes or cancer) (Oh et al., 2018). Still, 70-88% of patients are alive 25 years after clinical onset, and the median time from onset to death ranges from 24 years to > 45 years. In recent decades, MS patients in the developed countries have experienced increased life expectancy and decreased mortality, resulting from better prognosis, and progressive advances in the therapeutic area. At the same time, however, the incidence of MS has increased over the years, at least in women, which means a constant growing number of patients who need healthcare to delay disease progression (Howard et al., 2016).

1.1.2. Risk Factors

The acquisition of new and expanding knowledge about risk factors is of the utmost importance in guiding the exploration of pathological and biological pathways, disease prevention with complementary therapeutic strategies and, potentially, lifestyle recommendations (Nourbakhsh & Mowry, 2019; Waubant et al., 2019).

There is a strong genetic component to the disease, but accounts for only a portion of the increased risk of MS. The majority of risk factors are actually non-genetic, where lifestyle and environmental factors are significant contributors. Established MS-associated risk factors include high latitude, female sex, smoking, low vitamin D levels, EBV infection and BMI (Alfredsson & Olsson, 2019; Olsson et al., 2017; Tarlinton et al., 2019). The

aforementioned factors may act years before the onset of clinical symptoms, which is referred to as the subclinical phase of the disease. This is supported by the fact that many individuals at the clinical onset already have several lesions that are detected through magnetic resonance imaging (MRI) (Alfredsson & Olsson, 2019; Olsson et al., 2017).

On the other hand, the chronic dysregulation of immune homeostasis of MS is caused by a combination of genetic predispositions, pro-inflammatory factors and infectious exposures (Waubant et al., 2019). Regarding the genetic aspects, the human leukocyte antigen (HLA) class II and I genes are relevant modifiers of disease risk. The HLADRB1*15:01 variant is the primary genetic risk factor for MS since the binding to this allele may allow optimal connection and presentation of CNS-related autoantigens, which drives T cells to attack the CNS. It appears that the proliferation of type 1 T helper (Th1) lymphocytes is elevated in HLA-DR15-positive individuals, and this process is mediated by memory B cells, whereas the HLA*02 variant is associated with a protective effect (Alfredsson & Olsson, 2019; Olsson et al., 2017; Tarlinton et al., 2019; Waubant et al., 2019). Genetic platforms, such as Genome-wide association studies (GWAS), have led to the identification of over 200 non-HLA single-nucleotide polymorphisms (SNPs) and many of these are located near genes involved in adaptive or innate immunity (Nourbakhsh & Mowry, 2019; Olsson et al., 2017; Tarlinton et al., 2019).

The heritable nature of MS has been well characterized: monozygotic twins have a significantly higher clinical concordance rate (25%–30%) versus dizygotic twins (3%–7%). Family history of MS is reported in 15%–20% of MS cases, a much higher prevalence than the general population, whereas the lifetime risk of MS in first-degree relatives of MS subjects is estimated at 3%, 10–30 times larger than the age-adjusted risk in the general population (Patsopoulos & De Jager, 2020).

1.1.2.1. Sun Exposure and Vitamin D

The hypothesis that lower exposure to ultraviolet radiation (UVR) and subsequently lower blood concentration of 25-hydroxyvitamin D - vitamin D status -increases the risk of MS was proposed based on the observation that disease prevalence increases with greater distance from the equator (Waubant et al., 2019). Indeed, several studies have demonstrated an inverse correlation between vitamin D and MS risk, suggesting that higher

vitamin D levels may be associated with a reduced risk (Alfredsson & Olsson, 2019). Along with its metabolites, this vitamin modifies the phenotype and function of various immune cells via interaction with the vitamin D receptor, which plays roles in lymphocyte activation and proliferation, T-helper cell differentiation, exerting regulatory effects on the immune response. Moreover, vitamin D suppresses the production of pro-inflammatory cytokines by Th1, thereby promoting the innate immune system response and subduing adaptive immune activity (Nourbakhsh & Mowry, 2019; Tarlinton et al., 2019).

Genetic evidence has also supported the role of vitamin D in MS, where polymorphisms in the vicinity of the vitamin D metabolism enzyme gene cytochrome P27B1 (CYP27B1) have been linked to a higher risk of developing MS (Nourbakhsh & Mowry, 2019). Moreover, two case-control studies have demonstrated that genes regulating vitamin D levels have a notable effect on disease risk. Moreover, *in vitro* studies have proposed vitamin D as the first example of a gene-environment interaction involving the strongest genetic risk factor, HLA DRB1*15:01 (Handunnetthi et al., 2010).

Additionally, sex-specific differences have been observed, with women exhibiting a lower level of vitamin D compared to men. In pregnant women, studies have indicated an association between maternal sunlight exposure and the vitamin D status of the mother during pregnancy, as well as a potential link between maternal vitamin D deficiency and the vitamin D status of the fetus (Nourbakhsh & Mowry, 2019; Tarlinton et al., 2019).

1.1.2.2. Epstein-Barr virus

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus. The presence of antibodies indicating prior exposure to this virus, or EBV seropositivity, has been consistently demonstrated to be associated with both adult and pediatric onset MS (Soldan & Lieberman, 2023). Indeed, almost all adult patients with MS display serological evidence of prior EBV infection, namely elevated titers of antibodies against Epstein-Barr nuclear antigen 1 (EBNA1). While infection with EBV in childhood is typically asymptomatic, infection with this virus later in life can result in clinical infectious mononucleosis (IM) (Nourbakhsh & Mowry, 2019; Soldan et al., 2023) The epidemiological evidence for the involvement of EBV in MS is based on studies demonstrating that the risk is 2 to 3-fold higher in individuals who have suffered from IM (Alfredsson & Olsson, 2019; Tarlinton et

al., 2019). Furthermore, antibodies and T cells that respond to proteins of the lytic phase of EBV, which indicate a recent infection or reactivation of the virus, are also associated with MS (Tarlinton et al., 2019). Evidence demonstrated an additive interaction also exists between EBNA1 titer and HLA antigen genetic variants. In particular, the presence of a higher EBNA1 titer or a history of IM represents a more significant risk factor in individuals who carry HLA antigen MS risk alleles. It has been demonstrated that IM and increased EBNA1 antibody titers interact with HLA MS risk genetic variants, and that IM interacts with HLA DRB1*15:01, acting in a synergistic manner (Nourbakhsh & Mowry, 2019; Olsson et al., 2017).

1.1.2.3. Obesity and Body Mass Index

Several high-quality observational studies have reported that obesity in adolescence and early adulthood is associated with approximately double the risk of developing pediatric and adult MS (Nourbakhsh & Mowry, 2019; Olsson et al., 2017; Waubant et al., 2019). The prevalence of MS is even higher in the developed countries, where people have access to high-calorie foods and adopt increasingly sedentary lifestyles. A large study that followed approximately a quarter of a million nurses demonstrated that having a BMI greater than 30 was associated with a more than double the MS risk (Munger et al., 2009).

There is evidence that obesity is associated with the stimulation of Th1-promoting immune responses, which involve the production of cytokines and a decreased function of T regulatory (Treg) cells. This results from an elevation in leptin levels, that enhance phagocytosis and cytokine secretion in macrophages, promoting $CD4^+$ T cell proliferation and survival, as well as favoring Th1 and type 17 T helper (Th17) cells activity while inhibiting Treg-mediated responses (Jörg et al., 2016).

Mendelian randomization studies show that BMI interacts with HLA genetic variants: individuals with a high BMI who carry DRB1*15:01 and do not have the protective HLA-A*02 have a ~14-fold increased risk of developing MS (Hedström et al., 2014). The largest genome-wide association study, which employed 336.603 MS cases, corroborated the previously identified BMI-gene interactions and revealed 70 different SNPs associated with BMI, which collectively lead to an increased odds ratio of developing MS (Mokry et al., 2016).

1.1.2.4. Smoking and Oral Tobbaco

The MS risk associated to tobacco exposure appears to be a dose-response relationship, with a higher cumulative dose associated with a greater likelihood of developing the disease (Alfredsson & Olsson, 2019; Jakimovski et al., 2019; Nourbakhsh & Mowry, 2019; Olsson et al., 2017; Waubant et al., 2019). Passive smoking is also considered a risk factor, suggesting that the increased disease risk resulting from exposure to tobacco smoke is due to non-specific lung irritation and inflammation (Alfredsson & Olsson, 2019; Nourbakhsh & Mowry, 2019; Olsson et al., 2017; Waubant et al., 2019). Both active and passive smoking have been associated with a faster progression of MS and an earlier transition to the progressive phenotype (Jakimovski et al., 2019). A study found that a pack of cigarettes per day would result in an increase of 27% in the number of relapses, and patients who ceased smoking demonstrated a reduced risk of Expanded Disability Status Scale (EDSS) progression in comparison to those who continued to smoke (Petersen et al., 2018). In people under the age of 26, levels of cotinine, a nicotine metabolite found in the blood, were significantly associated with an increased risk of MS (Alfredsson & Olsson, 2019; Jakimovski et al., 2019; Nourbakhsh & Mowry, 2019; Olsson et al., 2017). Furthermore, children who grew up in an environment associated with parental smoking had more than double the risk of a first episode of MS. Furthermore, the specific HLA-DBR1*15:01 alleles confer a higher probability of developing MS, about 14 times, when exposed to passive smoking (Jakimovski et al., 2019; Nourbakhsh & Mowry, 2019).

It is becoming increasingly evident that the lungs and their microenvironment may play a crucial role in the pathogenesis of MS. A recent EAE study has demonstrated that during the initial stage of the disease, T cells are not fully equipped to migrate across the blood-brain barrier (BBB) and trigger a CNS inflammatory response, having to undergo significant gene expression alterations in alveolar spaces, for an upregulation of migratory properties (Nourbakhsh & Mowry, 2019). The findings of a related study, where EAE mice were exposed to the condensate of cigarette smoke for 14 days suggest that this exposure prior to disease onset exacerbates early symptoms, largely due to exacerbated neuroinflammatory processes such as microglial activation, proliferation, and macrophage infiltration (Jakimovski et al., 2019). A subsequent investigation identified acrolein, a highly

cytotoxic compound, as a major mediator of the described harmful effects. Acrolein is a byproduct of oxidative stress and a known component of cigarette smoke, capable of inflicting damage on cellular membranes, proteins, and DNA. The partial benefits seen with its pharmacological inhibition suggest that neutralizing the compound's can help mitigate the progression of EAE (Jakimovski et al., 2019).

Finally, smoking status has been associated with worse outcomes both cross-sectionally and longitudinally, as observed through MRI studies. In particular, the comparison between "ever-smokers" and non-smokers in MS patients reveals that smoking is linked to an increase of the number of gadolinium-enhancing lesions and to a greater tissue damage, both in inflammatory (T2 lesions) and degenerative (T1 lesions) aspects of MS (Healy et al., 2009; Jakimovski et al., 2019; Zivadinov et al., 2009).

1.1.2.5. Nutrition and Lifestyle

Diet is a major source of environmental interaction, and dietary metabolites exert broad systemic effects, making diet an attractive candidate as a potential environmental mediator in MS (Katz Sand, 2018).

In vitro experiments have shown that high-salt conditions activate serum glucocorticoid-regulated kinase 1, promoting T-cell differentiation into pathogenic Th17 cells, promoting a more severe course of EAE (Katz Sand, 2018; Nourbakhsh & Mowry, 2019; C. Wu et al., 2013). Nevertheless, this study is inimitable to humans, because the used amount of salt would correspond to a human intake of over 500 grams per day. Nevertheless, in a small study involving the Argentinian population, individuals with MS who had a high salt intake had markedly more relapses and MRI-evidenced disease activity than those with a low salt consumption (Nourbakhsh & Mowry, 2019; Olsson et al., 2017).

Epidemiological studies have also demonstrated an increased incidence of MS in populations with high intake of saturated fats, that directly impact the innate and adaptive immune system through activation of pro-inflammatory toll-like receptors, leading to downstream consequences including increased nuclear factor kappa B (NF-κB). In an animal model of MS, mice fed a Western high fat diet (HFD) exhibited worsened clinical scores in association with increased T cell and macrophage infiltration, and spinal cord expression of proinflammatory cytokines IL-1β, IL-6, and IFN-γ. In contrast, short-chain fatty acids (SCFAs)

have been demonstrated to favor differentiation of regulatory T cells with resulting production of anti-inflammatory cytokines (Jakimovski et al., 2019; Katz Sand, 2018).

A review of food consumption data also found that vegetables, nuts, and fish - rich in Ω-6 and Ω-3 polyunsaturated fatty acids (PUFAs) were associated with a reduced risk of MS (Jakimovski et al., 2019). In 1950, Swank et al. observed geographic variations in MS incidence that could be related to intake of fats, particularly fats derived from animal products such as meat and dairy. The same population was later followed for an additional 34 years and the research showed that the group with the lowest consumption of dietary saturated fat (<= 20g/day) had a mortality rate three times lower than those with dietary fat intake ranging between 24 and 42 g/day (Jakimovski et al., 2019; Katz Sand, 2018). Accordingly, EAE mice fed a high-fat diet demonstrated increased gene expression of the brain components from the renin-angiotensin system (RAS), a finding that coincided with increased vascular endothelial permeability, recruitment of inflammatory cells, and upregulation of adhesion molecules (Timmermans et al., 2014). The high-fat diet has also been implicated in increasing brain inflammation, decreasing protective neurotrophic factors, and lowering the occurrence of neural plasticity, learning and regeneration (Katz Sand, 2018).

1.1.2.6. Caffeine and Alcohol Consumption

Studies on the role of alcohol and coffee consumption on MS have been inconsistent (Alfredsson & Olsson, 2019). In two cohort studies of female nurses, the investigators did not find any impact of caffeine and alcohol on MS risk (Massa et al., 2013). Contrastingly, in animal models, caffeine decreases the risk of developing neuroinflammation, demonstrating neuroprotective and anti-inflammatory properties (Jakimovski et al., 2019). According to an experimental study in EAE rat model, caffeine's protective effect appears to be due to an improvement in the integrity of the BBB, possibly via the caffeine-induced activation of adenosine 1A receptors (Jakimovski et al., 2019; Ruggiero et al., 2022). A cross-sectional survey in humans showed that coffee consumption, at least in the relapsing form of MS, has positive effects on the progression and disease course (D'Hooghe M et al., 2012). Despite all these observations, few works about caffeine supplementation as a therapeutic modality for MS are available in the literature, and the cellular mechanism involved in the

protective responses of this molecule have not yet been investigated (Ruggiero et al., 2022). As a result, the currently available data are insufficient to support any recommendations regarding coffee consumption in the context of MS (Olsson et al., 2017).

On another hand, epidemiological studies have shown that MS is inversely related to alcohol consumption, depending on the dose (Alfredsson & Olsson, 2019). One of the studies reported that Danish women with low alcohol consumption had a 44% lower risk of developing MS than non-drinkers, suggesting that a moderate alcohol intake might exert a protective effect in the context of the disease (Andersen et al., 2019).

1.1.3 Clinical Definition

MS can present with a wide variety of neurological signs and symptoms reflecting demyelination within the brain and spinal cord. Common symptoms include visual disturbance, motor weakness, sensory changes, incoordination, gait disturbance and bladder dysfunction (Buzzard et al., 2017). In clinical terms, MS is defined by relapses and brain MRI activity by the presence of gadolinium-enhancing lesions or unequivocally enlarging T2 lesions (Oh et al., 2018). MS relapses usually develop sub acutely over hours to days, reach a plateau lasting several weeks, then gradually recover. Gross clinical recovery from relapses often appears complete in early MS, however most of them leave behind some damage (Dobson & Giovannoni, 2019).

Traditionally, MS has been categorized into four distinct clinical phenotypes: relapsing – remitting (RRMS), secondary-progressive (SPMS), primary progressive (PPMS), and progressive relapsing (PRMS) (Oh et al., 2018).

The clinical course in MS is extremely variable. Most diagnosed patients begin with a more common relapsing-remitting course, which affects 85-90% of the cases and in which patients experience acute exacerbations of neurological dysfunction, lasting at least 24 hours, with partial or complete recovery and alternative periods of relative clinical stability. There is evidence that the incidence of RRMS may be increasing, particularly in women (Brownlee et al., 2017; Hauser & Cree, 2020; Oh et al., 2018).

Natural history studies tell us that within 20 years of diagnosis, the majority of patients diagnosed with RRMS will develop SPMS (Buzzard et al., 2017; Oh et al., 2018). SPMS typically develops 10 -15 years after RRMS onset, with a gradual evolution from

discrete relapses to slowly progressive disease, and occurs in up to 40% of patients by 20 years after the initial event (Dobson & Giovannoni, 2019; Oh et al., 2018). A much smaller proportion of patients are diagnosed with PPMS whereby neurological disability is slowly progressive from the onset and some patients may additionally experience discrete relapses, sometimes with unnoticed symptoms for months or even years (Brownlee et al., 2017; Buzzard et al., 2017; Hauser & Cree, 2020; Oh et al., 2018). Despite these distinctions, all clinical forms of MS appear to reflect the same underlying disease process. And although inflammation is typically associated with relapses, and neurodegeneration with progression, it is now recognized that both components are present in essentially all patients across the entire disease continuum (Hauser & Cree, 2020). Disability in MS is primarily measured by the EDSS. This scale allows clinicians to assess neurological disability in each of 8 functional systems - pyramidal, sensory, cerebellar, visual, bladder/bowel, brainstem, cerebral/mental and 'other') - as well as give an assessment of ambulation (Buzzard et al., 2017).

In addition to providing essential information for the diagnosis, MRI scans are also an important component to evaluate the disease activity. This method frequently shows subclinical disease activity in the form of new T2 lesions with or without gadolinium enhancement (Buzzard et al., 2017; Hauser & Cree, 2020). The diagnosis is based on 2 discrete episodes of neurologic dysfunction at least 30 days apart in different locations of the CNS or in those with one relapse who show evidence of dissemination in time (DIT) and dissemination in space (DIS) on MRI. Patients with a single attack that does not meet formal criteria for MS are considered to have a clinically isolated syndrome (CIS), whereas those with imaging consistent with MS are considered to have a radiographically isolated syndrome (RIS). Therefore, the diagnosis consists in the demonstration of multiple lesions DIT and DIS. DIS is recognized by one or more T2 lesions in at least 2 out of 4 areas of the CNS - periventricular, juxtacortical, infratentorial, or spinal cord - while DIT is recognized by either new T2 and/or gadolinium-enhancing lesion on follow-up MRI, with reference to a baseline scan, irrespective of the timing of the baseline MRI. (Howard et al., 2016).

1.1.4 Pathophysiology

The pathology of MS is characterized by inflammation, demyelination, reactive gliosis and neuroaxonal damage (Buga et al., 2023). Although research in the field of MS has not fully elucidated the processes responsible for its initiation and development, there is consensus among investigators and clinicians that the immune system plays a critical role in the development of this disorder, particularly in the acute early phases marked by relapses (Wootla et al., 2012; Zéphir, 2018).

1.1.4.1. Autoimmunity

The most widely accepted hypothesis regarding MS pathogenesis is the autoimmune one (Nakahara et al., 2012). It can be speculated that the MS lesion is initiated by $CD4^+$ T cells, but the amplification and damage are mediated by $CD8^+$ T cells that express various cytotoxic molecules. This idea is further supported by data showing that $CD8^+$ T cells target other CNS resident cells, including microglia, astrocytes and neurons, suggesting their pathogenic potential (Podbielska et al., 2018).

MS is considered an autoimmune condition initiated by auto-reactive immune cells that cross the BBB and target the CNS through a possible mechanism of molecular mimicry. In this scenario, auto-reactive $CD4^+$ T cells become activated by cross-reacting with antigens, such as microbial agents, in the peripheral immune compartment (Buga et al., 2023; Korn, 2008). Upon antigenic stimulation, naive $CD4^+$ T cells activate, expand, and differentiate into distinct subsets (Th1, Th2, Th17), each characterized by the production of specific cytokines. $CD4^+$ pro-inflammatory Th1 cells release mediators, such as tumor necrosis factor α (TNF- α), IFN-γ, leading to a self-directed inflammatory attack that results in myelin degeneration (Podbielska et al., 2018). These T cells also secrete pro-inflammatory cytokines that activate vascular endothelial cells, further disrupting the BBB and allowing the recruitment of additional immune cells into the CNS. This cascade of events increases inflammation and promotes disease progression. Furthermore, activated microglia release free radicals, NO (nitric oxide), and proteases, which further contribute to tissue damage and axonal loss (Gold & Wolinsky, 2011). Th1 and Th17 cells create a pro-inflammatory environment that attracts peripheral monocytes and promotes neuronal

damage (Buga et al., 2023). Th1 cells are responsible for producing INF-γ, which activates macrophages and also induces the production of reactive oxygen and nitrogen intermediates that damage the surrounding tissues. Th1 cells also produce IL-12, which stimulates the production of INF-γ and TNF-α, contributing to tissue damage. IL-17 levels are increased in both the blood and the CNS of MS patients, and Th17 cell numbers significantly increase during relapses. *In vitro* studies have shown that Th17 cells pass through the BBB more effectively than any other T cell subset, being considered central effector cells in chronic MS inflammation due to their capacity to infiltrate tissues and cause severe inflammation (Korn, 2008; Wang et al., 2022). IL-17 causes BBB damage, leading to increased infiltration of other cytokines, neutrophils, and monocytes into the CNS. Th17 cells also produce pro-inflammatory cytokines IL-21 and IL-22, which can directly damage neurons (Podbielska et al., 2018). In contrast, Th2 cells mediate humoral immunity and clear extracellular pathogens (Korn, 2008). Recognized by the secretion of IL-4, IL-5 and IL-13, these cells may also have regulatory functions. Current findings suggest that pro-inflammatory Th1 and Th17 cells predominate during relapse, while anti-inflammatory Th2 and Tregs drive remission (Podbielska et al., 2018).

Abnormalities in immune mechanisms have been suggested as key factors in the pathogenesis of MS. This assumption is based on findings of a reduced number and activity of circulating Treg cells, which correlate with an exacerbation of the disease symptoms (Liu et al., 2022).

Th9 cells are the most recently discovered lymphocyte subset, being characterized by a substantial production of IL-19, as well as IL-10 and IL-21. It was demonstrated that mice with EAE receiving Th9 cells had reduced lymphocyte infiltration in the meninges as compared to those receiving Th1 and Th17 cells. Its protective role was also shown by using IL-9 Receptor knockout mice with EAE, which presented a severe disease course (Liu et al., 2022).

More evidence suggests that B cells are also involved in the pathogenesis of MS through presenting antigenic peptides to T cells and driving the self-proliferation of brain homing T cells, possibly through memory B cells, producing regulatory cytokines and chemokines and contributing to differentiation of lymphocyte subpopulations (Liu et al., 2022). MS involves various other immune cells, including macrophages, natural killer cells, and microglial cells (Korn, 2008; Liu et al., 2022). Microglia and astrocytes play crucial roles

in MS development and progression through neurotoxic cascade. Astrocytes regulate glutamate level by controlling key enzymes involved in their production. Astrocytic glutamate transporters 'expression is altered in MS patients, leading to glutamate-mediated excitotoxicity and neuronal death (Buga et al., 2023).

Activated microglia play a dual role in the pathogenesis of MS, particularly during active demyelination, where these cells release pro-inflammatory mediators like NO and ROS. They play a crucial role in antigen T cell presentation; in the phagocytosis of myelin and cytokine production (Lassmann et al., 2012). Microglia can also switch to a more reparative phenotype, promoting remyelination by clearing myelin debris and producing growth factors (IGF-1; TGF- β; BDNF), creating a more supportive environment for OPCs to migrate, differentiate and form new myelin (Durafourt et al., 2012; Kotter et al., 2006; Lampron et al., 2015; Miron, 2017; Neumann et al., 2009; Olah et al., 2012; Pu et al., 2018; Rawji et al., 2018).

1.1.4.2. Demyelination and Remyelination: A Focus on the Dual Role of Inflammation

MS is a complex neurodegenerative disorder, but its two primary hallmarks are demyelination and axonal degeneration. The disease manifests as demyelinated plaques in both the white and gray matter of the brain, affecting areas like the cerebral cortex, cerebellar cortex and brainstem nuclei (Höftberger & Lassmann, 2017; Pivneva, 2009). Demyelination is marked by the selective destruction of oligodendrocytes (OLGs), the cells responsible for myelin production, and the activation of astrocytes during the phase of active tissue injury. Over time, this leads to the formation of gliotic scars in areas where lesions have become inactive (Höftberger & Lassmann, 2017; Lassmann, 2018). At the cellular level, early demyelination involves the production of reactive molecules, including oxygen radicals, NO, and reactive nitrogen species, largely by macrophages and activated microglia. These species are critical drivers of damage to myelin and OLGs (Höftberger & Lassmann, 2017; Lassmann, 2002).

Remyelination is a natural regenerative process following primary demyelination, where the exposed axon is re-coated with a new myelin sheath. This process is crucial as it restores saltatory conduction, provides axonal support, and aids in functional recovery

(Franklin & Simons, 2022). However, the efficiency of remyelination diminishes with time, significantly contributing to the currently untreated progressive phase of demyelinating diseases, such as MS. As a result, there is a growing interest in developing therapies that can promote or sustain remyelination (Cunha et al., 2020; Franklin & Simons, 2022). The failure of remyelination is primarily due to the insufficient proliferation of OLGs and their inability to migrate or extend processes into injury areas (Sharief, 1998). For effective remyelination, oligodendrocyte progenitor cells (OPCs) must be activated, migrate to the demyelinated lesions, and differentiate into mature myelinating OLGs. OPCs respond to injury by secreting cytokines and chemokines like C-C Motif Chemokine Ligand 2 and TNF-α. Additionally, OPCs can present antigens via major histocompatibility complex (MHC) classes I and II, influencing T cell proliferation and phagocytosing myelin debris (Zveik et al., 2023).

Cytokines play a pivotal role in MS pathogenesis by regulating aberrant autoimmune responses and mediating damage of OLGs and myelin. These molecules can have both detrimental and beneficial effects within inflammatory brain lesions (Sharief, 1998).

TNFα is abundantly present in the serum, cerebrospinal fluid (CSF) and active lesions of MS patients (Sharief, 1998). TNFα signaling occurs through two receptors: Tumor Necrosis Factor Receptor 1 (TNFR1), which primarily promotes neurotoxicity, and Tumor Necrosis Factor Receptor 2 (TNFR2), which fosters neuroprotection and reparative effects (Cunha et al., 2020; Zveik et al., 2023). TNF-α exerts multiple pro-inflammatory effects, contributing to OLG damage by inhibiting potassium channels, inducing apoptosis, and damaging microglia and astrocytes through upregulation of MHC-I and II gene products. Additionally, TNF-α triggers the release of NO and reactive oxygen species (ROS) (Sharief, 1998). Despite these damaging effects, TNFα also has neuroprotective properties. It can protect neurons by inducing the production of superoxide dismutase, an enzyme that shields neurons from the harmful effects of ROS. These neuroprotective properties are attributed to signaling via TNFR2, which counteracts the detrimental effects of IFN-γ on OPC differentiation (Sharief, 1998; Zveik et al., 2023).

A recent study demonstrated that EAE mice lacking oligodendroglial TNFR2 exhibited peripheral immune cell infiltration, increased demyelination, and impaired remyelination compared to wild-type mice (Madsen et al., 2020). Another study showed promising results from the dual modulation of TNFR1 and TNFR2 in an EAE model, which effectively mitigated

EAE symptoms while decreasing demyelination and axonal degeneration (Fiedler et al., 2023). It is important to note that the pivotal function of TNF-α in maintaining a regenerative milieu within the CNS is further underscored by instances where patients treated with anti-TNF agents developed demyelinating syndromes (Zveik et al., 2023).

Accordingly, investigators observed that exposure to an anti-inflammatory environment, represented by IL4 and IL10, resulted in decreased OPC differentiation, reduced phagocytic activity, MHC-II expression and cytokine production (Zveik et al., 2023).

A study involving mice and zebrafish lacking myeloid differentiation primary response 88 highlights the crucial role of microglia/macrophage activation in the remyelination process. This activation is essential for clearing myelin debris and initiating the secretion of pro-regenerative molecules necessary for myelin repair. By removing repulsive signals, this process facilitates OLG differentiation and remyelination (Cunha et al., 2020).

These insights could lead to the development of innovative targeted therapeutic approaches for MS, that encompass the full spectrum of OPC functions (Zveik et al., 2023).

1.1.4.3. Current Pharmacological Approaches

MS treatment has been primarily based on the prescription of immunosuppressive and immune-modulating agents. Nevertheless, a number of disease-modifying treatments (DMTs) that reduce the attack rate, delay progression and mainly target inflammation settings have been developed (Gholamzad et al., 2019). Currently, there are more than 10 compounds approved by the European Medicines Agency (EMA) and the United States Food and Drug Administration (FDA) for the treatment of MS, and new therapeutic approaches are currently being investigated in clinical trials (Konen et al., 2023).

The advent of second-generation molecules, which have been approved since 2018, has brought about a new era in MS therapeutics, due to reduced side effects and a more tailored approach. Although considerable progress has been made in the development of drugs for RRMS and for delaying its conversion to SPMS, treatment of the progressive forms remains unsatisfactory. This unmet clinical need is related to the complexity of the pathophysiological mechanisms involved in this stage of the disease (Amin & Hersh, 2023; Baecher-Allan et al., 2018; Faissner et al., 2019; Goldschmidt & McGinley, 2021). However,

a monoclonal antibody, ocrelizumab, has been approved for the treatment of PPMS (Amin & Hersh, 2023; Baecher-Allan et al., 2018; Goldschmidt & McGinley, 2021).

Interferon beta (IFN-β) and Glatiramer Acetate are the longest established injectables for MS, having the greatest depth of safety data (Amin & Hersh, 2023; Goldschmidt & McGinley, 2021; Travers et al., 2022). Nevertheless, the use of injectable treatments has declined due to the development of alternative DMTs with improved tolerability and higher efficacy (Amin & Hersh, 2023; Goldschmidt & McGinley, 2021). This is the case for Fingolimod, Siponimod and Ozanimod, which are sphingosine 1-phosphase (S1P) receptor modulators, that through their action on S1P receptor block the capacity of lymphocytes to egress from lymph nodes to CNS, thus reducing the number of circulating naive and central memory T and B cells into the peripheral circulation to start the inflammatory cascade associated with myelin destruction (English & Aloi, 2015; Gholamzad et al., 2019; Konen et al., 2023). In addition, S1PR modulators exert anti-inflammatory and neuroprotective effects through interaction with astrocytes and the S1P5 receptor on OLGs (Amin & Hersh, 2023; Faissner & Gold, 2022; Travers et al., 2022). Nevertheless, Fingolimod has notable adverse effects, including bradycardia, atrioventricular conduction disturbances, decreased peripheral lymphocyte count, increased transaminases, macular edema, and infection (English & Aloi, 2015), implying a considerably long monitoring period (Amin & Hersh, 2023; English & Aloi, 2015; Gholamzad et al., 2019; Goldschmidt & McGinley, 2021; Travers et al., 2022). Interestingly, in a model of demyelination in Xenopus tadpoles, treatment with Siponimod led to strong remyelination, suggesting that the medication might be also effective in progressive forms of MS (Baecher-Allan et al., 2018; Faissner & Gold, 2022).

Teriflunomide composes another example of an orally administered DMT halting T and B lymphocytes' proliferation, due to the reversible inhibition of dihydroorotate dehydrogenase, a mitochondrial enzyme responsible for pyrimidine synthesis of nucleic acids (Amin & Hersh, 2023; English & Aloi, 2015; Goldschmidt & McGinley, 2021; Konen et al., 2023). Nevertheless, teratogenic effects have been observed, requiring the use of reliable contraception measures for women within reproductive age (Travers et al., 2022).

The immunomodulatory effect of dimethyl fumarate, another orally administered DMT, is thought to be mediated by the activation of various cytoprotective signaling pathways (Konen et al., 2023). It is known that its metabolite, monomethyl fumarate,

activates the nuclear factor erythroid-derived 2 pathways, which is involved in the cellular response to oxidative stress (Amin & Hersh, 2023; Gholamzad et al., 2019). Diroximel fumarate**,** a second-generation fumarate, represents an improvement due to its reduced gastrointestinal (GI) side effects and improved overall tolerability (Faissner & Gold, 2022).

A series of monoclonal antibodies have also been approved for MS treatment. Alemtuzumab and natalizumab appear to have strong anti-inflammatory effects as measured by reducing relapses and affecting inflammation in forms of RRMS (Baecher-Allan et al., 2018). Natalizumab targets the alpha-4-integrin, interrupting leukocyte migration from the peripheral blood into the CNS by inhibiting the binding of leukocytes to the vascular cell adhesion molecule located on endothelial cells (Amin & Hersh, 2023; Gholamzad et al., 2019; Konen et al., 2023). On the other hand, Alemtuzumab, by targeting CD52, depletes monocytes and B and T lymphocytes, leading to long-lasting changes in adaptive immunity, and reduces the pathogenesis of inflammatory response in MS (Amin & Hersh, 2023; English & Aloi, 2015; Goldschmidt & McGinley, 2021).

By depleting pre- and mature B cells and subsets of $CD3^+$ T lymphocytes, anti-CD20 monoclonal antibodies such as ocrelizumab, rituximab and ofatumumab, are reasonable treatment options for first-line use given their favorable safety profiles, and second-line use in relapsing MS patients who have demonstrated breakthrough disease, especially in the setting of positive John Cunningham Virus antibody serology, in whom other highly effective treatments (e.g., natalizumab) may carry higher risks (Amin & Hersh, 2023; Faissner & Gold, 2022; Gholamzad et al., 2019; Goldschmidt & McGinley, 2021; Konen et al., 2023).

In terms of future perspectives, on one hand the potential role of immunoablation and autologous hematopoietic stem cell transplantation in treatment-resistant relapsing disease is currently under investigation (Goldschmidt & McGinley, 2021; Travers et al., 2022). On the other hand, Ublituximab and inhibitors of Bruton's Tyrosine Kinase are expected to be developed, which will compose a new class of medications acting on both B cells and myeloid cells (Faissner & Gold, 2022). Currently, oral inhibitors of Bruton's Tyrosine Kinase are under investigation in several phase III trials (Amin & Hersh, 2023; Faissner & Gold, 2022). The Ublituximab antibody is at the next stage of anti-CD20 therapy, targeting a unique epitope on the CD20 antigen and is glycoengineered for enhanced B-cell

targeting through antibody-dependent cellular cytotoxicity (Amin & Hersh, 2023; Faissner & Gold, 2022).

The current available medications for MS do not directly promote myelin sheath repair. Therefore, many researchers have attempted to achieve better therapeutic effects by promoting remyelination (Yu et al., 2019). Many dietary and natural products, including polyphenols, have been extensively studied for the treatment of autoimmune disorders (Khan et al., 2019; Shakoor et al., 2021). Olive leaf polyphenols have been thoroughly investigated for their antioxidant properties and ability to reduce oxidative stress, which plays a significant role in the pathogenesis of MS (Giacometti & Grubić-Kezele, 2020). Ellagic acid is another polyphenol being explored for its neuroprotective effects in MS. Researches have focused on its ability to reduce disease severity, influence the expression of genes involved in the pathogenesis of MS, and modulate the levels of related cytokines (Jafari Karegar et al., 2023). At last, Quercetin, a well-known polyphenol, has been highlighted for its non-pharmacological antioxidant properties in preclinical studies. This compound has demonstrated promising effects as a therapeutic agent in MS by reducing oxidative damage and inflammation (Javanbakht et al., 2023).

1.2. The Gut-Brain-Axis in Multiple Sclerosis

The gut-brain-axis (GBA) is a complex system of bidirectional interaction between the gut microbiota (GM) and the brain. This system encompasses 3 components: neuronal connections, the neuroendocrine and humoral pathways, and the immune system. Emerging research has revealed the involvement of GM in the pathophysiology of MS, offering a fresh viewpoint on the GBA, although the exact interaction of these components is still unknown (Altieri et al., 2023; Ordoñez-Rodriguez et al., 2023; Wang et al., 2022).

1.2.1. GM Dysbiosis

Studies carried out in different populations around the world reported that MS patients exhibit marked changes in their GM composition, described by the decrease or increase of determined bacterial *phylae* and species compared to control subjects (Torres-Chávez et al., 2023).

Berer et al. observed that mice colonized with microbiota from MS patients exhibited a higher rate and severity of EAE induction compared to mice colonized with microbiota from healthy individuals, highlighting the influential role of the GM in MS pathogenesis (Berer et al., 2017).

A study was conducted to investigate the microbiome composition during disease progression in both the EAE and cuprizone (CPZ)-induced demyelination models, where fecal samples from C57BL/6 mice were collected at specific intervals throughout the disease course. This study revealed rapid microbial changes in both models, with more pronounced alterations in EAE, likely due to its immune-mediated nature. In contrast, the microbiota shifts in the CPZ model, which involves non-T cell-mediated demyelination, were less clear, suggesting a more complex interaction (Moles et al., 2021). Both EAE and CPZ animals exhibited a rise in the *Firmicutes phylum* and a decline in *Bacteroidetes* at disease onset, mirroring findings in MS patients. *Actinobacteria* was decreased in both models, though its exact role in MS remains unclear, as it has been associated with health-related effects in other contexts. *Akkermansia*, a genus within the *Verrucomicrobia phylum*, was significantly elevated in CPZ mice but decreased during EAE progression. This difference might reflect *Akkermansia's* involvement in demyelination rather than inflammation, or variations in host responses between EAE and CPZ models of MS. Interestingly, *Prevotella*, a genus typically decreased in MS patients, was increased in both models, possibly due to the controlled diet given to the mice. Other genera like *Faecalibacterium* and *Ruminococcus* were increased during mild EAE but behaved differently in CPZ, which may indicate distinct microbiota responses in the two models (Moles et al., 2021).

Regarding humans, a study with 20 MS patients and 40 healthy controls performed in Japan demonstrated that altered intestinal microbiota in patients with RRMS was characterized by increased levels of *Actinobacteria*, *Bifidobacterium*, and *Streptococcus* and decreased amounts of *Bacteroides*, *Faecalibacterium*, *Prevotella*, *Anaerostipes*, and *Clostridia XIVa* and *IV clusters* (Fengna Chu et al., 2018). Similarly, biopsy samples of brain white matter showed a higher abundance of *Actinobacteria* in RRMS, which decreased in cases of PPMS, also characterized by elevated numbers of *Proteobacteria* (Fengna Chu et al., 2018).

A recent study of dysbiosis in PPMS patients reported alterations in the relative abundance of two dominant genus: *Gemmiger* and unclassified *Ruminococcaceae*. Interestingly, they also showed the relative increase of *Akkermansia muciniphila*, suggesting there may be a common mechanism in the association of gut dysbiosis and disease pathogenesis between RRMS and PPMS (Brown et al., 2021).

A recent investigation analyzed the microbiota changes in small intestinal tissues from MS patients in the active phase. The authors showed the ratio of *Firmicutes* to *Bacteroidetes* and presence of *Streptococcus* were increased, whereas the presence of *Prevotella* strains was diminished in patients with active MS compared to healthy controls and patients in remission. Moreover, the relative presence of *Prevotella* strains was negatively correlated with the proportion of Th17 cells, which is positively associated with the disease activity (Wang et al., 2022).

Interestingly, a number of therapies currently use to treat MS, such as IFN-β and glatiramer acetate, have shown to impact GM composition, increasing *Prevotella* and *Sutterella* and reducing *Sarcina* compared to untreated patients (Jangi et al., 2016; Katz Sand et al.).

1.2.2. Microbiota and Immunity

The gut immune system (GIS) contains various innate type lymphocytes: invariant natural killer cells, mucosal associated invariant T cells, γδ T cells and innate lymphoid cells. Interactions between these cells and the GM are determinant for their development and to ensure the homeostasis of the GIS (Chiba et al., 2018; Malik et al., 2016; Rodríguez Murúa et al., 2022; Rojas et al., 2019; Saligrama et al., 2019; Yamamura et al., 2007).

The GM can regulate several CNS properties, including modulating BBB permeability, limit astrocyte pathogenicity, activate microglia, and alter myelin genes' expression (F. Chu et al., 2018). The GM also participates in the fermentation of indigestible carbohydrates into SCFAs, the most common being acetate, propionate and butyrate. These molecules activate the brain's immune response by influencing the production of cytokines and interact with the G-protein-coupled receptor 43 to elicit anti-inflammatory effects (F. Chu et al., 2018). SCFAs also have an immunomodulatory function: rising the number of Treg

cells and suppressing the collaborative T cells, Th17 and Th1 (Ordoñez-Rodriguez et al., 2023).

A recent investigation demonstrated that more than 70% of MS patients displayed increased intestinal permeability. This increase might further facilitate GM-mediated activation of immune cells in the periphery, especially gut-associated lymphoid tissue (GALT) (Wang et al., 2022). GM dysbiosis leads to a decrease of anti-inflammatory metabolites and the enhancement of pro-inflammatory signals, suppressing the differentiation of Treg cells and promoting the expansion of Th1 and Th17 ones. In addition, several toxic microbiota components and metabolites, including lipopolysaccharide and lipoteichoic acid, might enter into circulation and compromise the integrity of BBB. Subsequently, Th1, Th17 and auto-reactive T cells can infiltrate the CNS, resulting in the inflammation and the injury of myelin and neurons. In turn, the CNS inflammation may also recruit more inflammatory immune cells and cytokines, aggravating the injury (Wang et al., 2022).

Studies have shown that antibiotic treatment in animal models EAE can have significant effects on T cell responses in both the CNS and the GI tract, such as the reduction of pro-inflammatory Th1 and Th17 cells levels and secretion of respective cytokines: IFN- γ and IL-17A. This kind of treatment has also shown to enhance the anti-inflammatory immune responses marked by elevated levels of Tregs expressing FoxP3, as well as increase secretion of anti-inflammatory cytokines, IL-10 and IL-13 (Fengna Chu et al., 2018).

A number of studies have suggested that specific bacterial strains have beneficial effects on EAE, protecting mice from disease exacerbation, the most common being *Bacteroides Fragilis*. *B. fragilis* been reported to induce the production of inflammatory cytokines, including IFN-γ, IL-1β, IL-6, TNF-α, IL-17 AND IL-2. However, it can also exert an anti-inflammatory role by promoting the conversion of $CD4^+$ T cells into $FoxP3^+$ Treg cells and inducing the production of IL-10 through polysaccharide A, which is produced specifically by this specie, protecting mice from CNS demyelinating diseases (Fengna Chu et al., 2018; Wang et al., 2022). Additionally, recent studies indicated that *Clostridia XIVa* and *IV* may also have the ability to induce $FoxP3^+$ Tregs and be able to suppress the inflammatory response in EAE (Fengna Chu et al., 2018). *Prevotella histicola* can likewise suppress EAE by inducing $CD4^+$ $FoxP3^+$ regulatory T cells and tolerogenic dendritic cells, while suppressing macrophages (Fengna Chu et al., 2018).

Interestingly, researchers have observed that the GM influences the interaction between T-cell C-C chemokine receptor type 9 (CCR9) and its ligand chemokine (CCL25), which when blocked leads to a reduction of $CCR9^+$ $CD4^+$ T cells in peripheral blood. In patients with RRMS and SPMS a decrease of CCR9 functionality was observed (Kadowaki et al., 2019). In order to study the influence of GM on $CCR9^+$ memory T (Tm) cells, $CD4^+$ Tm cells were analyzed in the peripheral blood of C57BL/6G mice maintained in specific pathogen-free (SPF) conditions and in C57BL/6 N germ-free mice (Kadowaki et al., 2019). In germ free mice a decrease in levels of $CCR9^+$ was verified. On the other hand, SPF mice were subsequently treated with a mixture of antibiotics and this treatment promoted an increase of $CCR9^+$ Tm cells and reduced EAE severity (Kadowaki et al., 2019). These results suggest that an alteration of the gut–systemic immune axis may be involved in the pathogenesis of MS (Kadowaki et al., 2019).

1.2.3. Microbiota and Neurodegeneration

The impact of the GM on the development of MS has been rooted in several preclinical studies in EAE (Fengna Chu et al., 2018). Only the top of the iceberg is known, and the role of the GM in neuroinflammation and neurodegeneration has remained to be fully elucidated (Torres-Chávez et al., 2023).

Bacteria secrete and consume a plethora of neuromodulators and neurotransmitters, including SCFAs, serotonin (5-HT), dopamine, gamma-aminobutyric acid (GABA), tryptophan, epinephrine, histamine and norepinephrine. These molecules have been shown to protect GI barrier integrity from the disruptive effects of proinflammatory cytokines (Fengna Chu et al., 2018; Dicks, 2022; Ordoñez-Rodriguez et al., 2023). Several studies have addressed the possible immunomodulatory potential of the neuroendocrine system in susceptibility and severity of autoimmune diseases (Deckx et al., 2013). The regulation of the immune system by neuroendocrine pathways is accomplished by hormones from the hypothalamic-pituitary-adrenal and the hypothalamic-pituitary-gonadal axes (Deckx et al., 2013).

Because maternal milk regulates microbiota composition and protects microbiota from metabolic diseases, it is important to mention that it has been documented that prolactin, a hormone secreted by the pituitary gland, has immunostimulatory effects such

as increasing the production of IFN-γ and IL-12 and the proliferation of T cells (Deckx et al., 2013; Luzardo-Ocampo et al., 2023).

Studies have shown a decrease in the functionality of the serotonergic system in MS. This decrease may be due to elevated pro-inflammatory cytokines' levels resulting from dysbiosis, activating enzymes that trigger the kynurenine pathway and deplete tryptophan for serotonin synthesis (Torres-Chávez et al., 2023). In addition, in animal models, the GM has been shown to have an influence on the synthesis of serotonin receptors outside the gut, highlighting serotonin as a critical mediator of the GBA in MS (Torres-Chávez et al., 2023). Similarly, a decrease in GABA brain transporters is expressed in MS patients. GABA is the main inhibitory neurotransmitter in the CNS and downregulates pro-inflammatory T-cell mediators. Its production is regulated by the GM, with *Lactobacillus brevis* being the most effective producer (Galland, 2014; Torres-Chávez et al., 2023).

A change in GM diversity may cause differential hormonal responses, which influence brain activity and instigate pathological processes. Intake of probiotic supplementation in MS patients has shown reduction in oxidative stress and inflammatory processes, facilitating modifications in hippocampal synapse by increasing the expression of brain-derived neurotrophic factor (BDNF) and increasing hypothalamic neuronal activity, which translates into better cognitive and learning processes (Cryan et al., 2019).

1.3. Polyphenols as nutraceuticals

1.3.1. Polyphenol Classification and Dietary Sources

Polyphenolic compounds (PCs) are a diverse group of bioactive molecules known for their significant health benefits, especially those linked to the Mediterranean diet (C. Ferreira et al., 2024). The phenolic ring, a basic monomer in PCs, grants them strong free radical scavenging abilities, contributing to their biological antioxidant responses. PCs are also capable of inhibiting the formation and activation of free radicals (Abbas et al., 2017; Zhang et al., 2022).

These compounds are divided into various classes based on their chemical structure, origin, and biological function. The largest classes are flavonoids and phenolic acids, while narrower classes include tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids (C. Ferreira et al., 2024; Singla et al., 2019).

Flavonoids are responsible for the coloring and aroma of flowers and fruits, and the majority are found as glycosides. Their typical structural backbone is C6-C3-C6, and the subgroups are determined by the position of the B ring attachment to the C ring. When the B ring is attached at position 3, they are isoflavones, and when this link happens in position 4 they are called neoflavonoids. Other subgroups include flavanones, flavanonols, flavones, flavonols, flavanols, and anthocyanins, depending on the structure of the C ring (C. Ferreira et al., 2024; Scalbert et al., 2005; Tsao, 2010).

Phenolic acids possess one carboxylic acid group, and can be divided into two major subtypes: benzoic acids whose skeletal structure is C6-C1, and cinnamic acids in which it is C6-C3 (Abbas et al., 2017). The most common hydroxycinnamic acid and hydroxybenzoic acids are chlorogenic acid (CGA), and gallic, vannilic, ellagic, syringic acid, p-hydroxybenzoic and protocatechuic acids, respectively (Di Lorenzo et al., 2021; C. Ferreira et al., 2024; Singla et al., 2019).

PCs are abundantly found in a variety of plant-based foods, including olive oil, herbs, vegetables, fruits, seeds, nuts, whole-grains cereals, and wine. Each of these food groups is enriched with specific polyphenolic classes. For example: phenolic acids are predominant in cereals and whole grains such as wheat, oats, rice, corn and triticale; flavones and hydroxycinnamic acids are abundant in dried herbs like oregano and peppermint;

catechins, hydroxycinnamic acids, anthocyanins, and proanthocyanidins are found in red wine; flavonoids, phenolic acids, and dihydrochalcones are present in fruits like apples, mangos, and pomegranates; and finally anthocyanins, which give berries their unique pigmentation and aroma (C. Ferreira et al., 2024).

The bioavailability of PCs is influenced by several factors, including their chemical structure, absorption, distribution, metabolism, and excretion, as well as food matrix and form of administration (Di Lorenzo et al., 2021; C. Ferreira et al., 2024). PCs classes vary in bioavailability, typically ranked as follows: phenolic acids > isoflavones > flavonols > catechins > flavanones, proanthocyanidins > anthocyanins (Di Lorenzo et al., 2021; C. Ferreira et al., 2024). The polyphenolic content of plants and fruits can be affected by external factors such as climate, cultivation methods, ripeness, storage, cooking, and processing (Arfaoui, 2021; Cory et al., 2018; C. Ferreira et al., 2024). The presence of specific macro and micronutrients in food can also modify PCs bioavailability and bioactivity (C. Ferreira et al., 2024).

Despite their low oral bioavailability (5-10%), PCs exhibit numerous scientifically proven, dose-dependent beneficial effects, including: antioxidant properties, strong free radical scavenging capacity, anti-inflammatory effects, antimicrobial activities, and anti-adipogenic properties (Abbas et al., 2017; Di Lorenzo et al., 2021). These beneficial effects make PCs promising candidates for incorporation into functional foods, nutraceuticals, and dietary supplements. Their immunomodulatory and anti-inflammatory activities are influenced by their chemical structure, particularly the number, positions, and types of substitutions, as well as the degree of polymerization of the chromane ring. For instance, high hydroxylation in the B ring of catechins and anthocyanidins enhances their metabolic reprogramming and bioactivity (C. Ferreira et al., 2024).

Therefore, PCs play a crucial role in promoting health and preventing disease, making them essential components of a healthy diet, particularly the Mediterranean dietary pattern. Their wide distribution in plant-based foods and their diverse bioactive properties underscore their importance in nutrition and health (C. Ferreira et al., 2024).

1.3.2. A Focus on Blueberries

Blueberries (BB) are renowned for their high content of polyphenols, which contribute to numerous health benefits (Wood et al., 2019). The primary polyphenols in BB are anthocyanins, followed by proanthocyanidins, flavonols and phenolic acids (Diaconeasa et al., 2015; Pap et al., 2021).

1.3.2.1. Blueberry Polyphenols' Therapeutic Potential

BB polyphenols exhibit a wide range of beneficial effects, including antioxidant, anti-inflammatory, antihypertensive, anticarcinogenic, and antidiabetic properties. Additionally, they can improve visual acuity and cognitive behavior. These potential health benefits have been evaluated through *in vitro* and *in vivo* studies in varied pathological contexts (Daniela D. Herrera-Balandrano et al., 2021; Pap et al., 2021), which will be further described in the sections ahead.

1.3.2.1.1. Metabolic Regulation

Studies on mice fed high-fat diets supplemented with BB have demonstrated significant metabolic benefits (DeFuria et al., 2009; Wu et al., 2016; T. Wu et al., 2013). BB-fed mice showed lower body weight and blood glucose levels (Wu et al., 2016). In addition, this study also revealed that whole BB and BB juice have been effective in improving insulin resistance and reducing serum insulin and leptin levels, as well as liver cholesterol, triacylglycerol, and low-density lipoproteins (LDL) levels (T. Wu et al., 2013).

Anthocyanins, major components of BBs, play a significant role in managing Type 2 Diabetes Mellitus (T2DM), by inhibiting oxidative stress, enhancing insulin secretion and improving insulin sensitivity through upregulation of insulin-regulated glucose transporter 4 translocation and activation of AMP-activated protein kinase (D. D. Herrera-Balandrano et al., 2021; Kurimoto et al., 2013; Takikawa et al., 2010). Accordingly, a study employing a diabetic mouse model demonstrated that the supplementation of a BB-Anthocyanin extract for 5 weeks reduced the animals' blood glucose levels and ameliorated their glucose tolerance (D. D. Herrera-Balandrano et al., 2021).

There are increasing evidence showing that proanthocyanidins (PACs) can also attenuate the progression of metabolic syndrome, including T2DM and obesity. Pre-clinical studies have suggested different mechanisms by which PACs regulate metabolic health, ranging from immunomodulatory signaling, stimulation of glucose and lipid metabolism, as well as through inhibition of digestives enzymes (Rodríguez-Daza et al., 2020).

Resveratrol, another significant polyphenol found in BB, has demonstrated various neuroprotective effects, such as anti-hyperglycemic action by lowering blood glucose levels and protecting pancreatic β-cells (Naz et al., 2023).

In cuprizone-treated C57BL/6 mice, resveratrol has therapeutic potential by diminishing mitochondrial dysfunction, oxidative stress and activating the NF-kB signaling (Ghaiad et al., 2017).

BB polyphenols have also shown to suppress body weight gain in HFD-fed C57BL/6J mice, decreased total cholesterol and triglycerides in the liver and feces, regulated lipid metabolism-associated genes, and modulated GM composition, indicating their potential as natural prebiotics for targeting obesity and its complications (Rodríguez-Daza et al., 2020).

1.3.2.1.2. Gut Health and Microbiota

Polyphenols can positively modify the GM by increasing beneficial bacteria like *Bifidobacterium* and *Lactobacillus*, or by inhibiting potentially pathogenic bacteria (Dolara et al., 2005; Lear et al., 2019; Queipo-Ortuño et al., 2012; Roudsari et al., 2019; Tzounis et al., 2011; Tzounis et al., 2008).

Recent research highlights the significant role of berry polyphenols, especially wild BB, in modulating GM (Ntemiri et al., 2020). A human cross-over dietary intervention study showed that wild BB promotes an increased relative abundance of *Bifidobacterium spp.*, which are beneficial for gut health (Rodríguez-Daza et al., 2020). Furthermore, in a murine model of diet-induced obesity and insulin resistance, wild BB polyphenolic extract improved glucose tolerance in association with changes in bacterial families, such as *Coriobacteriaceae* and *Verrucomicrobiaceae*, along with maintaining the colonic mucus layer (Rodríguez-Daza et al., 2020).

In healthy C57BL/6J mice, BB polyphenol extract inhibited body weight gain, normalized lipid metabolism, and altered GM composition, modulating specific bacteria such as *Deferribacteres*, *Actinobacteria*, *Proteobacteria*, *Desulfovibrio*, *Adlercreutzia*, *Bifidobacterium*, *Helicobacter*, *Flexispira*, and *Prevotella* (Jiao et al., 2019).

Rats fed HFD with whole BB powder showed increased abundance of *Porphyromonadaceae*, *Gammaproteobacteria*, *Proteobacteria* and *Fusobacteria*, and lower *Firmicutes* and *Bacteroidetes* compared to control groups (Lee et al., 2018). Lowbush wild blueberry powder led to higher *Actinobacteria*, *Coriobacteriaceae* and *Bifidobacteriaceae* abundance, while decreasing *Lactobacillus* and *Enterococcus* numbers (Lacombe et al., 2013). Interestingly, freeze-dried BB powder had sex-specific effects on GM composition in mice, with notable changes in *Corynebacterium*, *Clostridium*, *Facklamia*, *Turicibacter*, *Mogibacteriaceae*, *Coprococcus*, *Adlercreutzia* (Lacombe et al., 2013).

1.3.2.1.3. Neurodegeneration

Increasing the consumption of antioxidants, particularly polyphenols, has been shown to delay the onset of neurodegenerative diseases and improve the quality of life for patients. Fruit polyphenols, such as grapes and BB, possess strong antioxidant properties and can cross the BBB, accumulating in the CNS to various extents depending on their structural and chemical properties (Bensalem et al., 2019). Research involving long-term BB supplementation has evidenced that anthocyanins can cross the BBB and localize in brain regions crucial for learning and memory, such as the cortex, hippocampus, striatum, and cerebellum (Andres-Lacueva et al., 2005). In human studies, polyphenolic extracts have demonstrated to improve memory functions in healthy elderly volunteers with lower baseline memory performance (Bensalem et al., 2019; Nurk et al., 2009). Another study showed significant cognitive improvements in both healthy individuals and those with mild cognitive deficits after 12 weeks of polyphenol intake (Krikorian et al., 2010).

BB extract prevents mitochondrial damage associated with amyloid-beta (Aβ) peptide, reduces Aβ aggregation, and decreases neuroinflammation by regulating NF-κB (Paris et al., 2011). Likewise, BB anthocyanins reduce the production of pro-inflammatory cytokines (e.g., TNF-α, IL-1β) and the expression of enzymes involved in inflammation (e.g.,

NOS, COX) through the suppression of NF-κB activation (Carey et al., 2013; Lau et al., 2007). BB polyphenols can also prevent over-activation of microglia, which can otherwise lead to excessive production of inflammatory proteins such as TNF-α, contributing to neurodegeneration (Figueira et al., 2017).

BB exposure in neurons increases glutathione, ascorbic acid, and the activity of antioxidant enzymes such as catalase and superoxide dismutase. This antioxidant activity reduces the production of ROS and lipid peroxidation (Jeong et al., 2013; Papandreou et al., 2009). BB polyphenols enhance synaptic plasticity, memory, and cell survival by increasing hippocampal neurogenesis, extracellular signal-regulated kinases activation, and levels of insulin-like growth factor 1 (Casadesus et al., 2004). These effects also involve changes in calcium homeostasis and stress signaling (Joseph et al., 2007). BB supplementation increases BDNF, which protects neurons and areas of axonal injury, a suggested mechanism of action for the treatment of MS (Xin et al., 2012). In fact, dietary BB supplementation has been suggested to reduce the incidence and clinical severity of EAE, an animal model for MS (Xin et al., 2012). On another hand, BB supplementation has improved the survival and efficacy of transplanted embryonic dopamine neurons in a rat model of Parkinson's disease by reducing inflammation and providing direct neuroprotective properties (Xin et al., 2012).

Overall, the exposed results highlighted dietary supplementation with BB as a promising approach to alleviate neurodegenerative diseases and enhancing cognitive health.

1.3.3. Blueberry Leaves as a Valuable Byproduct for Disease Management

BB leaves, particularly from highbush BB (*Vaccinium corymbosum L.*), have been shown to possess significant antioxidant properties due to their high polyphenolic content, including anthocyanins, chlorogenic acid, quercetin glycosides, and notably, feruloylquinic acid, which is the most abundant (Ștefănescu et al., 2020). Highbush BB leaf extracts exhibit substantial antimicrobial properties, particularly against *Salmonella typhimurium* and *Enterococcus faecalis*. The leaf extracts also exhibit antimutagenic properties, contributing to their potential use in preventing genetic mutations and associated diseases (Ștefănescu et al., 2020). The presence of quercetin, kaempferol derivatives, and ellagitannins in BB

leaves further confer them anti-inflammatory and hypoglycemic effects, making them highly valuable for various health applications (Calixto et al., 2004; Hämäläinen et al., 2007).

As an example, blueberry leaf extract has been shown to suppress body weight gain and improve insulin resistance in HFD-induced obese mice. It reduces liver triglyceride levels and inhibits leptin secretion, suggesting its potential as an anti-obesity agent (Lee et al., 2014).

The leaves contain higher concentrations of these compounds than the fruits, offering potential for alternative or supplementary treatments for neurodegenerative diseases associated with aging (Debnath-Canning et al., 2020; Ștefănescu et al., 2020; Wang et al., 2015).

In a clinical study with nine older adults, the neurocognitive benefits of BB leaf extracts were attributed to their high anthocyanin content, which enhanced neuronal signaling, mediated memory function, and delayed neurodegeneration (Jeong et al., 2013; Krikorian et al., 2010). These extracts have also shown neuroprotective effects *in vitro*, increasing the viability of brain cells exposed to toxic stimuli (Ma et al., 2018). In accordance, rat brain mixed cell cultures of glial cells and neurons exposed to a leaf extract after glutamate toxicity displayed an increased number of viable cells, indicating a neuroprotective effect. Similarly, the same treatment in cells exposed to α-synuclein resulted in a greater percentage of control cells (Lee et al., 2010). A study demonstrated that BB flavonoids suppressed TNF-α via the NF-κB signaling pathway, making them promising candidates for managing conditions related to chronic inflammation (Shi et al., 2017).

Chapter II | MATERIAL AND METHODS

2.1. Sample characterization

Colon and brain samples were collected from 12-week-old male C57BL/6 mice subjected to CPZ-induced demyelination. Animals were daily subjected to oral gavage with 0.2% (w/w) CPZ (bis(cyclohexanone)oxaldihydrazone, C9012, Sigma-Aldrich) dissolved in 1% methylcellulose (Sigma, M7027/100G). To investigate acute demyelination, CPZ was administered daily for 5 weeks. For remyelination, CPZ intoxication was suspended for an additional 2 weeks.

The animals were divided into six groups (n=10): controls (CTR), receiving vehicles only for 5 and 7 weeks, respectively; intoxicated groups (CpzW5 and CpzW7) receiving CPZ for 5 or 7 weeks and sacrificed at the end of W5 (demyelination peak) or W7 (early remyelination); and treated groups, which orally received 500 mg/kg of BB daily from week 2.5 to 5 (CpzW5+BB) or 7 (CpzW7+BB).

At the end of weeks 5 and 7, animals were anaesthetized by intraperitoneal injection of ketamine chloride (1 g/mL; Imalgene®) in chlorpromazine 2.5% (Largactil®) and perfused postmortem transcardially with ice-cold phosphate buffer. Gut and brain were isolated, washed and weighted. Brains were divided into left and right hemispheres following excision of left and right cerebella. Left hemispheres (n=5) were stored in OCT CryoMatrix (6769006, ThermoScientific) for fluorescence microscopy or 10% neutral buffered solution (n=5) to be used for histological analysis. Following Peyer patches excision for flow cytometry, small intestine and colon were divided into four sections and properly stored for gene expression, biochemical assays, fluorescence microscopy and histology.

Animal experiments were conducted according to the National and European Communities Council Directives of Animal Care and received approval (#12/2018) by the local (iCBR) Animal Welfare Body (ORBEA). Biological samples were collected prior to the beginning of present study.

2.2. Determination of BB Biomass Total Phenolic Content and Total Antioxidant Capacity

BB biomass was obtained from senescent leaves of a combination of cultivars of Vaccinium shrubs. Total Phenolic Content (TPC) and Total Antioxidant Capacity were assessed by the Folin–Ciocalteu method and 2,2' -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacity assay, as previously described by our group (G. Ferreira et al., 2024). Results are expressed as milligrams of gallic acid equivalents (GAE mg/g) and as miligrams of ascorbic acid equivalents (AAE mg/mg) per gram of BB biomass, respectively snd presented as mean ± S.E.M of 4 assays.

2.3. Colonic Histopathological Analysis

Samples of the colon were collected for histological examination. Tissues included entire sections of colon prepared as "Swiss rolls" allowing for the evaluation of the entire longitudinal section. Hematoxylin & Eosin (HE) staining of intestinal tissue was performed.

Tissue samples were formalin-fixed and embedded in paraffin wax (n=2 each experimental group). One HE-stained cryosection (5 µm) from each block was reviewed. Tissue sections were deparaffinized in xylene and hydrated to a decrescent series of ethanol until distilled water. Thereafter, the tissue sections were immersed in hematoxylin stain Solution, Gill 1 (Sigma Aldrich; Missouri, USA) for 2 minutes and washed in tap water. Then, they were counterstained with 0,5% aqueous eosin (Sigma Aldrich; Missouri, USA) for 30 seconds and after that dehydrated, cleared, and mounted.

Image acquisition was carried out by light microscopy with a Zeiss microscope Mod. Axioplan 332 2 (Zeiss, Jena, Germany).

Tissue pathophysiology was characterized by the presence of inflammatory cells, mucosa layer hyperplasia, and goblet cell (GC) reduction. Mucosa thickness and GC number were determined using the free ImageJ® software (version 1.54f, https://imagej.net/). For each sample, the layer thickness was measured in 5 different locations and the goblet cell number in 5 different images, and averaged. For GC counting, only crypts that were visible throughout their entirety were considered.

Adapting from Erben et al. (/ISSN:1936-2625/IJCEP0001236), a scoring system was applied where scores from 0 to 3 were attributed to each of the three histological features mentioned above in a blind manner, according to the increasing severity of the damage. A final score entitled “colonic inflammation score” was obtained by adding the three previous scores.

2.4. Brain Tissue Preparation and Myelination Analysis

Kluver-Barrera (KB) staining of brain tissue was performed. Brain samples were formalin-fixed and embedded in paraffin wax. Tissue sections were deparaffinized in xylene and hydrated with ethanol, followed by overnight staining with 0.1% Luxol Fast Blue solution. Thereafter, the sections were washed in tap water and differentiated with 0.05% lithium carbonate solution (Sigma Aldrich; Missouri, USA) and 70% ethanol. After another washing step, sections were stained with 0.1% Cresyl Violet solution (Sigma Aldrich; Missouri, USA) for 10 minutes and two more steps of differentiation followed, with 95% ethanol and 10% Acetic Acid solution (Sigma Aldrich; Missouri, USA). Finally, sections were dehydrated, cleared, and mounted.

Image acquisition was carried out by light microscopy with a Zeiss microscope Mod. Axioplan 332 2 (Zeiss, Jena, Germany).

Quantification of the percentage of myelinated area was carried out in the corpus callosum and cerebellum in 3 to 7 images per group. Briefly, microphotographs of KB-stained histological sections were processed and analyzed using publicly available ImageJ software (version 1.54f, https://imagej.net/). Firstly, the regions of interest (ROIs) were manually drawn in each image excluding the Purkinje cells and cerebellar cortex and were subsequently added to the ROI Manager. Secondly, Color Deconvolution was performed in order to separate the different staining components. Then, all images regarding LFB staining were processed into a gray-scale 8-bit image. Afterwards, the images’ threshold was adjusted. To define threshold values, Auto Threshold was performed and the best match for the real blue intensity of the LFB image, in order to retain as many true pixels representing myelin as possible, was selected.

Myelination was determined by calculating the percentage of myelinated area within the ROIs.

2.5. Microglia Reactivity

2.5.1. Immunohistochemistry

Fixed brains were equilibrated in 30% sucrose in PBS overnight at 4ºC prior to being transferred to fresh fixative solution and prepared for histological processing. The tissues were then embedded in OCT CryoMatrix (6769006, ThermoScientific) and serial 30 µm sagittal sections (between bregma −0.45 and −1.85) were cut on a cryostat. The sections were stored as free floating at – 20ºC in cryopreservation solution, consisting of 30% glycerol, 30% ethylene glycol in phosphate buffer 0.4M. To label microglia, sections were fixed with 4% paraformaldehyde, washed 3 times for 5 minutes in PBS and then blocked with 5% FBS and 0.25% Triton X-100 in PBS pH 7.3 at RT for 2 hours. Slices were then incubated overnight at 4ºC on a shaker in primary antibody anti-IBA1 diluted in 1% FBS (1:200, Wako chemicals, 019–19741) After washing 3 times for 10 mins in PBS, slices were incubated at RT for 2 hours on a shaker in goat anti-rabbit Alexa Fluor 488 (1:1000, Invitrogen, A11034) secondary antibody prepared in PBS with DAPI (1:10000, Invitrogen, D1306). Slices were then washed 3 times in PBS and mounted on glass slides using Dako S3023 Fluorescence Mounting Medium. After removing the excess of mounting medium, a coverslip was positioned on top and the edges sealed with clear nail polish.

2.5.2. Image Acquisition

Digital imagining was carried out on a Zeiss microscope Mod. Axioplan 332 2 (Zeiss, Jena, Germany) using a 40x objective. To analyze the microglial morphology, *Z*-stack-multifocal images were generated with a 0.75 µm step size. 4 sagittal brain sections per animal were imaged for each region of interest: corpus callosum, hippocampus and the cortical layer 6.

2.5.3. Skeleton Analysis

A total of 4 images per brain area were selected from each animal. Microglia skeleton analysis was performed as described (DOI: 10.3791/57648). Briefly, each image was processed in a systematic way to obtain a filled image and its counterpart outlined shape. For this purpose, a series of steps were performed using ImageJ free software (https://imagej.net/) and appropriate plugins: (1) the images were transformed to 8-bit grayscale to best visualize all positive staining; (2) were filtered to soften the background and (3) enhance the contrast; (4) despeckeled to remove salt-and-pepper noise generated in the previous steps; (5) binarized to obtain a black and white image by applying an established threshold, (6) despeckeled again to eliminate noise resulting from binarization; (7) subjected to the close function to connect dark pixels separated by up to 2 pixels; (8) targeted for outliers and (9) converted to skeletonized images. **Figure 1A** illustrates the workflow process to convert an entire original photomicrograph to a skeletonized image, using IBA-1-1stained tissue as an example. The Analyze Skeleton Plugin (developed by and maintained here: https://imagej.net/plugins/analyze-skeleton/) was then applied to the skeleton image, tagging skeletal features relevant to microglia ramification, including the number of endpoint (blue) and junction (purple) voxels (**Figure 1B**), process length and process number.

The number of process endpoints, process length and number from the Analyze Skeleton data output was normalized by the number of microglia cell somas in each image, accessed through the CellCounter plugin in ImageJ (available here https://imagej.net/ij/plugins/cell-counter.html).

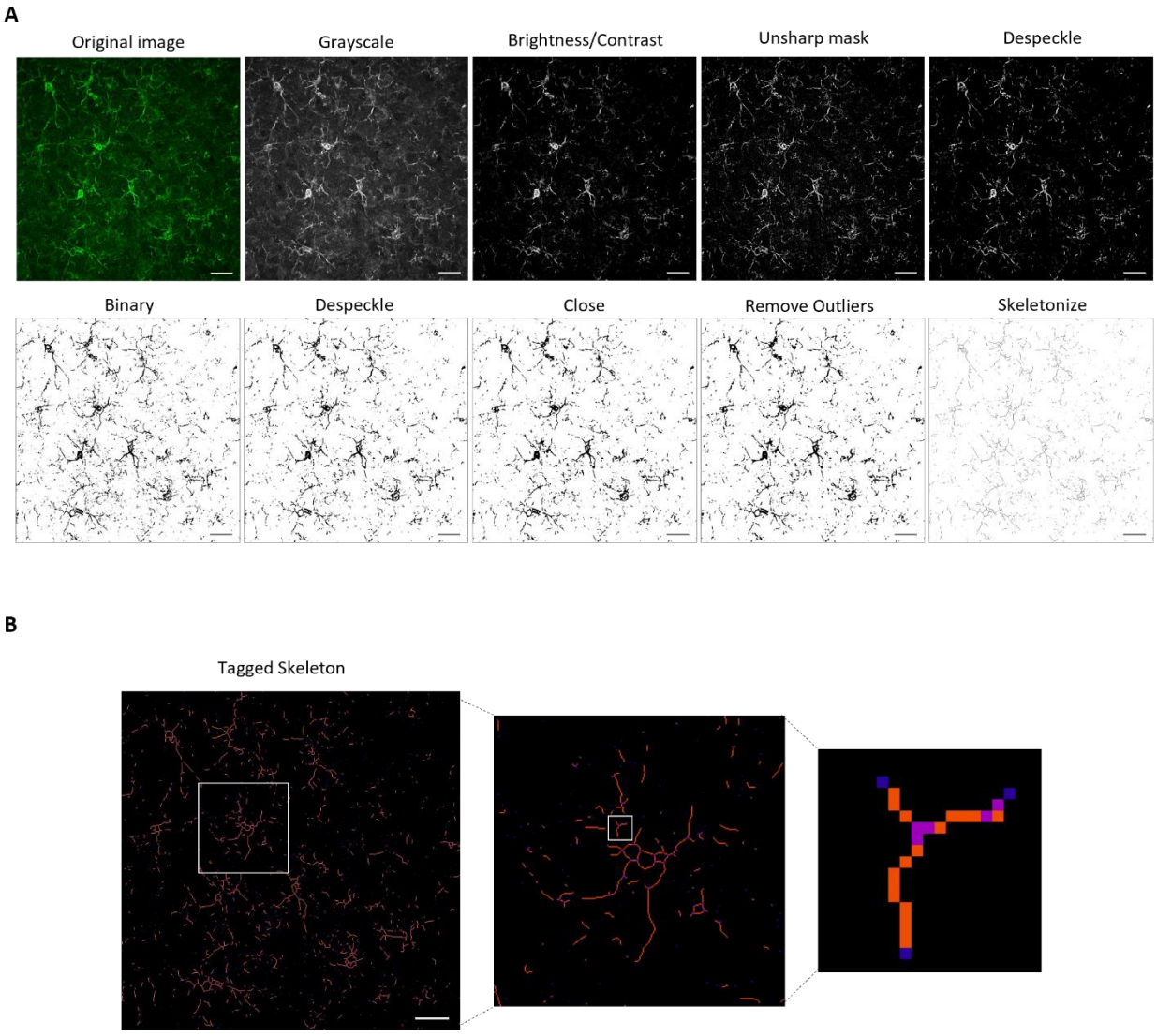

Figure 1. Skeleton analysis of microglia morphology in Iba-1-stained tissue. A) Photomicrograph conversion for skeleton analysis. Original photomicrographs were subjected to a series of ImageJ plugin protocols prior to conversion to binary images, which were then skeletonized. B) Processed skeletonized images using the Analyze Skeleton plugin identify skeletonized processes as orange, endpoints as blue, and junctions as purple. Scale bar = 20 µm.1

2.6. Statistical Analysis

All data are given as arithmetic means ± S.E.M. Differences between groups were statistically tested using the software GraphPad Prism (version 9.5.0, GraphPad Software, Inc., La Jolla, CA, USA) with confidence intervals of 0.05. *p*-values of ≤ 0.05 were considered to be statistically significant. Significant outliers were excluded from the densitometrical analyses using the Grubbs' test (alpha = 0.05). The distribution of continuous variables was analyzed using the Kolmogorov-Smirnov test to assess significant deviations from normality. One-way analysis of variance (ANOVA, followed by Bonferroni's test for multiple comparisons) or the nonparametric Kruskal-Wallis test (followed by the Dunn's test for multiple comparisons) were used for normally or non-normally distributed data, respectively.

Chapter III | RESULTS

3.1. Total Phenolic Content and Total Antioxidant Capacity

Following 1 year of storage at room temperature, the observed total phenolic content was 237,90 ± 22,87 mg of GAE/g BB biomass and total antioxidant capacity was 50.95 ± 0,06 mg of AAE/g BB biomass. These observations hint for BB biomass stability upon storage at room temperature.

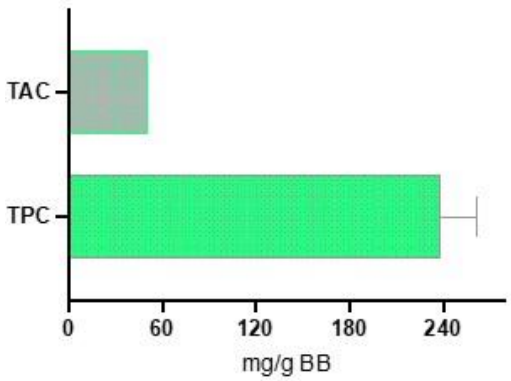

Figure 2. Total phenolic contents (TPC) and total antioxidant capacity (TAC) of blueberry leaves biomass (BB) following 1 year of storage at room temperature. TPC was determined upon ABTS assay and expressed in terms of mg of gallic acid equivalents per 1g BB; TAC was determined upon Folin assay and expressed in terms of mg of ascorbic acid equivalents per 1g BB. Results are expressed as mean ± S.E.M of 4 assays.

3.2. Colonic Inflammation

The degree of gut inflammation in the colon was evaluated during the demyelination (W5) and remyelination (W7) peaks, using four parameters: goblet cell (GC) loss, mucosal hyperplasia, cellular infiltration, and overall colonic inflammation.

A tendency for increased GC loss and mucosal hyperplasia (Figure 3b-c) was found following five weeks of CPZ, which persisted upon BB treatment. However, these increases did not reach statistical significance. In the remyelination phase, these two parameters showed the same tendency (Figure 3g-h).

CPZ intoxication seemed to promote cellular infiltration in the colon, as demonstrated by the increased number of infiltrating cells in the CPZ groups in relation to controls.

Despite the lack of statistical significance, a substantial reduction in the number of inflammatory cells was observed in the BB-treated group during both disease stages, as

illustrated by Figure 3-d. The tendency for decreased cellular infiltration was found more pronounced following seven weeks of BB treatment (Figure 3i.).

Finally, the significantly higher colonic inflammation scores of the CPZ-intoxicated animals appeared to be counteracted by BB, highlighting its protective effect against inflammation (Figure 3e-j).

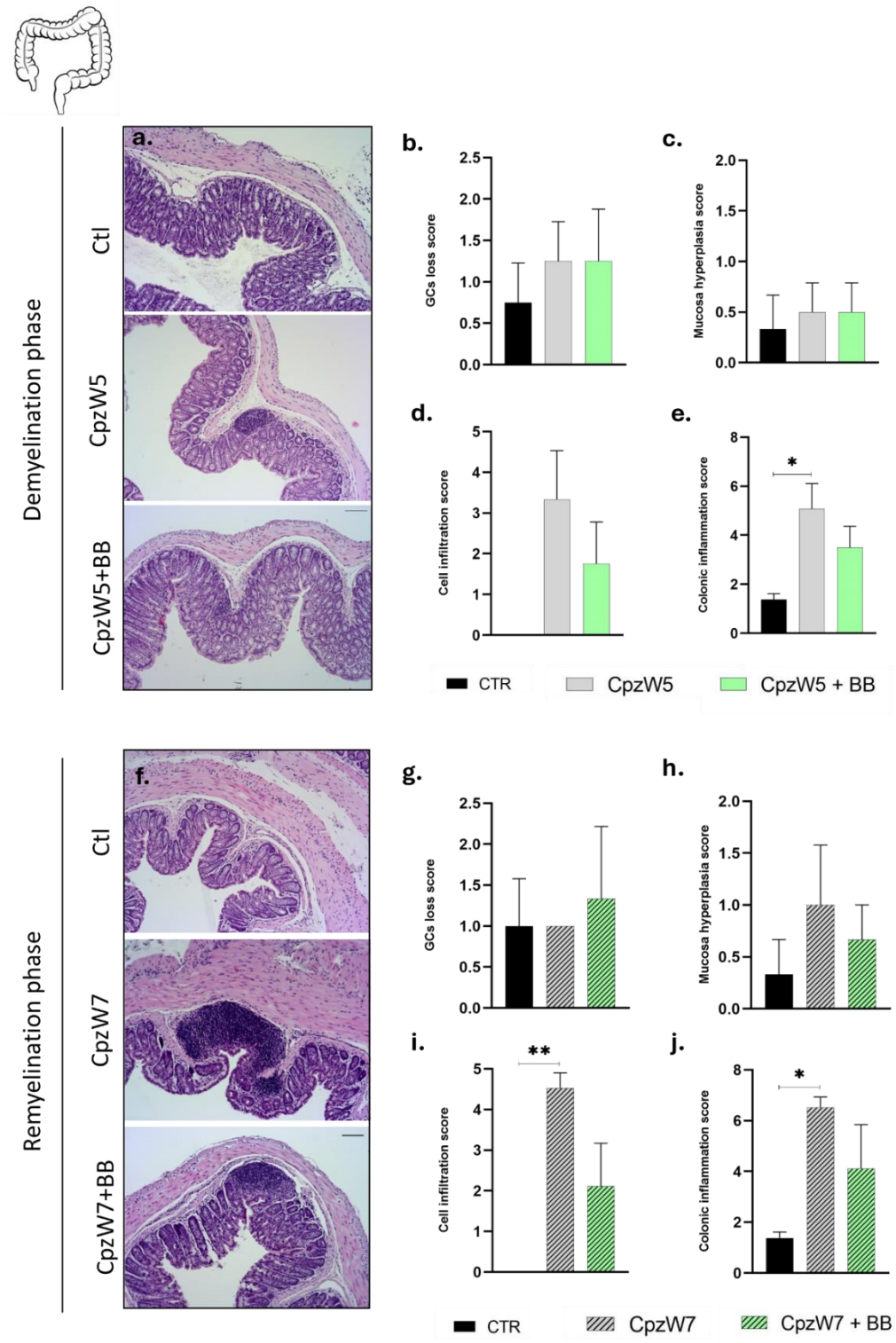

Figure 3. Gut inflammation score in the colon. Representative HE images of colon samples of the control (Ctl), cuprizone-fed (CpzW5 and CpzW7) and BB-administered (CpzW5+BB and CpzW7+BB) groups at the demyelination peak (**a**) and remyelination phase (**f**) (scale bar = 100 µm). Goblet cell loss score at the demyelination peak (**b**) and remyelination phase (**g**); Mucosa hyperplasia score at the demyelination peak (**c**) and remyelination phase (**h**); Cell infiltration score at the demyelination peak (**d**) and remyelination phase (**i**); Colonic total inflammation score at the demyelination peak (**e**) and remyelination phase (**j**); . Results are expressed as mean ± S.E.M of 3-4 animals per group. One-way ANOVA * $p<0.05$ vs CTR; ** $p<0.01$ vs CTR.

3.3. Myelinated Area Percentage in Central Nervous System

In the cerebellum, it was observed that during the demyelination phase (Figure 4b), the percentage of myelinated area was relatively low compared to the CTR group, with a significant difference observed between the CpzW5 and the Ctl groups ($p<0,05$ compared to Ctl). It is also important to mention that there was a slight increase in myelin percentage in the mice treated with BB biomass. In the remyelination phase (Figure 4e), the observed increase in myelin percentage within the CPZ group, despite being lower than the controls, suggests a partial recovery when compared to the more pronounced loss during the demyelination phase. The fact that this reduction in myelin levels does not reach statistical significance implies that the remyelination process is relatively robust, showing a trend toward recovery. Additionally, the introduction of BB appears to facilitate or accelerate this process, as it brings the myelin closer to basal levels, further indicating its potential role in promoting remyelination.

Although the CPZ-induced myelin loss in the corpus callosum persists in the intoxicated groups at both disease stages (Figure 4c,f), it is less intense when compared to the observed in the cerebellum, suggesting that different brain regions display distinct myelination patterns. Furthermore, in this cerebral region the beneficial effects of BB on promoting myelin recovery seem to be diminished or less effective, pointing to a potential region-specific effect from BB.

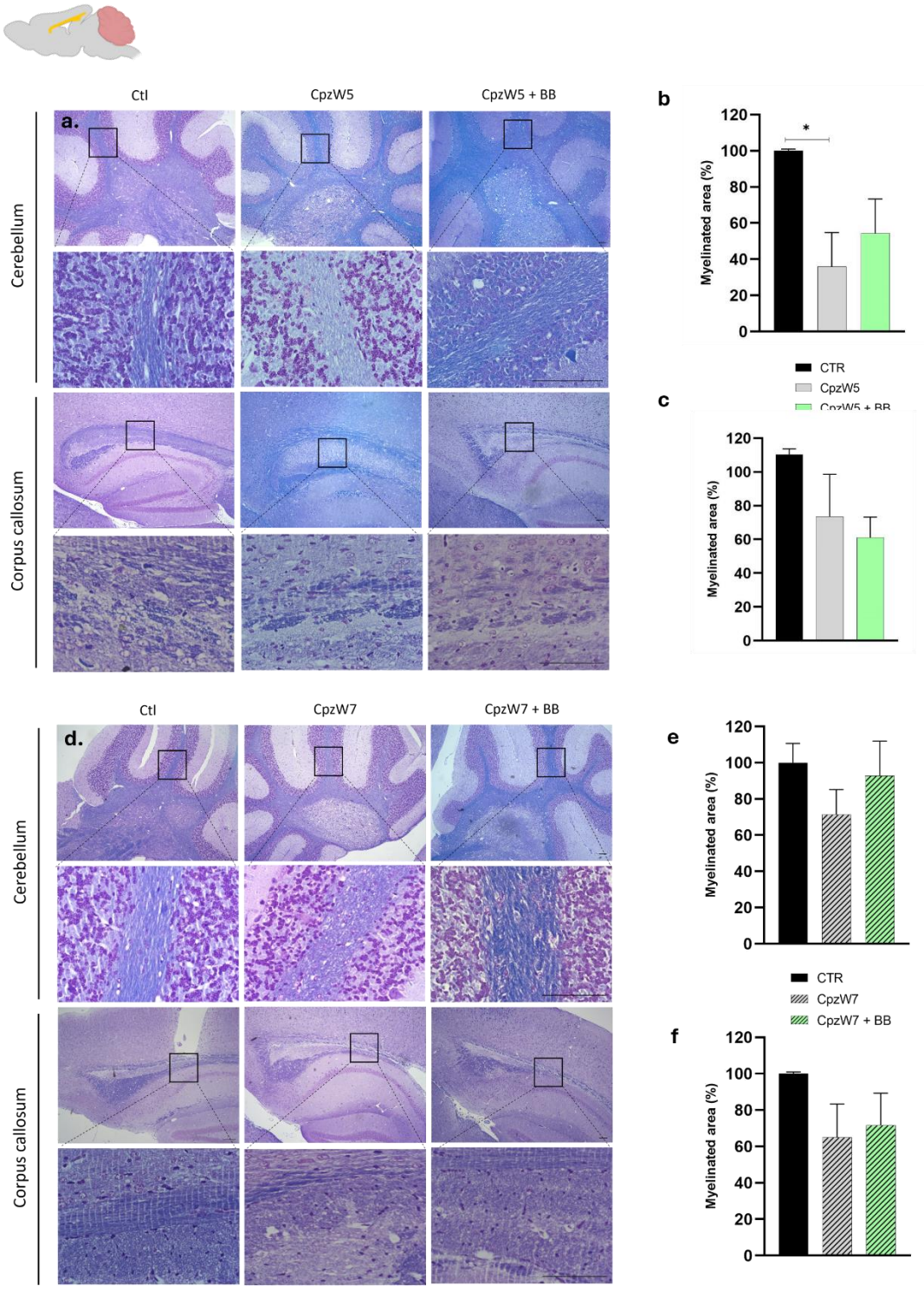

Figure 4. Myelination score. Kluver-Barrera (KB) staining of sagittal brain slices of the control (Ctl), cuprizone-fed (CpzW5 and CpzW7) and BB-administered (CpzW5+BB and CpzW7+BB) groups in the cerebellum **(red)** and corpus callosum **(orange)** (scale bar = 100 µm) at **(a)** the demyelination peak and **(d)** remyelination phase. Percentage of myelinated area of the cerebellum and corpus callosum in the demyelination peak (**b and c**, respectively) and remyelination phase (**e and f**, respectively). Results are expressed as mean ± S.E.M of 3-6 animals per group. One-way ANOVA * $p<0.05$ vs CTR.

3.4. Microglia Reactivity in Central Nervous System

Microglia reactivity was assessed during the demyelination and remyelination phases, using three microglial skeletal measures: process number, process length, and endpoint voxels. Morphological analysis was performed in the corpus callosum, cortex and hippocampus.

A substantial difference was observed between these two stages of treatment. After five weeks, there were no significant differences in any of the analyzed parameters between the three experimental groups in any of the evaluated regions (Figures 5-7; b-d). However, during the remyelination phase, there was an exponential increase in these measures upon treatment with BB biomass, showing extremely significant differences in the three analyzed brain areas, these effects being more expressive in the cortex and in the hippocampus regions (Figures 5-7; f-h). The observed outcomes highlight that microglia cells are highly reactive in response to BB treatment in the remyelination phase, suggesting they are in an inflammatory state, favorable for myelin debris' phagocytosis and consequent remyelination.

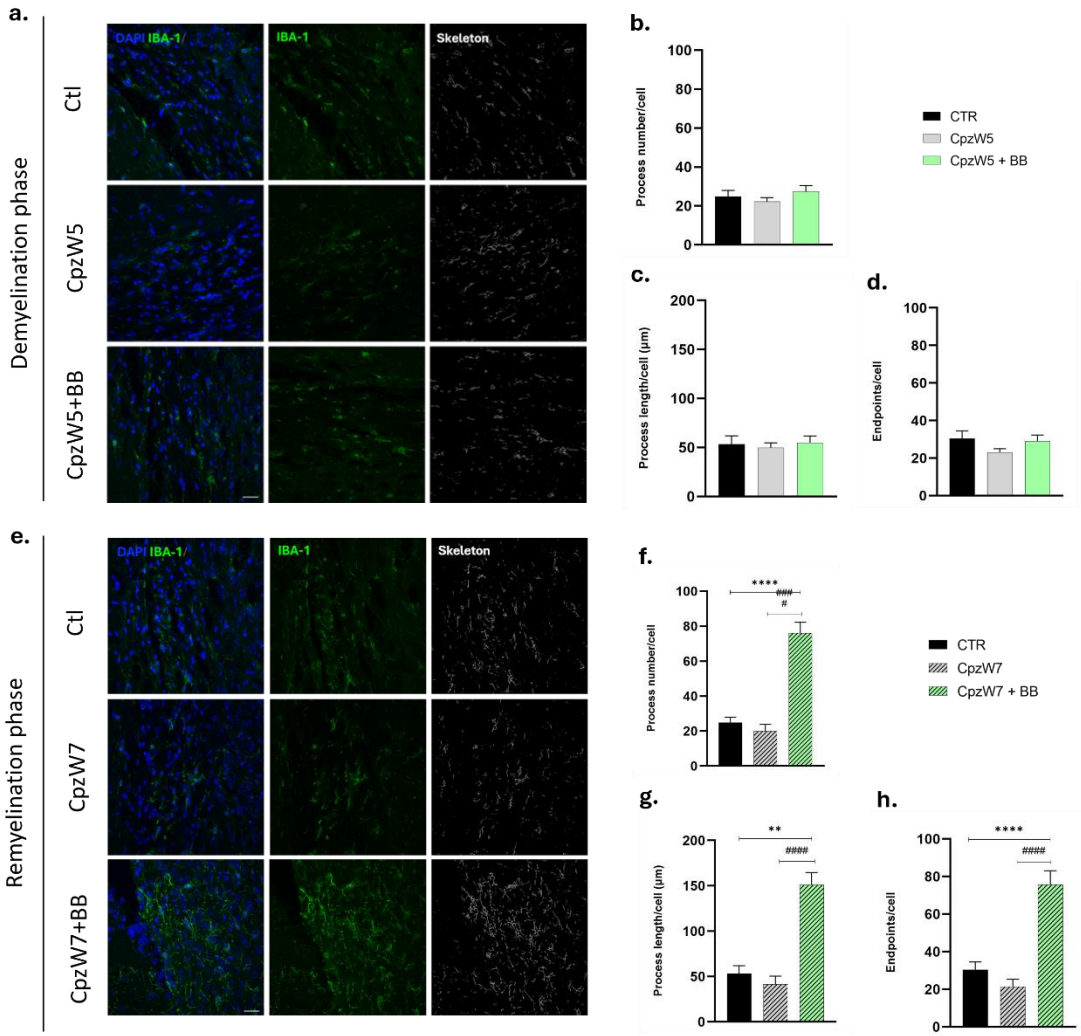

Figure 5. Microglia reactivity in corpus callosum. Specimen confocal images acquired with 40X objective of cryostat brain slices of the control (Ctl), cuprizone-fed (CpzW5 and CpzW7) and BB-administered (CpzW5+BB and CpzW7+BB) groups immunolabelled for Iba-1, showing the evolution of microglial activation in the corpus callosum at the demyelination peak **(a)** and remyelination phase **(e)** (scale bar = 20 µm). Quantification of microglial skeletal parameters, including process number at demyelination **(b)** and remyelination **(f)**; process length at demyelination **(c)** and remyelination **(g)**; and endpoints at demyelination **(d)** and remyelination **(h)** in the corpus callosum. Results are expressed as mean ± S.E.M of 3 animals per group. One-way ANOVA * p<0.05 vs CTR; ** p<0.01 vs CTR.

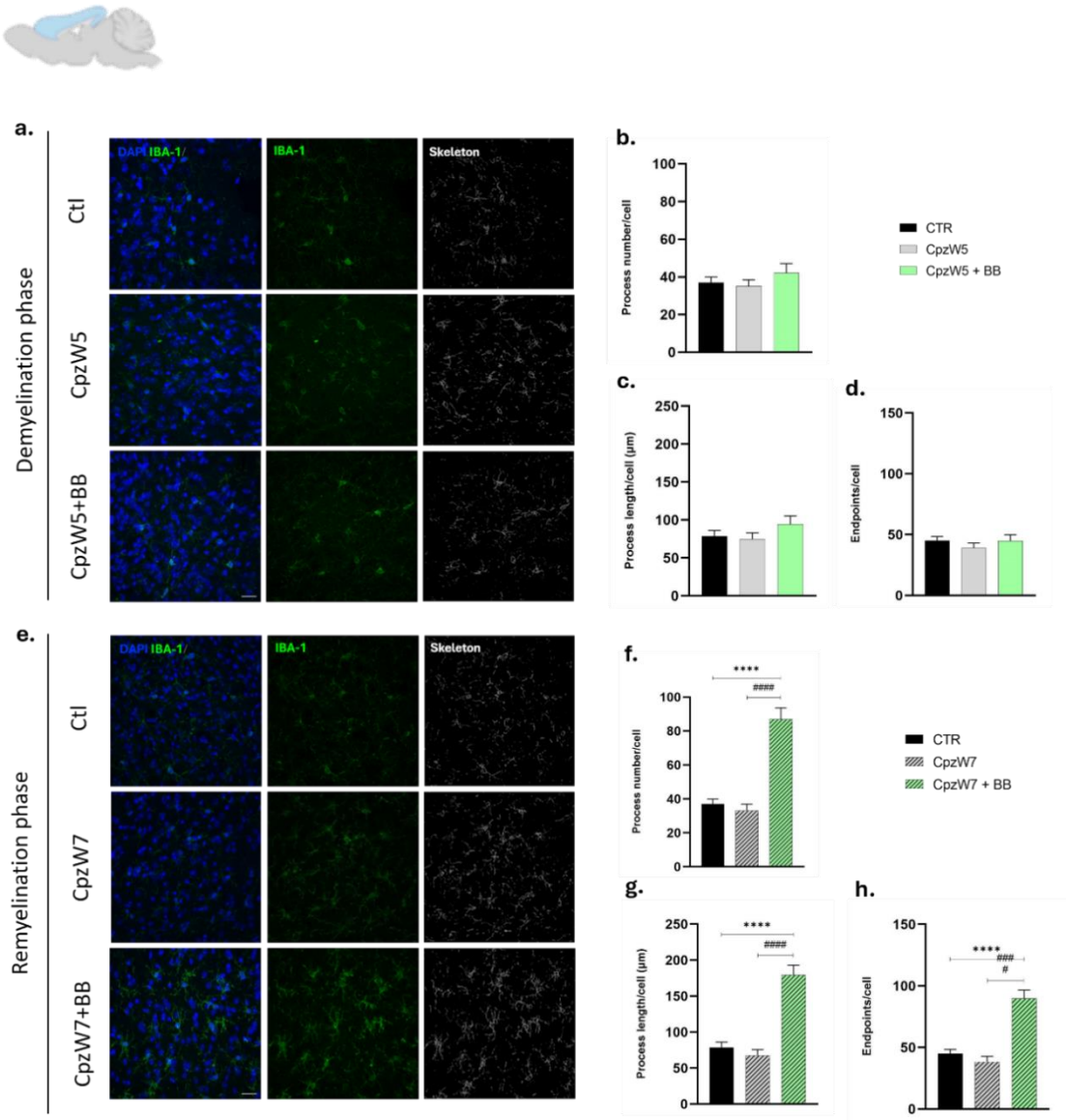

Figure 6. Microglia reactivity in cortex. Specimen confocal images acquired with 40X objective of cryostat brain slices of the control (Ctl), cuprizone-fed (CpzW5 and CpzW7) and BB-administered (CpzW5+BB and CpzW7+BB) groups immunolabelled for Iba-1, showing the evolution of microglial activation in the cortex at the demyelination peak **(a)** and remyelination phase **(e)** (scale bar = 20 µm). Quantification of microglial skeletal parameters, including process number at demyelination **(b)** and remyelination **(f)**; process length at demyelination **(c)** and remyelination **(g)**; and endpoints at demyelination **(d)** and remyelination **(h)** in the cortex. Results are expressed as mean ± S.E.M of 3 animals per group. One-way ANOVA * p<0.05 vs CTR; ** p<0.01 vs CTR.

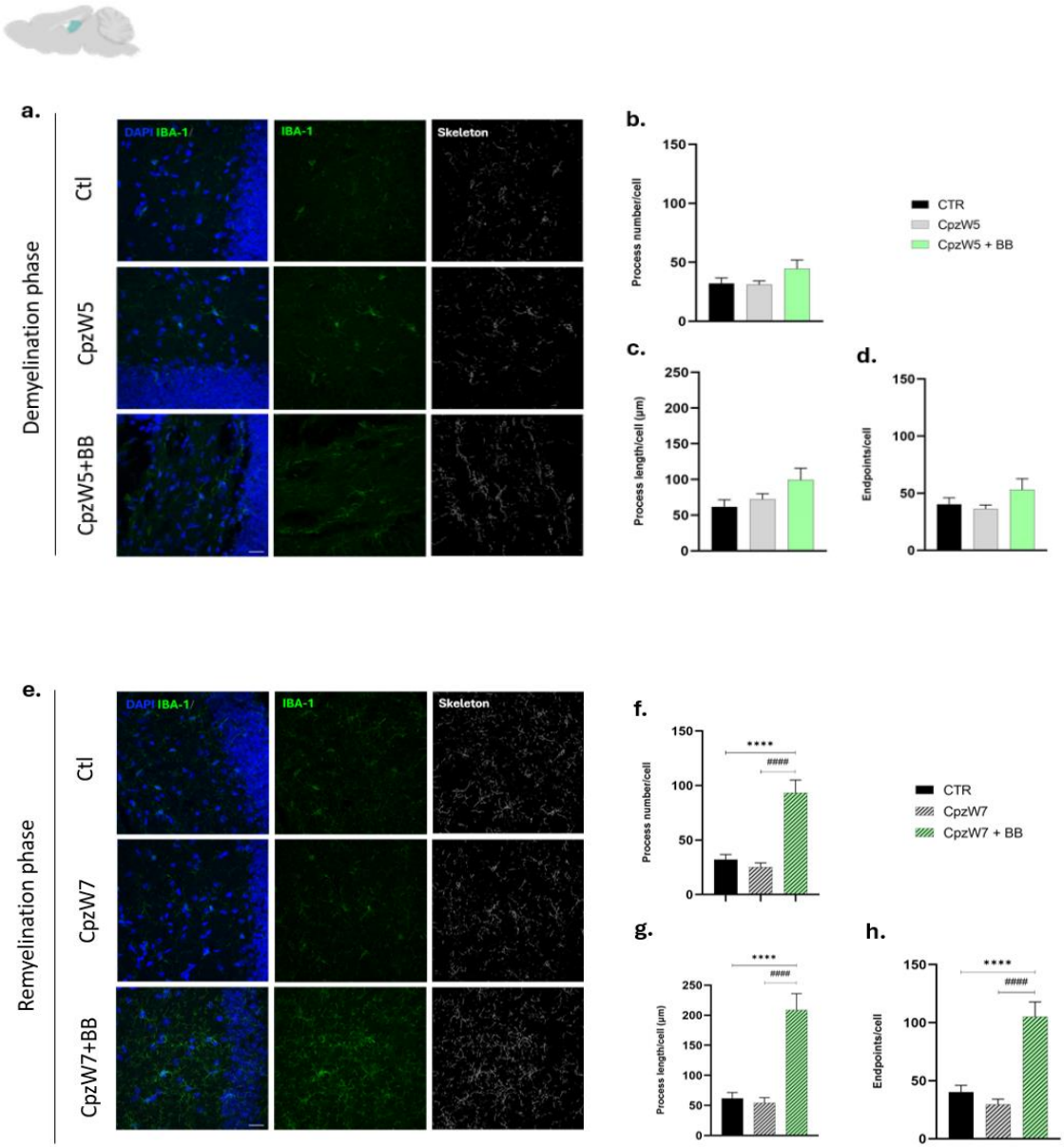

Figure 7. Microglia reactivity in hippocampus. Specimen confocal images acquired with 40X objective of cryostat brain slices of the control (Ctl), cuprizone-fed (CpzW5 and CpzW7) and BB-administered (CpzW5+BB and CpzW7+BB) groups immunolabelled for Iba-1, showing the evolution of microglial activation in the hippocampus at the demyelination peak **(a)** and remyelination phase **(e)** (scale bar = 20 µm). Quantification of microglial skeletal parameters, including process number at demyelination **(b)** and remyelination **(f)**; process length at demyelination **(c)** and remyelination **(g)**; and endpoints at demyelination **(d)** and remyelination **(h)** in the hippocampus. Results are expressed as mean ± S.E.M of 3 animals per group. One-way ANOVA * p<0.05 vs CTR; ** p<0.01 vs CTR.

Chapter IV | DISCUSSION AND CONCLUDING REMARKS

Discussion and Concluding remarks

While current MS therapies target symptom relief, modulating immune responses, and slowing disease progression, a true solution that effectively addresses the core issue of demyelination and promotes remyelination remains elusive.

Nutraceuticals, particularly plant-based compounds, offer a promising avenue for managing neurodegenerative diseases due to their broad range of bioactive properties.

For example, blueberries have been studied for their ability to prevent oxidative stress, inhibit inflammation, and improve vascular health. These benefits could theoretically extend to protecting neural tissue and promoting remyelination, crucial for managing diseases like MS (Erlund et al., 2008; Nair et al., 2014; Sweeney et al., 2002).

Interestingly, in addition to the fruit, other parts of the plant, such as the leaves—especially the senescent ones—are proving to be valuable sources of bioactive compounds with therapeutic benefits. However, human consumption of leaves is generally limited to infusions and decoctions due to their high content of indigestible components (such as structural polysaccharides in the plant cell wall), which hinders the absorption of phytochemicals in the gastrointestinal tract. Since PCs are sensitive to heat, these methods come with various drawbacks, including the need for high temperatures that can lead to PC degradation and only a limited portion of the compounds being accessible to the body. To overcome these challenges, a biotechnological process for leaf treatment was developed, resulting in a leaf biomass with an enhanced range of bioactive compounds that offer significant health benefits. Additionally, this novel process preserves heterosidic forms of PCs, such as quercetin-3-O-rutinoside (rutin), which are known for their efficient transport across the blood-brain and blood-retinal barriers, improving central bioavailability. Previous results from our group have confirmed that BB displays increased anti-oxidant capacity and prebiotic potential in comparison to the fruit. Furthermore, its rich content in PCs with increased central bioavailability highlights its potential as a nutraceutical in the context of neurodegenerative diseases such as MS.

Thus, the aim of this study was assessing the BB as a nutraceutical during the remyelination phase resorting to the cuprizone-induced demyelination model, involving BB

administration at a strategic timepoint - the peak of oligodendrocyte precursor cells proliferation – in order to evaluate its pro-remyelinating effects.

MS is highly heterogenous, and no single animal model perfectly replicates all aspects of the disease. However, there are three main types of animal models that have provided critical insights into different components of MS pathogenesis: Experimental Autoimmune Encephalomyelitis (EAE); Viral-induced Models (Theiler's Murine Encephalomyelitis Virus – TMEV), and Toxin-Induced Models (Cuprizone (CPZ)-induced demyelination) (Procaccini et al., 2015; Torkildsen et al., 2008).

Indeed, the EAE model for MS is the most extensively studied, where autoimmunity is induced by immunizing genetically susceptible mice with CNS-derived self-antigens, such as myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG). These proteins are the key components of the myelin sheath, and their presence in the immunization triggers an immune response that mimics the autoimmune attack seen in MS (Procaccini et al., 2015). A study using this model highlighted the significant role of the intestinal mucosal barrier in the progression of EAE (Nouri et al., 2014). This research demonstrates that intestinal changes, such as increased permeability, overexpression of the tight junction protein zonulin, and alterations in intestinal structure (e.g., increased crypt depth and submucosal and muscularis thickness), occur before the onset of neurological symptoms. The reported changes were accompanied by an infiltration of proinflammatory Th1/Th17 cells and a reduction in regulatory T cells in critical immune sites such as the gut lamina propria, Peyer's patches, and mesenteric lymph nodes. Interestingly, these intestinal changes occurred both through active immunization and adoptive transfer of autoreactive T cells, indicating that they were a direct result of immune system activation in EAE (Nouri et al., 2014).

Due to the presence of intestinal dysfunction in the EAE model, in this study, we chose to investigate the intestinal component in the Cuprizone (CPZ) model, as no prior research has explored this area. It should also be noted that the CPZ model is particularly valuable because it has a biphasic nature: replicates both demyelination and remyelination processes (Wergeland et al., 2012). Given this completeness, the Cuprizone model offers a unique opportunity to explore both the degenerative and regenerative phases of disease, extending our understanding of how peripheral systems, such as the intestinal component,

might influence these processes over time. While it primarily targets the CNS, the intestinal aspect remains unexamined. The intestinal component of the CPZ-induced demyelination model still remains controversial, since some studies suggest that CPZ does not pass through the intestinal mucosa (Benetti et al., 2010; McMahon et al., 2002),while others have reported otherwise (Berghoff et al., 2017);(Abakumova et al., 2023);(Zhang et al., 2023). Given the growing evidence of the gut-brain axis and the involvement of the intestinal barrier in neuroinflammatory diseases like MS, our investigation sought to determine whether intestinal alterations accompany the central impairments characterizing the disease model.

In our experimental study, we assessed inflammation in the colon in both disease phases: demyelination and remyelination. In the demyelination peak, increased inflammation and loss of GCs were observed in the CPZ-intoxicated animals in comparison to controls. Although inflammation persisted in the remyelination phase there was some recovery of GCs. Regarding the action of our biomass, BB did not seem to protect against the loss of GCs nor prevent hyperplasia, but significantly reduced intestinal inflammation in both phases. This suggests that while BB may not directly prevent damage to certain cell types (e.g., GCs) or prevent structural changes like hyperplasia, it has anti-inflammatory properties that could be beneficial in managing the overall inflammatory response in the colon.

The subsequent stage of the study focused on central components, namely the percentage of demyelination, with a particular emphasis on two regions: the corpus callosum and the cerebellum. The corpus callosum is referred to as the classic region to access myelination due to its high myelin content, while the cerebellum was selected based on the findings of previous studies conducted by technical colleagues which highlighted the significance of examining this region (Palavra et al., 2022). Myelin quantification revealed a reduction in percentage in the cerebellum at the demyelination peak, as anticipated. With regard to BB, it was observed that it does not protect against demyelination, so it’s not beneficial in the episode of outbreak. However, in the remyelination phase, BB seems to enhance recovery, a trend that has been corroborated by complementary experimental techniques.

Finally, from a pathophysiological perspective, it is crucial to investigate microglia cells, as they are integral to neuroinflammation, a process that is markedly evident in MS. One of the objectives of our study was to characterize the state of microgliosis in the two phases. We started by looking at the fluorescence intensity, however, this is a very limited parameter in terms of obtaining information, so we further analyzed cell morphology to provide a deeper understanding of the state of cell activation, especially in contexts like neuroinflammation or remyelination.

Microglia in healthy adult brain tissue typically display a ramified morphology, characterized by small somas and highly branched processes. However, they are capable of undergoing significant and rapid morphological changes in response to various stimuli, such as injury, infection, or neurodegeneration (Savage et al., 2019). Microglia can adopt several morphologically distinct forms: ramified; bushy (reactive); active; amoeboid; and rod-shaped, each reflective of specific states of activity and functions(Taylor et al., 2014). In steady-state conditions, ramified microglia are the most common, where they are highly dynamic, constantly surveying their environment through thin, branched processes. Following mild to moderate insults, microglia transition into bushy, reactive forms with thickened and retracted processes, suggesting early activation. In the event of more severe damage, microglia fully activate, retract their processes further, and adopt an amoeboid shape, becoming indistinguishable from macrophages. Finally, typically seen in response to brain injuries, rod-shaped may reflect specific polarized states associated with certain neurodegenerative processes. This extensive morphological plasticity suggests microglia serve as crucial indicators of CNS health, shifting their form and function in response to different challenges (Savage et al., 2019; Taylor et al., 2014; Young & Morrison, 2018).

Microglia's remarkable plasticity allows them to dynamically shift between pro-inflammatory (M1-like) and anti-inflammatory (M2-like) phenotypes, each playing a vital role in different phases of remyelination. In the early stages of demyelination, M1-like adopt a more activated, producing cytokines like TNF-α, IL-1β, and IL-6. While these can initially exacerbate neuroinflammation, they also help to clear myelin debris and stimulate the recruitment of OPCs. As the repair process progresses, microglia transition toward a more anti-inflammatory phenotype, secreting factors like IL-10 and TGF-β that help resolve inflammation and promote tissue repair. They also produce growth factors, which support

OPC survival and differentiation into mature oligodendrocytes, facilitating remyelination (Chari, 2007; Mahmood & Miron, 2022). Given the multifaceted influence of microglia on remyelination, modulating their activation states becomes a promising therapeutic approach. Drug screens targeting microglial phenotypes could help identify compounds capable of enhancing their pro-regenerative functions while mitigating excessive inflammation (Mahmood & Miron, 2022).

In the remyelination phase, BB was found to significantly promote microglia reactivity as reflected in terms of increased branch number, length and endpoints. Conversely, no differences were observed in the demyelination phase. This pattern remained consistent across all analyzed cerebral regions, underscoring BB's ability to influence the entire brain. These morphological changes suggest that neuroinflammation is occurring.

The evidence strongly supports the idea that inflammation, particularly in the early stages, plays a pivotal role in promoting remyelination. The interaction between the immune system and remyelination is intricate, with inflammatory signals contributing to both the initiation of repair and the recruitment of cells necessary for myelin regeneration (Chari, 2007; Graça & Blakemore, 1986). The early and robust inflammatory responses in active lesions appear to be critical for efficient remyelination. This is seen in both experimental models and human MS lesions, where active inflammation is linked to better remyelination outcomes (Chari, 2007; Graça & Blakemore, 1986). The release of pro-inflammatory cytokines and chemokines at this stage helps to clear debris and activate endogenous repair processes. In a study using Affymetrix microarrays, inflammatory gene expression was notably upregulated during remyelination (Arnett et al., 2003). This suggests that specific inflammatory pathways are integral to initiating and sustaining the repair process (Arnett et al., 2003). The inflammatory environment likely serves as a cue for OPCs to differentiate and for the clearance of myelin debris, creating conditions conducive to remyelination. The impairment of remyelination in experimental models lacking lymphocytes, MHC-II, inflammatory cytokines, or macrophages indicates that immune components are not just contributors to tissue damage but are also essential for repair (Arnett et al., 2001; Arnett et al., 2003; Bieber et al., 2003; Kotter et al., 2001; Kotter et al., 2005; Mason et al., 2001). This dual role is especially evident in how certain immune

cells, like T cells and macrophages, contribute to the remyelination process (Bieber et al., 2003).

Our results suggest that neuroinflammation may play a beneficial role in the remyelination process, having synergistic effects. Regarding our biomass, BB's effects on inflammation might intersect with this aspect as well. If BB promotes beneficial microglial activation and enhances debris clearance, it could mitigate the inhibitory effects of myelin debris on OPC differentiation, potentially improving remyelination efficiency. Further studies will be necessary for a more in-depth investigation of these hypotheses, to better understand the mechanisms behind the observed effects.

Chapter V | REFERENCES

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