CHAPTER 1

General Introduction
Index

Multiple sclerosis
  Clinical symptoms and disease course
  Aetiology
  Pathology
  Treatment

Astrocytes
  Astrocytes: history and function
  Astrocytes in MS
  The role of Gilenya® and sphingolipids in astrocytes

Brain bioenergetics
  Glycolysis
  Mitochondria: TCA cycle and oxidative phosphorylation
  Transcriptional regulation of glucose metabolism
  Metabolic alterations in MS; evidence from imaging studies
  Metabolic alterations in MS; pathological evidence

Thesis aim and outline
MULTIPLE SCLEROSIS

Clinical symptoms and disease course

Multiple sclerosis (MS) is a chronic inflammatory disease, characterized by focal accumulation of immune cells which form demyelinated lesions throughout the central nervous system (CNS). MS is the leading cause of non-traumatic neurological disability among young adults affecting more than 2.5 million people worldwide. Commonly, first symptoms appear early in adulthood around the age of 20-40 years. These initial symptoms can vary and include sensory disorders, optic neuritis, double vision and limb weakness. As the disease progresses, symptoms also comprise fatigue, bladder dysfunction, paralysis and cognitive impairment. From the moment of diagnosis, average survival is reported to range from 20 to nearly 45 years.

MS can be divided into 3 distinct subtypes depending on the clinical progression of symptoms. Relapsing-remitting (RR) MS is characterized by periods of neurological deficits followed by recovery and is found in 80% of MS cases. In time, 65% of the RR patients develop the secondary progressive (SP) form of MS, in which neurological deficits become progressive and permanent. In 20% of the cases, the initial RR phase is absent and patients are characterized by a progressive disease course from onset, named primary progressive (PP) MS. Figure 1 illustrates the hallmarks of MS disease progression. There is a marked predominance of females among patients with MS. The sex ratios are 3:1 in RR and 3:2 in SP MS and females are predisposed to higher relapse activity. In contrast, the majority of primary progressive MS patients are male. Diagnosis of MS is based on clinical grounds, which includes neurological examinations and the clinical history of the patient. If the available information is inconclusive, magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis can be used for diagnosis. MRI visualizes MS lesions in the CNS, however, in order to diagnose MS, there needs to be a dissemination of MS lesions in both time and space. In addition, CSF analysis can be used to evaluate the presence of CNS-derived antibodies.

Aetiology

The aetiology of MS remains elusive, however several environmental, genetic and ethnic risk factors have been identified over the years. In general, Caucasians are more affected compared to Hispanic, Black, and Asian populations although a recent report suggests that the incidence of MS is increasing in black individuals. Familial studies have shown that the risk of developing MS is higher for relatives of MS patients although the exact numbers vary. Studies in monozygotic twins indicate that genetic factors contribute 20% to 60% to disease susceptibility depending on the region and study design. There are several genes associated with an increased risk of developing MS, the strongest associations include genes which are part of the major histocompatibility complex II (MHCII). Recently, 110 established MS risk variants were identified outside the MHC II loci, most of which are associated with the immune system. The impact of these factors is, however, in general low.

Interestingly, the lowest prevalence of MS is found around the equator, and the highest in Northern Europe and Canada affecting up to 1:500 people. The risk of developing MS decreases when people migrate from high-risk regions to low-risk regions during childhood and vice versa.
Based on the low prevalence of MS around the equator, it was hypothesized that exposure to sunlight might be associated with a reduced risk of developing MS. Sunlight is essential to metabolize vitamin D into its bioactive form after it is taken up by the diet. Vitamin D is immunosuppressive and high vitamin D serum levels early in life are associated with a reduced risk of developing MS in white populations. Moreover, treatment with bioactive vitamin D alleviates the symptoms in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Although vitamin D supplements are often prescribed to MS patients, properly designed clinical trials are needed to determine whether vitamin D supplements actually may be an effective treatment.

Viral factors may also contribute to MS susceptibility. Infection with Epstein-Barr virus (EBV) in the first two decades of life increases the risk of developing MS. Moreover, EBV seropositivity in MS patients is 99-100% compared to 90-95% in controls. Other viruses associated with MS onset, include human endogenous retroviruses (HERV). HERVs have accumulated in the DNA of most mammalian species over time and nowadays make up 8% of the total human DNA. Most of these viruses are inactive and no longer capable of producing virus. However, some HERVs can be reactivated by other viral infections including EBV, leading to the production of new viral particles. HERV-derived viral particles have been found in MS brain and when detected in MS cerebrospinal fluid, it correlates with clinical progression and prognosis. Enhanced expression of these viral particles leads to immune activation and as such may contribute to MS development and progression.

Interestingly, it has been proposed that infectious agents, which are more common in low risk areas, rather dampen than worsen the clinical course of MS. For instance, it is known that parasites, such as helminths, secrete immunosuppressive factors and thereby influence disease symptoms. In fact, clinical trials with eggs of the helminth Trichuris suis are currently ongoing. Finally, environmental factors, including cigarette smoking and diet can also influence the risk of developing MS.

Pathology
MS is characterized by the formation of demyelinated lesions throughout the CNS in both white and grey matter. Depending on which brain area is affected, newly formed lesions may lead to a wide variety of clinical symptoms. Histopathologically, several distinct MS characteristics can be distinguished. The normal appearing white matter (NAWM) encompasses the myelinated white matter.
matter of MS patients without obvious signs of inflammation. Nevertheless, subtle changes in the NAWM, including microglial activation, have been detected. In many cases, clusters of activated microglia can be found in the NAWM, which have been suggested to indicate new lesion formation and are therefore called pre-active lesions. However, this concept is still debated and most of the microglial clusters are thought to resolve without forming a lesion.

Active demyelinated lesions are characterized by blood brain-barrier breakdown, accumulation of myelin-laden macrophages and T cells throughout the lesion area. T-cells are generally considered the most important immune cell in the pathogenesis of MS. Mostly because a MS-like disease can be induced in animal models by adoptive transfer of myelin-reactive T cells. Moreover, autoreactive T-cells against myelin components are found in MS patients. However, it is still debated whether entry of autoreactive T-cells is a primary event leading to lesion formation or secondary as a consequence of oligodendrocyte and axonal degeneration. Macrophages and activated microglia in active MS lesions produce large amounts of pro-inflammatory cytokines and reactive oxygen species (ROS), which contribute to cellular injury (Figure 2). ROS are highly reactive compounds secreted by immune cells to kill pathogens, but when produced in excessive amounts can lead to oxidative damage to lipids, proteins and DNA of surrounding cells. ROS can be synthesized by dedicated enzyme systems like the membrane bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADH oxidase generates superoxide by transferring electrons from NADPH to oxygen molecules. Increased levels of ROS contribute to oligodendrocyte- and axonal loss in active lesions.

Active lesions can be divided in early active and late active lesions, which are distinguished by the presence of myelin protein positive inclusions in early active lesions and macrophages containing neutral lipids in late active lesions. Axon transaction is found throughout the brain of MS patients, even in the NAWM, but occurs mainly in active lesions leading to the formation of terminal axon ovoids or bulbs. Acute axonal injury as indicated by accumulation of proteins such as amyloid precursor proteins (APP) is also best correlated with inflammation.

In time, active lesions convert into chronic active lesions in which ongoing demyelination by macrophages and microglia is limited to the rim of the lesion (Figure 2). In the centre of chronic active lesions, immune cells are absent and demyelination is complete. The chronic lesion center is populated with large hypertrophic astrocytes, which form a dense network referred to as the glial scar (Figure 3E). This gliotic scar forms a physical barrier to avert further demyelination but

Figure 2. MS lesion classification. Active lesions are characterized by loss of myelin (proteolipid protein (PLP) staining) and abundant MHCII positive immune cells (B). Chronic active lesions are also demyelinated (C), but MHCII positive macrophages are mainly found at the rim of the lesion and absent in the demyelinated lesion center (D)
also blocks remyelination\(^{42}\). Axonal degeneration continuous in chronic lesions albeit at a slower rate, and is considered the best pathological hallmark to correlate with disease disability\(^{39}\). MS lesions can be remyelinated over time thereby restoring axonal function, although the amount of remyelination can vary between patients. Remyelination, together with brain functional reorganization, the process in which healthy brain regions take over the function of damaged areas, may explain why most symptoms do not remain permanent in the initial stage of the disease\(^{43}\). It is thought that the ability of the brain to remyelinate and reorganize, decreases over time contributing to the progressive nature of the disease\(^{44,45}\).

Grey matter (GM) lesions lack obvious blood-brain barrier breakdown, leukocyte infiltration and astroglisis, therefore these lesions are discriminated on the basis of location\(^ {46}\). Type I GM lesions are also known as leukoaraiotic lesions and encompass both white and grey matter. Type II GM lesions do not touch the pial surface nor the WM border. Type III lesions are subpial and type IV lesions cover the whole width of the cortex\(^ {47,48}\). Particularly in the progressive phase of the disease GM atrophy becomes prominent. The histopathological correlate of cortical thinning remains unclear, but loss of axons, neurons and astrocyte processes throughout the cortex have been reported\(^ {49-51}\). To date, it remains unknown what drives GM lesion formation and consequent neurodegeneration. WM inflammation may contribute to GM pathology as correlations between WM lesion load and GM atrophy have been found\(^ {52,53}\). Moreover, cortical atrophy is most profound in areas that are highly interconnected with other brain areas. These brain areas depend on functional WM tracts to connect to other areas. As such, WM lesions will have a greater effect on neuronal function of these brain areas compared to areas which mainly process local input\(^ {54}\). Over time the extent of GM atrophy increases whereas the number of newly formed lesions diminishes. As a result, GM atrophy correlates better with disease progression than WM lesion load\(^ {55}\). It is therefore likely that disease processes, other than WM pathology, contribute to cortical atrophy\(^ {56}\). Meningeal inflammation, characterized by large numbers of B-cells and macrophages, is apparent in several histopathological studies and might contribute to GM pathology\(^ {57-60}\). In fact, the extent of meningeal inflammation was shown to correlate with both microglial activation and cortical demyelination. Leukocytes in the meninges release pro-inflammatory molecules which can diffuse into the GM and induce microglial activation and demyelination\(^ {61}\). Finally, astrocytes may also contribute to GM atrophy since a loss of astrocytes and astrocyte end-feet is observed in MS GM. Moreover, astrocyte loss is most prominent in patients with substantial meningeal inflammation\(^ {59}\). The role of astrocytes in MS will be discussed in more detail in section 2.

**Treatment**

Although the aetiology of the disease remains unknown a wide range of treatment options are currently available. Generally as a first line disease modifying compound (DMC), interferon-β (INF-β) or Copaxone® (Glatiramer acetate) are prescribed, both immunomodulatory drugs\(^ {62}\). If these treatments are ineffective, Tysabri® (Nataluzimb) can be administered; a monoclonal antibody against adhesion molecule very late antigen-4 (VLA-4), which prevents entry of immune cells into the brain. Although Nataluzimb is highly effective in reducing lesion formation and relapses it increases the risk of opportunistic brain infections. Recently, Gilenya® (FTY-720P) and Tecfidera® (dimethyl fumarate) have been approved for the treatment of RR MS. Gilenya® blocks
the egression of lymphocytes from the lymph nodes and Tecfidera® modulates immune cell responses and suppresses pro-inflammatory cytokine production through unknown mechanisms. Additionally, Gilenya® and Tecfidera® may also target the brain directly. Tecfidera® can activate nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor which activates genes with an antioxidant redox element (ARE) thereby increasing the cell’s antioxidants capacity which has been shown to be neuroprotective63. The effects of Gilenya® on the CNS will be discussed in more detail in section 2.3. Most therapies currently available are aimed at dampening the inflammatory response in MS and can be effective in the RR phase of the disease. Although some drugs also diminish axonal damage, reduce and stall progression, most patients still progress to SP MS64. Therefore, better diagnostic tools which would allow early treatment may improve the clinical outcome considerably. Nevertheless, treatments aimed at neuroprotection seem vital to limit disease progression.

ASTROCYTES

Astrocytes: history and function

Neuroglia are non-neuronal cells of the brain and were first described in 1858 by Virchow. He was the first to realize that in order to study and understand the CNS it is important to obtain knowledge on the substance (neuroglia) which lies between the neurons. Interestingly, to date, the neuroscience community remains mainly focused on neurons, whereas the importance of glial cells for the function of the CNS is repeatedly overlooked. In 1893, the term astrocytes (astron meaning a star, while kytos means a hollow vessel or a cell; thus, star-like cell) was first proposed by von Lenhossek65. At the end of the 19th century, drawings of various pathologists, including Golgi and Cajal, provided a detailed cellular morphological analysis of the brain which nicely illustrated the diversity of astrocytes in shape and size (Figure 3)65. Subsequently, different types of astrocytes were identified based on morphology and localization. Protoplasmic astrocytes are generally found in the GM. They contain many fine processes and contact blood vessels and neurons and form the glia limitans, the outermost layer of neural tissue. Fibrous astrocytes reside predominantly within the WM. These cells possess long processes, albeit not as elaborate as the protoplasmic astrocytes. Fibrous astrocytes contact blood- vessels and axons at the node of Ranvier. Other astrocyte populations are localized to specific brain regions, including; Müller cells in the retina. Bergmann astrocytes in the cerebellum, interlaminar astrocytes in the cerebral cortex of higher primates, and tanyocytes in periventricular areas66. Golgi postulated that astrocytes not only provide structure but also metabolic support to the brain because of their close contact with blood vessels. This was an excellent observation, as we now know that astrocytes cover >99% of the vasculature with their end feet and are important regulators of the blood-brain barrier67. Furthermore, astrocytes are in close contact with all cell types within the CNS to provide them with essential nutrients and maintain homeostasis68. Cajal was the first to develop an astrocyte specific staining by visualizing the expression of glial fibrillary acidic protein (GFAP), an intermediate filament. To date, GFAP is still the most widely used astrocyte marker, although the abundance of GFAP in astrocytes differs widely between different astrocyte populations. For example, only 15–20 percent of astrocytes in the cortex express GFAP.
Cajal also strongly believed that astrocytes participated in important brain functions and speculated that astrocytes were involved in sleep and wakefulness and even in executive brain functions such as attention. During evolution from mammals to primates, there is an enormous increase in density and complexity of astrocytes. The glia to neuron ratio has increased from 0.3 in rodents to 1.65 in humans. Moreover, astrocytes have become more complex and up to 30 times larger in the human brain compared to mice. This suggests that astrocytes could be important for the development of higher cognitive functions. Indeed, astrocytes are essential for proper neuronal function e.g. by the uptake and metabolism of neurotransmitters. Astrocytes form an extensive network through gap junctions allowing them to communicate swiftly via calcium waves, which is important to orchestrate synchronized neuronal firing. Moreover, several studies identified astrocytes as important players in sleep and memory formation, as Cajal predicted. Finally, it was recently shown that transplantation of human astrocytes into mouse brain resulted in improved learning across a range of behavioural tests, illustrating the significance of astrocytes to maintain higher cognitive functions. Thus, astrocytes are the most numerous and diverse cells in the brain. They provide structure, metabolic support and are also

Figure 3. Astrocyte morphology. A. Original astrocyte drawing by Ramón y Cajal (Cajal Legacy Institutò Cajal (CSIC)). B Original drawing by Retzius (1894-1916) of different cortical astrocytes in the frontal lobe from a 42 year old woman illustrating the heterogeneous astrocytic morphology (adapted from 12). C-E GFAP staining of post-mortem obtained MS brain. C. In the NAWM of MS patients anti-GFAP predominantly stains astrocytic processes. D Large reactive astrocytes in an active MS lesion have marked GFAP expression. E. In chronic MS lesions astrocytes form an astrocytic scar, where the thick processes are highly GFAP positive.
important for cognitive functions. We have only just started to unravel some of the essential functions astrocytes fulfil under normal conditions. Moreover, most studies focus on the relative simple rodent proteoplastic astrocytes, hence even less is known about the role of astrocytes in the human brain. Importantly, astrocytes also play a pivotal role under pathological conditions, which will be discussed in the following section.

**Astrocytes in MS**

Astrocytes play an eminent but dual role in MS. In active MS lesions, astrocytes become reactive, illustrated by increased GFAP expression (Figure 3). Reactive astrocytes can aggravate inflammation by secreting inflammatory molecules, thereby increasing vascular activation, which leads to enhanced expression of adhesion molecules and reduced expression of tight-junction proteins. This allows patrolling leukocytes to attach to the brain endothelium and migrate into the brain. Astrocytes have also been reported to be involved in myelin phagocytosis76,77. In chronic MS lesions, astrocytes form a hypertrophic scar, consisting of a fine network of tightly connected astrocyte processes surrounded by extracellular matrix (ECM) (Figure 3)78. The astrogial scar acts as a barrier that prevents inflammation to spread but it may also impede tissue repair. Astrocytes are actively involved in modelling the ECM by deposition of ECM proteins, such as fibronectin which inhibits remyelination79.

On the other hand astrocytes produce a plethora of protective proteins. They are a major cellular source of antioxidants and are thus essential for scavenging free radicals in MS lesions80. Moreover, astrocytes secrete factors that promote axonal regeneration and oligodendrocyte maturation and play an important role in clearing excess glutamate to minimize glutamate excitotoxicity81. It is nowadays accepted that reactive astrogliosis is initially beneficial and not merely detrimental as often implied. Various studies, aimed at untangling the role of reactive astrocytes in diverse disorders and models, have demonstrated that reactive astrocytes facilitate blood-brain barrier repair and secrete immunosuppressive and neuroprotective molecules82-84.

Astrocytes are also fundamental for the regulation of brain energy metabolism. Increasing evidence suggests there are widespread metabolic alterations in the brain of MS patients which likely contribute to the ongoing neurodegeneration, but the role of astrocytes in these processes remains elusive. Therefore, in the second part of this chapter, the role of astrocytes in brain bioenergetics during normal homeostasis and in MS will be described. Thus, astrocytes are involved in many different protective but also detrimental processes in MS. Hence, the dual role of astrocytes in MS makes them a complex, but nonetheless enthralling cell type to investigate.

**The role of Gilenya® and sphingolipids in astrocytes**

Gilenya® is a sphingosine-1-phosphate (S1P) analogue which binds S1P and initially activates S1P receptors (S1P1), but is paradoxically followed by S1P1 functional antagonism, whereby receptors are internalized85. Binding of S1P to the S1P1 receptor is an important signal for lymphocytes to egress from the lymph nodes. Interestingly, Gilenya can readily cross the blood-brain barrier and S1P receptors are widely distributed throughout the brain and particularly expressed by reactive astrocytes in MS lesions86,87. Moreover, Gilenya® was more effective in reducing the clinical score in EAE if astrocytes expressing S1P receptors are present88. These results imply that Gilenya®
may also target astrocytes directly. S1P signalling in astrocytes is increased under inflammatory conditions thereby enhancing proliferation, survival, migration, astrogliosis and nitric oxide (NO) production. Treatment of astrocytes with the bioactive compound of Gilenya®, FTY720P, increases migration and survival, but decreases astrogliosis, calcium release, production of the inflammatory cytokines and NO production by blocking nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) nuclear translocation. Thus, FTY720P seems to both agonize and antagonize S1P signalling. These effects are likely caused by inhibition of external S1P receptor activation while sustaining internal S1P−signalling.

S1P is not only a signalling molecule but is also part of cellular membranes and myelin. S1P is a sphingolipid, derived from the aliphatic amino alcohol sphingosine and displays great structural diversity and complexity. Changes in acetylation and substituents on the head group catalyzed by specific enzymes can form different types of sphingolipids, including sphingomyelin, ceramide, sphingosine and S1P, which together form the sphingomyelin cycle (Chapter 2, Figure 3). Similar to S1P, the different components of the sphingomyelin cycle can induce different cellular responses. Ceramide and sphingosine are, in contrast to S1P, associated with cell growth arrest, stress responses and apoptosis. Interestingly, alterations in the sphingolipid composition have been observed in MS tissue samples. Since Gilenya® modifies S1P signalling, it may also be involved in the regulation of sphingomyelin cycle products. Thus, besides its anti-inflammatory effects in the periphery, Gilenya® also represents an interesting astrocyte modulatory drug, which can manipulate both S1P receptor signalling and the sphingomyelin cycle.

BRAIN BIOENERGETICS

Glycolysis

Although the brain composes 2% of the body weight it utilizes 25% of total glucose levels, indicating the brain has an exceptionally high energy demand. Glucose is the main substrate to produce adenosine tri-phosphate (ATP) in the brain via three complex pathways: glycolysis, tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OxPhos). First, glucose needs to be taken up from the blood and actively transported by glucose transporters over the blood-brain barrier to enter the brain. Since astrocytes cover most of the vessel surface with their endfeet, they take up most of the glucose that enters the brain via specific glucose transporters. Inside the cell, glucose is phosphorylated by hexokinases, converting glucose into glucose-6-phosphate (Glu-6-P). Glu-6-P can be stored as glycogen in astrocytes or converted via multiple glycolytic steps into pyruvate which neto gains two ATP and two NADH molecules. The glycolytic end product pyruvate can be transported into the mitochondria or reduced by the lactate dehydrogenase (LDH) complex into lactate. LDH is encoded by two different genes namely LDHA and LDHB. LDHA convert pyruvate into lactate, whereas LDHB predominantly produces pyruvate. Lactate is secreted by specific monocarboxylate transporters (MCT) and also taken up via MCTs expressed on neighbouring cells. Inside the cell, lactate can be oxidized into pyruvate by LDHB thereby producing NADH. This metabolic coupling is also known as the astrocyte-neuron lactate shuttle (ANLS). Although increasing evidence supports this hypothesis, the amount of lactate produced by astrocytes (10-60% from total glucose) and the utilization by neurons in vivo is still heavily debated and may strongly depend on local demands. Interestingly, two recent
studies show that lactate, produced by oligodendrocytes and secreted via MCT1, is essential for proper axonal functioning\textsuperscript{109,110}. Recent evidence suggests that lactate is also involved in cell-signalling\textsuperscript{111,112}. Figure 5 provides an overview of the metabolic coupling between astrocytes, oligodendrocytes and neurons.

**Mitochondria: TCA cycle and oxidative phosphorylation**

The main glycolytic product pyruvate is transported from the cytosol into mitochondria. Here, pyruvate enters the TCA cycle, generating electron donors that are eventually utilized by the OxPhos chain to produce ATP. First, pyruvate is metabolized into acetyl CoA by the pyruvate dehydrogenase complex (PDH) to fuel the TCA cycle. Acetyl CoA is condensed with oxaloacetate by citrate synthase to citrate, which is subsequently converted into iso-citrate, $\alpha$-ketoglutarate ($\alpha$-KG), succinyl-CoA, succinate, fumarate, malate and oxaloacetate (OAA), respectively. The reactions which are catalyzed by isocitrate dehydrogenase (IDH), $\alpha$-ketoglutarate dehydrogenase ($\alpha$-KGDH), malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) produce NADH and FADH$_2$ (Figure 6). FADH$_2$ and NADH, produced during glycolysis and in the TCA cycle, donate electrons to the OxPhos chain, which consists of five complexes (I-V). Electrons donated to complex I and II are transported through the complexes of the OxPhos chain generating a proton gradient over the inner mitochondrial membrane. This proton gradients is used by complex V, also known as ATP synthase, to produce ATP (Figure 6)\textsuperscript{102}. During the electron transport, some
Glucone Glucose Glucose Glucose Glucose
Pyruvate Lactate Lactate Glucose Lactate Lactate Pyruvate NADH Glycolysis LDHA LDHB

Glucone Glucose
Glycolysis

Pyruvate

LDHA

Lactate

Glucose Lactate

Lactate

Lactate

Pyruvate

NADH
Astrocytes take up most of the glucose and lactate in the brain via specific glucose (green) and monocarboxylate transporters (red). Glucose enters the glycolysis producing pyruvate. Pyruvate can enter the mitochondria to generate ATP via the TCA cycle and OxPhos. Alternatively pyruvate can be converted to lactate by lactate dehydrogenase A (LDHA). Lactate but also non-metabolized glucose can be transported to nearby cells including oligodendrocytes and neurons. Oligodendrocytes supply myelinated axons under normal conditions with lactate which they produce themselves or which is supplied by astrocytes. Inside the axon lactate can be converted back to pyruvate by LDHB gaining NADH and pyruvate can enter the TCA cycle to produce ATP.

Mitochondria are unique organelles with double membranes and they contain circular DNA. Mitochondrial DNA (mtDNA) is particularly vulnerable for ROS-mediated damage due to the absence of histones and limited repair mechanisms. Since mitochondria continuously produce ROS, mitochondria are equipped with an efficient antioxidant machinery to prevent oxidative damage. Firstly, superoxide dismutase 2 (SOD2) dismutates superoxide ($O_2^-$) into hydrogen peroxide ($H_2O_2$). Subsequently, $H_2O_2$ is reduced by peroxiredoxin 3 (Prx3), resulting in oxidation of its own cysteine residue. To regain antioxidant function, Prx3 needs to be reduced by thioredoxin 2 (Trx2), which in turn becomes oxidized. Lastly, thioredoxin reductase 2 (TrxR2) reduces Trx2 at the cost of NADPH. Under pathological conditions or prolonged stress, these protective mechanisms can be insufficient resulting in mitochondrial oxidative stress and cellular injury.

Mitochondria are the main source of energy, therefore proper mitochondrial function is essential for all cells. Neurons are one of the most energy consuming cells of the body, making them exceedingly dependent on mitochondrial metabolism. The main processes contributing to the high brain energy needs include the maintenance of ion gradients and actively uptake and recycling.
of neurotransmitters. In contrast, astrocytes are generally considered to be more glycolytic, allowing them to produce high levels of pyruvate and lactate to support neighbouring cells.

**Transcriptional regulation of glucose metabolism**

It is important that cells quickly adapt their glucose metabolism to changes in the environment. Not surprisingly, many factors are involved in regulating glucose metabolism in order to ensure optimal energy production under various conditions. For example, hypoxia-inducible factor 1α (HIF-1α) senses oxygen levels and translocates to the nucleus under low oxygen conditions activating genes with a hypoxic responsive element, leading to enhanced anaerobic metabolism.

Another key player in metabolic regulation is peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α). When low levels of ATP or NADH are detected by AMP activated protein kinase (AMPK) or sirtuin 1 (SIRT1), PGC-1α is activated to meet the increased cellular energy demand. PGC-1α is a co-transcription factor which binds to different transcription factors, including nuclear respiratory factor 1 and 2 (NRF1 and -2), oestrogen-related receptors (ERRα, -β, -γ) and peroxisome proliferator-activated receptors (PPAR α, -δ, -γ). As such, PGC-1α can rapidly induce transcription of a wide set of genes involved in mitochondrial biogenesis, oxidative metabolism, fatty acid metabolism and mitochondrial antioxidants (Figure 7).

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**Figure 7. PGC-1α is a key metabolic regulator.** AMPK can sense the cell its need for energy and activates PGC-1α when ATP levels are low. PGC-1α can also be activated by Sirt1 which senses a shortage of NADH levels. Upon activation PGC-1α can bind to several DNA-bound transcription factors inducing transcription of genes involved in varies mitochondrial processes including fatty acid oxidation, oxidative phosphorylation and mitochondrial ROS defence. Adapted from 121.
Metabolic alterations in MS; evidence from imaging studies

Conventional imaging techniques, like MRI, have proven to be a valuable tool for diagnosing and monitoring MS by determining lesion formation and atrophy measurements. However, MRI provides only limited information about the underlying pathology, which requires more advanced MR techniques. Proton MR spectroscopy (MRS) can be used to measure and quantify different metabolites in specific brain areas. As such, MRS has been used to analyze n-acetyl-aspartate (NAA) and lactate in MS patients. The exact function of NAA is not fully understood, but since it is mainly found in axons and neurons and synthesized in the mitochondria from aspartate and acetyl-CoA, NAA levels are thought to reflect neuronal mitochondrial metabolism. NAA levels are reduced in the brain of MS patients, albeit most profoundly in lesions, and correlate with disease progression (Table 1). Conversely, lactate is highly increased in active MS lesions and to a lesser extent in NAWM and inactive lesions. Lactate levels are also increased in the cerebrospinal fluid (CSF) and serum of MS patients (Table 2). CSF lactate levels correlate with lesion activity and clinical exacerbations, whereas serum lactate levels correlate well with disease progression and patient disability.

Another important metabolic parameter, which can be analyzed in vivo with the use of different contrast agents, is brain perfusion. Brain perfusion is determined by 3 parameters, namely cerebral blood flow (CBF), cerebral blood volume (CBV) and mean transit time (MTT), which is the average time it takes blood to pass through a given region of brain tissue. In general, brain perfusion is reduced throughout the brain in both RR and SP MS patients, except for active MS lesions where perfusion rates are increased (Table 1). Reduced perfusion suggests there is reduced delivery of oxygen and glucose to the brain.

To visualize glucose uptake, a radioactively labelled glucose analogue (18F-FDG) can be used and analyzed with a positron emission tomography (PET) scan. Similar to normal glucose, 18F-FDG is taken up from the blood by glucose transporters. Inside the cell, it is immediately phosphorylated by the glycolytic enzyme hexokinase thereby preventing 18F-FDG from being released again. However, 18F-FDG can not be further metabolized until the radioactive label decays. In MS patients, there is reduced glucose uptake throughout most of the brain, except for the active lesions where increased 18F-FDG uptake is observed, most likely due to the disrupted blood-

<table>
<thead>
<tr>
<th>Metabolic changes compared to healthy control brain. NAWM = normal appearing white matter; Diffuse WM = WM including lesions; NAA = N-acetyl aspartate; Lac = lactate; Glu = glucose utilization; ↑ = increased; ↓ = decreased; ~ = similar; ( ) = number of studies analyzed. * lesion type not specified. See supplementary table 1 for references.</th>
<th>NAWM</th>
<th>Diffuse WM</th>
<th>Active WM</th>
<th>Inactive WM</th>
<th>WM lesion*</th>
<th>GM</th>
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<td>↓ (5)</td>
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<td>↓ (3)</td>
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<tr>
<td>Lac</td>
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<td>↑ (2)</td>
<td>↑ (3)</td>
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<td>Perfusion</td>
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<td>Glu</td>
<td>~ (1)</td>
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Table 1. Metabolic changes compared to healthy control brain.
brain barrier en leukocyte influx (Table 1). There are no obvious differences in CSF or serum glucose levels between MS and control samples (Table 2). Supplementary table 1 provides a complete overview of all the studies included in table 1 and 2.

Taken together, these data suggest glucose metabolism is slightly reduced in the NAWM, whereas in active MS lesions glucose metabolism is highly increased. The reduced levels of NAA in active lesions are likely the result of extensive axonal loss. Finally, in the GM of MS patients glucose metabolism is evidently reduced.

<table>
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<th>Lactate</th>
<th>Glucose</th>
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<tr>
<td>CSF</td>
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| ↓ (2)   |

Table 2. Metabolic changes compared to healthy control samples. See supplementary table 1 for references

**Metabolic alterations in MS; pathological evidence**

Imaging studies indicate that glucose metabolism in the cortex of MS patients is reduced. This can partially be explained by the loss of neurons, which are responsible for consuming most of the brain's energy\(^{117}\). Moreover, neurons in the cortex of MS brains exhibit reduced OxPhos activity and increased mitochondrial DNA deletions\(^{131,132}\). Recently, we showed that PGC-1α, a key metabolic regulator, is decreased in the cortex of MS patients. Moreover, PGC-1α mitochondrial target genes are decreased in NAGM and its reduced expression levels correlated with increased neuronal loss\(^{49}\). Taken together mitochondrial dysfunction is extensive in MS GM and may contribute to reduced glucose metabolism and neuronal cell death.

Examination of post-mortem derived brain tissue demonstrated altered expression of different glucose metabolism related genes in the NAWM. These changes include increased hypoxia-related genes, like glucose transporters, hif-1α and vascular endothelial growth factor (VEGF)\(^{133,134}\). Hypoxic markers are most profoundly upregulated in fulminant and early active MS lesions, but rarely found in late active or chronic MS lesions\(^{135-137}\). This could be due to the chronic nature of the disease as hypoxic responses are generally more acute. Interestingly, hypoxic markers were also found to be increased in EAE, and increasing O\(_2\) levels reduces clinical symptoms and lesion formation in experimental MS animal models\(^ {138}\). However, in active MS lesions, there is blood-brain barrier breakdown and imaging studies indicate that glucose and lactate levels are highly increased. The upregulation of hypoxic markers despite increased availability of nutrients suggests a largely increased energy demand in MS lesions. The increased energy demand in MS lesions can be caused by the influx of glucose consuming leukocytes and increased metabolism of reactive astrocytes and, most importantly, axons\(^{139-141}\). Under normal conditions, axons consume high amounts of energy which is even further increased after demyelination. In myelinated axons, conduction is saltatory and de- and repolarization only takes place at the nodes of Ranvier. During depolarization voltage-gated Na\(^+\) channels open, allowing Na\(^+\) influx. During repolarization, the increased intra-axonal Na\(^+\) concentration is restored by the energy dependent Na/K-ATPase. In demyelinated axons, which lack nodes of Ranvier, conduction is blocked. This conduction block can be restored by upregulation and redistribution of Na\(^+\) channels and Na/K-ATPase, which requires high amounts of ATP\(^ {139}\). In order to meet the increased energy demand, demyelinated axons in MS have increased mitochondrial content\(^{142}\). An increase in glucose metabolism often
General Introduction

The last decade, several new treatment options have been introduced for RRMS patients, although these drugs can be effective in RRMS there are several limitations. These drugs all target the immune system, resulting in a higher susceptibility to infections and other diseases. Clinical progression, chronic inflammation and neuronal- and axonal degeneration in the brain continue, despite current treatments. Therefore it is crucial to suppress inflammation in the brain specifically and to develop neuroprotective therapies. Astrocytes are actively involved in both neuroinflammatory and neuroprotective processes, as such we hypothesize that astrocytes play a cardinal role in MS pathogenesis. Identification of novel neuroprotective and anti-inflammatory mechanisms might reveal new therapeutic targets aimed at improving neuronal function under inflammatory and degenerative conditions. Hence, the aim of this thesis is to gain more insight in the role of astrocytes in neuroinflammation, ROS detoxification, metabolic coupling and associated neurodegeneration in MS.

In MS lesions astrocytes play a dual role by promoting as well as resolving inflammation. Therefore, in chapter 2 and 3, we investigate the role of astrocytes in ROS handling and the production of inflammatory molecules. In chapter 2 we show that reactive astrocytes produce large amount of the pro-inflammatory sphingolipid ceramide, which disrupts the blood-brain barrier and enhances leukocyte migration into the brain. This can be prevented by treating astrocytes with Gilenya®, thereby revealing a novel target of this recently approved MS therapeutic. Chapter 3 shows that astrocytes in MS lesions might dampen oxidative damage and inflammation by increasing PGC-1α levels. Increased astrocytic PGC-1α reduces production of ROS and inflammatory cytokines and protects surrounding neurons against an oxidative insult.

Although astrocytes play a key role in brain energy metabolism, so far most studies have focussed on axonal and neuronal mitochondrial function in MS lesions. Therefore in chapter 4 and 5 we aim to gain more insight in both astrocytic and axonal metabolic alterations in order to better understand the processes underlying axonal degeneration. Chapter 4 provides a comprehensive overview of glucose and monocarboxylate transporters in MS tissue. Our findings suggest there is
reduced uptake of glucose and lactate by demyelinated axons, but they obtain increased metabolic support from astrocytes. In chapter 5 we show that glucose metabolism in astrocytes and axons in MS lesions is altered and provide evidence for increased astrocyte-axon lactate shuttling. Chapter 6 provides an overall discussion and novel perspectives about the role of astrocytes in MS.

REFERENCES

32. Sty, PK, Zamponi, GW, van Minnen, J, et al. Will the real


78. Sofroniew, MV. Molecular dissection of reactive astrogliosis and glial scar formation. Trends in Neurosciences. 32:638-647.


91. Doorn van, R. Harmony of citizens is the wall of cities: orchestrating the neurovascular unit. Amsterdam, 2013.


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<td>8</td>
<td>3T 1H MRSI</td>
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<td>1.5T 1H MRSI</td>
<td>Sign ↓ NAA/Cr + NAA/Cho to HC</td>
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<td>SP + PP</td>
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<td>6</td>
<td>1.5T 1H MRSI</td>
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<td>Glutamate</td>
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<td>No difference in Glx to HC</td>
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<tr>
<td></td>
<td>16</td>
<td>4</td>
<td>3T 1H MRSI</td>
<td>Sign ↑ glutamate to HC (glutamine levels did not changed)</td>
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<tr>
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<td>1.5T 1H MRSI</td>
<td>Lactate resonance detected to NAWM of patient</td>
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**General Introduction**

**SUPPLEMENTARY DATA**


| Diffuse WM | NAA | \( \text{RR} \) | 11 | 20 | 1.5T 1H MRSI | Sign ↓ NAA/Cr to HC + further decrease in follow-up | De Stefano, N., et al. (1998). Axonal damage correlates with disability in patients with relapsing-remitting multiple sclerosis. Results of a longitudinal magnetic resonance spectroscopy study. Brain 121 (Pt 8), 1469-1477.
| NAA | \( \text{RR} \) | 18 | 10 | 3D 3T 1H MRSI | Gradual increase over time in follow up | Kirov, I.I., et al. (2012). Serial proton MR spectroscopy of gray and white matter in relapsing-remitting MS. Neurology.
| NAA | \( \text{SP} \) | 18 | 20 | 1.5T 1H MRSI | Sign ↓ NAA/Cr to HC no changes in follow up | De Stefano, N., et al. (1998). Axonal damage correlates with disability in patients with relapsing-remitting multiple sclerosis. Results of a longitudinal magnetic resonance spectroscopy study. Brain 121 (Pt 8), 1469-1477.


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<th>Perfusion</th>
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<td></td>
<td>SP</td>
<td>14</td>
<td>34</td>
<td>1.5T ASL MRI</td>
<td>Sign ↑ perfusion to HC</td>
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### WM lesions

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<th>Sign ↓ NAA/Cho in active lesions to HC</th>
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<td>16</td>
<td>3T 1H MRSI</td>
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### Glutamate

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<th>Glutamate</th>
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<td></td>
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<td>4</td>
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<td>(Glutamine levels did not change)</td>
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### Lactate

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<th>Lactate</th>
<th>RR</th>
<th>47</th>
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<th>Increased lactate in 37.8% active lesions (and in 16.7% inactive)</th>
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<td>RR</td>
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<td>0</td>
<td>1.5T 1H MRSI</td>
<td>Increased lactate in NAWM with progressive decrease during follow-up</td>
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### GENERAL INTRODUCTION


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### General Introduction

#### Whole Brain

<table>
<thead>
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<th>benign MS</th>
<th>reduced NAA, no correlation edas</th>
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<td>RR 18</td>
<td>11</td>
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<td>PP 43</td>
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<tr>
<td>RR 44</td>
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<td>1.5T DSC MRI, sign ↓ Gix in cortical GM to HC</td>
<td>SP 9</td>
</tr>
<tr>
<td>PP 14</td>
<td>11</td>
<td>3T DSC MRI sign ↓ Gix in cortical GM to HC and to RR MS</td>
<td>SP 14</td>
</tr>
<tr>
<td>PP 12</td>
<td>34</td>
<td>1.5T ASL MRI sign ↓ perfusion to HC</td>
<td>PP 12</td>
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<td>Gluc uptake</td>
<td>RR 20</td>
<td>18 FDG PET sign ↓ cortical CMRglu</td>
<td>RR 20</td>
</tr>
<tr>
<td>RR 25</td>
<td>6</td>
<td>18 FDG PET sign ↓ cortical CMRglu (9% global brain CMRglu ↓)</td>
<td>? 25</td>
</tr>
<tr>
<td>RR 22</td>
<td>7</td>
<td>18 FDG PET sign ↓ global cortical CMRglu 18F</td>
<td>? 22</td>
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<tr>
<td>? 10</td>
<td>0</td>
<td>FDG PET decreased over 2y follow-up</td>
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#### Serum

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<tr>
<td>RR 430</td>
<td>625</td>
<td>Increased, correlated to EDSS, increased for RR</td>
<td>RR 430</td>
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<td>PP 30</td>
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<td>Increased, correlated to EDSS, higher</td>
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<tr>
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<td>Increased, not separatedly measured, correlated to EDSS</td>
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<td>Increase RR and SP MS, most evident during clinical exacerbation of RR</td>
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<tr>
<td></td>
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<td>no difference (really high glucose patients removed)</td>
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