The role of mitochondria in axonal degeneration and tissue repair in MS

J van Horssen¹, ME Witte¹ and O Ciccarelli²

Abstract
Axonal injury is a key feature of multiple sclerosis (MS) pathology and is currently seen as the main correlate for permanent clinical disability. Although little is known about the pathogenetic mechanisms that drive axonal damage and loss, there is accumulating evidence highlighting the central role of mitochondrial dysfunction in axonal degeneration and associated neurodegeneration. The aim of this topical review is to provide a concise overview on the involvement of mitochondrial dysfunction in axonal damage and destruction in MS. Hereeto, we will discuss putative pathological mechanisms leading to mitochondrial dysfunction and recent imaging studies performed in vivo in patients with MS. Moreover, we will focus on molecular mechanisms and novel imaging studies that address the role of mitochondrial metabolism in tissue repair. Finally, we will briefly review therapeutic strategies aimed at improving mitochondrial metabolism and function under neuroinflammatory conditions.

Keywords
Axonal injury, mitochondria, multiple sclerosis, neurodegeneration, imaging, spectroscopy

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Axonal injury in multiple sclerosis

Multiple sclerosis (MS) is a chronic neuroinflammatory disorder of which the aetiology and pathogenesis are still enigmatic. Most MS patients experience an episodic course of the disease, with recurrent intervals of exacerbations followed by periods of remission. The relapsing–remitting phase is characterised by massive leukocyte infiltration and extensive microglial activation in the central nervous system (CNS). Histopathological analysis of MS brain tissue reveals the presence of focal white matter lesions distributed throughout the CNS. In the initial stages (acute), MS lesions are defined by primary demyelination with abundant inflammatory infiltrates that generally consist of lymphocytes, activated microglia and macrophages, which produce vast amounts of inflammatory mediators that break down the myelin sheet, with axonal conduction block as a consequence. As a bystander effect, inflammatory mediators also mediate transection of a subset of axons. These pathological processes are thought to underlie the acute clinical symptoms that characterise an MS relapse. More advanced stages of the disease, as seen in progressive MS, are mainly characterised by widespread cortical demyelination, neuronal damage and diffuse degenerative changes throughout the CNS. Taken together, evidence is accumulating that inflammation-induced axonal damage and demyelination occur in the initial stages of the disease, and over time degenerative processes take over, eventually leading to extensive axonal loss and brain atrophy.¹

Axonal damage was already recognised by Charcot, and it is nowadays well understood that axonal damage and loss are key features of MS pathogenesis that correlate with permanent neurological deficits in MS patients.² Although degenerating axons are mainly observed in active demyelinating lesions and associated with the number of infiltrated macrophages, activated microglia and CD8-positive T cells,³ axonal damage and loss are not restricted to early active lesions but also occur in more advanced stages of MS lesions.⁴ The invasion of blood-borne leukocytes in the

¹Department of Molecular Cell Biology and Immunology, VU University Medical Center, The Netherlands.
²Department of Brain Repair and Rehabilitation, UCL Institute of Neurology, UK.
J.V.H. and M.E.W. contributed equally to this study.

Corresponding author:
J. van Horssen, Department of Molecular Cell Biology and Immunology, VU University Medical Center Amsterdam, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands.
Email: j.vanhorssen@vumc.nl
Mitochondrial dysfunction in axonal damage: evidence from experimental and post-mortem studies

Pronounced mitochondrial defects have been reported in early and chronic white matter lesions. Mitochondrial density is increased in both axons and astrocytes in active demyelinating lesions and coincides with enhanced expression of mtHSP70, a well-characterised marker of mitochondrial stress. Complex IV activity is significantly decreased in fulminant MS lesions and correlates with the number of activated microglia and infiltrated macrophages. In an experimental MS animal model, Nikić et al. demonstrated that intra-axonal mitochondrial defects, which already occur in the absence of overt demyelination, are an essential step towards axonal degeneration and local accumulation of invading macrophages. Interestingly, they showed that exogenous antioxidants were able to reduce mitochondrial pathology and thereby reduce axonal degeneration. In search of targets involved in excessive reactive oxygen species (ROS) ROS formation in MS, we performed microarray analysis on initial lesion areas and found a marked increase in NADPH oxidase-1 and -2 expression, which coincided with increased mRNA levels of key mitochondrial proteins. Together, these findings indicate an important role for inflammation-derived ROS in axonal mitochondrial dysfunction in the initial stages of MS lesion formation.

In chronic MS lesions, and even in remyelinated lesions, mitochondrial alterations have been detected by biochemical and histochemical techniques. These reports revealed enhanced intra-axonal mitochondrial density and increased axonal complex IV activity in chronic MS lesions, which might be a compensatory mechanism for the observed complex I dysfunction. Likewise, increased mitochondrial density and complex IV activity have been observed in remyelinated axons. Axonal conduction, which is blocked by demyelination in highly inflammatory lesions, can be restored in chronic demyelinated axons by upregulation and reorganisation of voltage-gated Na⁺ channels. Simultaneously, Na⁺/K⁺ ATPase is upregulated to restore intra-axonal Na⁺ concentration, resulting in an enhanced metabolic demand, which, in turn, leads to increased mitochondrial content and complex IV activity, which is seen in half of large chronically demyelinated axons (Figure 1). However, when the energy demand exceeds the ATP-producing capacity of mitochondria, demyelinated axons become chronically energy deficient, which is called ‘virtual hypoxia’; this will result in excessive sodium influx, which, in turn, drives the reverse action of the sodium-calcium exchanger, leading to calcium influx. This increased calcium influx, in combination with calcium released from mitochondria and via the glutamate-mediated pathway, mediates the subsequent axonal degeneration. In addition, dysfunctional mitochondria in chronic lesions are likely to generate vast amounts of ROS, leading to impaired mitochondrial function thereby creating a vicious cycle, which will inevitably damage the demyelinated axons. Thus, mitochondria are initially needed to preserve demyelinated axons, but in time set off a cascade of deleterious events leading to axonal injury and loss.

Mitochondrial alterations are not restricted to white matter MS lesions, and nowadays evidence is emerging that mitochondrial changes also occur in MS grey matter. Dutta and colleagues performed microarray analysis on myelinated cortex samples of progressive MS patients and revealed a marked decrease in the expression of genes involved in oxidative phosphorylation (OxPhos), which coincided with reduced neuronal activity of complex I and III. They postulated that these mitochondrial changes contributed to axonal degeneration and progressive neurological disability in MS patients. Recently, Mahad’s research group reported the presence of mtDNA deletions and respiratory-deficient neurons throughout the MS cortex. These findings point towards impaired oxidative metabolism in neurons in normal-appearing grey matter (NAGM) in MS patients, which subsequently leads to local energy deficits and increased mitochondrial ROS production, which in turn is a likely cause for the mtDNA deletions. Although the cause of mitochondrial changes in neurons in MS cortex is unknown, they will inevitably have an impact on axonal mitochondrial function, and thus on axonal survival under inflammatory or demyelinated conditions.

Relationship between mitochondrial dysfunction and both acute and chronic neurological deficits: evidence from imaging

Acute neurological symptoms are thought to be due to conduction failure in demyelinated axons and axonal transection in acute inflammatory lesions. Irreversible (chronic)
symptoms, instead, are considered to be related to the degeneration of axons and possibly to the gliotic response to tissue injury. In addition to the evidence discussed above that points to mitochondrial impairment as a key element in the pathogenesis of MS, insights into the mitochondrial function in vivo have been provided by imaging studies. In particular, proton and phosphorus ($^1$H and $^{31}$P) magnetic resonance spectroscopy ($^1$H-MRS and $^{31}$P-MRS) allows detection of changes in the concentration of several proton- and phosphorus-containing compounds, which have high specificity for different pathological processes; $^{19}$ some of these metabolites may reflect ‘indirectly’ or ‘directly’ the status of mitochondrial metabolism within neurons or in any other cell type (Table 1).

N-acetyl-aspartate (NAA) is in a key position to be a $^1$H-MRS marker of neuronal health and density and is present
Table 1. Pathological specificity of imaging markers for mitochondrial metabolism and their clinical relevance.

<table>
<thead>
<tr>
<th>Imaging marker</th>
<th>Specificity for neurons</th>
<th>Specificity for mitochondrial metabolism</th>
<th>Specificity for axonal loss</th>
<th>Correlation with clinical disability</th>
<th>Use in clinical trials</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1H-MRS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAA</td>
<td>High (in adult brain)</td>
<td>Good, depending on its synthesis and role in neuronal mitochondria.</td>
<td>Good</td>
<td>Good</td>
<td>Reported</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Low</td>
<td>Theoretically good, but depending on the extent of the link between oxidative stress and mitochondrial dysfunction.</td>
<td>Low</td>
<td>Unknown</td>
<td>Not done</td>
</tr>
<tr>
<td>Lactate</td>
<td>Low</td>
<td>Good, but indirect, reflecting non-mitochondrial anaerobic metabolism which increases if mitochondrial function is impaired.</td>
<td>Low</td>
<td>Unknown</td>
<td>Not done</td>
</tr>
<tr>
<td><strong>31P-MRS</strong></td>
<td></td>
<td>High and direct.</td>
<td>Low</td>
<td>Unknown</td>
<td>Not done</td>
</tr>
<tr>
<td>Phosphorus metabolites</td>
<td>Low</td>
<td></td>
<td>Low</td>
<td>Unknown</td>
<td>Not done</td>
</tr>
</tbody>
</table>

1H-MRS: proton magnetic resonance spectroscopy; 31P-MRS: phosphorus magnetic resonance spectroscopy.

at very high concentration in the brain. Although the metabolic and neurochemical functions of NAA are not fully known, it is thought to be indirectly linked with ATP metabolism. NAA is synthesised in neuronal mitochondria and plays a role in enhancing mitochondrial energy production. Therefore, reduced NAA concentration reflects impaired neuronal mitochondrial metabolism in addition to neuronal cell loss; this indicates that the specificity of NAA for neuronal mitochondrial dysfunction is limited, but unique. Bearing in mind this important aspect, a significant decrease in the concentration of NAA (including NAA-glutamate) in the lezsional and normal-appearing white matter and in the NAGM of the brain of patients with MS when compared to healthy subjects has been often reported, with mean (SD) decreases of about 20% in the lesions and 7% in the normal-appearing brain tissue.

A decrease in NAA levels has also been described in the spinal cord of MS patients when compared to controls; this decrease varied between 39% at the level of an acute lesion at the onset of a spinal cord relapse and 21% (as ratio of NAA/total choline) in chronic spinal cord lesions. Furthermore, a significant reduction in NAA concentration was found in all types of MS, especially in the progressive forms and even in patients with clinically isolated syndrome. With regard to the clinical relevance of NAA, several MRS studies have described an association between reduced NAA and increased disability, as measured by the Expanded Disability Status Scale (EDSS) during an acute relapse and in the chronic stage of the disease. Additionally, NAA levels (or NAA/creatine ratio) correlate with fatigue and cognitive function and predict disease evolution in patients with clinically isolated syndrome.

As discussed above, axonal mitochondria are damaged by the vast amount of ROS produced by macrophages and activated microglia. Glutathione is a potent antioxidant that protects cells against the deleterious effects of ROS. Since glutathione is consumed in this protective task, a reduction in glutathione levels is expected to reflect oxidative stress and, therefore, subsequent impaired mitochondrial function. The levels of glutathione were found to be reduced in patients with MS when compared to healthy subjects in the frontal regions of the brain at 3 T and in the grey matter and in an MS lesion at 7 T, suggesting the presence of underlying oxidative damage in tissue, especially to mitochondria.

Another metabolite measured with 1H-MRS which provides a window for the study of mitochondrial dysfunction is lactate. In the presence of impaired mitochondrial oxidative metabolism, there is a shift from aerobic to anaerobic metabolism, and, therefore, the production of lactate increases. Increased lactate levels are present within the acute (enhancing) lesions, tend to decrease progressively over time, but may remain elevated for several weeks, suggesting an increased glycolytic activity by macrophages and reactive glia, even when the blood-brain barrier is repaired (i.e. no gadolinium-enhancing lesion is seen).

A more direct measure of mitochondrial function is provided by 31P-MRS. The major 31P-MRS peaks include high-energy phosphates, such as alpha, beta and gamma nucleotide triphosphates (reflecting ATP), phosphocreatine (PCr: an indicator of oxidative metabolism) and inorganic phosphate (Pi) (Figure 3). Therefore, the rate of mitochondrial metabolism and, in particular, of oxidative phosphorylation, is reflected by the ratio of Pi to PCr: reduced concentration of PCr (which transfers a phosphate group to ADP), together with increased Pi levels, in the presence of unchanged ATP levels. These metabolic changes suggest decreased intracellular energy metabolism, without specificity for cell type and independently from the cause of reduced energy metabolism, which may include decreased oxygen availability or blood flow, increased energy demand and impaired mitochondrial function. Both reduced and increased PCr ratios have been found within MS lesions and/or white matter, as recently summarised by Hattingen.
Horssen et al. and colleagues in agreement with the hypothesis that changes in energy metabolism may occur towards either reduced mitochondrial metabolism or increased energy production (in excess of demand). However, comparison between studies is challenging because of differences in patient cohorts and imaging protocols. A recent paper that combined $^1$H-MRS with $^{31}$P-MRS demonstrated that the ratio between PCr and Cr in the centrum semiovale did not change in MS when compared with controls (the increase in PCr was in fact proportional to the increase in total Cr); this finding suggests an overall, non-significant change in the cellular energy state of the underlying tissue.

Figure 2. Two representative $^1$H-MRS spectra on the right with the corresponding Chemical Shift Imaging grid on the left. On the top, spectrum obtained from a spectroscopic voxel localised on the normal-appearing white matter of a 42-year-old female patient with secondary progressive MS, showing reduced NAA peak compared to the spectrum on the bottom, which is obtained from a voxel localised on the normal white matter of a 38-year-old healthy control. Courtesy of Dr David Pailing (UCL Institute of Neurology). $^1$H-MRS: proton magnetic resonance spectroscopy; NAA: N-acetyl-aspartate; MS: multiple sclerosis.
In conclusion, despite the diversity of technical approaches and protocols, abnormalities consistent with mitochondrial dysfunction have been directly (or indirectly) detected in the brain and spinal cord in the acute and chronic phases of MS, although high specificity for neuronal cells is rarely achieved. Future studies will focus on combining $^1$H-MRS with $^{31}$P-MRS to provide a more specific approach to assess mitochondrial function and energy demand in order to develop biomarkers for evaluating therapeutic responses.

**Link between mitochondrial metabolism and tissue repair: evidence from post-mortem and imaging**

Axonal injury and neuronal damage are considered to be responsible for permanent, non-remitting, clinical deficits. Concomitant with the axonal degeneration that follows an acute inflammatory event, repair mechanisms spontaneously take place and lead to clinical improvement. For
Figure 4. $^{1}$H-MRS spectroscopic volumes and spectra from the same relapsing–remitting patient at onset of a spinal cord relapse (left) due to a lesion between C1 and C3 and after six months (right). The top row shows the MRS volume positioned between C1 and C3 on sagittal images at baseline (left) and follow-up (right). The concentration of NAA was 2.53 mM at study entry (left), and 2.24 mM after six months (right). The EDSS of this patient significantly improved by one point between baseline and six months.

$^{1}$H-MRS: proton magnetic resonance spectroscopy; NAA: N-acetyl-aspartate; EDSS: Expanded Disability Status Scale.
example, about 65% of MS patients with an acute relapse caused by a new cervical cord lesion improved clinically during a six-month follow-up study. Mechanisms of repair include resolution of inflammation, remyelination and possibly cortical adaptation. The increases in mitochondrial mass and complex IV activity within half of large chronically demyelinated axons\(^4\) may reflect a compensatory reaction to the increased energy demand (Figure 1). This post-mortem evidence is in agreement with the findings of increased mitochondrial number and activity in the white matter tracts of shiverer mutant mice, which lack CNS myelin.\(^4\) In this model, intra-axonal mitochondrial changes may be a direct response to the enhanced energy requirement in axons lacking myelin in a non-inflammatory environment.

A recent study carried out in patients with relapsing-remitting MS followed-up after an acute spinal cord relapse reported a reduction in NAA levels after one month from onset, and a sustained increase in NAA concentration from one to six months\(^4\) (Figure 4). This increase in NAA levels is significant when correcting for the concomitant development of spinal cord atrophy, which occurs during the same follow-up and suggests ongoing axonal loss. As discussed before, NAA concentration reflects both axonal integrity and axonal mitochondrial metabolism; therefore, an increase in NAA levels, occurring in parallel with atrophy development, may be driven by increased axonal mitochondrial metabolism. The contribution of remyelination to these NAA changes is thought to be negligible.\(^5\) Previous brain studies reported a similar reversible decrease in NAA concentration after an acute lesion.\(^6\)–\(^8\) An interesting clinical observation is that the observed increase in NAA over time correlates with clinical recovery, and is less evident in patients with longer disease duration.\(^4\) Although technical limitations are present in this type of MRS studies, these findings suggest that the increased energy production, which occurs in response to acute demyelination, may have an impact on clinical recovery. In addition, the repair mechanisms involving mitochondrial metabolism, and reflected by the increase in NAA levels, may become less efficient over time.

After six months from onset of a spinal cord relapse, patients who present a higher ‘metabolic’ component of the NAA are less likely to have greater disability and show better walking ability than those with a lower ‘metabolic’ component.\(^9\) This ‘metabolic’ component has been estimated statistically combining NAA with other imaging measures of axonal loss and degeneration. This novel approach allows an estimate of the NAA changes which cannot be explained by the imaging structural measures, and therefore likely reflects mitochondrial metabolism. Future studies will concentrate on extracting the metabolic component of NAA using more complex statistical models, and validating this novel method with experimental animal models.

**Therapeutic strategies aimed at restoring mitochondrial function**

The studies reviewed in this paper demonstrate that mitochondrial metabolism is essential in maintaining axonal integrity and survival during the initial and progressive stages of MS. As such, mitochondria as well as pathogenic mechanisms that drive mitochondrial dysfunction, such as oxidative stress, represent attractive therapeutic targets.\(^10\) There are several options to limit oxidative stress and associated mitochondrial dysfunction: 1) pharmacological inhibitors of enzymes involved in free radical formation, e.g. NADPH oxidase inhibitors; 2) activation of pathways inducing the production of endogenous antioxidant enzymes, e.g. fumaric acid esters or 3) mitochondria-targeted antioxidants, such as MitoQ.\(^11\) Although in vitro studies have clearly demonstrated the beneficial effects of these approaches, future research is needed to explore their efficacy in a human setting. Importantly, some compounds, including the Nrf2 activator BG12 and the mitochondria-targeted antioxidant MitoQ, have been shown to exert protective effects in clinical trials.\(^52,53\) 1H-MRS and 31P-MRS, in combination with structural imaging techniques, should be further explored in order to obtain in vivo information on mitochondrial energy metabolism, and introduce new imaging outcomes for treatment trials with agents that protect and modulate mitochondrial function.

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**Conflict of interest statement**

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**References**