

Original Paper

## Enhanced number and activity of mitochondria in multiple sclerosis lesions

Maarten E Witte,<sup>1\*</sup> Lars Bø,<sup>2</sup> Richard J Rodenburg,<sup>3</sup> Jeroen A Belien,<sup>1</sup> Rene Musters,<sup>4</sup> Thierry Hazes,<sup>1</sup> Liesbeth T Wintjies,<sup>3</sup> Jan A Smeitink,<sup>3</sup> Jeroen JG Geurts,<sup>1,5</sup> Helga E De Vries,<sup>6</sup> Paul van der Valk<sup>1</sup> and Jack van Horssen<sup>1,6</sup>

<sup>1</sup>Department of Pathology, VU University Medical Centre, Amsterdam, The Netherlands

<sup>2</sup>Department of Neurology, Haukeland University Hospital, University of Bergen, Norway

<sup>3</sup>Department of Paediatrics, Nijmegen Centre for Mitochondrial Disorders, Radboud University Nijmegen Medical Centre, The Netherlands

<sup>4</sup>Department of Physiology, VU University Medical Centre, Amsterdam, The Netherlands

<sup>5</sup>Department of Radiology, VU University Medical Centre, Amsterdam, The Netherlands

<sup>6</sup>Department of Molecular Cell Biology and Immunology, VU University Medical Centre, Amsterdam, The Netherlands

\*Correspondence to:

Maarten E Witte, Department of Pathology, VU University Medical Centre Amsterdam, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.  
E-mail: m.witte@vumc.nl

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### Abstract

**Mitochondrial dysfunction has been implicated in the development and progression of multiple sclerosis (MS) lesions. Mitochondrial alterations might occur as a response to demyelination and inflammation, since demyelination leads to an increased energy demand in axons and could thereby affect the number, distribution and activity of mitochondria. We have studied the expression of mitochondrial proteins and mitochondrial enzyme activity in active demyelinating and chronic inactive MS lesions. Mitochondrial protein expression and enzyme activity in active and chronic inactive MS lesions was investigated using (immuno)histochemical and biochemical techniques. The number of mitochondria and their co-localization with axons and astrocytes within MS lesions and adjacent normal-appearing white matter (NAWM) was quantitatively assessed. In both active and inactive lesions we observed an increase in mitochondrial protein expression as well as a significant increase in the number of mitochondria. Mitochondrial density in axons and astrocytes was significantly enhanced in active lesions compared to adjacent NAWM, whereas a trend was observed in inactive lesions. Complex IV activity was strikingly up-regulated in MS lesions compared to control white matter and, to a lesser extent, NAWM. Finally, we demonstrated increased immunoreactivity of the mitochondrial stress protein mtHSP70 in MS lesions, particularly in astrocytes and axons. Our data indicate the occurrence of severe mitochondrial alterations in MS lesions, which coincides with enhanced mitochondrial oxidative stress. Together, these findings support a mechanism whereby enhanced density of mitochondria in MS lesions might contribute to the formation of free radicals and subsequent tissue damage.**

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### Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) and the most common cause of non-traumatic disability in young adults [1]. Histopathological hallmarks of MS include inflammation, demyelination, loss of oligodendrocytes and neuroaxonal degeneration [2,3]. The clinical consequence of neuroaxonal injury has been demonstrated in several studies, which report a correlation between the amount of axonal loss, measured by N-acetylaspartate proton magnetic resonance spectroscopy, and clinical disability [4,5]. Additionally, post-mortem studies have reported a strong association between axonal damage and degree of inflammation in active MS lesions [3,6]. Remarkably, axonal loss is

also observed in non-inflammatory lesions and normal-appearing white matter (NAWM) [7,8], suggesting that other factors also contribute to axonal loss. Currently, it is widely accepted that during inflammation macrophages are responsible for tissue damage and axonal loss by production of nitric oxide (NO), proteolytic enzymes and reactive oxygen species (ROS) [9]. Under pathological conditions, high levels of ROS are produced by several enzymes, such as NADPH oxidase and myeloperoxidase, and by dysfunctional mitochondria [10].

Mitochondria play a central role in ATP production and cellular homeostasis and are involved in apoptotic and non-apoptotic cell death. ATP generation mainly occurs in the oxidative phosphorylation system

(OXPHOS), which is located in the inner mitochondrial membrane and consists of the electron transport chain (complexes I–IV) and ATP synthase (complex V). Under physiological circumstances, low levels of ROS are produced as a by-product of oxidative phosphorylation, mainly at complex I and III [11], which are efficiently removed by mitochondrial antioxidant enzymes. Mitochondrial alterations, however, can lead to increased ROS generation, which cause damage to proteins, lipids and nucleotides [12]. Upon exposure to ROS, cells up-regulate the production of endogenous protective proteins, such as antioxidant enzymes and mitochondrial heat shock protein 70 (mtHSP70) [13,14].

Recently, a limited number of studies focused on the involvement of mitochondria in the pathogenesis of MS [15–17]. Several studies have shown associations between mitochondrial DNA (mtDNA) polymorphisms and MS [18,19]. Similarly, a number of papers described the occurrence of MS in patients suffering from Leber's hereditary optic neuritis (LHON), which is caused by mutations in mtDNA [20,21]. However, primary LHON mutations do not commonly occur in MS, and MS is not caused by mtDNA mutations of pathogenic significance [22].

Oxidative damage to mitochondrial DNA (mtDNA) and impaired activity of complex I have been observed in chronic active MS lesions [16]. Mitochondrial dysfunction could occur as a response to chronic demyelination of axons. Upon demyelination, up-regulation and redistribution of sodium channels from the nodes of Ranvier along the entire demyelinated axon occurs in order to facilitate conduction [23]. This requires additional ATP to maintain a functional membrane potential, which is under control of  $\text{Na}^+/\text{K}^+$ -ATPase. As mitochondria provide 99% of ATP in eukaryotic cells through the OXPHOS system, the amount of mitochondria or OXPHOS enzyme activity per mitochondrion needs to be up-regulated to meet the enhanced energy demand in the demyelinated axon. Interestingly, increased numbers of mitochondria have been reported in non-myelinated axons of an experimental animal model, in which myelin formation is abnormal [24]. Enhanced OXPHOS activity and mitochondrial number lead to increased production of intracellular ROS and subsequent damage to mtDNA, mitochondrial proteins and neurofilaments [12] and could further contribute to neuroaxonal degeneration. Surprisingly, little is known about the distribution of mitochondria and mitochondrial activity in MS white matter lesions. Hence, we studied the density, distribution, cellular localization and enzyme activity of mitochondria in distinct MS lesion stages.

## Methods

### Brain tissue

Brain tissue from seven non-neurological controls and 26 patients with clinically diagnosed and

neuropathologically confirmed MS was obtained at rapid autopsy and immediately frozen in liquid nitrogen (in collaboration with The Netherlands Brain Bank). White matter MS tissue samples were selected under post-mortem magnetic resonance imaging, as published previously [25]. Relevant clinical information was retrieved from the medical records and is summarized in Table 1. The ages of MS patients at time of death were in the range 34–80 (mean 60) years, with a mean post-mortem delay of 9 h. The ages of the non-neurological controls were in the range 24–84 (mean 63) years, with a mean post mortem delay of 6 h. All donors or next-of-kin provided written informed consent for brain autopsy and use of material and clinical information for research purposes.

### Lesion classification

MS lesions were detected and classified by immunohistochemical analysis using antibodies directed against proteolipid protein (PLP), a myelin component, and anti-major histocompatibility complex class II, clone LN3, as described previously [26]. Active lesions are characterized by abundant perivascular and parenchymal leukocyte infiltration throughout the lesion area, whereas chronic inactive lesions are hypocellular and contain few infiltrating inflammatory cells throughout the lesion. White matter areas without any signs of demyelination and abundant microglial activation were classified as normal-appearing white matter (NAWM). Based on the above-mentioned classification, we identified 12 active and 30 chronic inactive lesions.

### Immunohistochemistry

For immunohistochemical stainings, 5  $\mu\text{m}$  serial cryosections of 12 active lesions, 25 chronic inactive lesions and seven control WM blocks were air-dried and fixed in acetone for 10 min. Next, sections were preincubated with rabbit serum (1:50 diluted in 1% bovine serum albumin in phosphate-buffered saline (PBS), pH 7.4; Dako, Copenhagen, Denmark) for 10 min. Then the sections were incubated for 60 min with the appropriate primary antibody and subsequently incubated with a biotinylated rabbit anti-mouse  $\text{F(ab')}_2$  (1:500; Dako, Copenhagen, Denmark) for 30 min. After incubating for 60 min with s-ABC-HRP complex (1:200; Dako), peroxidase labelling was visualized by 3,3-diaminobenzidine (Sigma, St. Louis, MO, USA). Finally, tissue sections were counterstained with haematoxylin and mounted with DEPEX (Gurr, BDH Laboratories, Poole, UK). Between all incubation steps, the sections were extensively washed in PBS, pH 7.4. All antibodies were diluted in PBS containing 1% bovine serum albumin, which also served as a negative control. Negative controls were essentially blank. Lesion and control tissue were processed simultaneously.

## Mitochondrial alterations in MS lesions

**Table 1.** Clinical data of MS patients and non-neurological controls

Case	Age (years)	Type of MS	Sex	Post-mortem delay (h : min)	Disease duration (years)	Cause of death
1	70	SP	f	6:29	27	Urinary tract infection
2	64	SP	f	7:45	25	Dehydration
3	70	SP	m	6:25	Unknown	Unknown
4	52	SP	f	8:25	22	Respiratory failure
5	34	ND	m	Unknown	Unknown	Unknown
6	69	SP	f	13:20	27	Viral infection
7	64	PP	m	7:30	Unknown	End stage MS
8	65	SP	m	10:35	25	Urosepsis
9	59	ND	f	8:45	24	Respiratory insufficiency
10	66	SP	f	6:20	43	Cancer metastasis in liver
11	80	SP	f	9:35	59	Acute leukaemia
12	53	SP	f	10:45	27	Euthanasia
13	43	SP	m	8:30	17	Pneumonia
14	77	PP	m	4:15	26	CVA
15	48	SP	f	5:50	21	Congestive cardiac failure
16	48	SP	f	4:50	25	Euthanasia
17	72	SP	f	6:05	13	Pneumonia
18	55	PP	f	17:00	19	CVA
19	59	SP	m	22:15	32	Myocardial infarction
20	50	PP	f	7:45	Unknown	Unknown
21	70	ND	f	7:45	51	Cardiac arrest
22	44	PP	f	10:15	Unknown	Decompensation
23	77	ND	f	5:43	Unknown	Respiratory insufficiency
24	61	ND	f	9:55	29	Peri-renal abscess
25	56	ND	m	8:00	27	Pneumonia
26	66	ND	m	7:45	Unknown	Sepsis, hyperthermia
Control	57	—	m	5:40	—	Unknown
Control	24	—	f	0:59	—	Cachexia
Control	82	—	f	Unknown	—	Unknown
Control	91	—	f	Unknown	—	Unknown
Control	84	—	f	4:45	—	Respiratory failure
Control	72	—	m	9:45	—	Unknown
Control	77	—	f	8:20	—	Cachexia, uraemia

SP, secondary progressive MS; PP, primary progressive MS; ND, not determined; m, male; f, female; CVA, cerebrovascular accident.

For double immunohistochemical staining, the sections were incubated overnight with the mitochondrial marker anti-endoplasmic reticulum-associated binding protein (anti-ERAB) and one of the following antibodies: glial fibrillary acidic protein (GFAP; astrocytes); neurofilament (NF; axons); Nogo (oligodendrocytes); and major histocompatibility complex (MHC) class II (inflammatory cells). ERAB immunopositivity was visualized with biotinylated rabbit anti-mouse F(ab')<sub>2</sub> (1:500) followed by 60 min incubation with Streptavidin Alexa-488 (1:400; Molecular Probes, Leiden, The Netherlands). The other antibodies were fluorescently labelled with Alexa-594-labelled goat anti-rabbit (1:400; Molecular Probes). Between all incubation steps, sections were extensively washed in PBS, pH 7.4. Negative controls for the fluorescent double-labelling experiments involved omission of both primary antibodies, and were essentially blank. A detailed description of the primary antibodies used in this article is provided in Table 2.

### Quantification of co-localization

To quantify the density of mitochondria within astrocytes and axons in different lesion stages, as well as the total density of mitochondria, we collected six active and six inactive lesions with adjacent NAWM

**Table 2.** Antibody details

Antigen	Dilution	Antibody type	Source
PLP	1:500	IgG2a	Serotec
MHC class II (LN3)	1:100	IgG2b	Dako
ERAB	1:250	IgG1	Abcam
Complex I subunit 20 kDA	1:50	IgG1	Invitrogen
Complex I subunit 30 kDA	1:100	IgG1	Invitrogen
Complex IV subunit I (COX-I)	1:50	IgG2a	Invitrogen
Complex IV subunit V (COX V)	1:50	IgG2a	Invitrogen
GFAP	1:500	Rabbit polyclonal	Dako
NF	1:100	Rabbit polyclonal	Sigma
NOGO	1:1000	Rabbit polyclonal	Gift from Dr Yan
mtHSP70	1:50	IgG1	Stressgen

from 12 patients. Cryosections were immunostained as described above to analyse the amount of mitochondria (ERAB-positive elements) in axons (NF-positive elements) and astrocytes (GFAP-positive elements). Images were acquired at random in lesions and adjacent NAWM using a standard confocal scanning

microscope (TCS SP2, Leica Microsystems, Heidelberg GmbH, Mannheim, Germany) equipped with an Ar/Kr laser (488 nm) and three HeNe lasers (543, 594 and 633 nm). Images were scanned in two channels, 488 for mitochondria and 594 for astrocytes and axons. Of each section, the maximum number of Z-images (average of 54 Z-images; Z-step was 0.12  $\mu\text{m}$ ) were taken at  $\times 630$  magnification with a double zoom. The basic premises and procedures of the quantification of co-localization were described previously [27,28].

In brief, the software is capable of analysing image series acquired in two- or multichannel experiments. Because the number of overlapping or touching objects in channel A compared to channel B can be different from the number of overlapping or touching objects when channel B is compared to channel A (example: six small objects in channel A all overlap on a single, large structure in channel B, and therefore only one object from channel B overlaps with six in channel A), the comparison of two channels was done in duplex by reversing the channels to be compared. Overlay of graphic output synchronized the results of the duplicate channel. The procedure involved in the software is as follows. The software first recognized three-dimensional (3D) objects in each channel in the Z-series of images. A 3D object was defined as an aggregate of at least 20 voxels with above-threshold intensity. Thresholds for both channels were set manually. Then the program continued by analysing which identified 3D objects in images belonging to the first channel showed (partial) voxel overlap with identified 3D objects in images belonging to the second channel. Next the software determined which of the objects from one channel did not overlap with any object from the other channel. These remaining 3D objects were then processed to determine which of them touched a 3D object present in the other channel. Output consisted of text data files and colour-coded bitmapped projection images of the recognized 3D objects. Finally, the number of co-localized objects was corrected for differences in section thickness.

### Complex IV enzyme histochemistry

Complex IV enzyme histochemistry on serial sections of 11 active and 20 chronic inactive lesions was performed as described previously [29]. Detection of complex IV activity was analysed in sections, which were also used for complex IV immunostainings. In short, freshly cut 5  $\mu\text{m}$  cryosections were immediately incubated for 1 h at 37 °C with substrate mix containing 10 mg cytochrome C (Sigma, St. Louis, MO, USA), 5 mg 3,3'-diaminobenzidine (Sigma), 0.5 mg catalase (Sigma) and 750 mg sucrose in 10 ml phosphate buffer, pH 7.4. Tissue sections were counterstained with haematoxylin and mounted with DEPEX.

### Complex IV and citrate synthase activity assay

For biochemical measurements of complex IV and citrate synthase activity in chronic inactive lesions and NAWM, 100 mg brain tissue of both lesion and NAWM was obtained from nine patients. Cryosections (5  $\mu\text{m}$ ) were stained for PLP and LN3 to locate and classify lesion area and surrounding NAWM. Lesion and NAWM tissue were cut out of consecutive 10  $\mu\text{m}$  cryosections. Hereafter, two additional cryosections were stained for PLP and LN3. In parallel, 100 mg white matter tissue was collected from seven non-neurological patients and used as controls. All samples were prepared in 10% w/v homogenate SEF buffer (0.25 M sucrose, 2 mM K-EDTA, 10 mM phosphate buffer, pH 7.4) using a Potter–Elvehjem homogenizer. Homogenates were stored at  $-80$  °C. Prior to complex IV and citrate synthase measurements, samples were freeze-thawed three times and subsequently diluted with two volumes of SEF buffer. Citrate synthase and complex IV activities in brain extracts were measured according to methods described previously [30,31]. All values are given as  $\mu\text{M}/\text{min}/\text{g}$  wet weight tissue.

### Statistical analysis

The Mann–Whitney U-test was used to compare complex IV activity and co-localization between lesion/NAWM and control. Student's paired *t*-test was used to compare between NAWM and lesion.

## Results

### Mitochondrial protein expression in NAWM and control white matter

To study mitochondrial protein expression in MS lesions and NAWM, we performed immunohistochemistry using different antibodies directed against distinct mitochondrial proteins. In control white matter brain tissue, COX V immunostaining displayed a diffuse parenchymal staining, whereas a more punctuate pattern, typical for mitochondria, was observed with ERAB, complex I 20 kDa and 30 kDa and COX I. In NAWM and control tissue, astrocyte cell bodies and processes showed immunopositivity for all mitochondrial antibodies. Microglia, oligodendrocytes and endothelial cells lining the cerebral blood vessels showed a moderate cytoplasmic staining.

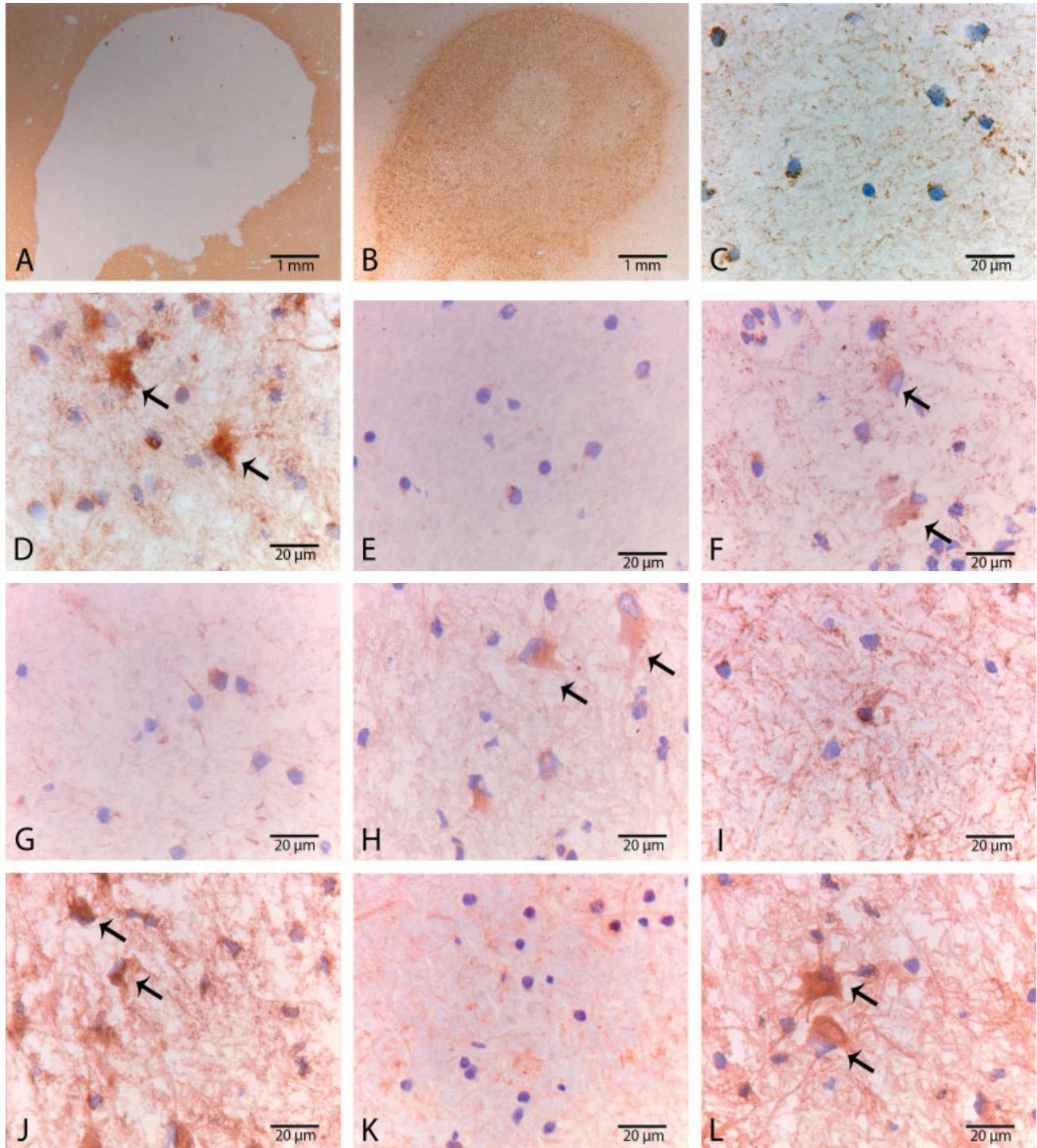
### Mitochondrial protein expression is enhanced in active and CIA MS lesions

In both active (Figure 1) and CIA (Figure 2) lesions we observed enhanced immunoreactivity of complex I 20 kDa and 30 kDa, COX I and V and ERAB compared to surrounding NAWM (Figures 1C–L, 2C–L). Particularly cells with an astrocyte-like morphology showed a marked increased staining in active demyelinating and CIA lesions (arrows in Figures 1C–L, 2C–H).

## Mitochondrial alterations in MS lesions

To identify the cellular localization of mitochondrial proteins in MS lesions, we performed colocalization studies using anti-ERAB with distinct cellular markers (GFAP, NF, MHC class II, and Nogo). In active lesions, a markedly increased co-localization of mitochondria with astrocytes and axons compared to adjacent NAWM was observed (Figure 3A–F). This

increase was also observed in CIA lesions, albeit to a lesser extent (Figure 3G–L). Oligodendrocytes showed a small perinuclear rim of mitochondria (see Supporting information, Figure S1), but no clear differences were observed in the amount of mitochondria comparing NAWM with lesion areas. Infiltrating leukocytes in active lesions contained



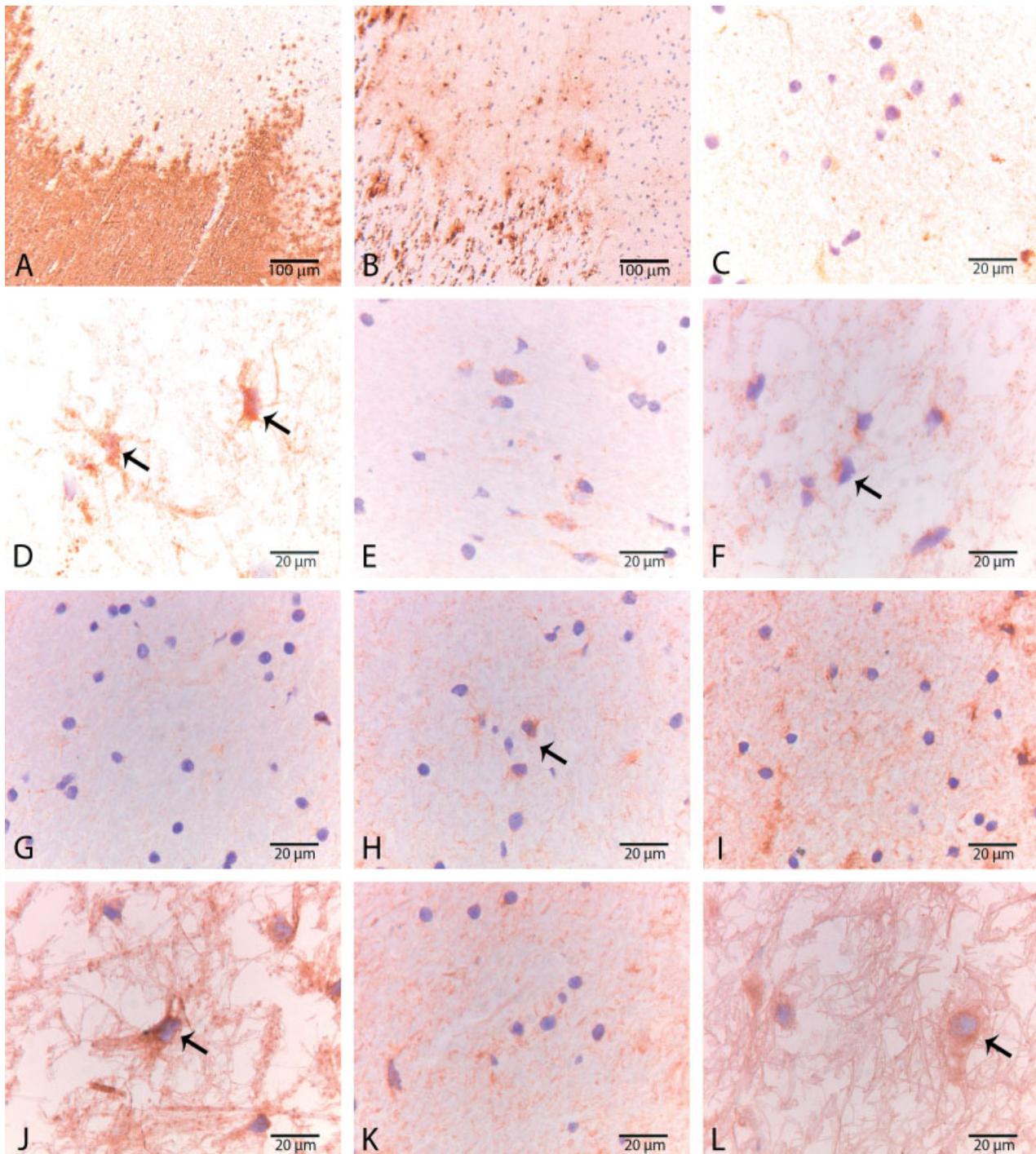
**Figure 1.** Immunohistochemical staining of active MS lesions for various mitochondrial proteins. Active lesions are characterized by demyelination (PLP, A) and contain abundant MHC class II-positive leukocytes (B). Increased expression of ERAB in active MS lesions (D) compared to adjacent NAWM (C) indicates increased mitochondrial density. In active lesions, complex I subunit 20 kDA (F) and 30 kDA (H) are enhanced compared to NAWM (E, G). Complex IV subunits I (J) and V (L) expression is increased in lesions compared to NAWM (I, K). Up-regulation of mitochondrial proteins was particularly observed in astrocyte cell bodies and processes (arrows). PLP, proteolipid protein; MHC, major histocompatibility complex; ERAB, endoplasmatic reticulum-associated binding protein; MS, multiple sclerosis; NAWM, normal-appearing white matter

multiple ERAB-positive mitochondria (see Supporting information, Figure S1).

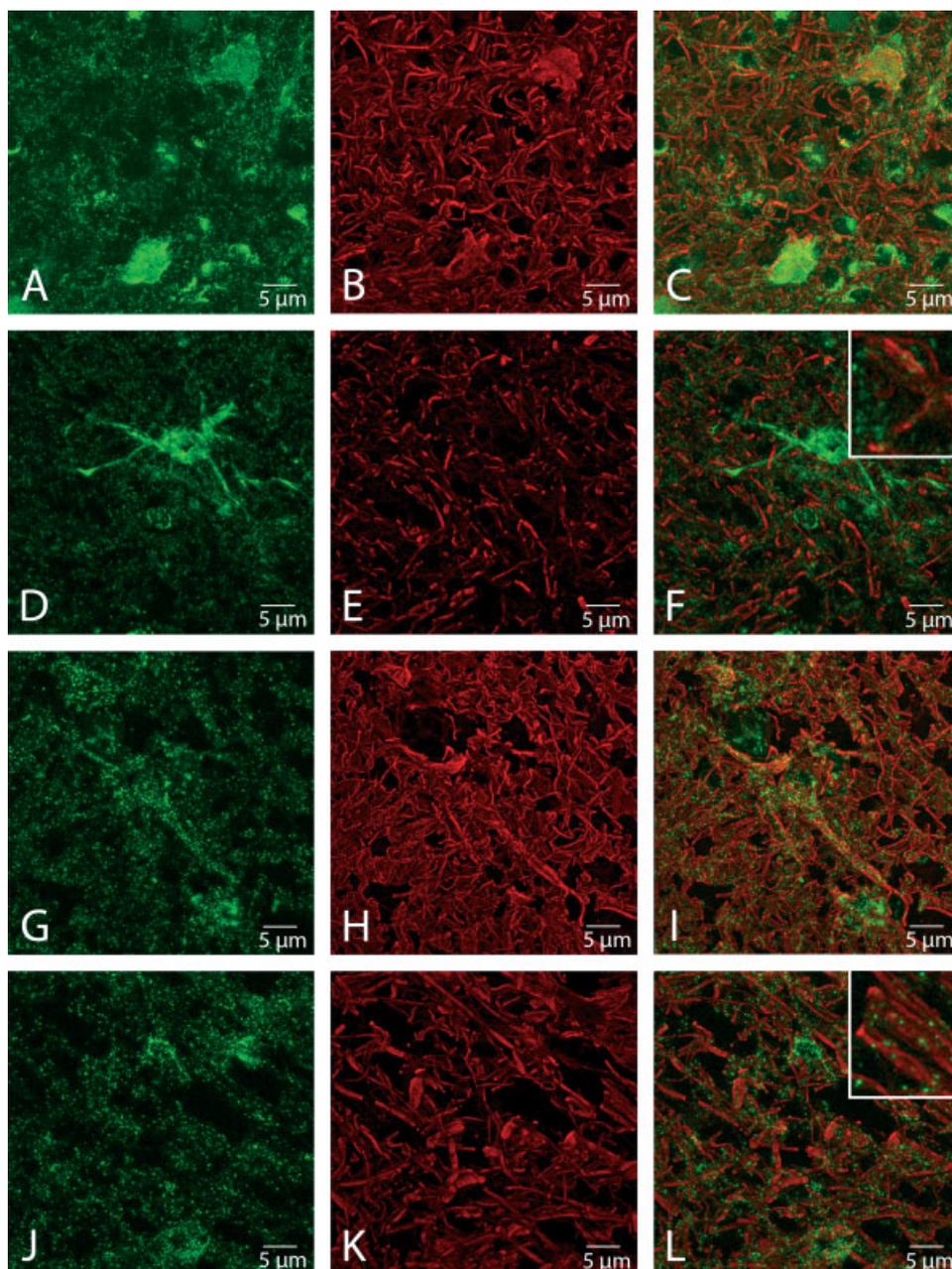
#### Mitochondrial density is significantly increased in astrocytes and axons in MS lesions

To quantify the mitochondrial density in astrocytes and axons, we studied co-localization of ERAB-positive

mitochondria with GFAP- and NF-positive structures in MS lesions and surrounding NAWM. In line with our immunohistochemical data, we demonstrated an increased mitochondrial density in active and CIA lesions compared to NAWM (Figure 4A). The number of mitochondria within axons and astrocytes was significantly enhanced in active lesions compared to



**Figure 2.** Immunohistochemical staining of chronic inactive MS lesions for various mitochondrial proteins. Chronic inactive lesions are characterized by demyelinated areas (PLP, A) with few MHC class II-positive cells (B). In chronic inactive lesions, an increase in expression of ERAB is observed (D) compared to NAWM (C). Expression of all mitochondrial proteins tested was up-regulated in chronic inactive lesions [complex I subunits 20 kDA (F), 30 kDA (H) and complex IV subunits I (J) and V (L)] compared to adjacent NAWM (E, G, I, K, respectively). Up-regulation of mitochondrial proteins was particularly observed in astrocyte cell bodies and processes (arrows). MS, multiple sclerosis; PLP, proteolipid protein; MHC, major histocompatibility complex; ERAB, endoplasmic reticulum-associated binding protein; NAWM, normal-appearing white matter



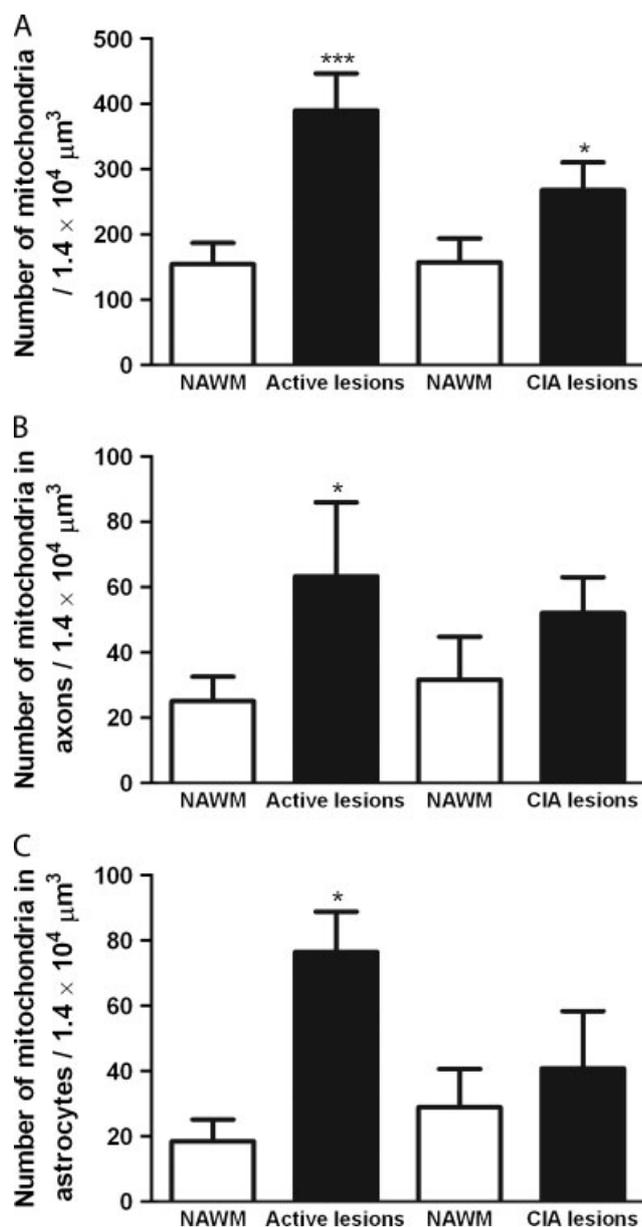
**Figure 3.** Confocal images representing an image stack of double immunofluorescence staining of mitochondria (anti-ERAB, green) and astrocytes (GFAP, red) and axons (NF, red) in active (A–F) and chronic inactive (G–L) MS lesions. In active lesions, mitochondria co-localize with astrocytes and astrocyte processes (A–C) and axons (D–F, inset). Similarly, mitochondria co-localize with astrocytes (G–I) and axons (J–L, inset) in chronic inactive lesions. MS, multiple sclerosis; ERAB, endoplasmatic reticulum-associated binding protein; GFAP, glial fibrillary acidic protein; NF, neurofilament

adjacent NAWM, whereas a trend was observed in CIA lesions (Figure 4B). Mitochondrial density in astrocytes was also significantly enhanced in active lesions and only slightly in CIA lesions (Figure 4C).

#### Complex IV enzyme activity is up-regulated in MS lesions

Histochemical analysis of complex IV activity in NAWM (Figure 5B–C) and control (data not shown) demonstrated a weak parenchymal staining and a clear perinuclear staining in cells with an astrocytic appearance. No difference was observed in staining intensity between NAWM and control white matter.

We revealed an enhanced complex IV enzyme activity in CIA lesions (Figure 5B, E) and active lesions (Figure 5D). Complex IV activity was predominantly increased in large hypertrophic astrocytes and axons in active as well as CIA lesions. Biochemical measurements of complex IV activity in CIA lesions were in line with the histochemical data (Figure 5F) and demonstrated a tendency of increased complex IV activity per mitochondrion in CIA lesions compared to NAWM. Interestingly, complex IV activity was significantly increased in CIA lesions compared to control white matter. Complex IV activity is depicted relative to citrate synthesis activity due to differences



**Figure 4.** Mitochondrial density in MS brain tissue, astrocytes and axons. The number of mitochondria (ERAB-positive elements) within active lesions and chronic inactive lesions is significantly increased compared to NAWM (A). The number of mitochondria within axons is significantly increased in active lesions compared to adjacent NAWM, whereas the number of mitochondria in axons in chronic inactive lesions is only slightly increased compared to adjacent NAWM (B). Similarly, within active lesions astrocytes contain significantly more mitochondria than NAWM. The number of mitochondria in astrocytes in chronic inactive lesions is not altered compared to NAWM (C). MS, multiple sclerosis; ERAB, endoplasmic reticulum-associated binding protein; NAWM, normal-appearing white matter

in mitochondrial number between control, NAWM and lesion tissue.

#### MtHSP70 expression is increased in MS lesions; predominantly in astrocytes

To examine whether the observed increase of mitochondria in lesions was associated with enhanced

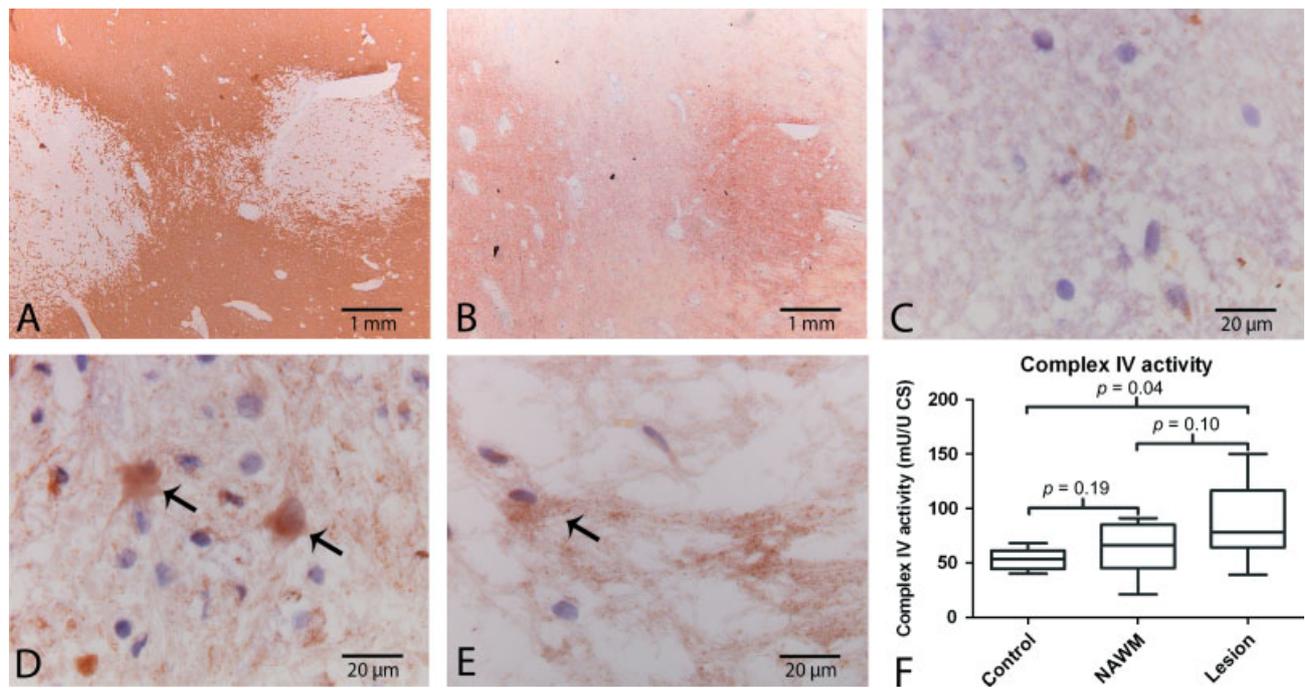
mitochondrial stress, we qualitatively assessed the expression of the mitochondrial heat shock protein 70 (mtHSP70), which is known to be up-regulated upon mitochondrial oxidative stress. In NAWM and control white matter, anti-mtHSP70 weakly stained mitochondria in astrocytes and no differences were observed between NAWM and control white matter (Figure 6A, B). Interestingly, mtHSP70 expression was markedly increased in mitochondria in both active and CIA lesions (Figure 6C, D). Confocal microscopical analysis demonstrated enhanced mtHSP70-immunoreactive mitochondria in astrocytes (Figure 6C, D, inset) and axons (Figure 6D, inset).

#### Discussion

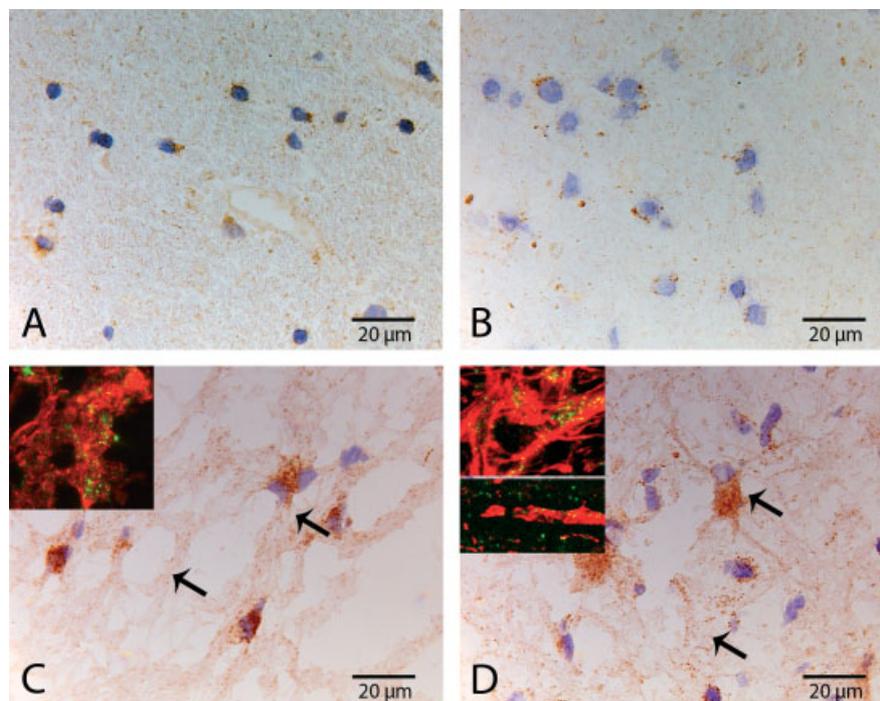
This study provides detailed information on the distribution and activity of mitochondria in active and chronic inactive MS white matter lesions. We show a significant increase in mitochondrial protein expression in different MS lesion stages. Moreover, we demonstrate that the activity of complex IV is markedly up-regulated in MS lesions compared to control white matter and surrounding NAWM, which correlates with the increase in complex IV protein expression. Enhanced numbers of mitochondria in axons and astrocytes are observed in active lesions and, to a lesser extent, in CIA lesions. Interestingly, increase in complex IV activity and mitochondrial density coincides with a marked up-regulation of the mitochondrial heat shock protein 70 (mtHSP70) in MS lesions, indicative of mitochondrial oxidative stress in MS lesions.

Recently, Mahad and colleagues reported a general reduction of mitochondria and a decrease in complex IV expression in pattern III MS lesions [32]. Type III lesions are characterized by extensive oligodendrocyte apoptosis and hypoxia-like tissue injury [33]. In the present study, we use brain tissue from patients with established MS, in which hypoxia-like damage and oligodendrocyte apoptosis are absent or very rare [34]. Although decreased mitochondrial activity might occur in a subset of early lesions, we evidently demonstrate enhanced density of mitochondria and complex IV activity in MS lesions in patients with established disease. Recently, Mahad *et al* [35] demonstrated decreased activity of complex IV in axons in the rim of chronic active lesions, whereas axons within inactive lesions displayed increased activity of complex IV. Furthermore, mitochondrial mass was found to be increased in axons in chronic inactive lesions, which is in line with our data [35]. We observed increased complex IV activity in active lesions, whereas they found a reduction of complex IV activity in the inflammatory rim of chronic active lesions. However, we have investigated complex IV activity within the whole active lesions, not specifically in axons. Hence, it is likely that even though axonal complex IV activity is decreased, total complex IV activity is increased in inflammatory MS lesions.

## Mitochondrial alterations in MS lesions



**Figure 5.** Complex IV activity is increased in MS lesions compared to control white matter. Complex IV enzyme activity (B) is increased in demyelinated areas (PLP, A) compared to NAWM. Complex IV activity is low in NAWM (C) and strikingly increased in active (D) and chronic inactive (E) lesions. Complex IV activity is predominantly observed in astrocyte-like cell bodies and processes (D, E, arrows). Biochemical analysis confirmed enhanced complex IV enzyme activity in chronic inactive MS lesions compared to NAWM and control white matter (F). NAWM, normal-appearing white matter; MS, multiple sclerosis; PLP, proteolipid protein



**Figure 6.** MtHSP70 expression is increased in MS lesions. MtHSP70 expression in control white matter (A) is comparable to NAWM (B). MtHSP70 expression is strikingly increased in active (C) and chronic inactive (D) lesions compared to NAWM and predominantly observed in astrocytes (C, D, arrows) as confirmed by double immunohistochemistry with GFAP (C, D, upper inset). Co-localization of mtHSP70 (green) with axons (NF, red) was also observed in MS lesions (D, lower inset). MS, multiple sclerosis; mtHSP70, mitochondrial heat shock protein 70; WM, white matter; NAWM, normal-appearing white matter; CIA, chronic inactive; GFAP, glial fibrillary acidic protein; NF, neurofilament

We demonstrate an increased number of mitochondria in demyelinated axons in MS lesions. Most likely, mitochondria are recruited to demyelinated axons to meet the increased energy demand needed for proper conduction [23]. In active inflammatory lesions, axons are subjected to elevated levels of ROS and NO, which leads to irreversible inhibition of complex I [36], and subsequent increased ROS production by mitochondria, ATP depletion, influx of  $\text{Ca}^{2+}$  through the reversed  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and eventually axonal injury [37]. The suggested role of mitochondria in axonal loss is further supported by our finding that the increase in mitochondrial density in axons is more evident in active compared to CIA lesions. This might explain the higher occurrence of axonal damage and loss in active lesions [3,6]. Similarly, the increase in the number of mitochondria in axons in CIA lesions might contribute to the 'slow-burning' axonal loss observed in these lesions.

The increase in mitochondria and complex IV activity in astrocytes likely reflects their active involvement in various pathological processes in MS pathology. Astrocytes produce a vast array of molecules involved in MS lesion formation and persistence, such as matrix metalloproteinases, extracellular matrix proteins, chemokines and cytokines (for review, see [38]). Furthermore, reactive astrocytes express high levels of proteins involved in glutamate metabolism, suggesting an important role in sequestering and metabolizing extracellular glutamate [39]. Taken together, astrocytes play an active role in various energy-demanding processes underlying MS pathology. As a consequence, enhanced mitochondrial activity in astrocytes, as reported in this study, might contribute to the formation of toxic free radicals.

In fact, reports demonstrated the occurrence of lipid peroxidation and nitrotyrosine residue formation within astrocytes in MS lesions indicating that astrocytes are a target of ROS-induced damage [40,41]. Lipid peroxidation and tyrosine residue formation can be mediated by peroxynitrite which is formed by a spontaneous reaction between superoxide and nitric oxide [42,43]. In active MS lesions, macrophages produce ROS [44], whereas both astrocytes and macrophages produce NO [45]. Peroxynitrite is able to decrease ATP production through irreversible inhibition of complexes I and III [46]. Previously, a decrease in the activity of mitochondrial respiratory chain complexes I and III was reported in non-demyelinated areas of the motor cortex of MS patients, although the cellular origin was not reported [15]. In chronic active WM lesions, a general reduced complex I activity and a slight increase in complex IV activity have been observed by Lu and colleagues [16], who postulate that enhanced complex IV activity is compensatory for decreased complex I activity; however this remains to be established. It is conceivable that the observed increase in mitochondria and complex IV activity might be a compensation for the reduced ATP production.

In contrast to complex IV, where increased immunopositivity coincides with increased activity, complex I immunoreactivity is increased and activity is decreased, as described previously [16]. Inhibition of complex I activity by either NO or peroxynitrite might explain this apparent contradiction.

Finally, we demonstrate enhanced immunoreactivity of mtHSP70 in MS lesions, particularly in reactive astrocytes. MtHSP70 is a chaperone protein involved in mitochondrial protein import (for review, see [47]) and is up-regulated upon oxidative stress [14]. Over-expression of mtHSP70 reduces ROS formation, improves mitochondrial function and protects astrocytes against ischaemic injury *in vitro* [48]. Therefore, up-regulation of mtHSP70 in astrocytes in MS lesions might function as a protective mechanism to counteract oxidative damage and mitochondrial stress. Remarkably, enhanced mtHSP70 immunoreactivity is also observed in CIA lesions, indicating that astrocytes in MS lesions are subjected to oxidative stress even in the aftermath of acute inflammation.

In summary, we demonstrate an increase in mitochondrial density and activity in active MS lesions and, to a lesser extent, in CIA MS lesions, particularly in astrocytes and axons. In addition, we show enhanced mtHSP70 immunoreactivity in MS lesions, indicating the occurrence of mitochondrial oxidative stress. We speculate that the mitochondrial alterations, as demonstrated in this study, play an important role in various processes underlying MS pathology such as axonal injury. To date, therapies that prevent neuroaxonal loss or promote axonal regeneration are scarce. We speculate that therapies based on enhancement of endogenous antioxidant expression or supplementation of mitochondria-targeted antioxidants might be an attractive strategy to reduce tissue damage in MS [49].

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### **SUPPORTING INFORMATION ON THE INTERNET**

The following supporting information may be found in the online version of this article.

**Figure S1.** Immunohistochemical staining of ERAB in MS lesions