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# Acetate Supplementation Reduces Disease Progression, Alters Cns And Myelin Lipid Content, And Influences Cns And Myelin Protein Content In Mice Subjected To Experimental Autoimmune Encephalomyelitis

Amber C. Chevalier

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ACETATE SUPPLEMENTATION REDUCES DISEASE PROGRESSION, ALTERS CNS AND MYELIN LIPID CONTENT, AND INFLUENCES CNS AND MYELIN PROTEIN CONTENT IN MICE SUBJECTED TO EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

By

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Bachelor of Arts, Concordia College, 2012

Dissertation

Submitted to the Graduate Faculty

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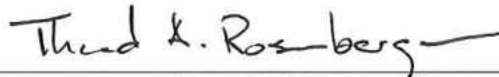
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This dissertation, submitted by Amber Christine Chevalier in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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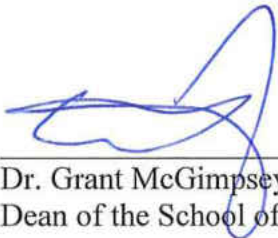
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**PERMISSION**

Title:        Acetate Supplementation Reduces Disease Progression, Alters CNS and Myelin Lipid Content, and Influences CNS and Myelin Protein Content in Mice Subjected to Experimental Autoimmune Encephalomyelitis

Department:   Pharmacology, Physiology, and Therapeutics

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Amber Christine Chevalier
December, 2017

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**ACKNOWLEDGEMENTS**

## DEDICATION

To my family and friends!

**ABSTRACT**

Acetate supplementation increases brain acetyl-CoA levels that influence inflammation, energy production, histone and non-histone protein acetylation, and purinergic signaling. Interestingly, acetate significantly increases fatty acid content in lipopolysaccharide (LPS)-stimulated BV2 (immortalized murine cell line) microglia compared to control-treated LPS-stimulated cells. This suggests that increasing brain acetyl-CoA metabolism may also influence lipid synthesis. We wanted to determine if treatment with glyceryl triacetate alters central nervous system (CNS) lipid content in mice subjected to experimental autoimmune encephalomyelitis (EAE), an autoimmune multiple sclerosis model. In addition, we performed experiments to determine whether treatment had an effect on disease progression, protein levels of enzymes involved in lipid metabolism, cytoskeletal structure, and myelin structure. We found acetate supplementation attenuated the onset of clinical symptoms in EAE mice based on a clinical score and hang time test. These experiments also showed treatment altered spinal cord phospholipid, fatty acid, cholesterol, and ganglioside content in mice subjected to EAE. In addition, treatment significantly increased total brain phosphatidylserine and choline glycerophospholipid as well as GD3 ganglioside levels in EAE mice compared to control-treated EAE mice. We also determined how treatment altered brain lipid levels within the myelin fraction, the membrane fraction lost in EAE. Acetate supplementation significantly increased brain myelin phosphatidylinositol and GM1 ganglioside levels in EAE mice compared to control-treated EAE mice. These data showed that increasing

acetyl-CoA metabolism altered CNS lipid content in mice subject to EAE to suggest that treatment may alter CNS lipid metabolism in this model. To test this hypothesis, we used Western blot analysis to measure protein levels of enzymes involved in lipid synthesis and lipid breakdown. EAE resulted in a significant increase of cytosolic phospholipase $A_2$ protein levels, but treatment returned those levels back to control to suggest treatment may modulate cytosolic phospholipase $A_2$-mediated lipid breakdown. In addition, we found treatment significantly decreased phosphorylated acetyl-CoA carboxylase protein levels in EAE mice, an enzyme involved in fatty acid synthesis. The phosphorylated acetyl-CoA carboxylase is the inactive form of the enzyme, which suggests acetate may shift this enzyme from the inactive to active form to promote fatty acid synthesis.

These data test the hypothesis that treatment may alter CNS lipid metabolism in mice subjected to EAE. This is significant regarding the treatment of demyelinating diseases because developing a therapy to promote lipid synthesis and/or reduce lipid breakdown may prevent, possibly replace, the lipid loss found in EAE. As well, we determined how acetate supplementation altered EAE-induced increases in cytoskeletal structure and myelin inhibiting protein levels. We found EAE injury resulted in a significant increase in cytoskeletal associated proteins, β-actin and merlin, but treatment was unable to return these levels to control. This suggests treatment may not alter all EAE disease pathologies. In addition, there was a significant increase in oligodendrocyte myelin glycoprotein, a myelin inhibiting protein, and acetate supplementation returned this protein back to control levels. This suggests treatment may alter myelin structure, and this was also shown in the FluoroMyelin$^{TM}$ staining. Treatment slightly increased FluoroMyelin$^{TM}$ intensity in EAE compared to control-treated EAE mice but not to same

extent as control levels. These studies demonstrate that acetate supplementation reduced

disease progression, altered CNS and myelin lipid content, and influenced CNS and

myelin protein content in mice subjected to EAE.

# CHAPTER I

## INTRODUCTION

### Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating neurodegenerative disease (Cusick *et al.* 2013, Lassmann & van Horssen 2011, McQualter & Bernard 2007) affecting more than 2.3 million people worldwide (McQualter & Bernard 2007). The inflammatory aspect of the disease involves a self-antigen T-cell attack on the central nervous system (CNS) (Cusick et al. 2013). In MS, the permeability of the blood brain barrier (BBB) decreases allowing peripheral immune cell infiltration, consisting of T-cell lymphocytes, into the CNS (Lassmann & van Horssen 2011). Antigen presenting cells like macrophages present an antigen to T cells, and as a result, T cells are activated to promote the release of pro-inflammatory cytokines and the recruitment of additional immune cells to target the foreign antigen (Cusick et al. 2013, Lassmann & van Horssen 2011, McQualter & Bernard 2007). With regards to MS autoimmunity, T-cells presented with a self-antigen against myelin promote the destruction of myelin via microglia and macrophage activation, pro-inflammatory cytokine release, and further peripheral immune cell infiltration. These events lead to chronic CNS inflammation (Cusick et al. 2013, Lassmann & van Horssen 2011, Nakahara *et al.* 2012, McQualter & Bernard 2007) and immune-mediated demyelination (Lassmann & van Horssen 2011). There is another mechanism by which demyelination can occur in MS, oligodendrocyte-deficient demyelination (Nakahara et al. 2012). This mechanism leads to demyelination either

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through mature oligodendrocyte cell death or dysfunction in oligodendrocyte differentiation and/or function (Lucchinetti *et al.* 2000).

Oligodendrocytes are glial cells within the CNS that are important in myelination (Mitew *et al.* 2014, Boulanger & Messier 2014), and myelin promotes axonal insulation to assist in normal neuronal signal transduction (Mitew et al. 2014, Aggarwal *et al.* 2011, Chrast *et al.* 2011).  Oligodendrocyte progenitor cells (OPCs) are precursor cells that differentiate into mature oligodendrocytes.  OPCs undergo activation and recruitment to the site of demyelination initiated by a stimulus like inflammation or the presence of myelin debris.  A stimulus caused by inflammation promotes the release of particular growth factors, cytokines, and matrix metalloproteinases promoting oligodendrocyte development, inhibiting differentiation, activating proliferation, promoting survival, and increasing recruitment to the site of demyelination.  For example, a growth factor, platelet-derived growth factor (PDGF) forms a complex with platelet-derived growth factor receptor A (PDGFRα) and NG2 expressed on OPCs to promote proliferation but not differentiation.  Once OPCs are recruited to the site of demyelination, OPCs undergo differentiation to become mature oligodendrocytes.  Differentiation from OPCs to mature nonmyelinating oligodendrocytes is based on the release of the thyroid hormone triiodothyronine, the decrease of PDGF, and the release of other growth factors like IGF-1 and TGF-β1.  At this point, mature oligodendrocytes attach to the axon becoming mature myelinating oligodendrocytes promoting myelination (Boulanger & Messier 2014).  It is suggested that in MS pathology, early demyelination leads to remyelination recovery, but as the disease progresses, remyelination fails leading to significant demyelination (Boulanger & Messier 2014) shown by changes in CNS lipid in MS

(Alling *et al.* 1971, Cumings 1969, Cumings 1953, Cumings & Goodwin 1968, Davison & Wajda 1962, Gerstl *et al.* 1970, Gerstl *et al.* 1961). These data suggest that there is a dysfunction in the oligodendrocytes that may be related to immune-mediated demyelination. Both immune-mediated and oligodendrocyte-deficient demyelination are present in different MS lesion patterns.

There are four MS lesion patterns. Types I and II relate to immune-mediated demyelination, and types III and IV relate to oligodendrocyte-deficient demyelination. T-cells and macrophages are present in Types I and II lesion patterns increasing inflammation contributing to demyelination. Interestingly, distinguishing Type II from Type I is based on the presence of immunoglobulins in particular IgG and complement proteins. Type III lesions show oligodendrocyte cell death suggesting no mature myelinating glial cells at the lesion site. As a result, there is a decrease in myelination leading to demyelination. On the other hand, Type IV lesion pattern is related to a dysfunction in the oligodendrocytes. Although there is no oligodendrocyte cell death observed in Type IV lesions, these cells show no myelinating function (Lucchinetti et al. 2000, McQualter & Bernard 2007), which in turn promotes demyelination. The pathological characteristic found in MS suggests two mechanisms for demyelination: immune-mediated and oligodendrocyte-deficient demyelination, both of which results in the loss of myelin and may contribute differently to various MS diagnoses.

MS patients are diagnosed with either relapse-remitting, primary progressive, or secondary progressive (Cusick et al. 2013, Lublin & Reingold 1996, Lublin *et al.* 2014). Most patients, approximately 80%, are diagnosed with relapsing-remitting (McQualter & Bernard 2007) who demonstrate symptoms for a period of time followed by periods of

recovery (Lublin & Reingold 1996).  Over time, 50% of patients with relapsing-remitting

develop secondary progressive MS (Lublin & Reingold 1996) that results in a net

decrease in the recovery period.  People who present with primary progressive MS show

symptoms with no recovery period (Lublin & Reingold 1996).  Understanding the

pathology of secondary progressive and primary progressive MS will provide insight as

to how remyelination in chronic MS fails that leads to significant demyelination

(Boulanger & Messier 2014).  Furthermore, demyelinating lesions within patients

suffering from progressive MS may display more oligodendrocyte-deficient

demyelination versus immune-mediated.  On the other hand, the pathology of relapse-

remitting MS may provide insight into the demyelination injury and remyelination

recovery periods possibly related to both immune-mediated and oligodendrocyte-

deficient demyelination.  In short, MS is a complex disease with differing pathology and

diagnoses.  Because of its complexity, different animal models have been developed to

study the different aspects of MS disease pathology and to determine the effectiveness of

treatments during different clinical courses.


**Animal Models of Multiple Sclerosis**

Different MS animal models allows us to understand the various aspects of MS

pathology, lesion patterns, and diagnoses.  These models include the experimental

autoimmune encephalomyelitis (EAE), Theiler's murine encephalomyelitis (TMEV),

cuprizone, and lyso-lecithin or also known as lyso-phosphatidylcholine.  These models

can resemble either relapse-remitting, primary progressive, or secondary progressive MS

and mimic types I and II lesion patterns or types III and IV (Denic *et al.* 2011, Simmons

*et al.* 2013, Basso *et al.* 2008, Batoulis *et al.* 2011, Hampton *et al.* 2008, Berard *et al.* 2010, Palumbo & Bosetti 2013).  Furthermore, some of these systems allow us to understand mechanisms involved in the demyelination and remyelination process (Denic et al. 2011).

TMEV is a viral-induced autoimmune MS model and has insisted in understanding mechanisms involved in viral infections as risk factors for developing MS (Denic et al. 2011).  In particular, associations between infections of either human herpes virus-6 or Epstein-Barr virus to the later development of MS has been proposed (Berti *et al.* 2002, Kakalacheva *et al.* 2011, Virtanen & Jacobson 2012).  Regarding human herpes virus-6, 12 out of 13 MS patients compared to no controls showed human herpes virus-6 expressing oligodendrocytes especially in regions of plaques (Challoner *et al.* 1995) suggesting viral-induced immune mediated demyelination.  In addition, the prevalence of human herpes virus-6 infection is higher in MS patients compared to other neurological diseases and controls (Chapenko *et al.* 2003).  As well, human herpes virus-6 positive cells in the brain were observed in 73% of MS patients within an area of active demyelination where only 2 out of 28 controls observed these cells.  Furthermore, 54% of MS versus 0% of control patients showed active human herpes virus-6 infection present in the blood (Knox *et al.* 2000), and the presence of brain human herpes virus-6 DNA was found more in MS (57.8%) compared to non-MS patients (15.9%) (Cermelli *et al.* 2003).  Each of these observations suggest a possible association between human herpes virus-6 infection and later development of MS.

In addition to the human herpes virus-6, there is a suggested association between Epstein -Barr virus and later development of MS (Kakalacheva et al. 2011, Virtanen &

Jacobson 2012). Epstein-Barr virus can become latent in B-cells during a person's life suggesting that memory B-cells that recognize the antigen are active in an individual's immune system (Kakalacheva et al. 2011, Virtanen & Jacobson 2012). Based on studies observing the correlation between developing infectious mononucleosis, the disease caused by Epstein-Barr virus, in MS versus non-MS patients, there is approximately a twofold higher risk for someone who developed infectious mononucleosis to later develop MS (Handel *et al.* 2010, Thacker *et al.* 2006). Epstein-Barr virus RNA and protein are found in plasma and B-cells in brains of MS patients, but it is not detected in patients with other inflammatory neurological diseases (Serafini *et al.* 2007). This suggests a strong association between Epstein-Barr infection and MS development. TMEV model may be useful to help understand the pathology of viral-induced immune-mediated demyelination. The induction of the TMEV RNA virus promotes CNS inflammation resulting in immune-mediated demyelination, and as a result of the chronic CNS inflammation, this model stimulates the chronic progressive model (Denic et al. 2011, Procaccini *et al.* 2015). In this regard, TMEV would not be an appropriate model to mimic relapse-remitting MS but may help in the understanding of the potential risk of viral infections leading to autoimmune-induced demyelination (Procaccini et al. 2015). The TMEV model mimics immune-mediated demyelination induced by a previous viral infection, but another MS model, the cuprizone model, mimics oligodendrocyte-deficient demyelination.

Cuprizone, given in the diet, is a copper chelating agent suggested to induce mature oligodendrocyte death through a dysfunction in mitochondrial metabolism (Denic et al. 2011, Procaccini et al. 2015). Cuprizone deactivates enzymes within the respiratory

chain leading to oxidative stress (Kipp *et al.* 2017).  Once administrated, the

demyelination process begins (Procaccini et al. 2015) primarily within the corpus

callosum and the white matter in the hippocampus (Ransohoff 2012) due to the loss of

mature oligodendrocytes (Procaccini et al. 2015).  On the other hand, once cuprizone is

removed from the diet, remyelination can occur through the differentiation of OPCs that

were unaffected by the diet.  Differentiation of OPC to mature oligodendrocytes results in

remyelination (Procaccini et al. 2015).  Cuprizone selectively targets mature

oligodendrocytes but not neurons, astrocytes, microglia, and OPCs.  Cuprizone

significantly decreases the number of mature oligodendrocytes whereas the number of

OPCs remain unaffected suggesting a decrease in the myelinating function due to

treatment (Benardais *et al.* 2013).  Cuprizone serves as a good model in understanding

the mechanisms involved regarding oligodendrocyte function during the demyelination

and remyelination process.  In addition, these data suggest the cuprizone model allows us

to understand mechanisms involved not only in the development of lesion pattern III, but

also oligodendrocyte-deficient demyelination versus immune-mediated demyelination

(Simmons et al. 2013, Procaccini et al. 2015, Ransohoff 2012, Palumbo & Bosetti 2013).

In addition to cuprizone, another toxin-induced demyelinating agent is lyso-lecthin.

The use of lyso-lecthin has helped to understand the demyelination and

remyelination processes.  Lyso-lecthin, injected into spinal cord (Denic et al. 2011) of

mice, induces direct demyelination within the white matter tracts (Procaccini et al. 2015,

Ransohoff 2012) but has no association with immune-mediated demyelination

(Procaccini et al. 2015).  Over time, remyelination can occur (Ransohoff 2012).  The big

drawback to this model is that it does not truly mimic MS pathology with either immune-

mediated or oligodendrocyte-deficient demyelination (Procaccini et al. 2015). Another

MS animal model widely used is the experimental autoimmune encephalomyelitis (EAE)

model, and this model mimics immune-mediated demyelination.

EAE is an autoimmune MS model mimicking the self-antigen attack on the CNS

resulting in immune-mediated demyelination (Simmons et al. 2013, Palumbo & Bosetti

2013). Depending on the protocol and mouse background, EAE can model different MS

diagnoses: relapse-remitting, primary progressive, or secondary progressive. EAE

relapsing-remitting models include SJL/J mice inoculated with proteolipid protein (PLP)

peptide ($PLP_{139-151}$), (Batoulis et al. 2011, Simmons et al. 2013) and C57BL/6 mice

inoculated with a low dose of myelin oligodendrocyte glycoprotein (MOG) peptide

(Batoulis et al. 2011, Simmons et al. 2013, Berard et al. 2010). A chronic progressive

EAE model involves C57BL/6 mice inoculated with high dose of MOG peptide ($MOG_{35-55}$) (Batoulis et al. 2011, Simmons et al. 2013, Berard et al. 2010) and followed by an

injection of pertussis toxin (Kipp et al. 2017, Procaccini et al. 2015). In addition, a

secondary progressive EAE model has been developed (Basso et al. 2008, Hampton et al.

2008, Simmons et al. 2013). In all these models, EAE induction results in an

autoimmune response via self-antigen attack of myelin components resulting in immune-

mediated demyelination (Denic et al. 2011, Simmons et al. 2013) through a Th1/Th17

response based on the release of IFN-γ and IL-17 (Constantinescu *et al.* 2011, Murphy *et al.* 2010, Robinson *et al.* 2014). IL-33 may also play a role in EAE pathogenesis as

inhibition of IL-33 decreases IFN-γ and IL-17 and improves symptoms in EAE model.

Inhibition of IL-33 in EAE results in a decrease of pro-inflammatory mediators such as a

transcription factor promoting Th1 cell differentiation (T-bet) and RAR-related orphan

receptor gamma t.  Furthermore, inhibiting IL-33 increases anti-inflammatory cytokines such as IL-10 and TGFβ in the EAE spinal cord (Li *et al.* 2012).

EAE allows us to understand the autoimmune pathology of MS, but there are some drawbacks to this model.  MOG-induced C57BL/6 EAE is a chronic progressive model but does not lend itself to study the relapse-remitting form of MS.  The majority of individuals with MS are diagnosed with relapse-remitting while the other fraction is diagnosed with primary progressive (McQualter & Bernard 2007).  As mentioned previously, about 50% of relapse remitting patients will develop the secondary progressive form of MS (Lublin & Reingold 1996).  Chronic EAE models may assist in the developing a treatment strategy for progressive MS.  In addition, the EAE disease is primarily found in the spinal cord (Batoulis et al. 2011, Ransohoff 2012, Simmons et al. 2013) whereas MS disease is significantly found within the brain (Procaccini et al. 2015).  Therapies effective in EAE have led to clinical trials and FDA approval.  On the other hand, there are plenty of therapies showing promising results in EAE but failed in clinical trials (Denic et al. 2011, Kipp et al. 2017, Procaccini et al. 2015).  We decided to use the chronic EAE model for our studies because it represents an autoimmune progressive model to investigate mechanisms related to both immune-mediated and oligodendrocyte deficient demyelination.  This model may allow us to understand a potential dysfunction in lipid metabolism in MS.  We can determine how EAE injury results in changes in CNS lipid content and protein levels of enzymes and metabolites involved in lipid synthesis and breakdown.  Furthermore, there is only one FDA approved therapy for primary progressive MS (Dargahi *et al.* 2017), diagnosed in 20% of patients (McQualter & Bernard 2007), and current therapies seem ineffective in treating the secondary

progressive form (Dargahi et al. 2017).  In this model, we can determine the effectiveness

of glyceryl triacetate in the progressive EAE course and determine a potential treatment

strategy for progressive MS.


**Current Available Therapies for Multiple Sclerosis**

Disease-modifying therapies (DMTs) are the current FDA approved MS

medications that modulate the immune response either by inhibiting immune cell

infiltration or by acting as anti-inflammatory agents (Lim & Constantinescu 2010).  First-

line medications include interferon-β and glatiramer acetate, and second-line medications

include mitoxantrone, fingolimod, dimethyl fumarate, natalizumab, and alemtuzumab

(Lim & Constantinescu 2010).  Each one has its own mechanism of action targeting

components within the immune response to produce its immunosuppressive effect.

Interferon-β (IFN-β) was the first FDA approved drug for the treatment of

patients with relapse-remitting MS (Dargahi et al. 2017) in 1993 (Delbue *et al.* 2017).

This treatment inhibits T-cell activation and decreases BBB permeability that in turn

decreases immune cell infiltration into the CNS (Lim & Constantinescu 2010).  This

treatment also increases anti-inflammatory cytokines like IL-10 and IL-4 while

decreasing pro-inflammatory cytokines such as IFN-γ, IL-17, and TNF-α (Dargahi et al.

2017, Oh & O'Connor 2015).  In clinical trials, IFN-β decreases relapse rate, the severity

of relapses, the number of lesions (Dargahi et al. 2017), and is safe in patients suffering

from MS (Oh & O'Connor 2015).  The other first line treatment that is FDA approved is

glatiramer acetate (Delbue et al. 2017).

Glatiramer acetate resembles the structure of myelin basic protein (MBP) (Dargahi et al. 2017, Lim & Constantinescu 2010), and importantly, MBP is a protein found within myelin (Campagnoni & Campagnoni 2004, Eichberg & Iyer 1996). Glatiramer acetate decreases the number of autoreactive T-cells (Lim & Constantinescu 2010) presented with a self-antigen against MBP.  By blocking the interaction between T-cells and the self-MBP peptide (Dargahi et al. 2017), antigen presenting cells cannot present the self-antigen to T-cells to promote autoreactive T-cell activation and immune-mediated demyelination.   In addition, this treatment decreases pro-inflammatory cytokines and increases anti-inflammatory cytokines (Dargahi et al. 2017) to decrease CNS inflammation.  In clinical trials, this treatment decreases the relapse rate by 30%, improves MRI scans, decreases new lesion formation (Dargahi et al. 2017, Lim & Constantinescu 2010), and is well-tolerated (Lim & Constantinescu 2010, Oh & O'Connor 2015).  IFN-β and glatiramer acetate are first-line, but there are numerous second line medications also effective in treating MS like natalizumab, mitoxantrone, and alemtuzumab.

Natalizumab is a monoclonal antibody treatment targeting α4 integrins such as α4β1 integrin (Dargahi et al. 2017, Delbue et al. 2017, Lim & Constantinescu 2010, Oh & O'Connor 2015).  The integrin, α4β1, is expressed on immune cells and interacts with vascular cell adhesion molecule 1 (VCAM-1) expressed on BBB endothelial cells (Dargahi et al. 2017, Lim & Constantinescu 2010).  Natalizumab blocks this interaction between α4β1 and VCAM-1 by binding to VCAM-1 resulting in the decrease of immune cell infiltration into the CNS (Dargahi et al. 2017, Delbue et al. 2017, Lim & Constantinescu 2010, Rice *et al.* 2005) decreasing CNS inflammation.  In particular,

peripheral T-cells are unable to cross the BBB and promote the self-antigen immune attack in the CNS contributing to immune-mediated demyelination.  In the EAE model, α4β1 integrins are essential in the trafficking of T-cells into the CNS to contribute to EAE pathogenesis (Bauer *et al.* 2009).  In the EAE model, targeting α4β1 integrins results in a decrease of immune cells within the CNS and the attenuation of clinical paralysis (Yednock *et al.* 1992).  Natalizumab decreases relapse rate by 68%, disability progression by 42%, number of lesions (Dargahi et al. 2017, Delbue et al. 2017, Polman *et al.* 2006), and clinically improves MRI scans (Delbue et al. 2017, Lim & Constantinescu 2010, Polman et al. 2006).  In addition, this treatment is well tolerated (Oh & O'Connor 2015), shows long term benefits, and increases the quality of life (Delbue et al. 2017) of patients suffering from MS.  This treatment is prescribed to patients with severe relapse-remitting MS (Lim & Constantinescu 2010) due to the increase risk of developing progressive multifocal leukoencephalopathy, a fatal demyelinating disease (Dargahi et al. 2017, Delbue et al. 2017).  This treatment was FDA approved (Dargahi et al. 2017, Delbue et al. 2017) until PML was detected in a few patients, and as a result, natalizumab was removed from the market until recently reinstated for special circumstances (Dargahi et al. 2017).

Mitoxantrone started as a cancer drug for patients with non-Hodgkin's lymphoma, breast cancer, acute myeloid leukemia, and prostate cancer by targeting type II topoisomerase to prevent DNA synthesis and repair that leads to the inhibition of cancer cell division.  Now, this drug is FDA approved for the treatment of MS (Delbue et al. 2017).  Using human MS peripheral blood mononuclear cells *in vitro*, mitoxantrone decreases immune cell proliferation, promotes immune cell death, and decreases dendritic

cell maturation (Neuhaus *et al.* 2005) suggesting this treatment modulates the immune response and possesses immunosuppressive properties (Dargahi et al. 2017) in MS. As well, this treatment prevents T-cell activation and antigen presentation, decreases the proliferation of immune cells such as T-cells, B-cells, and macrophages (Lim & Constantinescu 2010), and decreases pro-inflammatory cytokines (Dargahi et al. 2017, Lim & Constantinescu 2010). In preclinical studies, mitoxantrone attenuates clinical paralysis and disease onset in the EAE model (Lim & Constantinescu 2010, Ridge *et al.* 1985), and in clinical trials, treatment decreases the number of relapses by 69% (Lim & Constantinescu 2010) and disability progression by 80%. Due to serious side effects like cardiotoxicity and acute leukaemia (Lim & Constantinescu 2010), this treatment is not used often (Dargahi et al. 2017).

Alemtuzumab is a monoclonal antibody inhibiting CD52 expressed on lymphocytes and monocytes (Lim & Constantinescu 2010). As a result, activated T and B-cells are eliminated, and there is switch from a pro-inflammatory to an anti-inflammatory state (Dargahi et al. 2017). Anti-CD52 improves clinical symptoms based on a decrease in clinical score between control-treated EAE mice and anti-CD52-treated EAE mice. In addition, treatment decreases T-cell infiltration, pro-inflammatory cytokines, demyelination, and axonal loss (Turner *et al.* 2015). In clinical trials, this treatment delays the onset of disability by 74% (Lim & Constantinescu 2010) and decreases the relapse rate (Dargahi et al. 2017). Unfortunately, due to frequent and serious side effects, this drug is only prescribed when patients are unable to respond to other DMTs (Dargahi et al. 2017). The second line agents, natalizumab, mitoxantrone,

and alemtuzumab, have serious side effect profiles and so, these better tolerated second line agents, fingolimod and dimethyl fumarate, are prescribed more often.

Fingolimod was the first oral FDA approved drug for the treatment of patients with relapse-remitting MS (Dargahi et al. 2017). Fingolimod targets the sphingosine-1-phosphate receptor that interacts with T-cells promoting inflammatory signaling (Lim & Constantinescu 2010). As a result, this treatment decreases auto-reactive lymphocytes into the CNS (Dargahi et al. 2017). Fingolimod was effective in MS animal models and moved onto clinical trials (Lim & Constantinescu 2010). In the relapse-remitting EAE model, fingolimod significantly improves clinical symptoms showing an attenuation of relapses and an improvement in motor behavior. Interestingly, treatment is not effective in the secondary progressive EAE model (Al-Izki *et al.* 2011) suggesting immunomodulatory therapies might not be sufficient in the treatment of progressive MS. In clinical trials, treatment with fingolimod decreases the relapse rate by about 50%, lesion number and volume (Lim & Constantinescu 2010, Dargahi et al. 2017), the number of relapses (Dargahi et al. 2017), and is well tolerated (Lim & Constantinescu 2010).

Dimethyl fumarate is another oral FDA approved MS therapy (Dargahi et al. 2017, Delbue et al. 2017). Treatment shows neuroprotective properties through nuclear factor E2-related factor 2 activation decreasing oxidative stress in the CNS (Dargahi et al. 2017, Lim & Constantinescu 2010, Oh & O'Connor 2015). This treatment also displays anti-inflammatory properties by decreasing pro-inflammatory cytokines and cell adhesion molecules decreasing immune cell infiltration into the CNS (Dargahi et al. 2017, Lim &

Constantinescu 2010).  In clinical trials, treatment decreases the relapse rate (Dargahi et al. 2017) with moderate side effects and overall long-term safety (Oh & O'Connor 2015).

There are a few FDA approved DMTs on the market that also modulate the immune response.  Daclizumab binds to IL-2 receptor that decreases the activation of T-cells overall decreasing inflammation (Dargahi et al. 2017, Lim & Constantinescu 2010).  In clinical trials, treatment decreases relapse rate and lesion formation (Dargahi et al. 2017).  Teriflunomide is a leflunomide metabolite (Dargahi et al. 2017, Lim & Constantinescu 2010) that inhibits dihyro-orotate dehydrogenase (Dargahi et al. 2017, Lim & Constantinescu 2010, Oh & O'Connor 2015) preventing the proliferation and differentiation of lymphocytes (Dargahi et al. 2017, Lim & Constantinescu 2010).  In clinical trials, treatment decreases relapse rate, lesion number, and disability progression (Dargahi et al. 2017, Oh & O'Connor 2015).  Interestingly, ocrelizumab is the first FDA approved drug for primary progressive MS (Dargahi et al. 2017) and targets CD20 expressed on B-cells.  Treatment eliminates B-cells to suppress T-cell induced inflammation to inhibit the activation of CD4 T cells, cytokine release, and antibody release (Dargahi et al. 2017).  These therapies seem to be effective in relapse-remitting MS but not effective in treating secondary progressive (Dargahi et al. 2017).  This leads to the need for the development of new therapeutic strategies effective in treating progressive MS.

**Gaps in Knowledge**

There are two mechanisms by which demyelination can occur in MS, immune-mediated demyelination, Types I and II, and oligodendrocyte-deficient demyelination,

Types III and IV (Lucchinetti et al. 2000, Nakahara et al. 2012). Current DMTs are immunomodulatory (Lim & Constantinescu 2010) targeting the immune-mediated demyelination but not the oligodendrocyte-deficient demyelination. This suggests that current MS therapies do not treat the entire disease. Developing therapies to promote the differentiation of OPCs, increase the myelination function of oligodendrocytes, increase the production of lipid, or decrease the breakdown of lipid are mechanisms that may be effective in treating all forms of demyelination. Using different MS animal models may also lead to potential therapeutic targets leading to the recovery of myelin lipid and normal lipid turnover rates.

MS patients are diagnosed with either relapse-remitting, primary progressive, or secondary progressive course. Current DMTs are effective in the treatment of relapse-remitting (Dargahi et al. 2017, Lim & Constantinescu 2010) where there is only one FDA approved drug for primary progressive, and current DMTs seem to be ineffective for treating secondary progressive (Dargahi et al. 2017). Therefore, this suggest that developing therapeutic strategies in the treatment of progressive versus relapse-remitting MS is definitely needed.

Immune-mediated demyelination involves both the increase in CNS inflammation and the loss of myelin (Cusick et al. 2013, Lassmann & van Horssen 2011, McQualter & Bernard 2007). Understanding the inflammatory response in EAE and MS is fairly well-known, but research still focuses on how to down-regulate the immune response therapeutically. The understanding of the demyelination processes in EAE and MS and how to therapeutically promote the recovery of myelin is where research needs to switch its focus. Altering spinal cord lipid in EAE may lead to a therapy that in turn increases

myelin lipid deposition to treat demyelinating diseases.  Our studies set out to determine

how glyceryl triacetate alters CNS lipid content in mice subjected to EAE, an

autoimmune progressive MS animal model.  If treatment alters CNS lipid content in this

model, this will lead to determining how treatment alters lipid synthesis and/or

breakdown to show that recovery of lipid lost due to injury.

**Lipid Synthesis, Lipid Breakdown, and Myelin Lipid Composition**

Lipids are essential in membrane structure and stability (Schmitt *et al.* 2015,

Zhang & Liu 2015, Morell 1984), signaling regulation (Glade & Smith 2015, Kim *et al.*

2014), inflammation (Schmitt et al. 2015, Shimizu 2009), and normal cognitive function

(Glade & Smith 2015, Kim et al. 2014, Zhang & Liu 2015).  For example,

phosphatidylserine is important in maintaining normal nerve membrane and myelin

structure, regulating neurotransmitter metabolism, and regulating phospholipase

C/protein kinase C signaling (Glade & Smith 2015).  In addition, phosphatidylserine

supplementation improves learning and memory in the elderly showing various levels of

dementia suggesting lipids contribute to normal cognitive function (Glade & Smith 2015,

Kim et al. 2014).  Also, gangliosides, cholesterol, and plasmalogens promote myelin

stability (Saher *et al.* 2005, Schmitt et al. 2015), and plasmalogens contribute to

phospholipase $A_2$-mediated inflammation (Schmitt et al. 2015).  Very importantly, lipids

are essential in membrane structure for example myelin structure, comprised mostly of

lipid compared to protein (Morell 1984).  In order for lipids to incorporate into the myelin

structure, lipids are produced through the precursor, acetyl-CoA (Jaworski *et al.* 2016).

Acetyl-CoA is the precursor to fatty acid, cholesterol, and ceramide synthesis. Acetate is

converted to acetyl- CoA by acyl-CoA synthetase enzyme (Jaworski et al. 2016, Shimazu *et al.* 2010) and has been shown to incorporate into phospholipid (Smith 1964, Mehta & Namboodiri 1995), fatty acid (D'Adamo & Yatsu 1966), and cholesterol (Chakraborty *et al.* 2001).

Fatty acids are long-chain carboxylic acids that can be saturated, with no double bonds, or unsaturated, with one or more double bonds. First, malonyl-CoA is produced by the introduction of a carboxyl group to acetyl-CoA catalyzed by acetyl-CoA carboxylase, and the condensation of malonyl-CoA with another acetyl-CoA results in the formation of β-ketoacyl-CoA. From this point, elongation by two carbons per reaction results in fatty acids with 12 or more carbons. In addition, desaturase enzymes catalyze the insertion of a double bond into fatty acids to form unsaturated fatty acids. Overall, fatty acids are formed either by *de novo* synthesis with the acetyl-CoA precursor or through the elongations and desaturation of essential fatty acids from the diet including linoleic (18:2n6) or alpha-linolenic (18:3n3) acids (Barcelo-Coblijn & Murphy 2009, Cinti *et al.* 1992, Jaworski et al. 2016, Baenke *et al.* 2013, Benjamins *et al.* 2012). Once the appropriate fatty acids are formed, two fatty acids are incorporated into phospholipid at the sn-1 and sn-2 positions (Baenke et al. 2013).

Phospholipid contains a glycerol-3-phosphate backbone with fatty acids located at the sn-1 and sn-2 position indicated by X and R shown in Figure 1. Phosphatidic acid (PtdOH) is the precursor to the production of phospholipids. First, dihydroxyacetone phosphate (DHAP) is acylated to form acyl-DHAP, the precursor to ether lipids like plasmalogen (PlsEtn and PlsCho) and plasmanyl (PakEtn and PakCho) fractions of ethanolamine and choline glycerophospholipid (EtnGpl and ChoGpl). Additional

acylation results in the production of PtdOH to give rise to phosphatidylinositol (PtdIns)

or diacylglycerol (DAG). From there, DAG forms phosphatidylethanolamine (PtdEtn)

and phosphatidylcholine (PtdCho). Interestingly, PtdCho can be formed from PtdEtn

through methylation of the side group at the sn-3 position shown by letter Y in Figure 1.

Phosphatidylserine (PtdSer) can be formed from PtdEtn through the addition of a

carboxyl group at the sn-3 position (Baenke et al. 2013, Benjamins et al. 2012,

Braverman & Moser 2012, Brites *et al.* 2004, Hajra 1995, Kennedy 1986, Magnusson &

Haraldsson 2011, Kim et al. 2014).
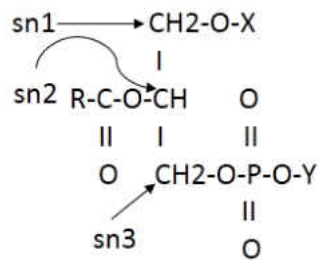


**Figure 1**: General structure of phospholipids. Sn-1, sn-2, and sn-3 positions are indicated by the arrows. X and R is the locations of a fatty acid, and Y is the location of the phospholipid side group.


Acetyl-CoA is also the precursor to cholesterol synthesis. The condensation of

three acetyl-CoAs result in the production of β-hydroxy-β-methylglutaryl (HMG)-CoA,

and further, HMG-CoA is reduced to mevalonic acid catalyzed by HMG-CoA reductase.

After the production of mevalonic acid, there is a series of reactions that results in the

production of cholesterol and cholesteryl esters, a cholesterol metabolite (Baenke et al.

2013, Benjamins et al. 2012, Saher *et al.* 2011, Jaworski et al. 2016). The synthesis of

cholesterol occurs primarily in the brain because cholesterol does not cross the blood

brain barrier (Morell & Jurevics 1996).

Sphingolipid synthesis requires the production of palmitoyl-CoA, a fatty acid-CoA and through a series of reactions results in the production of ceramide, the precursor to sphingolipids. Ceramide is the precursor to the production of sphingomyelin (Cer$P$Cho), sulfatides or galactocerebrosides, and gangliosides (Benjamins et al. 2012). Gangliosides also require the addition of mono- or polysaccharides and sialic acid (N-acetyl-neuraminic acid, NANA) to ceramide (Schnaar *et al.* 2014, Yu *et al.* 2011).

There is a natural turnover of lipid (Rosenberger *et al.* 2002, Ando *et al.* 2003, Moser *et al.* 1999) that requires a synthesis of lipid in conjugation with a breakdown of lipid involving phospholipase activity such as cytosolic phospholipase $A_2$ and phospholipase C. Phospholipases hydrolyze membrane phospholipids to promote inflammatory and signaling cascades. Cytosolic phospholipase $A_2$ (cPLA$_2$) releases arachidonic acid from membrane phospholipids at the sn-2 position shown in Figure 2. Arachidonic acid is used to form inflammatory eicosanoids like prostaglandins and thromboxanes catalyzed by cyclooxygenases or leukotrienes catalyzed by 5-lipoxygenase. In addition, cPLA$_2$ can hydrolyze membrane ether phospholipids to release arachidonic acid and inactive lyso-platelet-activating factor (lyso-PAF). PAF
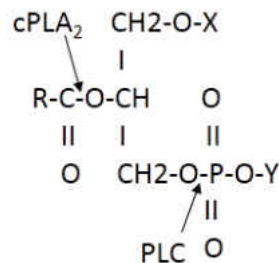


**Figure 2:**The phospholipase targets on phospholipids. Abbreviations include cPLA$_2$ (cytosolic phospholipase $A_2$) and PLC (phospholipase C).

becomes active after the acetylation of lyso-PAF, which then promotes inflammation (Shimizu 2009, Benjamins et al. 2012). In addition to cPLA$_2$, phospholipase C (PLC) cleaves the bond indicated in Figure 2 at the sn-3 position of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$) releasing DAG and inositol-1,4,5-trisphosphate (I(1,4,5)P$_3$). Important to note, PtdIns(4,5)P$_2$ is formed through the phosphorylation of PtdIns. DAG activates protein kinase C, an important signaling molecule, and I(1,4,5)P3 increases calcium release from the endoplasmic reticulum (Suh *et al.* 2008, Benjamins et al. 2012). Cytosolic PLA$_2$ is important in promoting pro-inflammatory lipid signaling, and PLC is important in regulating signal transduction.

Regarding the EAE model and MS, there are changes in lipid content and phospholipase levels (Kalyvas & David 2004, Soliman *et al.* 2013b, Cumings 1969, Cumings 1953, Cumings & Goodwin 1968, Gerstl et al. 1970, Gerstl et al. 1961). MS is considered in part, an autoimmune disease, and cPLA$_2$ promotes inflammation contributing to immune-mediated demyelination. Cytosolic PLA$_2$ is associated with an increase in immune cells in EAE lesions, and the inhibition of cPLA$_2$ results in the improvement of symptoms in this model. This suggests that an increase in cPLA$_2$ levels contribute to immune-mediated demyelination (Kalyvas & David 2004) in EAE. In addition, MS and EAE have been associated with changes in myelin lipid content (Alling et al. 1971, Cumings 1969, Cumings & Goodwin 1968, Gerstl et al. 1970, Salvati *et al.* 1990). Myelin is an electrical insulator to assist in directing the electrical current along the axon (Aggarwal et al. 2011, Chrast et al. 2011). Myelin composition (Morell 1984) is primarily composed of lipid (80%) where the other fraction is composed of protein (20%). The lipid portion is composed of cholesterol (28%), galactolipid (28%), and

phospholipid (43%). Within myelin galactolipid, galactocerebrosides occupy 23%, and

sulfatides occupy 4%. Myelin phospholipid portion is composed of PtdEtn (16%),

PtdCho (11%), Cer*P*Cho (8%), PtdSer (5%), PtdIns (0.6%), and ethanolamine

plasmalogen (PlsEtn, 12%). The protein fraction is composed of proteins primarily MBP

(Campagnoni & Campagnoni 2004, Eichberg & Iyer 1996) and PLP (Greer & Lees 2002,

Hudson 2004). Other myelin proteins include 2':3'-cyclic nucleotide 3'

phosphodiesterase (Eichberg & Iyer 1996), myelin-associated glycoprotein (MAG)

(Eichberg & Iyer 1996, Georgiou *et al.* 2004), MOG (Pham-Dinh *et al.* 2004), and

oligodendrocyte-myelin glycoprotein (OMgp) (Mikol *et al.* 1993, Mikol & Stefansson

1988). In the EAE model, injury decreases spinal cord phospholipid (Revina *et al.* 2011,

Taranova 1985) in particular PtdEtn, PtdCho, and PtdSer (Revina et al. 2011), spinal cord

and brain cerebrosides (Roth *et al.* 1982, Taranova 1985), spinal cord and brain sulfatides

(Maggio *et al.* 1972), and spinal cord gangliosides (Maggio et al. 1972). Regarding

myelin brain lipid, EAE injury decreases phospholipid content in particular CerPCho,

PtdSer + PtdIns, and PtdEtn (Salvati et al. 1990). EAE injury results in significant

decreases in CNS lipid that is comparable to lipid changes shown in MS. Regarding

white matter in MS patients, there are decreases in sphingomyelin (Davison & Wajda

1962), plasmalogen (Cumings 1969, Davison & Wajda 1962), phospholipid (Cumings

1953, Cumings & Goodwin 1968, Gerstl et al. 1970, Gerstl et al. 1961, Alling et al. 1971,

Cumings 1969), cerebrosides (Cumings 1969, Cumings 1953), sulfatides (Alling et al.

1971), gangliosides (Kishimoto *et al.* 1967), and cholesterol (Cumings 1953). There is a

decrease in lipid content and an increase in phospholipase activity to suggest an increase

in pro-inflammatory lipid signaling or possibly a metabolic issue resulting in a

dysfunction of lipid metabolism.  A therapy to promote lipid synthesis and/or decrease

lipid breakdown within the CNS may be a potential remyelination therapy to treat

patients suffering from demyelinating diseases like MS.  In this regard, increasing acetyl-

CoA metabolism may alter CNS lipid metabolism and be a therapeutic strategy for

treating MS.

**Glyceryl Triacetate**

Glyceryl triacetate currently approved as a food additive (Tsen *et al.* 2014)

metabolizes into acetate and glycerol when administered orally (Arun *et al.* 2010a, Long

*et al.* 2013, Tsen et al. 2014).  From there, acetate is available to enter the CNS to form

active acetyl-CoA utilized in energy production, acetylation, and lipid synthesis (Bhatt *et

al.* 2013, Bhatt & Rosenberger 2014, Soliman & Rosenberger 2011, Soliman *et al.*

2012b).  Acetate, a two carbon monocarboxylic acid, which is the shortest fatty acid and

again, is converted to the active form acetyl-CoA by acyl-CoA synthetase (Jaworski et al.

2016).  After a single dose of glyceryl triacetate given to control Sprague-Dawley rats,

brain acetyl-CoA increases 2.2 fold 30 minutes to 4 hours post treatment (Reisenauer *et

al.* 2011).  By increasing brain acetyl-CoA metabolism, treatment can alter inflammation,

histone acetylation, purinergic signaling, and energy stores.

It is suggested glyceryl triacetate is anti-inflammatory in animal models of

neuroinflammation and neuroborreliosis.  In a LPS-induced neuroinflammation rat

model, acetate supplementation reduces microglia activation, reactive astrocytes

(Reisenauer et al. 2011), and decreases IL-1β protein and mRNA levels (Soliman et al.

2012b).  In addition, in the rat model of neuroborreliosis, treatment decreases microglia

activation and brain IL-1β levels suggesting a decrease in the pro-inflammatory response (Brissette *et al.* 2012). Acetate shows anti-inflammatory effects *in vitro* as well as *in vivo*. In primary astrocytes, acetate treatment attenuated TNFα and IL-1β levels (Soliman *et al.* 2013a), and similarly in BV2 microglia, acetate treatment attenuated pro-inflammatory cytokine levels like IL-1β, TNFα, and IL-6 (Soliman *et al.* 2012a). Acetate also alters pro-inflammatory lipid signaling *in vitro*. In LPS-stimulated BV2 microglia cultures, treatment returned PLCβ1 levels to controls. However, in LPS-stimulated primary astrocytes, treatment significantly decreases phosphorylated cPLA$_2$ and PLCβ1 levels compared to sodium chloride-treated LPS-stimulated primary astrocytes. In LPS-stimulated BV2 and primary astrocytes, treatment significantly decreases cyclooxygenase-1 (COX-1) and COX-2 levels, but interestingly, only in LPS-stimulated astrocytes, acetate significantly decreases prostaglandin E2 levels compared to sodium chloride-treated LPS-stimulated cells (Soliman et al. 2013b). Acetate reduces pro-inflammatory lipid signaling *in vitro* and *in vivo* to suggest treatment displays anti-inflammatory effects. This shift from a pro-inflammatory to an anti-inflammatory state may be a result of changes in histone acetylation shifting gene and protein expression to increase anti-inflammatory compounds versus pro-inflammatory ones.

In a LPS-induced neuroinflammation model, acetate supplementation significantly increases histone acetylation and decreases histone deacetylase levels and activity compared to control-treated LPS-induced rats (Soliman et al. 2013a, Soliman et al. 2013b, Soliman et al. 2012a, Soliman & Rosenberger 2011, Soliman et al. 2012b). Treatment reverses the effects of MK-801, a drug which induces histone hypoacetylation, in mice to correlate with cognitive improvements shown by an increase in performance in

the elevated plus maze and novel object recognition tests (Singh *et al.* 2016). Acetate

supplementation reduces inflammation that may correlate to changes in histone

acetylation and levels of histone deacetylase, but treatment also alters purinergic

signaling *in vivo*.

In a LPS-induced neuroinflammation model, LPS exposure decreases brain CD73

levels and increases brain adenosine A2A receptor, and acetate supplementation returns

these levels back to control (Smith *et al.* 2014). These data suggest that treatment alters

purinergic signaling in the neuroinflammation model. As well, treatment increases brain

phosphocreatine levels suggesting treatment alters energy stores in the CNS (Bhatt et al.

2013). In addition, acetate regulated MAPK and NF-κB signaling in primary astrocytes

(Soliman et al. 2013a) and MAPK and JNK pathway in BV2 microglia (Soliman et al.

2012a). Acetate alters inflammation, acetylation state, purinergic signaling, energy

stores, and MAPK regulation.

Acetate supplementation is effective in rat models of neuroinflammation and

neuroborreliosis, but it is also effective in treating cancer, Canavan disease, and traumatic

brain injury. Canavan disease is an autosomal recessive demyelinating disease where

people suffering from this disease show a mutation in the gene that encodes for

aspartoacylase (ASPA) that releases acetate from N-acetylaspartate (NAA). Due to this

mutation, there is an increase in NAA and a decrease in acetate in the brain. As a result,

there is hypomyelination due to acetate unable to incorporate into myelin (Namboodiri *et

al.* 2006b, Namboodiri *et al.* 2006a). Acetate supplementation alters brain acetate levels

but not brain NAA levels (Mathew *et al.* 2005). Increasing brain acetate bypasses the

mutation, and acetate is utilized to produce myelin lipid in Canavan disease. In tremor

rats, a model for Canavan disease, treatment significantly increases motor performance

through the rotarod and increases myelin galactocerebroside compared to untreated

tremor rats (Arun *et al.* 2010b).  Glyceryl triacetate is safe and tolerable at both low and

high doses in tremor rats and infants suffering with Canavan disease (Madhavarao *et al.*

2009, Segel *et al.* 2011).  These data that suggest acetate supplementation is beneficial in

the treatment of Canavan disease.  Treatment prevented cell growth in glioma-derived

tumor cells (Long *et al.* 2015, Long et al. 2013, Tsen et al. 2014) and significantly

increases motor performance in a rat model for traumatic brain injury (Arun et al. 2010a).

Acetate supplementation is effective in the treatment of rat models of neuroinflammation,

neuroborreliosis, Canavan disease, and traumatic brain injury.  In particular, treatment

has been shown to be effective in tremor rats and infants suffering from Canavan disease,

a demyelinating disease, to suggest treatment may be beneficial in other demyelinating

diseases like MS.  Understanding acetate's role to promote lipid synthesis may treat

demyelination and lead to the development of a remyelination therapy to recover the lipid

lost due to demyelination.  Acetate supplementation may be an avenue to address this,

and interestingly, acetate significantly increases fatty acid content in LPS-stimulated BV2

microglia cultures (Bhatt & Rosenberger 2014).  With the knowledge from this previous

study, we wanted to investigate acetate's role in altering lipid metabolism in an injury

state.


**Overview of Dissertation**

We propose that acetate supplementation alters CNS lipid content in mice

subjected to EAE.  To test this hypothesis, spinal cord and brain lipid levels were

determined in control and EAE mice treated with either water or glyceryl triacetate.  We also determined how treatment alters disease progression, spinal cord protein levels of enzymes involved in lipid metabolism, cytoskeletal structure, and myelin structure.  We found that acetate supplementation attenuated the onset of clinical symptoms in EAE mice.  In addition, the treatment altered spinal cord phospholipid, fatty acid, cholesterol, and ganglioside content in these animals.  Acetate supplementation altered total brain and myelin brain phospholipid and ganglioside content in EAE mice.  These data suggest that treatment alters CNS lipid content in mice subjected to EAE.  EAE injury results in a significant increase in $cPLA_2$ levels, but treatment returned $cPLA_2$ levels to control levels to suggest a role in modulating the pro-inflammatory lipid signaling.  In addition, treatment returned OMgp, a myelin protein, back to control levels, and acetate supplementation slightly increased FluoroMyelin$^{TM}$ intensity, a myelin lipid-affiliated stain, in the EAE mice compared to control-treated EAE mice.  These data suggest that treatment may alter myelin structure in this model.  On the other hand, treatment did not alter cytoskeletal structure changes due to the EAE injury suggesting acetate may not have a role in reversing all EAE pathology.  Acetate supplementation attenuated disease progression, altered CNS and myelin lipid content, and influenced CNS and myelin protein content in mice subjected to EAE.

## CHAPTER II

## METHODS

### Materials

Reagents, reagent grade solvents, and resorcinol reagent were from EMD (Gibbstown, NJ, USA), Macron Fine Chemicals (Radnor, PA, USA), Sigma (St. Louis, MO, USA), Calbiochem (Billerica, Massachusetts, USA), and Mallinckrodt Chemicals (St. Louis, MO, USA). Thin layer chromatography plates (silica gel 60) were purchased from EMD (Gibbstown, NJ, US) and 1,2,3-triheptadecanoin, used as an internal standard for fatty acid analysis, was from Nu-Chek Prep (Elysian, MN, USA). Myelin oligodendrocyte glycoprotein ($MOG_{35-55}$) was purchased from AnaSpec (Fremont, CA, USA), and pertussis toxin was purchased from List Biological Laboratories (St. Campbell, CA, USA). A SuperSignal West Femto chemiluminescent substrate was purchased from Thermo (Waltham, MA USA), and a femtoLUCENT$^{TM}$ PLUS HRP kit was purchased from Biosciences (Cat # 786-056, San Jose, CA, USA). Materials and reagents needed for Western blot analysis were purchased from BioRad (Hercules, CA, USA). Antibodies were purchased from Cell signaling (Beverly, MA, USA), Santa Cruz (Dallas, TX, USA), or Sigma (St. Louis, MO, USA). Paraformaldehyde was purchased from Alfa Aesar (Haverhill, MA, USA), and microscope slides and coverslips were purchased from VWR (Chicago, IL, USA) and Brain Research Laboratories (Waban, MA, USA) respectively. PolyFreeze was purchased from Sigma (St. Louis, MO, USA),

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Triton X-100 was purchased from VWR (Chicago, IL, USA), and sucrose was purchased from EMD (Gibbstown, NJ, USA).

**Induction of EAE and Treatment**

All animal procedures were performed under an approved UND animal protocol based on the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.  C57BL/6 female mice (Charles River Laboratories, Portage, MI, USA) were divided into groups of four mice per cage by our animal facility personnel.  After a one-week acclimation period, the individual cages were assigned to 1 of 4 non-blinded treatment groups: EAE mice treated with water, EAE mice treated with glyceryl triacetate, control mice treated with water, and control mice treated with glyceryl triacetate in that order until all groups were represented.  These groups were arbitrarily assigned and not randomized.  In the morning of first experimental day within the animal facility, mice were treated with either water or glyceryl triacetate (4.0 g/kg body weight, once daily) by oral gavage.  Ten minutes after the initial treatment, EAE was induced with a subcutaneous injection of $MOG_{35-55}$ (50 µg/mouse) dissolved in complete Freund's adjuvant (Sigma, St Louis, MO, USA).  Two days after the inoculation, EAE mice received a second injection of pertussis toxin (200 ng, i.p.) (Kalyvas & David 2004). Control mice were treated with either glyceryl triacetate or water on the first experimental day however these mice did not receive the subcutaneous injection of complete Freund's adjuvant or pertussis toxin.  These groups of mice were included to determine if changes in lipid content was due to treatment alone.  There are limitations in using control mice instead of sham-treated mice in that the immunization procedure with

adjuvant followed by pertussis toxin may introduce potential complicating effects. All mice were treated at the same time daily with an oral gavage of either water or glyceryl triacetate (GTA, 4 g/kg body weight) until the end of the experimental timeframe. To insure access to food, all mice subjected to EAE were given moistened food in petri dishes on the day the first animal demonstrated tail weakness and/or paralysis. The moistened food was replaced daily until the end of the treatment period. At the end of the treatment period, mice were anesthetized with pentobarbital (50 mg/kg, i.p., Virbac, Fort Worth, TX, USA) and euthanized by cardiac perfusion using heparinized (Baxter, Deerfield, IL, USA) phosphate buffered saline (PBS: 137mM NaCl, 2.7 mM KCl, and 10mM phosphate ($Na_2HPO_4/KH_2PO_4$) at pH 7.4). Mice used for histochemical analysis were subsequently perfused with 4% paraformaldehyde to fix the spinal cord for myelin staining. The spinal cords and brains used for chemical analysis were dissected out of the animals, flash frozen in liquid nitrogen, and then stored at -80°C until use. Spinal cords fixed with paraformaldehyde were stored in a 4% paraformaldehyde solution at $4^0$C until analysis.

**Myelin Staining**

Before sectioning, spinal cords of control mice (n = 5), EAE mice treated with water (n = 4), and EAE mice treated with GTA (n = 5) were equilibrated overnight in 30% sucrose dissolved in PBS. 20µm sections of the lumbar region of the spinal cord were made using an IEC cryostat, and a myelin stain was performed using FluoroMyelin™ Green (1:300, Molecular Probes, Eugene, OR, USA) based on manufacturer's instructions. Images were collected using an Olympus BX50 fluorescent

microscope equipped with Spot Advanced Imaging software (Version 3.4.5). Values of the spinocerebellar and fasciculus length were reported as means ± SD for each group in units of mm.

**Behavioral Testing**

During the treatment period, mice were evaluated daily for signs of paralysis and assigned a clinical score. The assigned scores correspond to: 0; no symptoms, 1; flaccid tail, 2; mild hind limb weakness, 3; severe hind limb weakness, 4; hind limb paralysis, and 5; hind limb and forelimb paralysis (Kalyvas & David 2004). All mice were acclimated to the hanging wire test on days 3-5 post-inoculation then tested at 4-5 day intervals up to day 36 to determine the impact treatment had on motor strength, balance, and grip strength. The hanging wire test involves recording the duration (up to 60 sec) that an animal is able to hold onto to a wire mesh while being inverted approximately 15 cm above a cushioned mat (Sango *et al.* 1996). The sample size, described in Figure 3, was the total number of mice used for this analysis (n = 24). In Figure 3, values were reported as means ± SD for each group per testing day.

**Total Spinal Cord and Brain Lipid Extraction**

Frozen spinal cord and brain samples of control (n = 6, per treatment) and EAE mice (n = 8, per treatment) treated either water or GTA were weighed and then extracted in a glass Tenbroeck homogenizer using n-hexane: 2-propanol (3: 2 by volume) as described (Radin 1981, Bhatt & Rosenberger 2014). The lipid extracts were collected and stored in n-hexane: 2-propanol (3: 2, by volume) at -80°C until use.

**Myelin Brain Lipid Extraction**

Frozen brains of control (n=8), EAE mice treated with water (n=3), and EAE mice treated with GTA (n=8) were weighed and placed in large Wheaton homogenizers using a Telfon pestle. After allowing the tissue to thaw to avoid freeze artifacts, 5mL of 0.3M sucrose was added to homogenize tissue using Eberback Model 7265 ConTorque Tissue Homogenizer (Ann Arbor, MI). Using 0.83M sucrose, 15mL was added to Beckman (Indianapolis, IN, USA) polycarbonate centrifuge tubes (Lot A60327, Indianapolis, IN, USA). Then, the homogenized sample was slowly added to the centrifuge tubes with a 0.3M sucrose wash. Similar weighted tubes was placed in a Beckman L8-60M ultracentrifuge (Indianapolis, IN, USA) at a speed of 30,000rpm at 4°C for 30 minutes. Before that, a ghost run was performed to bring the chamber to the appropriate temperature to avoid warming the samples. Once the run was complete, the cloudy interface was removed and placed in a new Beckman centrifuge tube, and in these tubes, distilled water was added to wash out the sucrose. Tubes were weighted and accurately balanced with a corresponding tube by adding distilled water. Tubes were centrifuged at 30,000rpm at 4°C for 15 minutes, and supernatant fluid was discarded leaving the crude myelin pellet (Norton & Poduslo 1973, Autilio *et al.* 1964). 2mL of 20mM Tris HCl at pH 7.45 at 4°C was added to the tubes to dissolve the pellet, and the sample was transferred to 16mm screw top test tubes to perform a Folch lipid extraction as previously described (Folch *et al.* 1957). Lipid extracts were stored in hexane: 2-proponal (3:2 by volume) until further analysis.

**Thin Layer Chromatography**

Spinal cord and brain phospholipids were isolated using thin layer chromatography (20 x 20 cm, Merck silica gel 60 plates, EMD, Gibbstown, NJ) using a solvent system of chloroform/ methanol/ glacial acetic acid/ water (50: 37.5: 3: 2 by volume).  A second series of two-dimensional separations were performed to isolate the ethanolamine and choline plasmalogen from their parent glycerophospholipid fractions.  This separation was performed on 10 x 10 cm thin layer chromatography plate using the solvent system described above in the first direction and chloroform/ methanol/ ammonium hydroxide (65: 25: 4, by volume) system in the second direction.  Between dimensions, the plates were exposed to hydrochloric acid fumes to hydrolyze the vinyl ether linkage found in the plasmalogen fractions.  Bands corresponding to 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (ethanolamine plasmalogen or PlsEtn), 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine  and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine  (PtdEtn + PakEtn or phosphatidylethanolamine + plasmanylethanolamine), 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphocholine (choline plasmalogen or PlsCho), 1,2-diacyl-*sn*-glycero-3-phosphocholine  and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (PtdCho + PakCho or phosphatidylcholine + plasmanylcholine), 1,2-diacyl-*sn*-glycero-3-phosphoinositiol (PtdIns or phosphatidylinositol), 1,2-diacyl-*sn*-glycero-3-phosphoserine (PtdSer or phosphatidylserine), and sphingomyelin (CerPCho) were scraped from the plate and used to quantify either esterified fatty acid content or phospholipid content (Bhatt & Rosenberger 2014).  Neutral lipids were isolated using a solvent system of heptane/ isopropyl ether/ glacial acetic acid (60: 40: 4 by volume) on 20 x 20 cm silica gel 60

plates.  Bands corresponding to cholesterol and cholesteryl esters were scraped from the

plates and used to quantify content (Breckenridge & Kuksis 1968).  Gangliosides were

isolated on 20 x 20 cm silica gel 60 plates using a solvent system of chloroform/

methanol/ water (55:45:10 by volume).  Bands corresponding to standards were

visualized with a resorcinol-HCl reagent following a heating step where plates were

covered with a 20 x 20 cm glass plate and then heated to 105°C for 30 minutes (Ueno *et*

*al.* 1978, Svennerholm 1957).

**Quantifying Phospholipid Content**

        The individual phospholipid bands were visualized with iodine vapor, scraped

from the plate, and were quantified using a colorimetric lipid phosphorus assay (Rouser

*et al.* 1969).  Sample and standard absorbance was measured on a DU 530 UV/VIS

spectrophotometer (Beckman/Coulter) at 797 nm.  Sample concentration was assigned

based on an external standard curve (3.125 to 400 nmol) and reported as the means ± SD

in units of µmol/g wet weight.

**Quantifying Ganglioside Content**

        Individual ganglioside bands were scraped from the thin layer chromatography

plates and transferred to screw top test tubes. Ganglioside content was quantified based

on a resorcinol assay as previously described (Svennerholm 1957).  Absorbance was

measured on a DU 530 UV/VIS spectrophotometer (Beckman/Coulter) at 450nm.

Sample concentration was established based on an external standard curve of fructose

ranging in concentration from 14 to 1800nmol.  Results are reported as the means ± SD in units of µmol/g wet weight.

**Quantifying Esterified Fatty Acid Content**

Esterified fatty acid content from individual phospholipid bands were visualized using 6-*p*-toluidino naphthalene sulfonic acid (62.6 mg in 200 mL 50 mM Tris, pH 7.4) under UV light.  Bands were transferred into screw top test tubes and then methylated using 2% sulfuric acid in toluene/ methanol (1: 1, by volume) for 120 minutes at 65°C using triheptadecanoin as an internal standard (Bhatt & Rosenberger 2014).  Fatty acids were extracted in n-hexane and quantified on a gas liquid chromatograph (Shimadzu) equipped with a flame ionization detector and a SP-2330 capillary column (Supelco, Bellefonte, PA, USA).  Peak area analysis of individual fatty acids was used to quantify fatty acid concentration using Simadzu EZStart software (build 14, version 7.2.1 SP1, Kyoto, Japan).  Values are reported as the means ± SD in units of µmol/g wet weight.

**Quantifying Cholesterol and Cholesteryl Ester Content**

Individual cholesterol and cholesteryl ester bands were visualized using iodine vapor, were scraped from the plate, and were transferred into screw top test tubs. Cholesterol content was quantified using an iron binding colorimetric assay (Bowman & Wolf 1962).  Absorbance was measured on a DU 530 UV/VIS spectrophotometer (Beckman/Coulter) at 550 nm, and content was calculated based on an external standard curve (582-2.27µg to determine spinal cord lipid and 558.4-1.09 µg to determine brain lipid).  Values are reported as the means ± SD in units of µmol/g wet weight.

**Protein Isolation**

Frozen spinal cord samples of control mice (n = 8), EAE mice treated with water (n = 5), and EAE mice treated with GTA (n = 8) were transferred into small plastic vials containing ice-cold 50 mM Tris buffer pH = 8.0 containing 150 mM sodium chloride, 1.0% Triton X-100, 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate with protease inhibitors (complete EDTA-free, Roche, Mannheim, Germany).  The samples were homogenized using a polytron, allowed to incubate for 2 h at 4°C, and centrifuged at 12,500 x g for 20 min at 4°C using a microfuge 22R centrifuge (Beckman Coulter, Indianapolis, IN, USA).  The supernatant was collected, divided into equal aliquots, and then stored at -80°C until use.

**Total Protein Determination**

Protein concentration was determined based on a micro-Bradford assay (Bradford 1976).  The absorbance was measured using Labsystem Multiskan Plus plate reader from Fisher Scientific at 595 nm, and protein concentration was determined based on an external standard curve (0.375 µg to 6 µg, bovine serum albumin).

**Western Blot Analysis**

Phospholipase, β-actin, merlin (moesin-, ezrin-, radixin-like protein), oligodendrocyte myelin glycoprotein (OMgp), and lipid synthesis enzyme levels were measured using Western blot analysis (Soliman et al. 2013a, Soliman et al. 2013b, Soliman et al. 2012a, Soliman & Rosenberger 2011, Soliman et al. 2012b). The lipid synthesis enzyme protein levels analyzed were alkylglycerone phosphate synthase

(AGPS), acyl-CoA synthetase (AceCS1, cytoplasmic & AceCS2, mitochondrial), and phosphorylated acetyl-CoA carboxylase (p-ACC).  Samples were separated on 7.5, 10, or 12% SDS-PAGE gels using 25 µg of protein suspended in 6x loading buffer (10 % 2-mercptoenthanol, 250mM Tris at pH6.3, 60 % glycerol, 6 % SDS, and 0.02 % bromophenol blue) at 100V for 2 h and then transferred to nitrocellulose membranes at 100V for 1.5-2 h on ice.  To analyze OMgp protein, 100µg of protein was loaded onto a 10% gel using the same procedure as described above.  A solution of 5 % defatted milk (MP Biomedicals, Solon, OH, USA) in TTBS (Tween Tris Base Solution, 20mM Tris, 150mM NaCl, 0.05% Tween-20, pH 7.5) was used to block the membrane and during the incubation of primary antibodies targeting PLC $\beta$1, PLC $\delta$1, $\beta$-actin, AGPS, AceCS1, AceCS2, merlin, OMgp, and p-ACC (overnight at 4$^\text{o}$C).  Antibodies targeting cPLA$_2$ and pcPLA$_2$, membranes were incubated in 5% BSA in TTBS containing the primary antibody (overnight at 4$^0$C).  The primary antibodies used were pcPLA$_2$ (Cell signaling 2832, rabbit IgG, 1:1000), cPLA$_2$ (Cell signaling 2831, rabbit IgG, 1:1000), PLC $\beta$1 (sc9050, rabbit IgG, 1: 500), PLC $\delta$1 (sc30062, rabbit IgG, 1: 1,000), $\beta$-actin (A2228, mouse IgG$_{2a}$, 1: 2,000), AGPS (sc374201, mouse 1gG, 1:1000), AceCS1 (sc85258, rabbit IgG, 1:2000), AceCS2 (sc85256, 1:1000, rabbit IgG), merlin (sc331, rabbit IgG, 1:1000), OMgp (sc271704, mouse IgG, 1:500), and p-ACC (Millipore 07-303, rabbit IgG, 1:1000).  Membranes were washed with TTBS and then incubated with the secondary antibody for 2 hr at room temperature.  The secondary antibodies used were goat anti-rabbit IgG HRP for phospholipase (sc2004, 1: 10,000), goat anti-mouse IgG$_{2a}$ HRP for $\beta$-actin (sc2061, 1: 20,000), goat anti-mouse IgG HRP for AGPS (1:10,000) and OMgp (1:5000) (sc2005), goat anti-rabbit IgG HRP for AceCS2, merlin, and p-ACC (BioRad

170-5046, 1:10,000), and goat anti-rabbit IgG HRP for AceCS1 (BioRad 170-5046,

1:5,000).  Chemiluminescent protein bands were visualized with a femto substrate

(Thermo, Biosciences) using an Omega Lum G gel imaging station (Aplegen, San

Francisco, CA, USA), and protein bands were quantified using UltraQuant software

(Aplegen, Version 14.05.15).  The band intensity of all protein bands was normalized to a

GAPDH loading control and reported as the mean ± SD (GAPDH: primary sc25778,

rabbit IgG, 1:500 or 1:1000; secondary sc2004 or BioRad 170-5046, goat anti-rabbit IgG

HRP, 1: 10,000) (Liu *et al.* 2015, Markoullis *et al.* 2012, Schiffmann *et al.* 2012).

**Statistical Analysis**

A One-way ANOVA with a Tukey-Kramer multiple comparison posttest was

used to calculate statistical differences (p ≤ 0.05) between the mean ± SD values of

multiple groups.  Student's t-test was used to quantify statistical difference between the

mean ± SD values of intraday behavioral testing group.  All statistical analysis was

performed using GraphPad InStat software (Ver. 3.0 for Windows, San Diego CA, USA)

with statistical significance set at p ≤ 0.05.  To determine sample size for the experiments

used in this study, we used GraphPad StatMate software (Ver. 2.0 for Windows, La Jolla,

CA, USA).

# CHAPTER III

## STUDY I

**Increasing Acetyl-CoA Metabolism Attenuates Injury and Alters
Spinal Cord Lipid Content in Mice Subjected to Experimental
Autoimmune Encephalomyelitis**

**Acetate Supplementation Attenuates Clinical Symptoms in Mice Subjected to EAE**

Prior to and during the 40 day treatment period each mouse was assigned a daily

clinical score based on the level of paralysis displayed (Kalyvas & David 2004) and

weighed (Figure 3). Mice were also subjected to a hanging wire test during the treatment

period to test motor strength, coordination, and grip strength (Figure 3B). We found that

control-treated EAE mice developed tail paralysis on day 13 post-inoculation,

experienced ascending paralysis that peaked on day 26, and remained consistent through

the end of the treatment period having an averaged clinical score of 3.5 (severe hind limb

weakness to hind limb paralysis) (Figure 3A). Several of the glyceryl triacetate-treated

EAE mice demonstrated tail paralysis on day 15, however as a group, none progressed

beyond this stage of injury. Of the glyceryl triacetate-treated EAE mice that

demonstrated tail paralysis only one failed to regain full movement by the end of the

treatment period. On day 20 through the end of the treatment period, glyceryl triacetate-

treated EAE mice showed significantly less signs of injury and performed the hanging

wire test significantly better than control-treated EAE mice (Figure 3B). On days 20

through the end of the treatment period, glyceryl triacetate-treated EAE mice were able
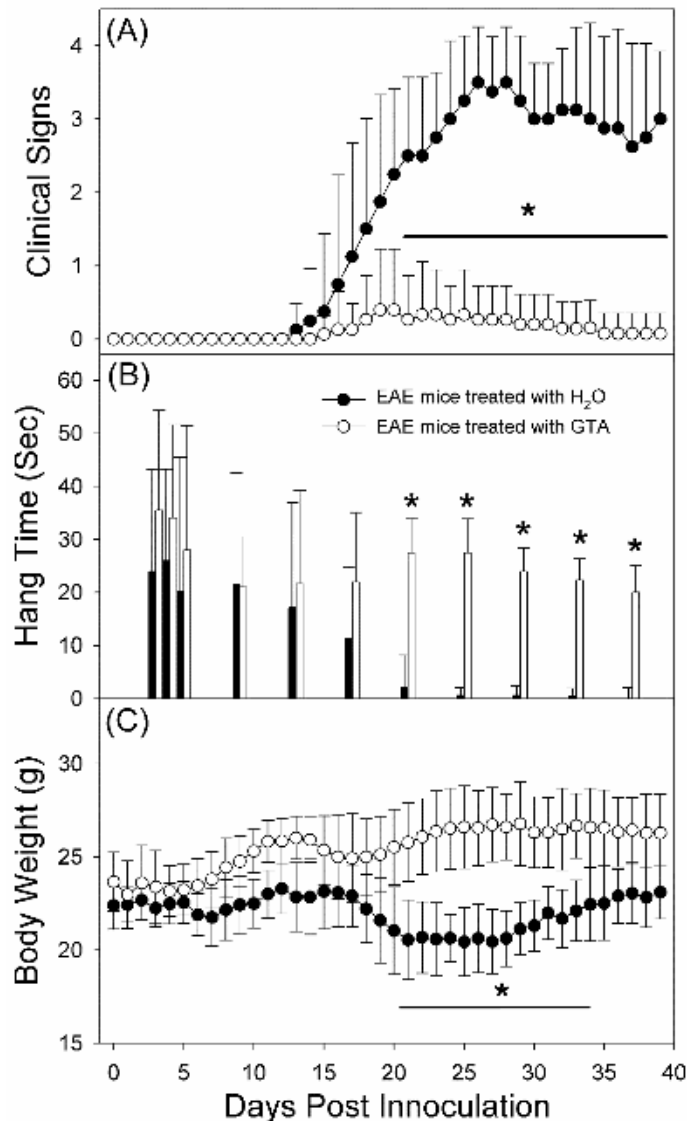
**Figure 3**: Behavioral analysis of mice subjected to EAE and treated with either water (H₂O) or glyceryl triacetate (GTA) (4 g/kg). Panel A shows the averaged effect treatment had on daily clinical scores, panel B shows the averaged effect treatment had on hanging wire test, and panel C shows the averaged changes in body weight measured throughout the treatment period. Clinical score criteria described in panel A represents: 0, no paralysis, 1, flaccid tail, 2, mild hind limb weakness, 3, severe hind limb weakness, 4, hind limb paralysis, and 5, hind limb and forelimb paralysis. All values report the means ± SD and the asterisks represents a statistical difference ($p \leq 0.05$) comparing water-treated to glyceryl triacetate-treated EAE mice (n = 24, total).

to support their body weight up-side-down for an average of 25 sec compared to 1-2 sec

for control-treated EAE mice.  In addition, there was a significant reduction in the body

weight between days 20 to 34 post-inoculation comparing control-treated to glyceryl

triacetate-treated EAE mice (Figure 3C).  None of the control animals demonstrated

changes in clinical symptoms associated with EAE (data not shown).  Therefore, these

animals were not subjected to the hanging wire test and their body weights were not

averaged into that data found in Figure 3C.  This body weight shift in EAE mice treated

with water paralleled the increase in clinical score and reduction in hang-time.  As a

whole, these data suggest acetate supplementation prevented the onset of clinical

symptoms in mice subjected to EAE.


**EAE Results in a Reduction of Spinal Cord Phospholipid That Was Not Found in EAE Mice Treated With Glyceryl Triacetate**

To investigate the impact treatment had  spinal cord lipid, we quantified

phospholipid content in whole spinal cords from control and EAE mice treated with

either water or glyceryl triacetate.  Table 1 shows the spinal cord phospholipid levels

(µmol/g wet weight) of the primary phospholipid groups: EtnGpl, PtdIns, PtdSer,

ChoGpl, and CerPCho.  Mice subjected to EAE and given a control treatment showed a

30% reduction in the levels of EtnGpl, ChoGpl, and CerPCho compared to control mice

treated with water or glyceryl triacetate.  Further, EAE resulted in a 24% reduction in

PtdSer compared to control mice given glyceryl triacetate (Table 1A).  More importantly,

the phospholipid levels in mice subjected to EAE and treated with glyceryl triacetate did

not differ compared to either group of control mice.  The levels of EtnGpl, PtdSer, and

**Table 1**: Spinal cord phospholipid content of control and EAE mice treated with either water or glyceryl triacetate.

| A | Control (n=6) | | EAE (n=8) | |
|---|---|---|---|---|
| Phospholipid | H$_2$O-treated | GTA-treated | H$_2$O-treated | GTA-treated |

| | μmol/g wet weight | | | |
|---|---|---|---|---|
| EtnGpl | 15.9 ± 1.1 | 15.7 ± 2.3 | [a]11.9 ± 1.7 | [c]16.1 ± 3.9 |
| PtdIns | 3.6 ± 0.7 | 3.9 ± 0.8 | 2.9 ± 0.5 | 3.5 ± 0.7 |
| PtdSer | 10.7 ± 1.1 | 11.9 ± 0.8 | [b]9.1 ± 0.7 | [c]12.2 ± 1.7 |
| ChoGpl | 20.3 ± 1.2 | 20.6 ± 1.9 | [a,b]13.5 ± 2.0 | [c]18.1 ± 3.9 |
| Cer*P*Cho | 8.6 ± 1.1 | 8.1 ± 0.9 | [a,b]6.3 ± 1.2 | 7.1 ± 1.0 |

| B | μmol/g wet weight | | | |
|---|---|---|---|---|
| PtdEtn+PakEtn | 7.4 ± 0.6 | 7.1 ± 1.0 | [a]5.4 ± 0.6 | [a,b,c]10.3 ± 2.0 |
| PlsEtn | 8.5 ± 1.2 | 8.6 ± 1.4 | 6.5 ± 1.6 | [a,b]5.8 ± 2.2 |
| PtdCho+PakCho | 14.8 ± 0.8 | 14.8 ± 1.9 | [a,b]10.8 ± 1.8 | [c]15.5 ± 3.7 |
| PlsCho | 5.5 ± 1.5 | 5.8 ± 0.8 | [a,b]2.7 ± 0.7 | [a,b]2.6 ± 0.6 |

Values represent the means ± SD in units of μmol/g wet weight. Abbreviations are: GTA, glyceryl triacetate, EtnGpl, ethanolamine glycerophospholipid, PtdEtn + PakEtn, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine or phosphatidylethanolamine and plasmanylethanolamine, PlsEtn, 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine or ethanolamine plasmalogen, PtdIns, phosphatidylinositol, PtdSer, phosphatidylserine, ChoGpl, choline glycerophospholipid, PtdCho + PakCho, 1,2-diacyl-*sn*-glycero-3-phosphocholine and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine or phosphatidylcholine and plasmanylcholine, PlsCho, 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphocholine or choline plasmalogen, and Cer*P*Cho, sphingomyelin. The letters indicate statistical significance ($p \leq 0.05$) where "a" represent comparisons made to water-treated control mice, "b" represent comparisons made to glyceryl triacetate-treated control mice, and "c" represent comparisons made to water-treated EAE mice.

ChoGpl in the EAE-glyceryl triacetate group were significantly higher compared to mice

subjected to EAE and treated with water (Table 1A). Analysis was performed to isolate

the plasmalogen fractions from EtnGpl and ChoGpl. Treatment significantly increased

the levels of PtdEtn + PakEtn compared to all groups and returned PtdCho + PakCho

fraction to control levels (Table 1B). Treatment did not result in the recovery of the

PlsEtn and PlsCho fractions to control levels. These data suggest that increasing CNS

acetyl-CoA metabolism may either prevent or reverse the loss of spinal cord phospholipid

in mice subjected to EAE.


**Acetate Supplementation Modulates Spinal Cord Fatty Acid Content in Mice
Subjected to EAE**

To determine if increasing acetyl-CoA metabolism altered fatty acid metabolism,

we measured esterified fatty acid content in control and EAE mice treated with either

water or glyceryl triacetate. Similar to that found *in vitro* (Bhatt & Rosenberger 2014),

acetate supplementation did not alter the esterified fatty acid content in control mice

(Tables 2 and 3). However, EAE resulted in a significant reduction in the content of

palmitic (16:0), stearic (18:0), oleic (18:1n-9 + n-7), arachidonic (20:4n-6), and

docosatetraenoic (22:4n-6) acids in the EtnGpl and ChoGpl fractions (Table 2).

Treatment with glyceryl triacetate however prevented the decrease in these fatty acids,

returning their levels to that found in control mice. Interestingly in mice subjected to

EAE, esterified fatty acid content in PtdSer demonstrated only reductions in palmitic

(16:0) and arachidonic (20:4n-6) acids with a significant increase in docosatetraenoic

**Table 2**: Spinal cord esterified fatty acid content in ethanolamine and choline glycerophospholipid from control and EAE mice treated with either water or glyceryl triacetate.

| Fatty acid | Control (n=6) | | EAE (n=8) | |
|---|---|---|---|---|
| | H$_2$O-treated | GTA-treated | H$_2$O-treated | GTA-treated |

| **EtnGpl** | µmol/g wet weight | | | |
|---|---|---|---|---|
| 16:0 | 1.3 ± 0.1 | 1.3 ± 0.2 | [a,b]0.7 ± 0.08 | [c]1.2 ± 0.2 |
| 18:0 | 4.2 ± 0.3 | 4.2 ± 0.4 | [a,b]2.9 ± 0.4 | [c]4.2 ± 0.7 |
| 18:1n-9 + n-7 | 9.5 ± 0.6 | 10.1 ± 1.1 | [a,b]6.3 ± 0.8 | [c]9.2 ± 1.5 |
| 20:1n-9 | 3.7 ± 0.6 | 3.8 ± 0.5 | 3.0 ± 0.6 | [c]4.8 ± 1.0 |
| 20:4n-6 | 2.2 ± 0.1 | 2.2 ± 0.2 | [a,b]1.7 ± 0.2 | [c]2.3 ± 0.4 |
| 22:4n-6 | 1.7 ± 0.1 | 1.6 ± 0.1 | [a,b]1.2 ± 0.2 | [c]1.8 ± 0.4 |
| 22:6n-3 | 3.2 ± 0.2 | 3.0 ± 0.4 | 2.3 ± 0.4 | [c]3.4 ± 0.9 |
| Total | 31.3 ± 2.0 | 31.9 ± 3.2 | [a,b]20.9 ± 2.6 | [c]31.0 ± 5.2 |

| **ChoGpl** | µmol/g wet weight | | | |
|---|---|---|---|---|
| 16:0 | 5.0 ± 1.7 | 5.1 ± 1.6 | [a,b]3.1 ± 0.3 | 4.6 ± 0.8 |
| 18:0 | 4.1 ± 0.4 | 4.3 ± 0.7 | [a,b]3.0 ± 0.3 | [c]4.4 ± 0.8 |
| 18:1n-9 + n-7 | 9.2 ± 0.5 | 9.6 ± 1.1 | [a,b]6.6 ± 0.5 | [c]9.8 ± 1.5 |
| 20:1n-9 | 0.9 ± 0.1 | 1.0 ± 0.3 | 0.7 ± 0.1 | [c]1.2 ± 0.03 |
| 20:4n-6 | 0.6 ± 0.1 | 0.6 ± 0.09 | 0.6 ± 0.04 | 0.6 ± 0.13 |
| 22:4n-6 | 0.1 ± 0.1 | 0.09 ± 0.02 | 0.08 ± 0.007 | 0.1 ± 0.03 |
| 22:6n-3 | 1.0 ± 0.2 | 1.0 ± 0.2 | 0.8 ± 0.07 | [c]1.1 ± 0.2 |
| Total | 22.9 ± 1.2 | 24.2 ± 2.2 | [a,b]16.1 ± 1.1 | [c]23.8 ± 3.3 |

Values represent the means ± SD in units of µmol/g wet weight. Abbreviations are: GTA, glyceryl triacetate, EtnGpl, ethanolamine glycerophospholipid and ChoGpl, choline glycerophospholipid. The letters indicate statistical significance ($p \leq 0.05$) where "a" represent comparisons made to water-treated control mice, "b" represent comparisons made to glyceryl triacetate-treated control mice, and "c" represent comparisons made to water-treated EAE mice.

**Table 3**: Spinal cord esterified fatty acid content in phosphatidylserine and sphingomyelin from control and EAE mice treated with either water or glyceryl triacetate.

| Fatty acid | Control (n=6) | | EAE (n=8) | |
|---|---|---|---|---|
| | H$_2$O-treated | GTA-treated | H$_2$O-treated | GTA-treated |

| **PtdSer** | μmol/g wet weight | | | |
|---|---|---|---|---|
| 16:0 | 0.2 ± 0.04 | 0.2 ± 0.06 | [a,b]0.04 ± 0.01 | [a,b]0.08 ± 0.03 |
| 18:0 | 5.7 ± 0.7 | 5.8 ± 1.0 | 6.5 ± 0.4 | [a,b,c]8.4 ± 1.5 |
| 18:1n-9 + n-7 | 4.0 ± 0.5 | 4.4 ± 0.7 | 4.0 ± 0.4 | [a,c]5.5 ± 1.0 |
| 20:1n-9 | 1.2 ± 0.2 | 1.3 ± 0.4 | 1.9 ± 0.4 | [a,b,c]2.8 ± 0.7 |
| 20:4n-6 | 0.2 ± 0.04 | 0.2 ± 0.04 | [a,b]0.04 ± 0.06 | [a,b]0.4 ± 0.07 |
| 22:4n-6 | 0.3 ± 0.08 | 0.3 ± 0.08 | [a]0.4 ± 0.07 | [a,b]0.5 ± 0.1 |
| 22:6n-3 | 0.8 ± 0.2 | 0.7 ± 0.2 | 0.9 ± 0.2 | [a,b]1.2 ± 0.3 |
| Total | 15.7 ± 3.0 | 16.2 ± 2.3 | 17.7 ± 1.6 | [a,b,c]22.9 ± 4.7 |

| **Cer*P*Cho** | μmol/g wet weight | | | |
|---|---|---|---|---|
| 16:0 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.2 |
| 18:0 | 0.9 ± 0.1 | 1.0 ± 0.2 | [a,b]0.4 ± 0.06 | [a,b]0.6 ± 0.07 |
| 18:1n-9 + n-7 | 0.4 ± 0.1 | 0.4 ± 0.07 | 0.3 ± 0.07 | 0.4 ± 0.2 |
| 24:0 | 0.4 ± 0.2 | 0.5 ± 0.1 | [a,b]0.1 ± 0.03 | [a,b]0.2 ± 0.03 |
| 24:1n-9 | 0.8 ± 0.3 | 1.0 ± 0.3 | [a,b]0.3 ± 0.06 | [a,b]0.5 ± 0.08 |
| Total | 4.5 ± 1.0 | 5.0 ± 1.1 | [a,b]2.1 ± 0.3 | [a,b]3.0 ± 0.6 |

Values represent the means ± SD in units of μmol/g wet weight. Abbreviations are: GTA, glyceryl triacetate, PtdSer, phosphatidylserine and Cer*P*Cho, sphingomyelin. The letter indicates statistical significance (p ≤ 0.05) where "a" represent comparisons made to water-treated control mice, "b" represent comparisons made to glyceryl triacetate-treated control mice, and "c" represent comparisons made to water-treated EAE mice.

(22:4n-6) acid (Table 3).  Moreover, treatment with glyceryl triacetate resulted in a

significant 15% increase in all fatty acids reported with the exception of palmitic acid

which remained significantly decreased compared to control mice.  With regard to

CerPCho, EAE resulted in the significant reduction in stearic (18:0), lignoceric (24:0),

and nervonic (24:1n-9) acids compared to control mice.  The content of these fatty acids

remained significantly lower in EAE mice treated with glyceryl triacetate (Table 3),

suggesting an increase in acetyl-CoA metabolism did not alter the incorporation or

turnover of N-acyl derived fatty acids associated with ceramide synthesis.  Overall, these

data suggest EAE resulted in a significant decrease in lipid and fatty acid content which

parallels injury severity.  Treatment with glyceryl triacetate however returned the

esterified fatty acid content in EtnGpl and ChoGpl to control levels, significantly

increased content of fatty acids in PtdSer, but had no effect on fatty acid content found in

CerPCho in EAE mice.  All of which suggests increasing acetyl-CoA metabolism in mice

subjected to EAE may result in an increase in acetyl-CoA being utilized for the synthesis

of esterified fatty acid that may possibly offset that lost to injury.


**Acetate Supplementation Prevents the Loss of Cholesterol in EAE Mice but Does Not Alter the EAE-Induced Decrease in Cholesteryl Esters**

Similar to that found with measuring spinal cord phospholipid content, EAE

significantly decreased the content of spinal cord cholesterol (Figure  4A) and cholesteryl

esters (Figure 4B) compared to control mice treated with either water or glyceryl

triacetate.  Treatment with glyceryl triacetate significantly increased the content of

cholesterol in mice subjected to EAE compared to EAE mice treated with water but was

not significantly different to that found in control animals.  On the other hand, both
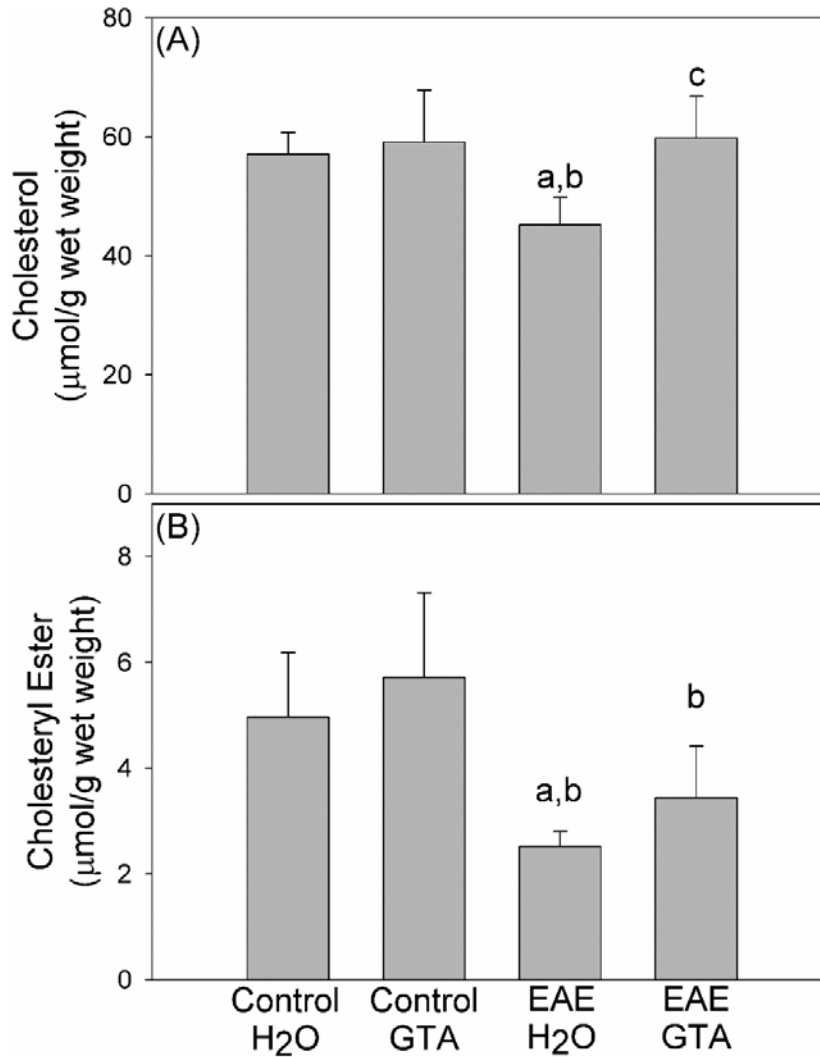
**Figure 4**: Spinal cord cholesterol and cholesteryl ester content in control mice (n=6, per treatment) and EAE (n=8, per treatment) mice treated with either water ($H_2O$) or glyceryl triacetate (GTA) (4g/kg). Panel A shows spinal cord content of cholesterol, and panel B shows spinal cord content of cholesteryl ester. Significant differences ($p \leq 0.05$) are indicated with a letter where "a" represent comparisons made to control mice treated with water, "b" represent comparisons made to control mice treated with glyceryl triacetate, and "c" represent comparisons made to EAE mice treated with water.

groups of mice subjected to EAE and treated with either water or glyceryl triacetate demonstrated a significant reduction in cholesteryl ester levels compared to control mice treated with either water or glyceryl triacetate. These data suggest, similar to the esterified fatty acid content studies, increasing acetyl-CoA metabolism may act to restore spinal cord cholesterol content to control levels but had no effect on cholesteryl ester content.

**Acetate Supplementation Alters Spinal Cord Ganglioside Content in Mice Subjected to EAE**

Figure 5 shows spinal cord ganglioside content in control and EAE mice treated with either water or glyceryl triacetate. GD3 content was significantly decreased in response to EAE when compared to control mice (Figure 5A). Acetate supplementation on the other hand resulted in a significant increase in GD3 content when compared to control mice treated with glyceryl triacetate and EAE mice treated with water. Further, GD1a content was significantly decreased in animals subjected to EAE when compared to control animals (Figure 5B). Acetate supplementation significantly increased GD1a levels in EAE mice compared to mice subjected to EAE and treated with water, but this increase was significantly less than that found in control animals. GM1 levels were significantly decreased in both EAE groups (Figure 5C). In addition, there were significant decreases in GT1a, GD2, GD1b, and GQ1b levels in mice subjected to EAE that remained decreased with treatment (Figure 5D). Overall, acetate supplementation altered GD3 and GD1a ganglioside content in mice subjected to EAE.

48
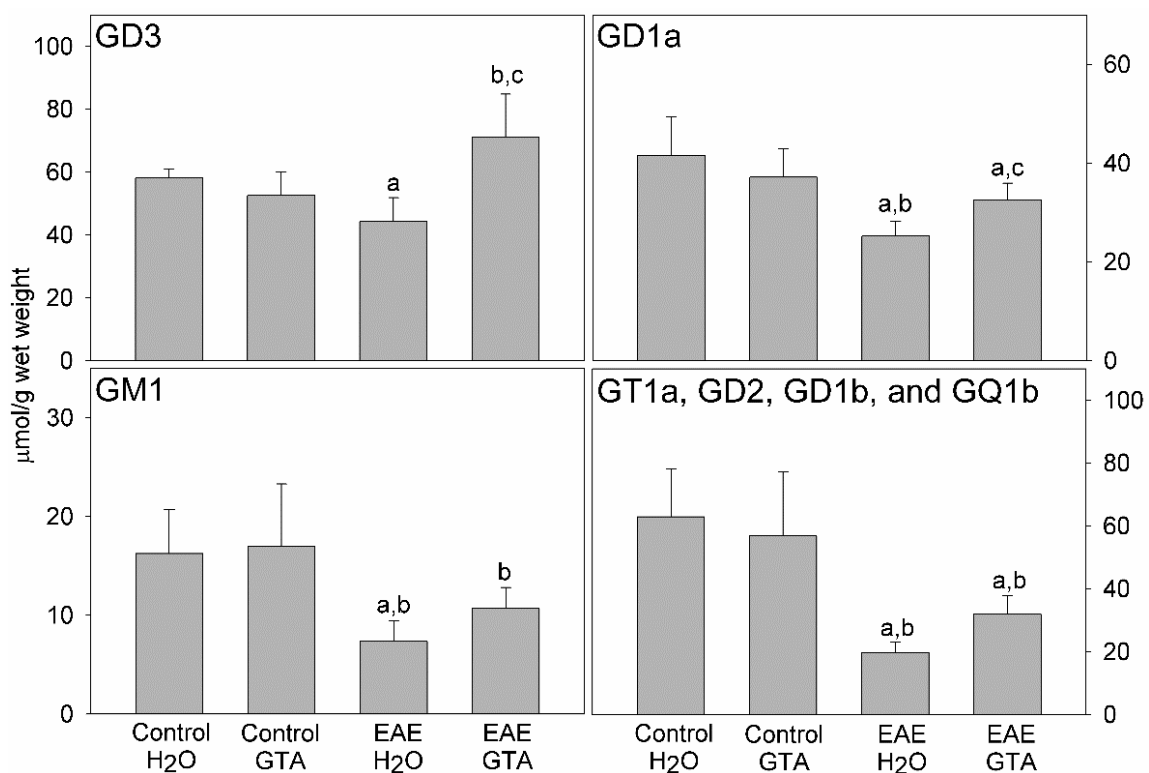
**Figure 5**: Spinal cord ganglioside content in control (n=6, per treatment) and EAE (n=8, per treatment) mice treated with either water ($H_2O$) or glyceryl triacetate (GTA) (4g/kg). Panel A shows the spinal cord content of GD3, panel B shows the spinal cord content of GD1a, panel C shows spinal cord content of GM1, and panel D shows the spinal cord content of GT1a, GD2, GD1b, and GQ1b. Significant differences ($p \leq 0.05$) are indicated with a letter where "a" represent comparisons made to control mice treated with water, "b" represent comparisons made to control mice treated with glyceryl triacetate, and "c" represent comparisons made to EAE mice treated with water.

**Acetate Supplementation Returns Cytosolic Phospholipase A$_2$ (cPLA$_2$) to Baseline but EAE and Treatment Did Not Alter Phospholipase C (PLC) Levels**

In peripheral nerve cell cultures, an alteration in actin polymerization is associated with myelin fragmentation (Jung et al. 2011) and is increased in Lewis rats subjected to EAE during the chronic injury phase (Smerjac & Bizzozero 2008). To determine the impact injury and treatment had on β-actin in the mouse, we measured its levels in spinal cord of control and EAE mice treated with either water or glyceryl triacetate. We found mice subjected to EAE and treated with water resulted in a significant 2-fold increase in β-actin levels when normalized to GAPDH (Figure 6F) consistent with the demyelinating injury. In EAE animals treated with glyceryl triacetate, we found a slight decrease in β-actin levels that was not significantly different from control or EAE mice treated with water. These data, as expected, suggest EAE in the mouse increases β-actin levels (Jung et al. 2011, Smerjac & Bizzozero 2008). However, the decrease in β-actin in the glyceryl triacetate-treated EAE mice suggests that treatment may not completely reverse all aspects of injury associated with EAE despite the clear reduction in the onset of clinical signs in this model. β-actin alone is not a direct measure of neuronal injury, and future experiments will understand changes in other structural proteins like Cofilin 1 (Tilve et al. 2015) in this model.

Figure 6A-E shows spinal cord phospholipase protein levels in control mice and mice subjected to EAE treated with either water or glyceryl triacetate. These data show that in control-treated EAE mice, there was a significant increase in phosphorylated (pcPLA$_2$) and non-phosphorylated cPLA$_2$ when normalized to GAPDH (Figure 6A and 6B). Similar to that found in cell culture (Soliman et al. 2013b), acetate supplementation prevented the increase in spinal cord cPLA$_2$ resulting in levels similar to that found in
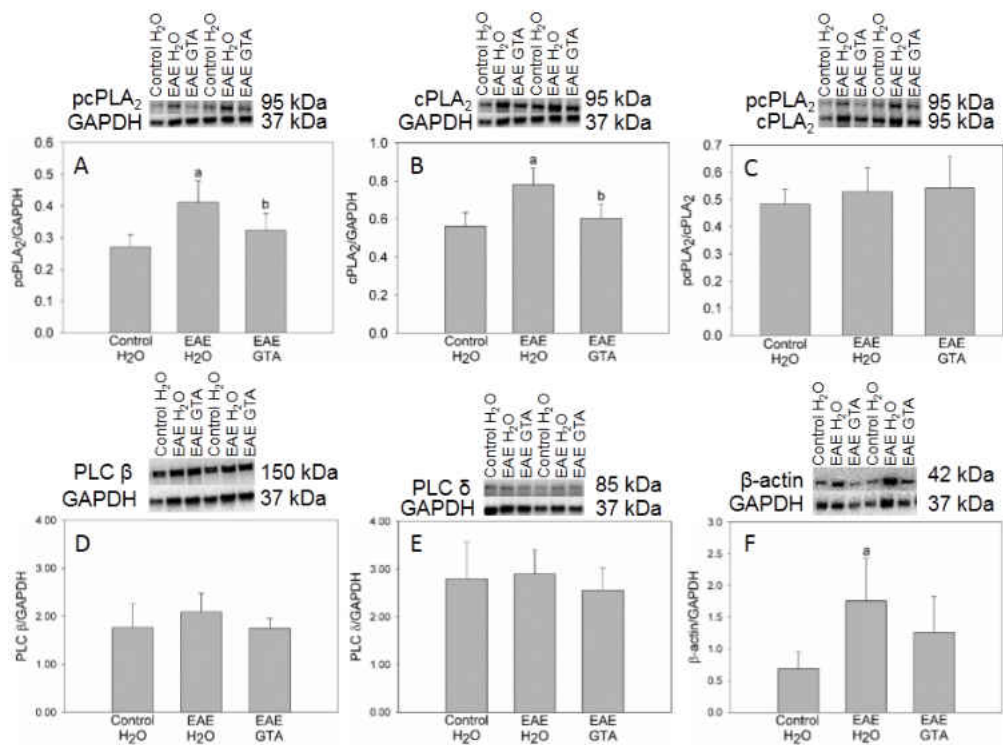
**Figure 6**: Changes in spinal cord phospholipase and β-actin levels normalized to GAPDH in control and EAE mice. Panel A shows changes in spinal cord phosphorylated cytosolic phospholipase $A_2$ (pcPLA$_2$) levels, panel B shows changes in cPLA$_2$ levels, panel C shows the ratio of pcPLA$_2$ to total cPLA$_2$, panel D shows changes in phospholipase C (PLC) β levels, panel E shows changes in PLC δ levels, and panel F shows changes in β-actin levels. Sample size is n=8 for control, n=5 for EAE mice treated with water ($H_2O$), and n=8 for EAE mice treated with glyceryl triacetate (GTA). Significance differences (p ≤ 0.05) are indicated by a letter where "a" represent comparisons made to control mice and "b" represent comparisons made to EAE mice treated with water.

control animals.  In addition, the ratio of pcPLA$_2$ to total cPLA$_2$, did not change between the three groups analyzed suggesting at 40 days post-inoculation the phosphorylation state of cPLA$_2$ was not increased (Figure 6C).  Because the ratio of pcPLA$_2$/cPLA$_2$ was not changed in those samples measured, the physiological significance regarding total PLA$_2$ enzymatic activity and cellular localization within the spinal cord remains to be determined and a subject for future studies.  Further, neither EAE nor treatment altered spinal cord protein levels of phospholipase C (PLC) β and PLC δ when normalized to GAPDH as shown in Figures 6D and E, respectively.  These results suggest, similar to that reported (Kalyvas & David 2004), cPLA$_2$ may play a greater role in EAE-mediated demyelination compared to PLC and that acetate supplementation with glyceryl triacetate may attenuate PLA$_2$-mediated lipid signaling.

**Acetate Supplementation Slightly Increases FluoroMyelin$^{TM}$ Intensity in Mice Subjected to EAE**

A histochemical analysis was performed using FluoroMyelin™ green to visualize changes in myelin content from lumbar regions of the spinal cord in control, EAE, and EAE mice treated with glyceryl triacetate.  Low magnification (40x) results show very distinct FluoroMyelin™ staining along the outer edges of the spinal cord in the spinocerebellar and fasciculus regions that was decreased in intensity in samples taken from mice subjected to EAE (Figure 7A-C).  High magnification (400X) analysis of control samples show that fluorescent staining is confined within a tight band along the outer surface on the spinal cord (ventral spinocerebellar track) with distinct vertical striations (Figure 7D).  The intensity of fluorescent staining was reduced in animals subjected to EAE in this region having a diffuse outer band with no apparent vertical

striations (Figure 7E).  EAE animals treated with glyceryl triacetate demonstrated

fluorescent intensity similar to that found in control animals having an outer band with

some vertical striations (Figure 7F).  Figure 7G shows the average length measured of the

spinocerebellar and fasciculus region of control, EAE mice, and EAE mice treated with

glyceryl triacetate.  These data show a decrease in the spinocerebellar/fasciculus length

comparing control to EAE mice that had an insignificant recovery with glyceryl triacetate

treatment.  These data suggest glyceryl triacetate may be positively influencing myelin

content in mice subjected to EAE, but at a degree lower than that found in control

animals.
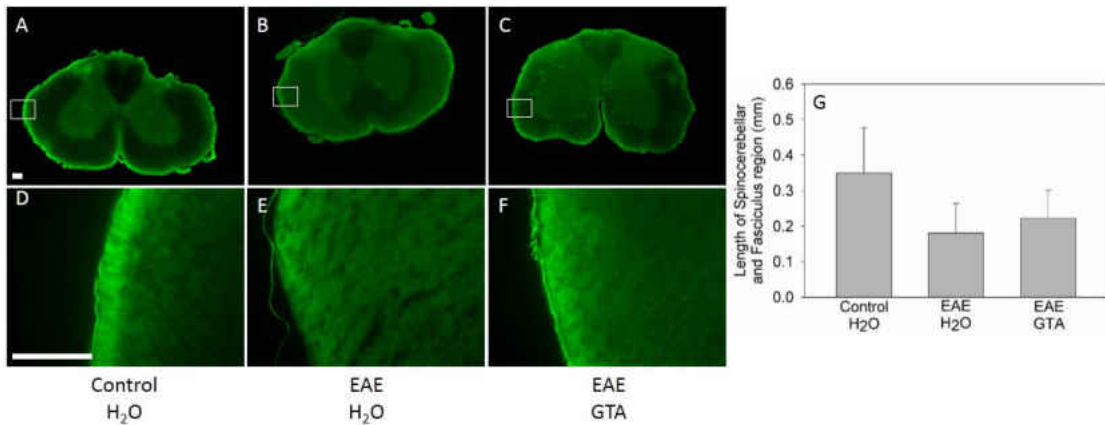


**Figure 7**: FluoroMyelin™ stain of 20 µM lumbar spinal cord sections taken from control
(Panels A & D), EAE mice (Panels B & E), and EAE mice treated with glyceryl triacetate
(GTA, Panels C & F).  In Panel A, the bar indicates a length of 0.1mm at 40x
magnification that is applicable to panels A-C.  In panel D, the bar indicates a length of
0.1mm at 400x magnification (panels D-F).  Panel G shows the average length of the
spinocerebellar and fasciculus region in control (n = 5), EAE mice (n = 4), and EAE mice
treated with glyceryl triacetate (n = 5).

# CHAPTER IV

# STUDY II

**Increasing Acetyl-CoA Metabolism Alters Spinal Cord Phosphatidylinositol Palmitic and Oleic Acid Levels in Mice Subjected to Experimental Autoimmune Encephalomyelitis**

**Acetate Supplementation Alters Esterified Fatty Acid Content in Phosphatidylinositol of EAE Mice**

Acetate supplementation altered spinal cord saturated and unsaturated fatty acid content in EtnGpl, ChoGpl, and PtdSer in EAE mice (Chevalier & Rosenberger 2017) (Tables 2 and 3). Table 4 shows spinal cord esterified fatty acid content in PtdIns from control and EAE mice treated with either water or glyceryl triacetate. Treatment with glyceryl triacetate showed a significant increase in palmitic acid (16:0) in EAE mice compared to control mice treated with water (102%), control mice treated with glyceryl triacetate (34%), and EAE mice treated with water (36%). In addition, treatment showed a significant 47% increase in oleic acid (18:1n-9) in EAE mice compared to EAE mice treated with water. The EAE injury resulted in a significant decrease in stearic acid (18:0) compared to control mice treated with water (59%) and glyceryl triacetate (54%). However, treatment was not able to recover levels back to that found in controls. Furthermore, there was a significant 55-58% decrease in total fatty acid content due to the EAE injury, which again was not returned to control levels following treatment. Overall, these data suggest acetate supplementation alters PtdIns palmitic and oleic acid

content in mice subjected to EAE suggesting that treatment alters spinal cord fatty acid

content in mice subjected to EAE.

**Table 4**: Spinal cord esterified fatty acid content in phosphatidylinositol from control and EAE mice treated with either water or glyceryl triacetate.

| | Control (n=6) | | EAE (n=8) | |
|---|---|---|---|---|
| Fatty acid | $H_2O$-treated | GTA-treated | $H_2O$-treated | GTA-treated |

| **PtdIns** | µmol/g wet weight | | | |
|---|---|---|---|---|
| 16:0 | 0.08 ± 0.02 | 0.1 ± 0.03 | 0.1 ± 0.02 | [a,b,c]0.2 ± 0.04 |
| 18:0 | 1.7 ± 0.2 | 1.5 ± 0.2 | [a,b]0.7 ± 0.1 | [a,b]0.9 ± 0.1 |
| 18:1n-9 + n-7 | 0.4 ± 0.1 | 0.5 ± 0.1 | [b]0.3 ± 0.05 | [c]0.5 ± 0.1 |
| 20:1n-9 | 0.1 ± 0.01 | [a]0.04 ± 0.03 | [a]0.02 ± 0.003 | [a]0.03 ± 0.008 |
| 20:4n-6 | 0.6 ± 0.2 | 0.5 ± 0.2 | 0.5 ± 0.1 | 0.6 ± 0.1 |
| 22:4n-6 | 0.05 ± 0.01 | 0.05 ± 0.02 | 0.04 ± 0.009 | 0.05 ± 0.009 |
| 22:6n-3 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.03 | 0.2 ± 0.04 |
| Total | 6.2 ± 1.1 | 5.8 ± 1.6 | [a,b]2.6 ± 0.3 | [a,b]3.5 ± 0.5 |

Values represent the means ± SD in units of µmol/g wet weight. Abbreviations are: GTA, glyceryl triacetate, and PtdIns, phosphatidylinositol. The letter indicates statistical significance ($p \leq 0.05$) where "a" represent comparisons made to water-treated control mice, "b" represent comparisons made to glyceryl triacetate-treated control mice, and "c" represent comparisons made to water-treated EAE mice.

# CHAPTER V

## STUDY III

### Increasing Acetyl-CoA Metabolism Increases Total Brain and Brain Myelin Lipid Content in Mice Subjected to Experimental Autoimmune Encephalomyelitis

**Acetate Supplementation Increases Total Brain Phospholipid Levels in Mice Subjected to EAE**

Table 5 shows total brain phospholipid in control (n=6) and EAE mice (n=8) treated with either water or glyceryl triacetate. There was no significant difference in EtnGpl, PtdSer, ChoGpl, and CerPCho content between the two control groups, also shown in the EAE spinal cord phospholipid data (Chevalier & Rosenberger 2017) (Table 1). There was no significant decrease in these phospholipid levels due to the EAE injury. Regarding total brain PtdIns, there was a significant decrease in phospholipid content in EAE mice treated with water and control mice treated with glyceryl triacetate compared to control mice treated with water, but treatment was unable to significantly alter PtdIns content between the two EAE groups. We did not expect to show significant changes in brain lipid content due to the EAE injury because this injury is considered a spinal cord disease (Batoulis et al. 2011, Ransohoff 2012, Simmons et al. 2013). Regarding Cer*P*Cho content (Table 5A), there was no significant differences between all groups. EAE resulted in a significant decrease in PtdIns compared to water- treated control mice, but treatment was unable to increase lipid levels (Table 5A). Interestingly, acetate supplementation significantly increased PtdSer in EAE mice compared to EAE mice

**Table 5**: Total brain phospholipid content of control and EAE mice treated with either water or glyceryl triacetate.

| A | Control (n=6) | | EAE (n=8) | |
|---|---|---|---|---|
| Phospholipid | H$_2$O-treated | GTA-treated | H$_2$O-treated | GTA-treated |

| | µmol/g wet weight | | | |
|---|---|---|---|---|
| EtnGpl | 15.3 ± 1.4 | 15.3 ± 1.6 | 15.6 ± 1.3 | 16.9 ± 1.0 |
| PtdIns | 2.0 ± 0.1 | [a]1.7 ± 0.2 | [a]1.7 ± 0.2 | 1.9 ± 0.1 |
| PtdSer | 5.8 ± 0.4 | 5.7 ± 0.7 | 5.6 ± 0.3 | [c]6.3 ± 0.3 |
| ChoGpl | 16.3 ± 1.0 | 16.3 ± 1.5 | 16.7 ± 1.2 | [a,b,c]18.2 ± 0.5 |
| Cer*P*Cho | 3.4 ± 0.6 | 2.7 ± 0.7 | 2.9 ± 0.6 | 3.3 ± 0.45 |

| B | µmol/g wet weight | | | |
|---|---|---|---|---|
| PtdEtn+PakEtn | 7.1 ± 0.7 | 7.3 ± 0.7 | 7.1 ± 0.5 | 7.9 ± 0.5 |
| PlsEtn | 8.2 ± 0.8 | 8.0 ± 1.0 | 8.5 ± 0.9 | 8.9 ± 0.6 |
| PtdCho+PakCho | 14.4 ± 0.7 | 14.6 ± 1.3 | 14.8 ± 0.9 | [a,b,c]16.5 ± 0.5 |
| PlsCho | 1.9 ± 0.9 | 1.7 ± 0.3 | 1.9 ± 0.4 | 1.7 ± 0.2 |

Values represent the means ± SD in units of µmol/g wet weight.  Abbreviations are: GTA, glyceryl triacetate, EtnGpl, ethanolamine glycerophospholipid, PtdEtn + PakEtn, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine  and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine or phosphatidylethanolamine and plasmanylethanolamine, PlsEtn, 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine or ethanolamine plasmalogen, PtdIns, phosphatidylinositol, PtdSer, phosphatidylserine, ChoGpl, choline glycerophospholipid, PtdCho + PakCho, 1,2-diacyl-*sn*-glycero-3-phosphocholine  and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine or phosphatidylcholine and plasmanylcholine, PlsCho, 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphocholine or choline plasmalogen, and Cer*P*Cho, sphingomyelin.  Statistical significance ($p \leq 0.05$) is indicated by a letter:  "a" significant to water-treated control mice, "b" significant to glyceryl triacetate-treated control mice, and "c" significant to water-treated EAE mice.

treated with water (Table 5A). There was no significant difference in EtnGpl, PtdEtn + PakEtn, and PlsEtn between all control and EAE animals. However, treatment with glyceryl triacetate resulted in a significant increase in ChoGpl levels in EAE mice. Regarding subgroups of ChoGpl, treatment significantly increasedPtdCho + PakCho levels in mice subjected to EAE but had no effect on PlsCho (Table 5A and 5B) similar to the spinal cord data (Chevalier & Rosenberger 2017) (Table 1). Acetate supplementation increased total brain PtdSer and ChoGpl in particular PtdCho + PakCho content in mice subjected to EAE.

**Acetate Supplementation Increases Brain Myelin Phosphatidylinositol in Mice Subjected to EAE**

Table 6 shows myelin brain phospholipid in control mice (n=8), EAE mice treated with water (n=3), and EAE mice treated with glyceryl triacetate (n=8). There was no significant difference between all groups in PtdSer and CerPCho (Table 6A). Treatment with glyceryl triacetate significantly increased PtdIns in EAE mice compared to water-treated EAE mice. EAE injury resulted in a significant decrease in PlsEtn and ChoGpl in particular the PtdCho + PakCho fraction, but treatment was unable to significantly increase lipid levels (Table 6A and 6B). EAE injury resulted in a significant decrease in particular brain lipids (Table 6B) even though EAE is considered a spinal cord disease (Batoulis et al. 2011, Ransohoff 2012, Simmons et al. 2013). One possible explanation is that PlsEtn is prominently found in myelin (Morell 1984), and we would expect the EAE injury would result in a decrease in myelin lipid. Acetate supplementation increased brain myelin PtdIns in mice subjected to EAE.

**Table 6**: Myelin brain phospholipid content of control, EAE mice treated with water, and EAE mice treated with glyceryl triacetate.

**A**

| Phospholipid | H$_2$O-treated Control | H2O-treated EAE | GTA-treated EAE |
|---|---|---|---|
| | μmol/g wet weight | | |
| EtnGpl | 5.6 ± 0.8 | 4.0 ± 0.3 | 5.0 ± 1.0 |
| PtdIns | 0.4 ± 0.1 | 0.3 ± 0.1 | [b]0.5 ± 0.1 |
| PtdSer | 1.7 ± 0.2 | 1.2 ± 0.1 | 1.7 ± 0.4 |
| ChoGpl | 5.2 ± 0.9 | [a]3.5 ± 0.2 | 4.3 ± 0.8 |
| Cer*P*Cho | 0.8 ± 0.1 | 0.6 ± 0.1 | 0.8 ± 0.2 |
| **B** | μmol/g wet weight | | |
| PtdEtn + PakEtn | 1.8 ± 0.3 | 1.3 ± 0.01 | 1.7 ± 0.5 |
| PlsEtn | 3.8 ± 0.6 | [a]2.7 ± 0.3 | 3.3 ± 0.6 |
| PtdCho + PakCho | 4.1 ± 0.7 | [a]2.7 ± 0.1 | 3.4 ± 0.8 |
| PlsCho | 1.1 ± 0.3 | 8.4 ± 0.08 | 8.8 ± 0.2 |

Values represent the means ± SD in units of μmol/g wet weight.  Abbreviations are: GTA, glyceryl triacetate, EtnGpl, ethanolamine glycerophospholipid, PtdEtn + PakEtn, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine  and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine or phosphatidylethanolamine and plasmanylethanolamine, PlsEtn, 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine or plasmalogen ethanolamine, PtdIns, phosphatidylinositol, PtdSer, phosphatidylserine, ChoGpl, choline glycerophospholipid, PtdCho + PakCho, 1,2-diacyl-*sn*-glycero-3-phosphocholine  and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine or phosphatidylcholine and plasmanylcholine, PlsCho, 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphocholine or plasmalogen choline, and Cer*P*Cho, sphingomyelin.  Statistical significance ($p \leq 0.05$) is indicated by a letter:  "a" significant to water-treated control mice and "b" significant to water-treated EAE mice.

**Acetate Supplementation Had No Effect on Brain Cholesterol and Cholesteryl Ester Content in Mice Subjected to EAE**

Figure 8 shows brain myelin cholesterol and cholesteryl esters in control mice (n=8), EAE mice treated with water (n=3), and EAE mice treated with glyceryl triacetate (n=8). This figure also shows total brain cholesterol and cholesteryl esters in control (n=6) and EAE (n=8) mice treated with either water or glyceryl triacetate. There was no significant difference in myelin cholesterol and cholesteryl esters between all groups (Figure 8A & 8C). In addition, there was no significant difference in total brain cholesteryl esters between all groups (Figure 8D). There was a significant increase in total brain cholesterol in the EAE groups compared to water-treated control mice, but treatment did not alter total brain cholesterol between the two EAE groups (Figure 8B). Acetate supplementation did not alter brain cholesterol and cholesteryl ester levels in mice subjected to EAE.

**Acetate Supplementation Increases Total Brain GD3 Ganglioside and Brain Myelin GM1 Ganglioside Content in Mice Subjected to EAE**

Figure 9 shows total brain ganglioside content in control (n=6) and EAE (n=8) mice treated with either water or glyceryl triacetate. There was no significant difference in GM1, GD1a, and the remaining fraction (GT1a, GD2, GD1b, and GQ1b) between all groups (Figure 9B-D). Treatment with glyceryl triacetate significantly increased total brain GD3 ganglioside content in EAE mice compared to control-treated EAE mice (Figure 9A). Figure 10 shows myelin brain ganglioside content in control mice (n=8), EAE mice treated with water (n=3), and EAE mice treated with glyceryl triacetate (n=8).
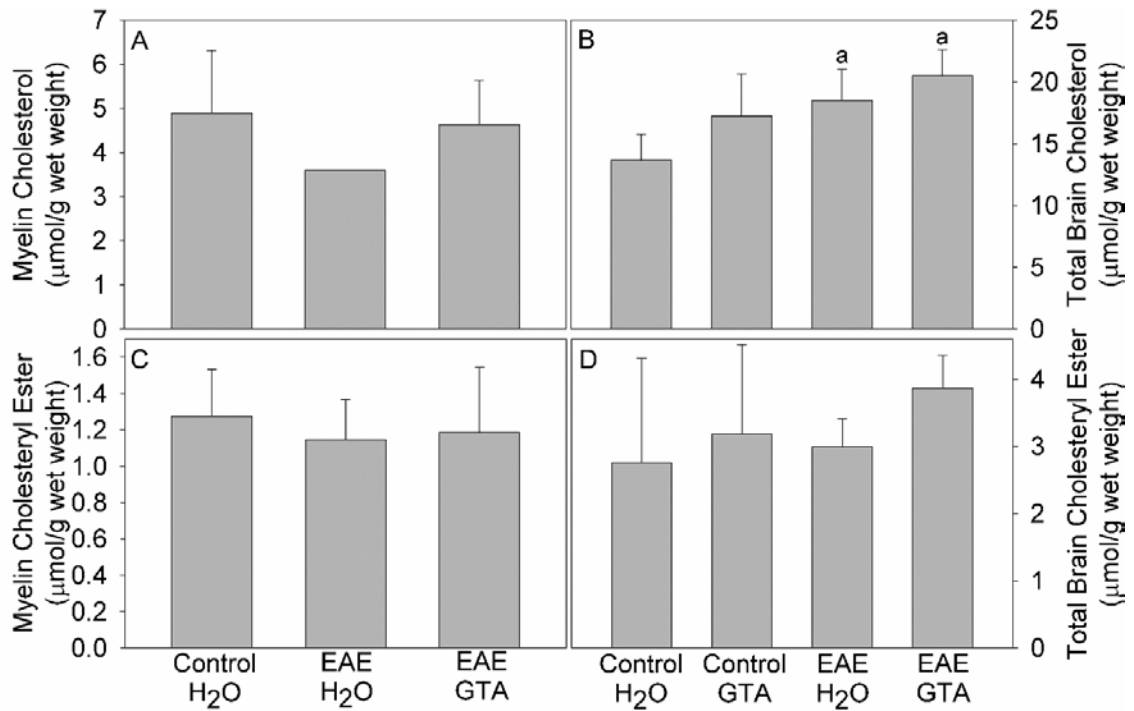
**Figure 8**: Brain myelin (A and C) and total brain (B and D) cholesterol (A and B) and cholesteryl ester (C and D) content in control and EAE mice. Sample number for panels A and C are n=8 for control mice, n=3 for EAE mice treated with water ($H_2O$), and n=8 for EAE mice treated with glyceryl triacetate (GTA) (4g/kg). Sample number for panels B and D are n=6 for each control group and n=8 for each EAE group. Regarding panels A and C, significance (p≤0.05) is indicated by a letter: "a" significant to control mice treated with water and "b" significant to EAE mice treated with water. Regarding panels B and D, significance (p≤0.05) is indicated by a letter: "a" significant to control mice treated with water, "b" significant to control mice treated with glyceryl triacetate, and "c" significant to EAE mice treated with water.
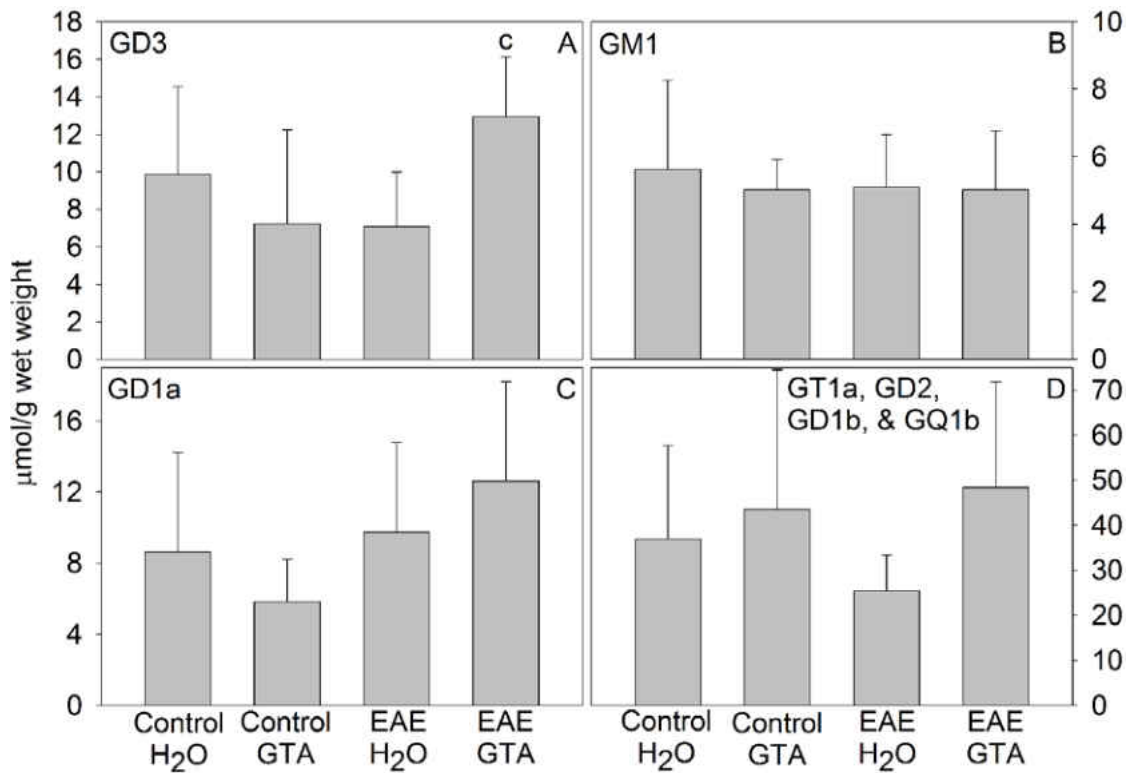
**Figure 9**: Total brain ganglioside content in control (n=6, per treatment) and EAE (n=8, per treatment) mice treated with water (H₂O) and glyceryl triacetate (GTA) (4g/kg). Panel A shows GD3, panel B shows GM1, panel C shows GD1a, and panel D shows the remaining fraction (combination of GT1a, GD2, GD1b, and GQ1b). Significance (p≤0.05) is indicated by a letter: "a" significant to control mice treated with water, "b" significant to control mice treated with glyceryl triacetate, and "c" significant to EAE mice treated with water.
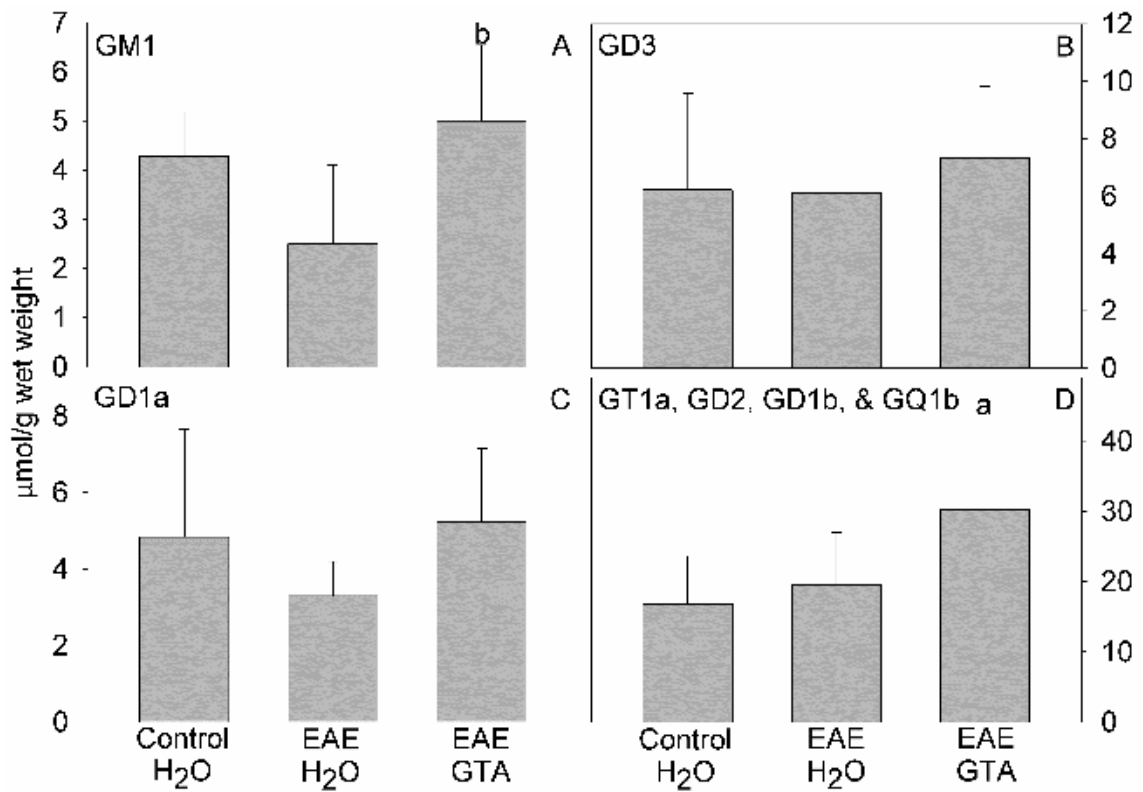
**Figure 10**: Brain myelin ganglioside content in control mice (n=8), EAE mice treated with water (n=3, $H_2O$), and EAE mice treated with glyceryl triacetate (GTA, n=8) (4g/kg). Panel A shows GM1, panel B shows GD3, panel C shows GD1a, and panel D shows the remaining fraction (combination of GT1a, GD2, GD1b, and GQ1b). Significance ($p \leq 0.05$) is indicated by a letter: "a" significant to control mice treated with water and "b" significant to EAE mice treated with water.

There was no significant difference in GD3, GD1a, and the remaining fraction between

all groups (Figure 10B-D).  Treatment with glyceryl triacetate significantly increased

myelin brain GM1 ganglioside content in EAE mice compared to control-treated EAE

mice (Figure 10A).  Overall, acetate supplementation significantly increased total

brainGD3 and brain myelin GM1 ganglioside content in EAE mice.


**Acetate Supplementation Alters Spinal Cord Oligodendrocyte Myelin Glycoprotein and Phosphorylated Acetyl-CoA Carboxylase Protein Levels in Mice Subjected to EAE but Had No Effect on Alkylglycerone Phosphate Synthase and Acyl-CoA Synthetase**

Figure 11 shows the protein levels of alkylglycerone phosphate synthase (AGPS),

acyl-CoA synthetase (AceCS1 & AceCS2), merlin (moesin-, ezrin-, radixin-like protein),

oligodendrocyte myelin glycoprotein (OMgp), and phosphorylated acetyl-CoA

carboxylase (p-ACC) in control mice (n=7), EAE mice treated with water (n=5), and

EAE mice treated with glyceryl triacetate (n=8).  AGPS is the enzyme important in ether

phospholipid biosynthesis meaning the production of the plasmanyl and plasmalogen

fractions (Braverman & Moser 2012, Rosenberger et al. 2002, Schmitt et al. 2015).

There was no significant difference between control and EAE animals (Figure 11A).

AceCS1, the cytoplasmic enzyme, and AceCS2, the mitochondrial enzyme, convert

acetate into acetyl-CoA (Jaworski et al. 2016, Shimazu et al. 2010).  Similar to AGPS

protein levels (Figure 11A), there was no significant difference between control and EAE

animals (Figure 11B-C).
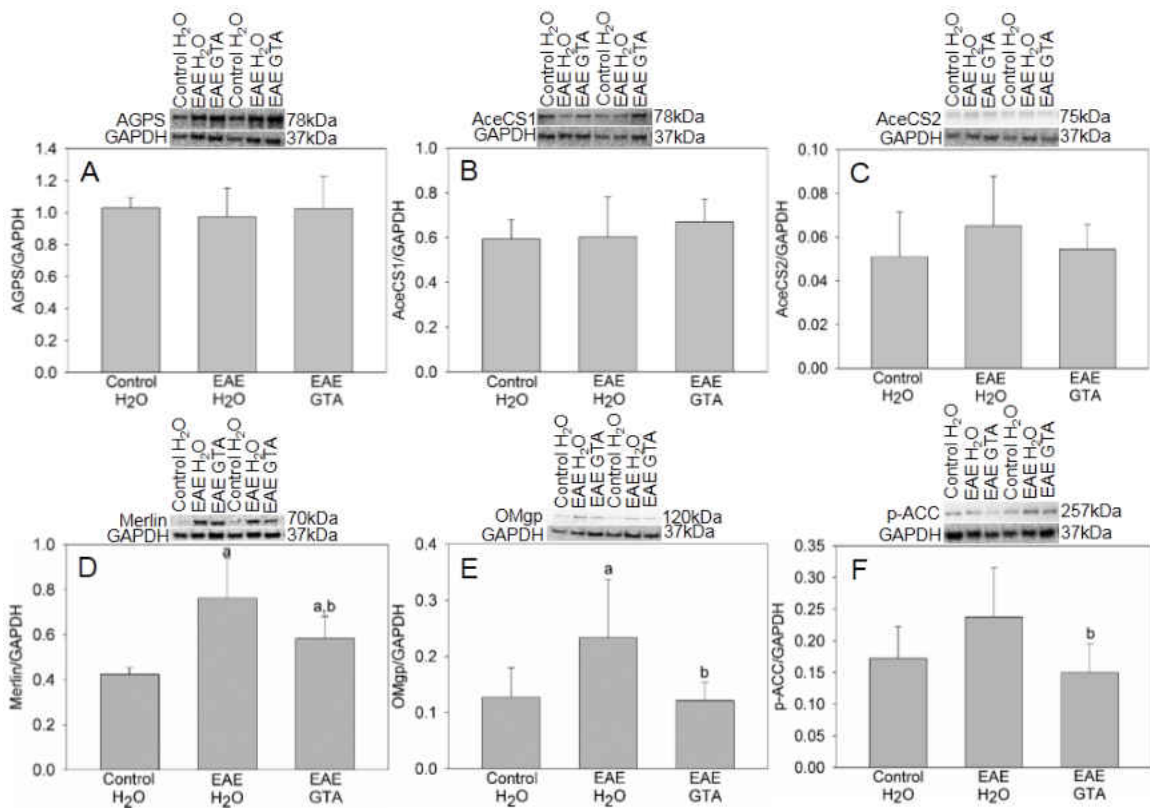
**Figure 11**: Spinal cord protein levels of alkylglycerone phosphate synthase (AGPS), acyl-CoA synthetase (AceCS1,cytoplasmic & AceCS2, mitochondrial), merlin (moesin-, ezrin-, radixin-like protein), oligodendrocyte myelin glycoprotein (OMgp), and phosphorylated acetyl-CoA carboxylase (p-ACC) in control mice (n=7), EAE mice treated with water (n=5, $H_2O$), and EAE mice treated with glyceryl triacetate (n=8), GTA) (4g/kg). Significance ($p \leq 0.05$) is indicated based on a letter: "a" significant to control mice treated with water and "b" significant to EAE mice treated with water.

We showed no significant differences in AGPS, AceCS1, and AceCS2 protein levels (Figure 11A-C) between control and EAE animals. At 40 days post inoculation and treatment, we showed treatment prevented the loss of spinal cord lipid components in EAE mice (Chevalier & Rosenberger 2017). Since we showed this recovery of lipid at this time point, we may need to determine protein levels of these enzymes early in the injury and treatment period. AGPS is an enzyme at the beginning of ether phospholipid biosynthesis (Braverman & Moser 2012, Robinson et al. 2014, Schmitt et al. 2015), and AceCS1/2 catalyzes the conversion of acetate to acetyl-CoA (Jaworski et al. 2016) preceding the production of fatty acid and cholesterol. All these data suggest that protein levels of these enzymes need to be analyzed earlier in the injury where acetate converts into acetyl-CoA and pushes that pool into the production of lipid.

Merlin is a cytoskeleton-associated protein that interacts with β-actin, for example, to link it to the plasma membrane (Hovens & Kaye 2001, Pecina-Slaus 2013). In the EAE model, there is a significant increase in β-actin (Jung et al. 2011, Smerjac & Bizzozero 2008), and in addition, we showed a significant increase in protein levels in EAE compared to control animals (Chevalier & Rosenberger 2017) (Figure 6). We would expect an increase in merlin as well due to EAE injury. EAE injury resulted in a significant increase in merlin protein levels when compared to control (Figure 11D). Treatment with glyceryl triacetate significantly decreased protein levels in EAE compared to control-treated EAE but was unable to completely return to control levels (Figure 11D) suggesting treatment does not alter cytoskeleton structure in EAE injury.

OMgp is a myelin inhibitor protein (Kan *et al.* 2016) that is suggested to inhibit remyelination and axonal growth (Cafferty *et al.* 2010, Huang *et al.* 2005, Kan et al.

2016, Vourc'h & Andres 2004, Wang *et al.* 2002, Mikol & Stefansson 1988).  OMgp

along with NogoA and MAG bind to receptors such as NgR1, p75 neurotrophin receptor

(p75NTR), and LINGO-1 to activate RhoA/ROCK pathway promoting the inhibition of

remyelination and axonal growth (Kan et al. 2016).  Spinal cord mRNA levels of NogoA,

NgR, p75NTR, LINGO-1, RhoA, and ROCK increases in rats subjected to EAE (Kan et

al. 2016), and OMgp is suggested to be involved in this pathway as well (Cafferty et al.

2010, Kan et al. 2016, Vourc'h & Andres 2004, Wang et al. 2002).  We expect OMgp

significantly increases due to the EAE injury, and in fact, the EAE injury resulted in a

significant increase in OMgp protein levels compared to control.  Interestingly, treatment

with glyceryl triacetate returned OMgp protein levels back to control levels (Figure 11E).

Acetate supplementation returned OMgp protein in EAE back to control levels to suggest

treatment may alter myelin protein structure.

Phosphorylated ACC is the enzyme that converts acetyl-CoA to malonyl-CoA,

the first step in the production of fatty acid (Baenke et al. 2013), and the phosphorylated

form is inactive.  Protein levels of p-ACC significantly decreased in EAE mice treated

with glyceryl triacetate compared to EAE mice treated with water (Figure 11F).  The next

step is to determine protein levels of the non-phosphorylated form of ACC to determine if

acetate is shifting ACC from the inactive to active form promoting fatty acid synthesis.

Treatment decreased p-ACC protein levels in EAE mice to suggest a potential shift from

an inactive to active form of the enzyme to promote fatty acid synthesis.  In Figure 11,

acetate supplementation returned OMgp protein back to control levels and significantly

decreased merlin and p-ACC levels in EAE mice compared to control-treated EAE mice.

# CHAPTER VI

## DISCUSSION

### Overall Conclusion of Dissertation

Acetate supplementation reduced disease progression, altered CNS and myelin lipid content, and influenced CNS and myelin protein content in mice subjected to EAE. We tested the hypothesis that acetate supplementation alters CNS lipid metabolism in mice subjected to EAE. Treatment with glyceryl triacetate altered spinal cord phospholipid, fatty acid, cholesterol, and ganglioside content in EAE, and acetate supplementation altered brain phospholipid and ganglioside levels in these animals. In addition, treatment attenuated the onset of clinical symptoms and returned cPLA$_2$ levels back to control levels in EAE mice. We also determined protein levels of enzymes involved in lipid synthesis, for example the inactive form of phosphorylated acetyl-CoA carboxylase. This enzyme was significantly decreased in glyceryl triacetate-treated EAE mice compared to control-treated EAE mice. These data suggest acetate may shift this enzyme from the inactive to active form to promote fatty acid synthesis. Furthermore, OMgp, a myelin inhibitor protein, significantly increased due to the EAE injury, but treatment returned protein levels back to control levels suggesting acetate may alter myelin structure. In addition, we found treatment slightly increased FluoroMyelin$^{TM}$ intensity in EAE mice compared to control-treated EAE mice, but the intensity level did not compare to control animals. These data suggest treatment may alter myelin structure but not to the extent compared to control animals. Acetate supplementation may alter

CNS lipid metabolism and myelin structure in mice subjected to EAE, and this is significant regarding the development of future treatments for demyelinating diseases like MS.

**Multiple Sclerosis May Be a Metabolic Disorder**

MS may not be an autoimmune disorder but rather a metabolic disorder, a dysfunction in lipid metabolism (Corthals 2011). To support this idea, MS pathology includes both autoimmunity and oligodendrogliopathy (Nakahara et al. 2012) which is recognized through lesion patterns showing immune-mediated or oligodendrocyte-deficient demyelination (Lucchinetti et al. 2000). This suggest that MS may have an underlying metabolic dysfunction in lipid metabolism resulting in both types of demyelination.

MS is largely considered an inflammatory disease in which autoreactive T cells enter the CNS and destroy myelin resulting in the loss of myelin, chronic inflammation, and neuronal damage (Cusick et al. 2013, Zindler & Zipp 2010). As a result, current therapeutic strategies for the treatment of MS work to inhibit peripheral immune cell infiltration and to down regulate the CNS immune response (Lim & Constantinescu 2010, Minagar 2013). However, another factor involved in MS, as well as other demyelinating diseases, is the dysfunction in mature oligodendrocyte maturation and function (de Castro *et al.* 2013, Lucchinetti et al. 2000, Wolswijk 2000). Furthermore, in MS there is a significant decrease in overall lipid content within the white matter tracks and a significant increase in activity of the enzymes that metabolize membrane lipid (Cumings 1969, Kalyvas & David 2004, Alling et al. 1971, Cumings 1953, Cumings &

Goodwin 1968, Davison & Wajda 1962, Gerstl et al. 1970, Gerstl et al. 1961, Kishimoto et al. 1967).  Also, changes in energy metabolism in MS lesions may contribute to changes in myelin pathology (Rone *et al.* 2016).  The hypothesis MS may also be a metabolic disorder (Corthals 2011) may help explain the overall decrease in lipid content, which may be a result of a decrease in the rate of lipid synthesis.  It is well known that phospholipid, cholesterol, plasmalogen, and cerebroside levels are decreased in patients who suffer from MS (Cumings 1953, Cumings & Goodwin 1968, Alling et al. 1971, Cumings 1969, Davison & Wajda 1962, Gerstl et al. 1970, Gerstl et al. 1961).  While in the EAE model, there is a decrease in white matter PtdSer, PtdIns, EtnGpl, and CerPCho (Salvati et al. 1990).  The question remains whether this loss in lipid content is a result of a metabolic dysfunction or the result of immune-targeted degradation of myelin membranes. In this regard, the $MOG_{35-55}$-induced EAE model effectively models the self-antigen attack within the spinal cord resulting in demyelination, but this model may also be useful in quantifying the impact lipid metabolism has on injury progression.  We postulate that a metabolic dysfunction in lipid metabolism may contribute to myelin loss in patients who suffer from MS.

There are a number of demyelinating disorders associated with a dysfunction in lipid metabolism such as Canavan disease, peroxisomal disorders, and disorders of fatty acid metabolism.  Canavan disease results from a mutation in the gene that encodes for the enzyme ASPA, which releases acetate from NAA (Namboodiri et al. 2006b).  The lack of NAA-degrading enzyme activity leads to excess accumulation of NAA in the brain and in turn a deficiency in acetate necessary for myelin lipid synthesis.  This results in oligodendrocyte dysfunction, spongiform changes, and the absence of myelin

(Namboodiri et al. 2006b, Kumar *et al.* 2006, Gordon 2000, Namboodiri et al. 2006a). Currently, no treatment or cure is available for patients with this disease, but however, acetate supplementation is clinically beneficial and safe in clinical and preclinical studies for Canavan disease (Madhavarao et al. 2009, Segel et al. 2011). Infants with this disease were given a low dose of glyceryl triacetate, which was found to be tolerable and safe in these patients (Madhavarao et al. 2009). Acetate supplementation may be beneficial in treating demyelinating and metabolic disorders and in turn may be beneficial in the treatment of MS.

Peroxisomal disorders, Zellweger syndrome and X-linked adrenoleukodystrophy, show peroxisomal dysfunction in fatty acid metabolism. This results in a decrease in plasmalogen content that contributes to a decrease in myelin lipid synthesis (Steinberg *et al.* 2004, Steinberg *et al.* 2006, Wanders & Waterham 2006). Fatty acid supplementation like acetate supplementation is not a new concept in the treatment of demyelinating and metabolic disorders. Oleic acid supplementation is effective in patients who suffer from adrenoleukodystrophy, a disease characterized as having a decrease in very-long chain fatty acid (VLCFA) uptake into the peroxisome (Chrast et al. 2011) and an increase in plasma VLCFA levels (Chrast et al. 2011, Moser *et al.* 1981, Poll-The & Gartner 2012). The inability of VLCFA to be transported into peroxisomes results in a dysfunction in fatty acid metabolism resulting in a loss of short chain fatty acids, a disruption in membrane dynamics, and a decrease in fatty acid oxidation (Steinberg et al. 2004, Steinberg et al. 2006, Wanders *et al.* 2010, Wanders & Waterham 2006, Weller *et al.* 2003, Kihara 2012). Thus, it is important to maintain fatty acid homeostasis to maintain normal brain function (Kihara 2012). Oleic acid is an eighteen carbon monounsaturated

fatty acid which inhibits the elongation of VLCFA (Moser *et al.* 1987, Rizzo *et al.* 1987). Oleic acid supplementation, given to adrenoleukodystrophy patients as glyceryl trioleate, decreases plasma VLCFA, improves clinical symptoms (Moser et al. 1987), and potentially normalizes fatty acid ratios within the CNS (Deon *et al.* 2008). Alternative experimental therapies also include a combination of different triglycerides; glyceryl trioleate and glyceryl trierucate (4:1, by Vol., **Lorenzo's oil**) that may be more clinically beneficial in lowering VLCFA. In fact, Lorenzo's oil lowers plasma VLCFA, increases peroxisomal function (Moser et al. 1999, Deon et al. 2008, Moser *et al.* 2007), and improves peroxisomal fatty acid oxidation (Wanders et al. 2010, Wanders & Waterham 2006). Lorenzo's oil treatment inhibits ELOVL 1, an enzyme involved in VLCFA synthesis, (Sassa *et al.* 2014) suggesting a potential mechanism of action that may lead to an improvement in lipid metabolism in this disease. Fatty acid supplementation may provide the necessary components to compensate for the metabolic dysfunction.

In addition to the dysfunction in fatty acid metabolism, patients with adrenoleukodystrophy show an increase in the production of leukotrienes within the CNS (Mayatepek *et al.* 1993, Mayatepek & Tiepelmann 1996). Immune-mediated demyelination is present (Mayatepek et al. 1993, Mayatepek & Tiepelmann 1996), but there is also a dysfunction in fatty acid metabolism suggesting a similar trend to that in MS and EAE. Both may contribute to changes in lipid content, and in patients suffering with MS, there are changes in CNS lipid content (Cumings 1969, Cumings 1953, Cumings & Goodwin 1968, Gerstl et al. 1970, Gerstl et al. 1961). All this suggests MS may also have an underlying metabolic dysfunction in lipid metabolism on top of the immune-mediated demyelination.

Another fatty acid treatment, docosahexaenoic acid (DHA) supplementation, shows therapeutic promise in patients who suffer from another peroxisomal disorder, Zellweger syndrome. Zellweger syndrome results in a dysfunction in fatty acid oxidation that contributes to an accumulation of fatty acids, a decrease in plasmalogen levels, and hypomyelination (Chrast et al. 2011, Weller et al. 2003, Ferdinandusse *et al.* 2001, Poll-The & Gartner 2012). DHA supplementation improves neuronal function related to vision and muscle movement in young children and infants (Martinez 1996, Martinez *et al.* 1993, Martinez 2001, Martinez *et al.* 2000), returns plasma DHA and plasmalogen to normal (Martinez et al. 2000), and improves myelination shown in MRI scans of patients (Martinez & Vazquez 1998, Martinez et al. 2000). Fatty acid supplementation shows promise in the treatment of demyelinating and metabolic disorders, and acetate supplementation may be the ideal fatty acid treatment because acetate is the simplest fatty acid and can be utilized for fatty acid (D'Adamo & Yatsu 1966), phospholipid (Smith 1964, Mehta & Namboodiri 1995), and cholesterol (Chakraborty et al. 2001) synthesis.

In addition to Canavan disease and peroxisomal disorders, there are numerous disorders of fatty acid metabolism. For example, rhizomelic chondrodysplasia punctata results from AGPS deficiency contributing to a decrease in myelin shown in MRI scans of patients. The decrease in myelin is a result from a decrease in plasmalogen due to the AGPS deficiency (Sztriha *et al.* 1997), an enzyme important in ether phospholipid biosynthesis (Sztriha et al. 1997, Braverman & Moser 2012, Rosenberger et al. 2002). Sjogren-Larsson disease results from an increase in long-chain fatty alcohols, and Resfum disease shows a dysfunction in α-oxidation. Also, Niemann-Pick disease results in a glycosphingolipid accumulation. All of these diseases result in a disruption in

myelin stability, assembly, and compactness (Chrast et al. 2011) that in turn alters axonal function, axon-glia interactions, and disrupts lipid-mediated signaling (Chrast et al. 2011, Schmitt et al. 2015). These metabolic disorders show changes in lipid content and metabolism, and MS and EAE also show changes in lipid content and metabolism (Cumings 1969, Cumings 1953, Cumings & Goodwin 1968, Gerstl et al. 1970, Gerstl et al. 1961, Kalyvas & David 2004) further suggesting MS may be a metabolic disorder rather than or on top of an autoimmune disorder (Corthals 2011).

Smith-Lemli-Opitz syndrome displays a dysfunction in cholesterol metabolism due to a mutation in the enzyme involved in the final step of cholesterol synthesis resulting in an increase in cholesterol metabolites but an overall decrease in cholesterol (Chrast et al. 2011). In this disease, it is suggested that the inability to incorporate cholesterol into myelin results in the loss of myelin. Interestingly, treatment involving cholesterol supplementation in combination with HMG-CoA reductase inhibitors shows an increase in plasma cholesterol and a decrease in its metabolites (Chan *et al.* 2009, Haas *et al.* 2007), and cholesterol supplementation alone is clinically beneficial for Smith-Lemli-Opitz disease (Elias *et al.* 1997, Irons *et al.* 1997). This suggests there is a potential change in the rate of lipid deposition by supplying the system with dietary cholesterol. Similarly, lovastatin, a HMG-CoA reductase inhibitor, significantly increases spinal cord myelin lipid levels and augments the survival and differentiation of OPCs in Lewis rats subjected to EAE (Paintlia *et al.* 2005). All of which support the premise that a change in the rate of lipid deposition leads to injury, and treatment needs to focus on increasing the rate to reverse or prevent further injury. As well, these diseases show changes in CNS lipid composition (Chrast et al. 2011) like MS (Cumings

1969, Cumings 1953, Cumings & Goodwin 1968, Gerstl et al. 1970, Gerstl et al. 1961, Alling et al. 1971, Davison & Wajda 1962, Kishimoto et al. 1967) to further suggest MS may have a dysfunction in lipid metabolism.

In addition to fatty acid supplementation, steroid administration in a MS animal model is anti-inflammatory and may promote steroid synthesis. Cholesterol, an important myelin lipid (Morell 1984), is also important in the production of neurosteroids like pregnenolone, dehydroepiandrosterone (DHEA), progesterone, and androstenedione. In MS white matter, DHEA is significantly lower compared to non-MS white matter but no difference in pregnenolone levels is observed suggesting a dysfunction in the conversion of pregnenolone to DHEA. MS white matter shows significantly lower mRNA levels of the enzyme that converts pregnenolone to DHEA to further support the idea that MS may be a metabolic disorder. In this same study, EAE results in a significant decrease in DHEA and may be a result of a decrease in cholesterol availability. Once these animals were administered DHEA, spinal cord inflammation decreased, and spinal cord mRNA levels of enzymes involved in the production of DHEA returned to control levels (Boghozian *et al.* 2017). These data further suggest an underlying metabolic disorder, a dysfunction in lipid metabolism, may be present in MS and EAE.

In our studies, acetate supplementation prevented the loss of particular spinal cord lipid components in mice subjected to EAE. If treatment is primarily anti-inflammatory, we would expect all lipid components would be recovered with treatment. Treatment altered particular spinal cord lipid components suggesting EAE may have a metabolic dysfunction in lipid metabolism. Therefore since EAE is a mouse model for MS, it may

suggest that MS is also a metabolic disorder. Thus a potential effective strategy to prevent or slow demyelination may involve promoting myelin lipid synthesis. Acetate within the central nervous system is readily converted into its CoA derivative suggesting acetate supplementation may be a potential therapy to promote lipid synthesis, and within the central nervous system acetate and acetyl-CoA are important precursors in the synthesis of fatty acid (D'Adamo & Yatsu 1966), phospholipid (Chakraborty et al. 2001, Smith 1964, Mehta & Namboodiri 1995), and cholesterol (Chakraborty et al. 2001).

## An Inflammatory Stimulus May Be Necessary to Shift the Acetyl-CoA Pool into Producing Lipid

Acetate treatment results in a significant increase in fatty acid content in LPS-stimulated BV2 microglia compared to control-treated LPS-stimulated cells. However, in the non-LPS stimulated cells, there was no significant difference in fatty acid content between the two control groups (Bhatt & Rosenberger 2014). This suggests treatment may require a stimulus, like inflammation, to result in changes in lipid content between control-treated and acetate-treated injury groups. To further support this hypothesis, we determined spinal and brain lipid content in both control and EAE animals. The control group determined whether treatment alone altered CNS lipid content *in vivo* without the presence of an inflammatory stimulus. Again, these animals were not injected with the MOG peptide and pertussis toxin to result in the spinal cord immune-mediated demyelination (Palumbo & Bosetti 2013). The EAE group determined if treatment needs a stimulus, like EAE inflammation, to result in changes in lipid content similar to previous literature (Bhatt & Rosenberger 2014). We found that in control mice, there was no significant difference in spinal cord and brain lipid content between the two

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groups similar to previous *in vitro* work (Bhatt & Rosenberger 2014). However, we found significant differences in spinal cord lipid content between the two EAE treatment groups. Treatment significantly increased spinal cord lipid content in EAE mice compared to control-treated EAE mice, but again, there was no significant difference in lipid levels between the two control groups. This supports the hypothesis that treatment may require a stimulus, such as LPS-stimulated microglia or EAE, to result in significant changes in lipid content. Due to the presence of the inflammatory stimulus within the CNS, we hypothesized that treatment would alter brain lipid content even though EAE is a spinal cord injury (Batoulis et al. 2011, Ransohoff 2012, Simmons et al. 2013), and in fact, we found acetate supplementation increased brain phospholipid and ganglioside content in EAE compared to control-treated EAE mice. Since there were changes in brain lipid content due to EAE and treatment, this suggests an inflammatory stimulus is important to result in changes in CNS lipid content and for the acetyl-CoA to be predominantly utilized in lipid synthesis.

The above data on lipid levels in EAE mice also provides insight into a mechanism of action for glyceryl triacetate in normal and EAE states. In control healthy animals, there is a natural lipid turnover (Ando et al. 2003, Rosenberger et al. 2002, Moser et al. 1999) where the amount of lipid produced equals the amount of lipid broken down (Figure 12). During the EAE injury, the amount of lipid broken down is significantly higher compared to the amount of lipid produced (Figure 12) due to chronic immune-mediated demyelination through the cPLA$_2$ pathway (Kalyvas & David 2004). When EAE mice are treated with glyceryl triacetate, we suggest treatment either decreases lipid breakdown through the cPLA$_2$ pathway, promotes lipid synthesis, or does

a combination of both to return the lipid turnover rate to control levels. This then

recovers the lipid lost due to injury (Figure 12). Acetate supplementation recovered the

EAE-induced spinal cord lipid loss and returned cPLA2 levels back to control to suggest

treatment may promote lipid synthesis and/or reduces breakdown.
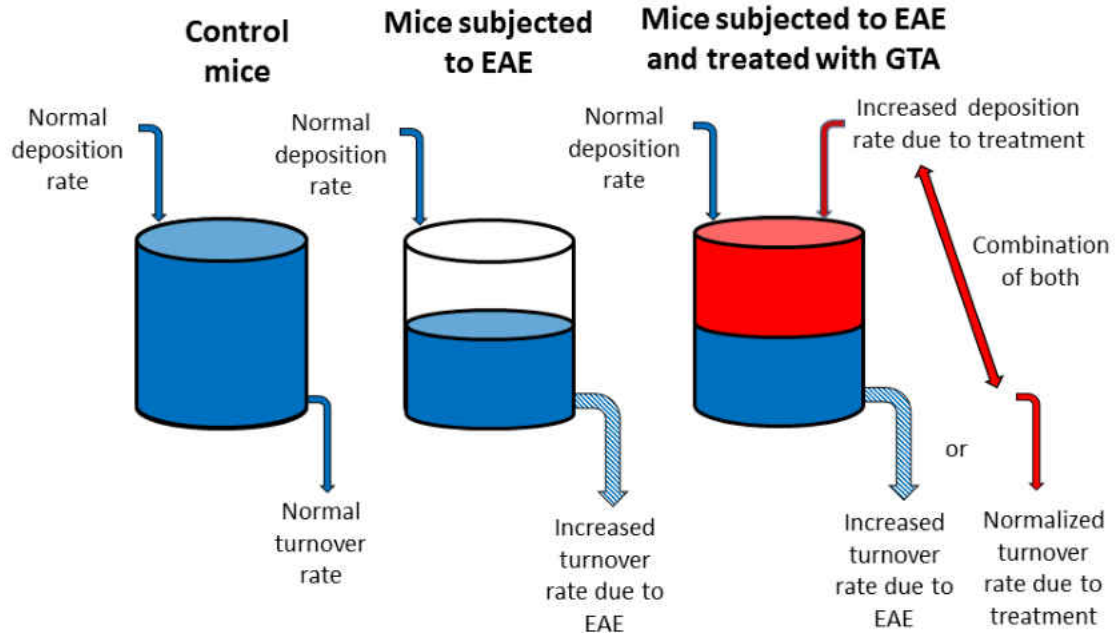


**Figure 12**: Our bucket theory regarding lipid turnover rate in control, EAE, and EAE mice treated with glyceryl triacetate. Abbreviations include EAE (experimental autoimmune encephalomyelitis) and GTA (glyceryl triacetate). The arrows indicating deposition rate refer to the amount of lipid produced, and the arrows indicating turnover rate refer to the amount of lipid broken down. Blue level indicates the level of lipid found within the spinal cord of these mice, and red level refers to the recovery of lipid due to treatment.

Acetate can be utilized in energy production, histone and non-histone protein

acetylation, and lipid synthesis (Bhatt et al. 2013, Bhatt & Rosenberger 2014, Soliman &

Rosenberger 2011, Soliman et al. 2012b). In a normal control state, acetate may be

utilized for the natural lipid turnover (Ando et al. 2003, Rosenberger et al. 2002, Moser et

al. 1999) to promote the production of lipid to compensate for lipid being broken down,

but there is no metabolic demand for the entirety of the acetyl-CoA pool to be used for lipid synthesis.  The remaining pool may also be used to increase energy production (Bhatt et al. 2013).  A single dose of glyceryl triacetate was administered to control rats.  Four hours later, treatment significantly increases brain phosphocreatine levels to suggest acetate supplementation promotes energy production in control rats (Bhatt et al. 2013).  In addition to acetate being utilized for energy production, acetate may alter histone and non-histone protein acetylation.  Acetylation, which was shown when male Sprague-Dawley rats were administered a single dose of glyceryl triacetate, and four hours post treatment, histone acetylation increases and histone deacetylase activity decreases (Soliman & Rosenberger 2011) to suggest treatment alters histone acetylation in control rats.  In a normal healthy state, acetate supplementation may alter lipid content to maintain the natural turnover (Ando et al. 2003, Rosenberger et al. 2002, Moser et al. 1999) but may have a greater effect on energy production (Bhatt et al. 2013) and histone acetylation (Soliman & Rosenberger 2011, Soliman et al. 2012b).  Although in an injury state, like EAE or LPS-stimulated inflammation, we suggest acetate may be predominately utilized for lipid synthesis.

In an injury state like EAE, increasing the amount of available acetyl-CoA may push the pool to be predominantly utilized in the production of lipid.  Treatment with glyceryl triacetate significantly increased spinal cord phospholipid, fatty acid, cholesterol, and ganglioside content in EAE mice compared to control-treated EAE mice.  These data suggest that the acetyl-Co pool is being utilized to recover the lipid lost due to injury.  This idea was supported by work completed by Bhatt and Rosenberger in LPS-stimulated BV2 microglia (Bhatt & Rosenberger 2014).  Acetate increases fatty acid content in LPS-

stimulated microglia compared to control-treated LPS stimulated cells. In addition, we found treatment with glyceryl triacetate significantly increased brain phospholipid and ganglioside content in EAE mice compared to control-treated EAE mice. Again, we showed changes in brain lipid content between the two EAE treatment groups. The EAE inflammatory stimulus is present within the CNS even though EAE is a spinal cord injury (Batoulis et al. 2011, Ransohoff 2012, Simmons et al. 2013). Therefore, acetate is being utilized in the production of brain and spinal cord lipid in the presence of the EAE injury.

Oligodendrocytes treated with radiolabeled acetate results in radiolabeled acetyl-CoA, which was not overly incorporated into TCA metabolites compared to cells treated with radiolabeled glucose. This suggests in a myelinating cell, acetate is essential in the production of myelin lipid rather than energy production (Amaral *et al.* 2016). This is not an obscure thought based on data from another study. Increasing acetyl-CoA metabolism through pyruvate or glycolysis pathway is not necessary for myelin stability in myelinating glia cells (Della-Flora Nunes *et al.* 2017). This suggests acetate may be predominantly utilized in lipid synthesis where there is that metabolic demand. In an injury state, acetate is predominantly utilized to increase acetyl-CoA metabolism for lipid synthesis, but there may also be a secondary pathway where acetate is important in promoting histone acetylation.

Acetate promotes histone and non-histone protein acetylation in an injury state to alter inflammation and also may change the expression of enzymes involved in lipid metabolism. In a neuroinflammation rat model, treatment with glyceryl triacetate results in an increase in histone acetylation and an increase in histone acetyltransferase activity to correlate with a decrease in IL-1β cytokine levels (Soliman et al. 2012b). Treatment is

anti-inflammatory by down regulating the expression of pro-inflammatory cytokines. In addition, acetate increases histone acetylation and decreases IL-1β and TNF-α protein levels in LPS-stimulated primary astrocytes (Soliman et al. 2013a) to suggest changes in histone acetylation may down-regulate the expression of pro-inflammatory cytokines both *in vivo and in vitro*. Down-regulating the pro-inflammatory response does not contradict the hypothesis that an inflammatory stimulus needs to be present to show changes in lipid content. When an inflammatory stimulus is present, acetate supplementation promotes histone acetylation to down-regulate inflammation (Soliman & Rosenberger 2011, Soliman et al. 2012b) but at the same time, alters lipid metabolism recovering the lipid lost due to injury (Bhatt & Rosenberger 2014, Chevalier & Rosenberger 2017). Acetate significantly increases fatty acid content in LPS-stimulated BV2 microglia, and in the same study, acetate increases histone acetylation in these cells (Bhatt & Rosenberger 2014) suggesting histone acetylation may promote the expression of enzymes involved in fatty acid synthesis. Furthermore, histone acetylation induced by treatment with glyceryl triacetate may down regulate the expression of enzymes involved in lipid synthesis and breakdown.

**Therapeutic Window Where Glyceryl Triacetate May Benefit Demyelinating and Metabolic Disorders**

Based on previous literature and current studies, there may be a therapeutic window where glyceryl triacetate may be clinically beneficial in demyelination and metabolic disorders. For example in patients with Canavan disease, acetate supplementation did not significantly improve or worsen clinical symptoms (Madhavarao et al. 2009). In preliminary studies, we administered glyceryl triacetate to EAE mice

after they exhibited hind limb paralysis, and acetate supplementation did not improve onset of paralysis but also did not worsen symptoms (data not shown). Comparing previous literature, preliminary results, and those outlined in these studies suggests there may be a therapeutic window where treatment may be effective in offsetting the loss of lipid due to injury. In these studies, some animals experienced tail paralysis that was reversed to full mobility due to treatment with glyceryl triacetate. In addition to acetate supplementation in Canavan disease and EAE, DHA supplementation in patients with Zellweger syndrome need to be treated early in development (Martinez 2001, Martinez et al. 2000) for treatment to be effective. One theory for this idea is once disease has progressed to the extent where mature oligodendrocytes and OPCs remain unviable, acetate supplementation may not be effective in preventing or reversing disease progression alone to promote myelin lipid deposition. OPCs differentiate into mature oligodendrocytes, and mature oligodendrocyte promote myelin and increase myelin lipid. When disease progresses to the point where there is a decrease in the number and/or functionality of OPCs and mature oligodendrocyte, fatty acid supplementation may not be effective in promoting myelination and reversing or preventing clinical onset in demyelinating diseases.

**Acetate's Role in Oligodendrocyte Maturation, Differentiation, and Function**

ASPA, the enzyme that releases acetate and aspartate from NAA (Namboodiri et al. 2006b), is found in both the cytosol and nucleus of oligodendrocytes (Baslow *et al.* 1999, Bhakoo *et al.* 2001, Kirmani *et al.* 2003, Madhavarao *et al.* 2002, Kirmani *et al.* 2002, Moffett *et al.* 2011). It has been suggested ASPA expression promotes acetate

utilization into lipid synthesis (Kirmani et al. 2003, Kirmani et al. 2002), and aspartate is used for energy production in oligodendrocytes especially during differentiation (Amaral et al. 2016, Amaral *et al.* 2017). In primary rat oligodendrocytes, NAA treatment increases ASPA and MBP protein suggesting that ASPA releases acetate into oligodendrocytes contributing to myelination. Furthermore, NAA treatment significantly increases mRNA levels of Tcf712, Olig2, Ugt8, and Sgms1 in primary oligodendrocytes. Tcf712 and Olig 2 are markers associated with oligodendrocyte differentiation, and the remaining are markers associated with lipid synthesis (Singhal *et al.* 2017). All these data suggest NAA contributes to ASPA releasing acetate, and acetate promotes myelination and oligodendrocyte differentiation. In this regard, acetate may alter lipid content in oligodendrocytes by promoting oligodendrocyte differentiation and myelin lipid synthesis.

Radiolabeled acetate incorporates into the lipid portion of primary pig oligodendrocytes. Radiolabeled acetate incorporates into 58.9% of the neural lipid fraction consisting of cholesterol and triglycerides, and the remaining portion consists of phospholipids like PtdCho (17.5%), EtnGpl (6.0%), PtdIns (3.2%), PtdSer (3.1%), and CerPCho (1.7%) (Burgisser *et al.* 1988). Within EtnGpl, radiolabeled acetate incorporates primarily within the plasmalogen fraction (71.6%) while the remaining 28.4% is PtdEtn + PakEtn (Burgisser et al. 1988). This is not surprising because oligodendrocytes promote myelination in the CNS (Boulanger & Messier 2014, Mitew et al. 2014), and PlsEtn is predominately found in myelin (Morell 1984). In addition, radiolabeled acetate incorporates into fatty acid and cholesterol (Warringa *et al.* 1987) in oligodendrocytes. The expression of AceCS1 and AceCS2 in oligodendrocytes provides

further support to the idea that acetate's role in oligodendrocytes is to promote lipid synthesis. AceCS1, localized in the cytoplasm and nucleus, is expressed in oligodendrocytes while AceCS2, localized in the mitochondria, is expressed in astrocytes. AceCS1 is associated with increasing histone acetylation in the nucleus and lipid synthesis in the cytosol where AceCS2 is associated with energy production in the mitochondria (Cahoy *et al.* 2008, Moffett et al. 2011, Moffett *et al.* 2013). To further support the hypothesis that acetate is predominately utilized for lipid synthesis over energy production within oligodendrocytes, it has been shown that radiolabeled acetate incorporates into TCA cycle metabolites at much lower rate compared to cells treated with radiolabeled glucose (Amaral et al. 2016). Also, acetyl-CoA produced through the glycolysis/TCA pathway is not essential for myelin maintenance (Della-Flora Nunes et al. 2017). Acetate incorporates into oligodendrocyte lipid content, and lipids, in general, promote oligodendrocyte development and function. In primary oligodendrocytes, cholesterol treatment is correlated with promoting oligodendrocyte differentiation (Berghoff *et al.* 2017), and DHA treatment promotes oligodendrocyte viability and protection from microglia activated-induced inflammation (Pu *et al.* 2013). Increasing acetyl-CoA metabolism to alter lipid content in oligodendrocytes may a result from promoting myelination and oligodendrocyte development and function.

Even though acetate may promote myelin lipid synthesis in oligodendrocytes, a small amount of acetate may be making its way to energy production for proper oligodendrocyte development and function leading to myelin lipid production. Myelin biosynthesis requires energy production in the form of ATP production and regulation of the glycolysis pathway. It is suggested that mature oligodendrocytes are less

metabolically active than OPCs, and both cell types require glycolysis to produce ATP as an energy source. Under injury-like conditions, mature oligodendrocytes decrease glycolysis and ATP metabolic processes to inhibit cell death, but this negatively affects the myelinating function. Reducing myelin production results in the preservation of energy for cell survival. There is no change in these processes in OPCs leading to cell death, and by decreasing the number of OPCs leads to a decrease in the differentiation to mature myelination oligodendrocytes. This results in the ability for oligodendrocyte to produce myelin lipid (Rone et al. 2016). Proper energy metabolism in oligodendrocytes leads to the production of lipid and normal function. Acetate may promote myelination and normal development and function in oligodendrocytes.

**The Significance of CNS Lipid Changes Due to EAE and Treatment Related to Myelin Structure, Function, and Stability**

Myelin composition is primarily comprised of lipid (80%) consisting of cholesterol (28%) and phospholipid (43%) while the remaining fraction is protein (20%) (Morell 1984). In these studies, we found treatment with glyceryl triacetate significantly increased spinal cord phospholipid and cholesterol in mice subjected to EAE compared to control-treated EAE mice, and treatment resulted in the levels of these lipids in EAE mice equivalent to that found in control mice. This suggests acetate supplementation may recover myelin lipid lost due to the demyelinating injury of EAE. Major myelin phospholipids include PtdEtn (16%), PtdCho (11%), and PtdSer (5%) (Morell 1984). We found acetate supplementation significantly increased spinal cord EtnGpl, ChoGpl, and PtdSer in EAE mice compared to control-treated EAE mice. Regarding the EtnGpl and ChoGpl groups, treatment altered PtdEtn + PakEtn and PtdCho + PakCho rather than the

plasmalogen fractions.  All these data suggest treatment may alter myelin lipid content in mice subjected to EAE.  PtdIns occupies 0.6% of the myelin phospholipid fraction (Morell 1984), and treatment with glyceryl triacetate significantly increased brain myelin PtdIns in EAE mice compared to control-treated EAE mice to further suggest treatment may alter myelin lipid content in EAE mice.  Treatment may promote myelin lipid deposition in demyelinating diseases, and this is significant in the development of a remyelination therapy for patients suffering from MS.

GD1a ganglioside levels significantly decreased due to the EAE injury compared to control levels.  Acetate supplementation significantly increased spinal cord GD1a ganglioside levels in EAE compared to control-treated EAE mice, but treatment was unable to completely return levels to control levels.  GD1a within the CNS is a ligand for MAG whose interaction promotes myelin stability and inhibits nerve regeneration by reducing nerve sprouting (Schmitt et al. 2015, Schnaar 2010) both of which are important in maintaining normal function within the CNS (Pan *et al.* 2005, Vyas *et al.* 2002, Vyas & Schnaar 2001, Yang *et al.* 1996, Chiavegatto *et al.* 2000, McKerracher *et al.* 1994, Mukhopadhyay *et al.* 1994, Schachner & Bartsch 2000, Schnaar et al. 2014).  EAE injury may potentially decrease myelin stability due to a decrease in GD1a/MAG interactions. Treatment may change the level of GD1a/MAG interactions to promote myelin stability, but this interaction may not be to the same extent as controls.  This potential reduction in the interaction of GD1a and MAG in glyceryl-triacetate EAE mice compared to control mice is possibly due to an increase in the inflammatory response that may limit GD1a/MAG interactions with an anti-ganglioside antibody attack to ganglioside content altering myelin stability (Mata *et al.* 1999, Sadatipour *et al.* 1998, Acarin *et al.* 1996).

Treatment increased spinal cord GD1a levels in EAE compared to control-treated EAE

mice to suggest treatment may promote myelin stability through MAG-GD1a interactions

but not to the same extent as compared to controls.

Acetate supplementation significantly increased total spinal cord and brain GD3

ganglioside levels in EAE mice compared to control-treated EAE mice. Thus the

dramatic loss of lipid due to the EAE injury may result in the metabolic need to increase

GD3 content compared to GM1 similar to a developing brain (Kracun *et al.* 1991, Rosner

*et al.* 1985). Showing changes in GD3 levels in EAE and treatment is significant because

in young mice, we may be promoting the continuation of myelination.

Acetate supplementation slightly increased FluoroMyelin$^{TM}$ intensity in EAE

mice compared to control-treated EAE mice, but the intensity did not reach the same

level as control mice. FluoroMyelin™ binds to myelin via lipophilic affiliation

indicating that an increase in intensity correlates with an increase in myelin lipid. We

showed acetate supplementation prevented the loss of spinal cord phospholipid and

cholesterol in mice subjected to EAE, but treatment was unable to completely return

GD1a ganglioside levels back to control. Between the FluoroMyelin$^{TM}$ staining and

spinal cord lipid data, treatment may increase myelin lipid and myelin stability in EAE

mice compared to control-treated EAE mice but not to the same extent as controls.

Cytosolic PLA$_2$ promotes the release of arachidonic acid from membrane

phospholipid to promote the production of inflammatory eicosanoids (Shimizu 2009,

Benjamins et al. 2012). EAE injury results in a significant increase in cPLA$_2$ levels

contributing to immune-mediated demyelination (Kalyvas & David 2004). We also

showed that the EAE injury resulted in a significant increase in cPLA$_2$ protein levels

compared to control mice, and treatment returned levels back to control levels. These

data suggest treatment may modulate pro-inflammatory lipid signaling to down regulate

the immune-mediated demyelination. Treatment may decrease the amount of lipid

broken down and potentially normalizing the lipid turnover rate in EAE mice.

EAE resulted in a significant increase in OMgp, a protein found within myelin

that inhibits remyelination and axonal growth. OMgp is suggested to act similar to

NogoA binding to NgR1, p75NTR, or LINGO-1 to activate RhoA/ROCK to inhibit

remyelination and axonal growth (Cafferty et al. 2010, Huang et al. 2005, Kan et al.

2016, Mikol et al. 1993, Vourc'h & Andres 2004, Wang et al. 2002). Interestingly,

treatment returned levels back to control levels in mice subjected to EAE indicating a

change in myelin structure with EAE and treatment. Acetate supplementation may

promote myelin lipid deposition by down-regulating pathways involved in remyelination

inhibition and up-regulating pathways involved in myelination. Treatment may alter

myelin composition, structure, and stability between control-treated and glyceryl

triacetate-treated EAE groups.


**The Importance to Develop Therapies that Promote Lipid Synthesis and/or Reduce Lipid Breakdown to Treat Multiple Sclerosis**

Acetate supplementation administered through glyceryl triacetate may potentially

promote lipid synthesis and/or reduce lipid breakdown based on results shown in these

studies. Treatment with glyceryl triacetate prevented the loss of spinal cord lipid

components in mice subjected to EAE suggesting treatment may stimulate lipid

deposition and/or prevent lipid breakdown. In addition, we showed treatment with

glyceryl triacetate returned $cPLA_2$ levels back to control levels in mice subjected to EAE

to suggest treatment may prevent lipid breakdown through the cPLA$_2$-arachidonic acid pathway.  We showed treatment significantly decreased p-ACC protein levels in mice subjected to EAE to suggest acetate may shift this enzyme from the inactive to active form to promote fatty acid synthesis.  Acetate supplementation is one potential lipid target, but there are other lipid supplementations that could potentially promote lipid synthesis and/or reduce lipid breakdown.

Previously, the therapeutic benefits of oleic acid supplementation in adrenoleukodystrophy (Moser et al. 1987), DHA supplementation in Zellweger syndrome (Martinez 1996, Martinez 2001, Martinez & Mougan 1999, Martinez et al. 1993, Martinez & Vazquez 1998, Martinez et al. 2000), and Lorenzo's oil in adrenoleukodystrophy (Moser et al. 1999, Moser et al. 2007) were presented.  There are additional lipid supplementation therapies to provide evidence that normalizing lipid turnover may relate to changes in clinical outcomes and pathology in various diseases. Hydrocortisone, a steroid, significantly increases radiolabeled acetate incorporation into fatty acid and cholesterol in primary oligodendrocytes (Warringa et al. 1987).  This suggests cholesterol synthesis is important for myelination in oligodendrocytes.  In a mouse model for traumatic brain injury, a diet consisting of omega-3 fatty acids, DHA and eicosapentaenoic (EPA, 20:5n-3), improves clinical outcomes in wire hanging, grid walking, foot fault, and platform locating tests to suggest a high omega-3 fatty acid diet compared to normal diet may promote an improvement in sensorimotor and cognition function.  A high omega-3 fatty acid diet is anti-inflammatory decreasing mRNA pro-inflammatory markers such as IL-1$\alpha$, IL-1$\beta$, TNF$\alpha$, and COX-2. This treatment may promote myelination increasing MBP (Pu et al. 2013).  A high omega-3 fatty acid diet is

clinically beneficial in patients with depression increasing brain white matter fatty acids as shown in MRI scans of patients (Chhetry *et al.* 2016). All these data suggest fatty acids are important in promoting myelin lipid production in the CNS, and acetate, being the simplest fatty acid, may also do the same. Supplementation of linolenic acid, an essential fatty acid, contributes to axonal survival in an animal model of sciatic nerve injury through regeneration and myelination. As well, treatment improves function which is shown through the toe spreading reflex test in this model (Ramli *et al.* 2017). Fatty acid supplementation may promote myelination and axonal survival to show clinically improvements in animal behaviors in disease processes.

In cuprizone and lyso-lecithin animal models, cholesterol supplementation may promote remyelination and oligodendrocyte differentiation. In the chronic cuprizone model, cholesterol decreases axonal damage, increases remyelination, and increases the number of OPCs and mature oligodendrocytes. Cholesterol can alter these conditions by gaining access to CNS due to the decreased permeability of the BBB. When cuprizone was removed from the diet, cholesterol increases OPC proliferation, the number of OPCs and mature oligodendrocytes, myelin content, and decreases axonal damage. In the lyso-lecithin model, cholesterol also increases remyelination and number of oligodendrocytes. All these data indicate cholesterol promotes remyelination and oligodendrocyte function in MS pathology (Berghoff et al. 2017). Lipids have a role in myelin stability and in turn promote cognitive function. All these data provide a potential therapeutic strategy, supplying the CNS with lipid, which promotes lipid synthesis and/or reduces lipid breakdown for demyelinating diseases. Ketogenic diet studies provide further support to this idea.

A case study tested the effectiveness of a ketogenic diet with a child showing a dysfunction in the mitochondrial aspartate-glutamate carrier isoform 1, and this dysfunction results in the inability to release aspartate into the cytosol from the mitochondria through the malate-aspartate shuttle. The child shows symptoms of hypotonia, deficits in psychomotor development, seizures, and hypomyelination. Hypomyelination is due to the inability of aspartate to form NAA and for NAA, to transport into oligodendrocytes to metabolize into acetate and aspartate. After the child was administered a ketogenic diet consisting of a high fat and low carbohydrate count, the child improves clinical symptoms and white matter volume. Improving white matter volume suggests treatment may promote myelination (Dahlin *et al.* 2015). GLUT1 deficiency syndrome results in a decrease in glucose uptake, and patients with this deficiency show mental impairment and motor dysfunction. Ketogenic diet given to a 3.5 year old boy with this deficiency shows clinical improvements in alertness and cognitive performance, and in addition, MRI scans indicate myelination as a result of the ketogenic diet (Klepper *et al.* 2007). A high fat diet may be beneficial in treating demyelinating diseases.

Recently, it is suggested a ketogenic diet may be therapeutically beneficial for patients suffering with progressive MS. Current MS therapies are immunomodulatory but do not decrease the progression of the disease leading to axonal loss and neurodegeneration. There is a change in mitochondrial function in MS pathology, a metabolic dysfunction related to neurodegeneration progression in MS. A dysfunction in mitochondrial function and a decrease in energy production through ATP is correlated with an increase in axonal loss and demyelination. In the EAE model, there is the

presence of mitochondrial injury to associate with an increase in inflammation and neurodegeneration. Administering an antioxidant shows to be neuroprotective in this model by decreasing inflammation, neuronal cell loss, and disease progression. Patients suffering with MS may also have glucose hypometabolism due to a dysfunction in mitochondrial function and energy production, which gives rise to the hypothesis that ketogenic diet may be beneficial in MS patients. In a MS animal model, the treatment with a ketogenic diet results in a decrease in inflammation and in clinical improvements related to learning, memory, and motor function. In a clinical trial with Alzheimer's patients, treatment with a ketogenic diet resulted in better cognition function (Storoni & Plant 2015). Supplying the CNS with lipid seems to be a potential therapeutic strategy to promote lipid synthesis and/or reduce lipid breakdown to maintain the normal lipid turnover rate and normal cognitive function in demyelinating and metabolic disorders.

There are two mechanisms by which demyelination occurs: immune-mediated and oligodendrocyte-deficient demyelination (Lucchinetti et al. 2000, Nakahara et al. 2012). Currently available MS therapies target the autoimmune aspect of the disease (Lim & Constantinescu 2010). However because it only targets one aspect of the disease process, it does not offer a complete treatment. Therapies need to be developed to promote lipid synthesis and/or reduce lipid breakdown to treat demyelinating diseases like MS. A combinational remyelination and immunomodulatory therapy would target both the oligodendrocyte-deficient and immune-mediated demyelination aspects of the disease process and therefore yield improved clinical results.

**Future Studies**

We will determine the effectiveness of glyceryl triacetate in other MS animal models like the cuprizone and relapse-remitting EAE models. The cuprizone model mimics oligodendrocyte-deficient demyelination (Palumbo & Bosetti 2013, Procaccini et al. 2015, Ransohoff 2012, Simmons et al. 2013), and relapse-remitting EAE will determine therapeutic potential in the treatment of this particular type of MS, which is present in the majority of patients (Delbue et al. 2017, McQualter & Bernard 2007). Regarding the cuprizone model, we can measure the width of the corpus callosum (Wergeland *et al.* 2012) to determine the extent of demyelination as a result of injury and how glyceryl triacetate recovers that width. As the amount of demyelination increases, the width of the corpus callosum decreases (Wergeland et al. 2012). We expect prophylactic treatment with glyceryl triacetate recovers that width back to control animals to suggest acetate has a role in myelination and oligodendrocyte differentiation. Discussed in the introduction, cuprizone directly affects mature oligodendrocytes but has no effect on OPCs (Benardais et al. 2013). If we show a change in the width of the corpus callosum with cuprizone and treatment, this suggests acetate may promote oligodendrocyte differentiation and function that in turn increases myelin lipid. Regarding the relapse-remitting EAE model, we will determine whether prophylactic treatment with glyceryl triacetate attenuates the onset of clinical symptoms similar to the progressive EAE model and reduces the number of relapses.

Once we perform the behavioral analyses, we will determine changes in brain lipid content with the cuprizone model and spinal cord lipid content in the relapse-remitting EAE model compared to control animals. We expect to show in both models,

treatment with glyceryl triacetate will significantly increase CNS lipid content compared

to control-treated injury animals, but similar to controls. All data will suggest treatment

is clinically beneficial in MS and potentially treats the entire disease. Also, the data will

suggest that treatment recovers myelin lipid lost due inury to treat all forms of

demyelination, both immune-mediated and oligodendrocyte-deficient demyelination.

The above analysis will, in addition, provide insight into the type of stimulus

necessary to show changes in lipid content. For example, a stimulus, in general like

oligodendrocyte death, in the cuprizone model is required for glyceryl triacetate to

promote the production of lipid, or an inflammatory stimulus, like EAE, is required for

treatment to promote the production of lipid. If treatment with glyceryl triacetate only

significantly increases lipid levels in the relapse-remitting EAE compared to control-

treated EAE animals but no difference in the cuprizone model, an inflammatory stimulus,

in particular, is essential for treatment to show changes in lipid content. On the other

hand, if treatment significantly increases lipid content in both the cuprizone and EAE

model compared to control-treated injury animals, a stimulus, in general, is required for

treatment to show changes in CNS lipid content. This is significant regarding the

treatment of both immune-mediated and oligodendrocyte-deficient demyelination in MS.

We will determine the effectiveness of using interventional treatments in the

progressive EAE, relapse-remitting EAE, and cuprizone models. An interventional

treatment being effective in MS animal models is significant in the treatment of MS

because patients are only prescribed medications when symptoms develop. In the

progressive EAE model, we can administer glyceryl triacetate when animals exhibit

flaccid tail and hind limb paralysis. We expect glyceryl triacetate will either reverse or

not worsen the clinical score in these animals. In the relapse-remitting EAE, we can administer glyceryl triacetate after the first relapse and determine if treatment reduces the number of relapses from that point. We expect interventional treatment reduces the number of relapses to show therapeutic benefits for the treatment of the majority of MS patients (Delbue et al. 2017, McQualter & Bernard 2007). In the cuprizone model, we can administer glyceryl triacetate 3, 7, or 14 days after cuprizone diet and determine how the width of the corpus callosum changes over time with cuprizone and treatment.

Regarding the cuprizone model, we can determine how continuing glyceryl triacetate treatment after withdrawal promotes remyelination. We can give cuprizone diet and glyceryl triacetate treatment for 30 days to mice, and after 30 days, we can remove the cuprizone diet and continue glyceryl triacetate treatment for 0, 7, 14, or 21 days. There will be nine groups: one control group, four non-treated cuprizone groups, and four treated cuprizone groups. The control group will be healthy mice euthanized on the day of the cuprizone withdrawal to determine a normal baseline for the width of the corpus callosum. The non-treated cuprizone groups will be mice given cuprizone but no glyceryl triacetate for 30 days and have cuprizone withdrawn for 0, 7, 14, or 21 days. These animals will give us the width of the corpus callosum over the traditional remyelination period. The treated cuprizone groups will be mice given both cuprizone and glyceryl triacetate and receive glyceryl triacetate for 0, 7, 14, or 21 days after withdrawal of cuprizone. Determine the width of the corpus callosum in this group will determine if treatment accelerates remyelination (Wergeland et al. 2012). We expect acetate supplementation accelerates the rate of remyelination by returning the width of

corpus callosum to control levels much earlier compared to non-treated cuprizone animals.

We want to determine protein levels of enzymes involved in lipid synthesis. We looked at AGPS, AceCS1/2, and p-ACC. AGPS is the enzyme involved in ether phospholipid biosynthesis (Braverman & Moser 2012, Rosenberger et al. 2002, Schmitt et al. 2015), and AceCS1/2 catalyzes the conversion of acetate to acetyl-CoA (Jaworski et al. 2016, Shimazu et al. 2010). We found there was no significant difference in these enzyme protein levels between control and EAE groups. We suspect that we are looking at protein levels too late in the injury, 40 days post inoculation and treatment. We need to understand how injury and treatment alter protein levels of these enzymes much earlier in the injury, a week or two post inoculation and treatment. In addition, at this time point, we can also determine protein levels of an enzyme involved in cholesterol synthesis, HMG-CoA reductase (Jaworski et al. 2016). We looked at the inactive phosphorylated ACC enzyme, important in fatty acid synthesis, (Baenke et al. 2013), and next, we need to determine protein levels of the non-phosphorylated form in EAE mice treated with either water or glyceryl triacetate. We found in these studies treatment with glyceryl triacetate significantly decreased p-ACC protein levels in EAE mice compared to control-treated EAE mice suggesting that acetate may shift ACC from an inactive to active form to promote fatty acid synthesis. We expect that acetate supplementation results in a significant increase in non-phosphorylated ACC protein levels in EAE mice compared to control-treated EAE mice.

In addition to understanding protein levels of these enzymes, we also want to determine how activity of these enzymes are altered due to EAE and administration with

glyceryl triacetate. With an end point of a week or two post inoculation and treatment, we expect treatment with glyceryl triacetate significantly increases protein and activity levels of these enzymes in EAE compared to control-treated EAE mice. In addition, we will determine how treatment alters histone acetylation in EAE to correlate with changes in enzyme protein, mRNA, and activity levels. We expect acetate supplementation results in an increase in histone acetylation similar to previous literature (Soliman & Rosenberger 2011, Soliman et al. 2012b) to correlate with a significant increase in enzyme protein, mRNA, and activity levels in EAE compared to control-treated EAE mice. All these data will test the hypothesis that acetate supplementation alters CNS lipid metabolism and promotes myelin lipid deposition in mice subjected to EAE.

We can further understand how treatment alters lipid breakdown through the $cPLA_2$ pathway. Again, we found acetate supplementation returned $cPLA_2$ back to control levels to suggest treatment may modulate pro-inflammatory lipid signaling. We can determine spinal cord and brain protein levels of enzymes and metabolites involved in this pathway, and this includes cyclooxygenases (COX-1 and COX-2), lipoxygenases (5-lipooxgenase), leukotrienes, thromboxanes, and prostaglandins in control and EAE mice treated with either water or glyceryl triacetate. We expect to find no significant difference in protein levels between the two control groups because there is no inflammatory stimulus present, but in control-treated EAE mice, we expect all protein levels will significantly increase due to the presence of chronic CNS inflammation. Acetate supplementation will then return levels back to control to support the idea that treatment modulates pro-inflammatory lipid signaling through the $cPLA_2$ pathway. In addition, we can determine CNS enzymatic activity of $cPLA_2$, COX, and lipoxygenases

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in control and EAE mice treated with either water or glyceryl triacetate. We expect

treatment decreases activity of cPLA$_2$, COX, and lipoxygenase enzymes compared to

control-treated EAE mice. Treatment will return the EAE-induced cPLA$_2$-mediated

inflammation to control levels. All these data will suggest treatment modulates pro-

inflammatory signaling and alters CNS lipid metabolism by reducing lipid breakdown.

Instead of using an *in vivo* model, it may be simpler and more cost effective to

move into an *in vitro* model to determine how acetate alters lipid synthesis and

breakdown to correlate with changes in lipid. We can use BV2 microglia, for example,

because we know acetate increases fatty acid content in LPS-stimulated BV2 microglia

(Bhatt & Rosenberger 2014). To continue the BV2 story, we can determine how acetate

treatment alters phospholipid, cholesterol, and ganglioside content in LPS-stimulated

cells. We can also determine how treatment alters cPLA$_2$ and PLC pathways looking at

mRNA, protein, and activity of these enzymes. In addition, we can determine mRNA

and protein levels of downstream effects within these two pathways. For example, in

cPLA$_2$-arachondic acid pathway, we can determine mRNA, protein, and activity levels of

COX and 5-lipooxygenase, and we can determine mRNA and protein levels of

inflammatory eicosanoids and leukotrienes. We expect acetate will decrease cPLA$_2$,

COX, and 5-lipooxygenase mRNA, protein, and activity levels in LPS-stimulated BV2

microglia. This will correlate with a decrease in inflammatory eicosanoids and

leukotrienes due to treatment in these cells to suggest that acetate reduces lipid

breakdown. As well, we can determine mRNA, protein, and activity levels of enzymes

involved in lipid synthesis. Enzymes of interest include the ones analyzed in Figure 11,

HMG-CoA reductase, and fatty acid synthase. Fatty acid synthase, in addition to ACC,

promotes the production of fatty acid (Jaworski et al. 2016). We need to do a time dependent experiment to determine the appropriate time to analyze these enzymes. We will have the following groups control BV2 cells treated with either sodium chloride or sodium acetate and LPS-stimulated BV2 cells treated with either sodium chloride or sodium acetate. We will collect cells 10 minutes, 15 minutes, 20 minutes, 30 minutes, 1 hour, 1.5 hours, and 2 hours post LPS and acetate treatment for protein analysis of these enzymes. The reason we will end at 2 hours is that Bhatt and Rosenberger showed acetate treatment increases fatty acid content in LPS-stimulated BV2 cells 2 hours post treatment (Bhatt & Rosenberger 2014). We need to determine at which time point we show a significant decrease in protein levels due to LPS, and acetate treatment returns levels back to control levels. Once that time point is determined, we will treat cells for that length of time with LPS and acetate and collect for mRNA, protein, and enzymatic analysis to determine whether acetate alters lipid synthesis. We can extend all these experiments in primary oligodendrocytes, and this is significant regarding promoting myelin lipid synthesis and/or reducing myelin lipid breakdown.

Also using *in vitro* BV2 microglia and oligodendrocyte models, we can determine if an inflammatory stimulus is necessary for glyceryl triacetate to alter lipid content or a stimulus, in general like myelin debris, is sufficient. We can treat cells with various levels of LPS or MOG peptide and determine lipid content. From that, if cells treated with LPS only show decreases in lipid content, this suggests an inflammatory stimulus is essential to show changes in lipid. Or, if cells treated with LPS or MOG show decreases in lipid content, this suggests any injury stimulus is essential to show changes in lipid. Understanding this concept is important in understanding MS pathology and therapeutic

development.  If inflammation needs to be present to show demyelination, then a combinational therapy of current immunomodulatory therapy with a therapy promoting lipid synthesis and/or reducing lipid breakdown is where therapeutic research needs to focus.  If an injury stimulus, in general, is necessary, then, therapeutic research needs to focus on a remyelination therapy that may potentially replace immunomodulatory therapies.  In addition, if MOG results in a significant decrease in lipid content in LPS-stimulated glia, we can treat cells with acetate to show the recovery of lipid and determine its role in promoting myelin lipid synthesis.

We hypothesized that treatment promotes lipid synthesis in EAE mice and that in turn stimulates myelin lipid deposition.  This can be tested using a radiotracer method previously described (Rosenberger et al. 2002).  C57BL/6 mice will undergo EAE inoculation induced by MOG peptide and pertussis toxin as described (Chevalier & Rosenberger 2017), and these animals will be administered prophylactic glyceryl triacetate treatment for 40 days as described (Chevalier & Rosenberger 2017).  Before animals are anesthetized and euthanized, these animals will be intravenously infused with radiolabeled hexadecanol ([1,1-3H]hexadecanol) using an infusion pump for 5 minutes at a rate of 0.4mL/min (Rosenberger et al. 2002).  Hexadecanol is a fatty alcohol and is incorporated into the ether phospholipid biosynthesis (Figure 13).  This fatty alcohol can cross the BBB and replace the acyl group on DHAP catalyzed by AGPS.  Then through a series of reactions described in Figure 13, PakEtn, PakCho, PlsEtn, and PlsCho are formed.  We can determine radioactivity and phospholipid content of these ether phospholipids by using thin layer chromatography with the 2D phospholipid separation and phosphorus assay described in the methods (Chevalier & Rosenberger 2017).  In
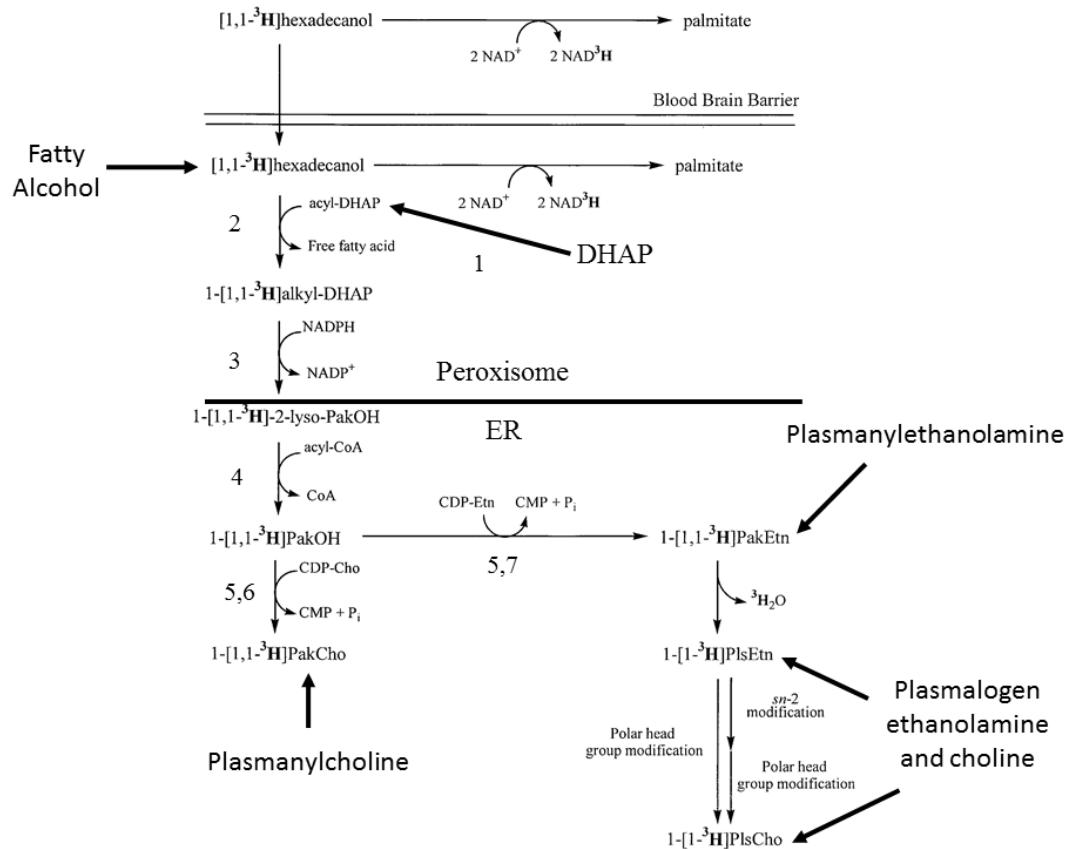
**Figure 13**: Ether phospholipid biosynthesis (Rosenberger et al. 2002, Figure reproduced with permission). Abbreviations include DHAP (dihydroxyacetonephosphate), 2-lyso-PakOH (1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate), PakOH (1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphate), PakCho (1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine or plasmanylcholine), PakEtn (1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine or plasmanylethanolamine), PlsEtn (ethanolamine plasmalogen), and PlsCho (choline plasmalogen). Enzymes involved include the following: 1) glyceronephosphate O-acyltransferase (GNPAT), 2) alkylglycerone phosphate synthase (AGPS), 3) alkyl-DHAP reductase, 4) 1-*O*-alkyl-2-acyl-*sn*-glycerol-3-phosphate acyltransferase, 5) 1-*O*-alkyl-2-acyl-*sn*-glycerol-3-phosphate phosphohydrolase, 6)1-*O*-alkyl-2-acyl-*sn*-glycerol choline phosphotransferase, and 7)1-*O*-alkyl-2-acyl-*sn*-glycerol ethanolamine phosphotransferase. Line indicates where in the synthesis that it transports from the peroxisome to the endoplasmic reticulum (ER).

addition, part of the sample separated on thin layer chromatography can be used to determine radioactivity of each ether phospholipid. We expect acetate supplementation significantly increases radiolabeled hexadecanol into PakEtn and PakCho content in EAE mice compared to control-treated EAE mice but similar to controls. In these studies, treatment altered PtdEtn + PakEtn and PtdCho + PakCho fractions in the EtnGpl and ChoGpl phospholipid groups in EAE mice. All these data will suggest treatment incorporates into the ether phospholipid biosynthesis to promote myelin lipid deposition.

In addition, we can determine the expression and activity of enzymes involved in the ether phospholipid biosynthesis (Figure 13). Enzymes of interest include glyceronephosphate O-acyltransferase (GNPAT), AGPS, alkyl-DHAP reductase, 1-*O*-alkyl-2-acyl-*sn*-glycerol-3-phosphate acyltransferase, 1-*O*-alkyl-2-acyl-*sn*-glycerol-3-phosphate phosphohydrolase, 1-*O*-alkyl-2-acyl-*sn*-glycerol choline phosphotransferase, and 1-*O*-alkyl-2-acyl-*sn*-glycerol ethanolamine phosphotransferase. These enzymes are involved in the production of PakEtn and PakCho, and the particular reaction of each enzyme is shown in Figure 13. GNPAT catalyzes the addition on an acyl group on DHAP and then, the acyl group is replaced with a fatty alcohol catalyzed by AGPS (Braverman & Moser 2012). The remaining enzymes are involved in the final steps leading to the production of plasmanyl ether phospholipids (Rosenberger et al. 2002). We expect acetate supplementation results in a significant increase in protein and activity of these enzymes involved in ether phospholipid biosynthesis in EAE mice compared to control-treated EAE mice but similar to controls.

After the above experiments, we can move into an *in vitro* model to determine how treatment directly affects the ether phospholipid biosynthesis. First, we can repeat

the above experiments in BV2 microglia and oligodendrocytes and determine

radioactivity and ether phospholipid content of PakEtn, PakCho, PlsEtn, and PlsCho.  In

addition, we can use enzyme inhibitors and determine how treatment alters ether

phospholipid content when particular enzymes are inhibited.  We can determine if acetate

alters the expression and activity of particular enzymes involved in this pathway or alters

all enzymes.  We expect that treatment in particular alters the rate limiting step in ether

phospholipid biosynthesis, AGPS (Braverman & Moser 2012, Rosenberger et al. 2002).

These suggest that treatment promotes myelin lipid deposition by showing changes in

ether phospholipid content and altering expression and activity of enzymes involved in

this pathway.

In the EAE model, we can determine how acetate supplementation alters the

peripheral and central nervous system immune responses.  We know that acetate

supplementation is anti-inflammatory in rat models of neuroinflammation and

neuroborreliosis as well as in *in vitro* neuroglia (Brissette et al. 2012, Reisenauer et al.

2011, Soliman et al. 2012b, Soliman et al. 2013a, Soliman et al. 2013b, Soliman et al.

2012a).  We can determine the anti-inflammatory effects of glyceryl triacetate in the EAE

model as well as in BV2 microglia and oligodendrocytes by using flow cytometry and

ELISA to determine immune cell count and cytokine levels.  Using flow cytometry, we

can determine CD4 T-cell, CD8 T-cell, and macrophage populations in blood, spinal

cord, and brain of control and EAE mice treated with either water or glyceryl triacetate.

We expect the populations of these cells are lower in glyceryl triacetate-treated EAE

mice compared control-treated EAE mice but similar to controls.  *In vitro*, we can also

use flow cytometry to determine the populations of these cells.  We will treat BV2

microglia and primary oligodendrocytes with LPS and acetate and collect samples for flow cytometry analysis to count immune cell populations. Using ELISA, we can determine cytokine levels in blood, spinal cord, and brain in control and EAE mice treated with either water or glyceryl triacetate. We expect acetate supplementation decreases pro-inflammatory cytokines in EAE mice compared to control-treated EAE mice but similar to non-EAE controls. If ELISA is unable to detect cytokine levels in the blood and CNS of these animals, we can also use flow cytometry to determine cytokine populations by targeting particular cytokines to separate from the rest of the population. The same experiments can be extrapolated to *in vitro* BV2 microglia and oligodendrocytes.

Acetate may promote myelination and oligodendrocyte differentiation. In primary oligodendrocytes, we expect acetate treatment increases lipid content in LPS-stimulated cells to correlate with changes in mRNA of markers associated with oligodendrocyte differentiation. These markers will include Tcf4 for immature oligodendrocytes, Mytl for mature oligodendrocytes, and Olig1 for oligodendrocytes in general (Emery 2010, Woodruff *et al.* 2001, Arnett *et al.* 2004). Furthermore, we can use different markers to determine the number of OPC, immature oligodendrocytes, and mature oligodendrocytes. OPC markers will include A2B5, a polysialoganglioside, NG2, PDGFRα, Sox5, Sox6, Sox 9, Hes 5, Id2, Id2, Id4, and E2A, and immature oligodendrocyte markers will include O1 antigen, galactosylcerebroside, and Tcf4. Mature oligodendrocyte markers will include MBP, PLP, YY1, Mytl, ZPF191, and MRF (Bansal *et al.* 1989, Emery 2010, Woodruff et al. 2001).

## CHAPTER VII

## SUMMARY

Multiple sclerosis (MS) is an inflammatory demyelinating neurodegenerative disorder (Cusick et al. 2013, Lassmann & van Horssen 2011, McQualter & Bernard 2007). There are two mechanisms by which demyelination and the loss of myelin lipid can occur, immune-mediated and oligodendrocyte-deficient demyelination (Lucchinetti et al. 2000, Nakahara et al. 2012). Immune-mediated demyelination, presented in MS lesion patterns I and II (Lucchinetti et al. 2000), result from a self-antigen attack on the central nervous system (CNS) (Cusick et al. 2013). There is the presence of autoreactive T-cells, activated microglia and macrophages, and pro-inflammatory cytokines to result in chronic CNS inflammation that contribute to demyelination (Cusick et al. 2013, Lassmann & van Horssen 2011, McQualter & Bernard 2007). Oligodendrocyte-deficient demyelination, presented in MS lesion patterns III and IV (Lucchinetti et al. 2000), results from a dysfunction in oligodendrocyte survival, maturation, and function (Lucchinetti et al. 2000). Oligodendrocytes are myelinating CNS glia cells (Boulanger & Messier 2014, Mitew et al. 2014), so a dysfunction in oligodendrocyte survival, maturation, and function also contributes to demyelination. Current MS available therapies only modulate the immune response of the disease (Lim & Constantinescu 2010) but do not treat the oligodendrocyte-deficient demyelination. Developing a therapy to stimulate lipid deposition and/or reduce lipid breakdown within the CNS may be a mechanism to treat MS in addition to current available therapies.

We hypothesized that acetate supplementation, administered as glyceryl triacetate, alters CNS lipid metabolism in mice subjected to experimental autoimmune encephalomyelitis (EAE), an autoimmune model for MS. We determined the effect of this treatment on disease progression, CNS lipid content, and CNS protein levels in mice subjected to EAE. We found acetate supplementation attenuated the onset of clinical symptoms in these animals shown by a daily clinical score and hang time test. In addition, treatment prevented the loss of spinal cord phospholipid, fatty acid, and cholesterol content in mice subjected to EAE. Treatment significantly increased spinal cord GD3 and GD1a ganglioside levels in EAE mice compared to control-treated EAE mice. Regarding total brain and myelin brain lipid content, acetate supplementation increased phospholipid and ganglioside content in EAE mice compared to control-treated EAE mice. We also determined protein levels of enzymes involved in lipid metabolism, cytoskeletal structure, and myelin structure. We found acetate supplementation returned cPLA$_2$ to control levels to suggest treatment modulates pro-inflammatory lipid breakdown. Treatment did not return the cytoskeletal proteins, β-actin and merlin, back to control levels in EAE mice suggesting that treatment does not alter all EAE pathology. Interestingly, acetate supplementation returned oligodendrocyte myelin glycoprotein (OMgp) to control levels in EAE mice suggesting that treatment may promote remyelination and axonal growth because OMgp is known to inhibit both (Cafferty et al. 2010, Huang et al. 2005, Kan et al. 2016, Mikol et al. 1993, Vourc'h & Andres 2004, Wang et al. 2002). Furthermore, treatment significantly decreased phosphorylated acetyl-CoA carboxylase (p-ACC) levels in EAE compared to control-treated EAE mice indicating acetate may shift this enzyme from the inactive phosphorylated to an active

non-phosphorylated form to promote fatty acid synthesis. Acetate supplementation attenuated disease progression, altered CNS and myelin lipid content, and influenced CNS and myelin protein content in mice subjected to EAE.

Future studies will address whether acetate supplementation stimulates myelin lipid deposition, reduces lipid breakdown, or combination of both. Using different MS animal models and LPS-stimulated neuroglia models, we will determine how treatment alters mechanisms involved in lipid synthesis and breakdown by determining protein expression, mRNA expression, and enzymatic activities of enzymes and proteins involved in these pathways. Furthermore, we will determine the effectiveness of prophylactic glyceryl triacetate treatment in other MS animal models like cuprizone and relapse-remitting EAE models. We will also determine the effectiveness of interventional glyceryl triacetate treatments in the cuprizone, relapse-remitting EAE, and progressive EAE models to determine the clinical benefits in the treatment of MS, since patients are only prescribed medications when symptoms develop. Future studies will determine whether acetate supplementation promotes myelin lipid deposition and/or reduces the breakdown to show that recovery of CNS lipid lost due to demyelination.

If acetate supplementation alters CNS lipid metabolism in MS animal models, this may start the conversation on whether MS may be a metabolic disorder (Corthals 2011). MS is considered an autoimmune disorder, but there may be an underlying dysfunction in lipid metabolism (Corthals 2011). As well, these studies provide insight into the potential mechanisms of action regarding glyceryl triacetate in mice subjected to EAE. In a healthy normal state, there is a natural lipid turnover (Ando et al. 2003, Rosenberger et al. 2002, Moser et al. 1999), but treatment may have a greater effect on energy production

and histone acetylation (Bhatt et al. 2013, Soliman & Rosenberger 2011, Soliman et al. 2012b). Regarding the EAE injury, lipid lost is significantly higher compared to lipid produced, where glyceryl triacetate-treated EAE mice show a normal lipid turnover rate either by promoting lipid synthesis, reducing lipid breakdown, or a combination of both. This is a result of the metabolic demand to recover the lipid lost due to injury.

**APPENDICES**

## List of Abbreviations

16:0: palmitic acid

18:0: stearic acid

18:1n-9: oleic acid

18:1n-7: vaccenic acid

18:2n-6: linoleic acid

18:3n3: alpha-linolenic acid

20:1n-9: eicosenoic acid

20:4n-6: arachidonic acid

22:4n-6: adrenic acid

22:6n-3: docosahexaenoic acid or DHA

24:0: lignoceric acid

24:1n-9: nervonic acid

2D: two-dimensional

2-lyso-PakOH: 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate

AceCS1/2: acyl-CoA synthetase

AGPS: alkylglycerone phosphate synthase

ASPA: aspartoacylase

ATP: adenosine tri-phosphate

β: beta

BBB: blood brain barrier

BV2: immortalized murine cell line

CD: cluster of differentiation

Cer$P$Cho: sphingomyelin

ChoGpl: choline glycerophospholipid

CNS: central nervous system

CoA: coenzyme A

COX: cyclooxygenase

cPLA$_2$: cytosolic phospholipase A$_2$

DAG: diacylglycerol

DHAP: dihydroxyacetone phosphate

DHEA: dehydroepiandrosterone

DMT: disease-modifying therapy

DNA: deoxyribonucleic acid

EAE: experimental autoimmune encephalomyelitis

EPA: eicosapentaenoic acid

ER: endoplasmic reticulum

EtnGpl: ethanolamine glycerophospholipid

FDA: food and drug administration

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GLUT1: glucose transporter

GNPAT: glyceronephosphate O-acyltransferase

GTA: glyceryl triacetate

H$_2$O: water

HDAC: histone deacetylase

HMG: β-hydroxy-β-methylglutaryl

HRP: horseradish peroxidase

I(1,4,5)P$_3$: inositol-1,4,5-trisphosphate

IFN: interferon

Ig: immunoglobulin

IGF-1: insulin-like growth factor 1

IL: interleukin

JNK: jun N-terminal kinase

LPS: lipopolysaccharide

MAG: myelin-associated glycoprotein

MAPK: mitogen-activated protein kinase

MBP: myelin basic protein

Merlin: moesin-, ezrin-, radixin-like protein

MHC: major histocompatibility complex

MK-801: drug induces hypoacetylation

MOG: myelin oligodendrocyte glycoprotein

MRI: magnetic resonance imaging

MS: multiple sclerosis

NAA: N-acetylaspartate

NANA: N-acetyl-neuraminic acid (sialic acid)

NF-κB: nuclear factor kappa B

OMgp: oligodendrocyte myelin glycoprotein

OPC: oligodendrocyte progenitor cell

p-ACC: phosphorylated acetyl-CoA carboxylase

PAF: platelet-activating factor

pcPLA$_2$: phosphorylated cytosolic PLA$_2$

PDGF: platelet-derived growth factor

PDGFRα: platelet-derived growth factor receptor A

PLC: phospholipase C

PLP: proteolipid protein

PlsCho: 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphocholine or plasmenylcholine or choline

   plasmalogen

PlsEtn: 1-*O*-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine or

   plasmenylethanolamine or ethanolamine plasmalogen

PtdCho: 1,2-diacyl-*sn*-glycero-3-phosphocholine or phosphatidylcholine

PakCho: 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine or plasmanylcholine

PtdEtn: 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine or phosphatidylethanolamine

PakEtn: 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanoline or plasmanylethanolamine

PakOH: 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphate

PtdIns: 1,2-diacyl-*sn*-glycero-3-phosphoinositol or phosphatidylinositol

PtdIns(4,5)P$_2$: 1,2-Diacyl-*sn*-glycero-3-phospho-(1-D-*myo*-inositol 4,5-bisphosphate) or

   phosphatidylinositol-4,5-bisphosphate

PtdOH: 1,2-diacyl-*sn*-glycero-3-phosphate or phosphatidic acid

PtdSer: 1,2-diacyl-*sn*-glycero-3-phosphoserine or phosphatidylserine

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TCA cycle: tricarboxylic acid cycle

TGF-β: transforming growth factor beta

Th1/Th17: T helper cell 1/17

TMEV: theiler's murine encephalomyelitis

TNF-α: tumor necrosis factor alpha

VCAM-1: vascular cell adhesion molecule-1

VLCFA: very long chain fatty acid

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# REFERENCES

Acarin, N., Rio, J., Fernandez, A. L., Tintore, M., Duran, I., Galan, I. and Montalban, X. (1996) Different antiganglioside antibody pattern between relapsing-remitting and progressive multiple sclerosis. *Acta Neurol Scand,* **93,** 99-103.

Aggarwal, S., Yurlova, L. and Simons, M. (2011) Central nervous system myelin: structure, synthesis and assembly. *Trends Cell Biol,* **21,** 585-593.

Al-Izki, S., Pryce, G., Jackson, S. J., Giovannoni, G. and Baker, D. (2011) Immunosuppression with FTY720 is insufficient to prevent secondary progressive neurodegeneration in experimental autoimmune encephalomyelitis. *Mult Scler,* **17,** 939-948.

Alling, C., Vanier, M. T. and Svennerholm, L. (1971) Lipid alterations in apparently normal white matter in multiple sclerosis. *Brain Res,* **35,** 325-336.

Amaral, A., Hadera, M. G., Kotter, M. and Sonnewald, U. (2017) Oligodendrocytes Do Not Export NAA-Derived Aspartate In Vitro. *Neurochem Res,* **42,** 827-837.

Amaral, A. I., Hadera, M. G., Tavares, J. M., Kotter, M. R. and Sonnewald, U. (2016) Characterization of glucose-related metabolic pathways in differentiated rat oligodendrocyte lineage cells. *Glia,* **64,** 21-34.

Ando, S., Tanaka, Y., Toyoda, Y. and Kon, K. (2003) Turnover of myelin lipids in aging brain. *Neurochem Res,* **28,** 5-13.

Arnett, H. A., Fancy, S. P., Alberta, J. A. et al. (2004) bHLH transcription factor Olig1 is required to repair demyelinated lesions in the CNS. *Science,* **306,** 2111-2115.

Arun, P., Ariyannur, P. S., Moffett, J. R., Xing, G., Hamilton, K., Grunberg, N. E., Ives, J. A. and Namboodiri, A. M. (2010a) Metabolic acetate therapy for the treatment of traumatic brain injury. *J Neurotrauma,* **27,** 293-298.

Arun, P., Madhavarao, C. N., Moffett, J. R. et al. (2010b) Metabolic acetate therapy improves phenotype in the tremor rat model of Canavan disease. *J Inherit Metab Dis,* **33,** 195-210.

Autilio, L. A., Norton, W. T. and Terry, R. D. (1964) The Preparation and Some Properties of Purified Myelin from the Central Nervous System. *J Neurochem,* **11,** 17-27.

Baenke, F., Peck, B., Miess, H. and Schulze, A. (2013) Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development. *Dis Model Mech,* **6,** 1353-1363.

Bansal, R., Warrington, A. E., Gard, A. L., Ranscht, B. and Pfeiffer, S. E. (1989) Multiple and novel specificities of monoclonal antibodies O1, O4, and R-mAb used in the analysis of oligodendrocyte development. *J Neurosci Res,* **24,** 548-557.

Barcelo-Coblijn, G. and Murphy, E. J. (2009) Alpha-linolenic acid and its conversion to longer chain n-3 fatty acids: benefits for human health and a role in maintaining tissue n-3 fatty acid levels. *Prog Lipid Res,* **48,** 355-374.

Baslow, M. H., Suckow, R. F., Sapirstein, V. and Hungund, B. L. (1999) Expression of aspartoacylase activity in cultured rat macroglial cells is limited to oligodendrocytes. *J Mol Neurosci,* **13,** 47-53.

Basso, A. S., Frenkel, D., Quintana, F. J. et al. (2008) Reversal of axonal loss and disability in a mouse model of progressive multiple sclerosis. *J Clin Invest,* **118,** 1532-1543.

Batoulis, H., Recks, M. S., Addicks, K. and Kuerten, S. (2011) Experimental autoimmune encephalomyelitis--achievements and prospective advances. *APMIS,* **119,** 819-830.

Bauer, M., Brakebusch, C., Coisne, C., Sixt, M., Wekerle, H., Engelhardt, B. and Fassler, R. (2009) Beta1 integrins differentially control extravasation of inflammatory cell subsets into the CNS during autoimmunity. *Proc Natl Acad Sci U S A,* **106,** 1920-1925.

Benardais, K., Kotsiari, A., Skuljec, J., Koutsoudaki, P. N., Gudi, V., Singh, V., Vulinovic, F., Skripuletz, T. and Stangel, M. (2013) Cuprizone [bis(cyclohexylidenehydrazide)] is selectively toxic for mature oligodendrocytes. *Neurotox Res,* **24,** 244-250.

Benjamins, J. A., Murphy, E. J. and Seyfried, T. N. (2012) *Lipids*: Basic neurochemistry principles of molecular, cellular, and medical neurobiology. Elsevier Academic Press, Amsterdam.

Berard, J., Wolak, K., Fournier, S. and David, S. (2010) Characterization of relapsing-remitting and chronic forms of experimental auotimmune encephalomyelitis in C57BL/6 mice. *Glia,* **58,** 434-445.

Berghoff, S. A., Gerndt, N., Winchenbach, J. et al. (2017) Dietary cholesterol promotes repair of demyelinated lesions in the adult brain. *Nat Commun,* **8,** 14241.

Berti, R., Brennan, M. B., Soldan, S. S., Ohayon, J. M., Casareto, L., McFarland, H. F. and Jacobson, S. (2002) Increased detection of serum HHV-6 DNA sequences during multiple sclerosis (MS) exacerbations and correlation with parameters of MS disease progression. *J Neurovirol,* **8,** 250-256.

Bhakoo, K. K., Craig, T. J. and Styles, P. (2001) Developmental and regional distribution of aspartoacylase in rat brain tissue. *J Neurochem,* **79,** 211-220.

Bhatt, D. P., Houdek, H. M., Watt, J. A. and Rosenberger, T. A. (2013) Acetate supplementation increases brain phosphocreatine and reduces AMP levels with no effect on mitochondrial biogenesis. *Neurochem Int,* **62,** 296-305.

Bhatt, D. P. and Rosenberger, T. A. (2014) Acetate treatment increases fatty acid content in LPS-stimulated BV2 microglia. *Lipids,* **49,** 621-631.

Boghozian, R., McKenzie, B. A., Saito, L. B., Mehta, N., Branton, W. G., Lu, J., Baker, G. B., Noorbakhsh, F. and Power, C. (2017) Suppressed oligodendrocyte steroidogenesis in multiple sclerosis: Implications for regulation of neuroinflammation. *Glia,* **65,** 1590-1606.

Boulanger, J. J. and Messier, C. (2014) From precursors to myelinating oligodendrocytes: contribution of intrinsic and extrinsic factors to white matter plasticity in the adult brain. *Neuroscience,* **269,** 343-366.

Bowman, R. E. and Wolf, R. C. (1962) A rapid and specific ultramicro method for total serum cholesterol. *Clin Chem,* **8,** 302-309.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem,* **72,** 248-254.

Braverman, N. E. and Moser, A. B. (2012) Functions of plasmalogen lipids in health and disease. *Biochim Biophys Acta,* **1822,** 1442-1452.

Breckenridge, W. C. and Kuksis, A. (1968) Specific distribution of short-chain fatty acids in molecular distillates of bovine milk fat. *J Lipid Res,* **9,** 388-393.

Brissette, C. A., Houdek, H. M., Floden, A. M. and Rosenberger, T. A. (2012) Acetate supplementation reduces microglia activation and brain interleukin-1beta levels in a rat model of Lyme neuroborreliosis. *J Neuroinflammation,* **9,** 249.

Brites, P., Waterham, H. R. and Wanders, R. J. (2004) Functions and biosynthesis of plasmalogens in health and disease. *Biochim Biophys Acta,* **1636,** 219-231.

Burgisser, P., Althaus, H. H., Rohmann, A. and Neuhoff, V. (1988) Lipid synthesis by oligodendrocytes from adult pig brain maintained in long-term culture. *Neurochem Int,* **13,** 111-118.

Cafferty, W. B., Duffy, P., Huebner, E. and Strittmatter, S. M. (2010) MAG and OMgp synergize with Nogo-A to restrict axonal growth and neurological recovery after spinal cord trauma. *J Neurosci,* **30,** 6825-6837.

Cahoy, J. D., Emery, B., Kaushal, A. et al. (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J Neurosci,* **28,** 264-278.

Campagnoni, A. T. and Campagnoni, C. W. (2004) *Myelin basic protein gene*: Myelin biology and disorders. Elsevier Academic Press, San Diego.

Cermelli, C., Berti, R., Soldan, S. S., Mayne, M., D'Ambrosia J, M., Ludwin, S. K. and Jacobson, S. (2003) High frequency of human herpesvirus 6 DNA in multiple sclerosis plaques isolated by laser microdissection. *J Infect Dis,* **187,** 1377-1387.

Chakraborty, G., Mekala, P., Yahya, D., Wu, G. and Ledeen, R. W. (2001) Intraneuronal N-acetylaspartate supplies acetyl groups for myelin lipid synthesis: evidence for myelin-associated aspartoacylase. *J Neurochem,* **78,** 736-745.

Challoner, P. B., Smith, K. T., Parker, J. D. et al. (1995) Plaque-associated expression of human herpesvirus 6 in multiple sclerosis. *Proc Natl Acad Sci U S A,* **92,** 7440-7444.

Chan, Y. M., Merkens, L. S., Connor, W. E., Roullet, J. B., Penfield, J. A., Jordan, J. M., Steiner, R. D. and Jones, P. J. (2009) Effects of dietary cholesterol and simvastatin on cholesterol synthesis in Smith-Lemli-Opitz syndrome. *Pediatr Res,* **65,** 681-685.

Chapenko, S., Millers, A., Nora, Z., Logina, I., Kukaine, R. and Murovska, M. (2003) Correlation between HHV-6 reactivation and multiple sclerosis disease activity. *J Med Virol,* **69,** 111-117.

Chevalier, A. C. and Rosenberger, T. A. (2017) Increasing acetyl-CoA metabolism attenuates injury and alters spinal cord lipid content in mice subjected to experimental autoimmune encephalomyelitis. *J Neurochem,* **141,** 721-737.

Chhetry, B. T., Hezghia, A., Miller, J. M., Lee, S., Rubin-Falcone, H., Cooper, T. B., Oquendo, M. A., Mann, J. J. and Sublette, M. E. (2016) Omega-3 polyunsaturated fatty acid supplementation and white matter changes in major depression. *J Psychiatr Res,* **75,** 65-74.

Chiavegatto, S., Sun, J., Nelson, R. J. and Schnaar, R. L. (2000) A functional role for complex gangliosides: motor deficits in GM2/GD2 synthase knockout mice. *Exp Neurol,* **166,** 227-234.

Chrast, R., Saher, G., Nave, K. A. and Verheijen, M. H. (2011) Lipid metabolism in myelinating glial cells: lessons from human inherited disorders and mouse models. *J Lipid Res,* **52,** 419-434.

Cinti, D. L., Cook, L., Nagi, M. N. and Suneja, S. K. (1992) The fatty acid chain elongation system of mammalian endoplasmic reticulum. *Prog Lipid Res,* **31,** 1-51.

Constantinescu, C. S., Farooqi, N., O'Brien, K. and Gran, B. (2011) Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol,* **164,** 1079-1106.

Corthals, A. P. (2011) Multiple sclerosis is not a disease of the immune system. *Q Rev Biol,* **86,** 287-321.

Cumings, J. N. (1953) The cerebral lipids in disseminated sclerosis and in amaurotic family idiocy. *Brain,* **76,** 551-562.

Cumings, J. N. (1969) The lipid composition of pure myelin in some demyelinating disorders. *Neuropatol Pol,* **7,** 255-260.

Cumings, J. N. and Goodwin, H. (1968) Sphingolopids and phospholipids of myelin in multiple sclerosis. *Lancet,* **2,** 664-665.

Cusick, M. F., Libbey, J. E. and Fujinami, R. S. (2013) Multiple sclerosis: autoimmunity and viruses. *Curr Opin Rheumatol,* **25,** 496-501.

D'Adamo, A. F., Jr. and Yatsu, F. M. (1966) Acetate metabolism in the nervous system. N-acetyl-L-aspartic acid and the biosynthesis of brain lipids. *J Neurochem,* **13,** 961-965.

Dahlin, M., Martin, D. A., Hedlund, Z., Jonsson, M., von Dobeln, U. and Wedell, A. (2015) The ketogenic diet compensates for AGC1 deficiency and improves myelination. *Epilepsia,* **56,** e176-181.

Dargahi, N., Katsara, M., Tselios, T., Androutsou, M. E., de Courten, M., Matsoukas, J. and Apostolopoulos, V. (2017) Multiple Sclerosis: Immunopathology and Treatment Update. *Brain Sci,* **7.**

Davison, A. N. and Wajda, M. (1962) Cerebral lipids in multiple sclerosis. *J Neurochem,* **9,** 427-432.

de Castro, F., Bribian, A. and Ortega, M. C. (2013) Regulation of oligodendrocyte precursor migration during development, in adulthood and in pathology. *Cell Mol Life Sci,* **70,** 4355-4368.

Delbue, S., Comar, M. and Ferrante, P. (2017) Natalizumab treatment of multiple sclerosis: new insights. *Immunotherapy,* **9,** 157-171.

Della-Flora Nunes, G., Mueller, L., Silvestri, N., Patel, M. S., Wrabetz, L., Feltri, M. L. and Poitelon, Y. (2017) Acetyl-CoA production from pyruvate is not necessary for preservation of myelin. *Glia,* **65,** 1626-1639.

Denic, A., Johnson, A. J., Bieber, A. J., Warrington, A. E., Rodriguez, M. and Pirko, I. (2011) The relevance of animal models in multiple sclerosis research. *Pathophysiology,* **18,** 21-29.

Deon, M., Garcia, M. P., Sitta, A. et al. (2008) Hexacosanoic and docosanoic acids plasma levels in patients with cerebral childhood and asymptomatic X-linked adrenoleukodystrophy: Lorenzo's oil effect. *Metab Brain Dis,* **23,** 43-49.

Eichberg, J. and Iyer, S. (1996) Phosphorylation of myelin protein: recent advances. *Neurochem Res,* **21,** 527-535.

Elias, E. R., Irons, M. B., Hurley, A. D., Tint, G. S. and Salen, G. (1997) Clinical effects of cholesterol supplementation in six patients with the Smith-Lemli-Opitz syndrome (SLOS). *Am J Med Genet,* **68,** 305-310.

Emery, B. (2010) Regulation of oligodendrocyte differentiation and myelination. *Science,* **330,** 779-782.

Ferdinandusse, S., Denis, S., Mooijer, P. A., Zhang, Z., Reddy, J. K., Spector, A. A. and Wanders, R. J. (2001) Identification of the peroxisomal beta-oxidation enzymes involved in the biosynthesis of docosahexaenoic acid. *J Lipid Res,* **42,** 1987-1995.

Folch, J., Lees, M. and Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem,* **226,** 497-509.

Georgiou, J., Tropak, M. P. and Roder, J. C. (2004) *Myelin-associated glycoprotein gene*: Myelin biology and disorders. Elsevier Academic Press, San Diego.

Gerstl, B., Eng, L. F., Tavaststjerna, M., Smith, J. K. and Kruse, S. L. (1970) Lipids and proteins in multiple sclerosis white matter. *J Neurochem,* **17,** 677-689.

Gerstl, B., Kahnke, M. J., Smith, J. K., Tavaststjerna, M. G. and Hayman, R. B. (1961) Brain lipids in multiple sclerosis and other diseases. *Brain,* **84,** 310-319.

Glade, M. J. and Smith, K. (2015) Phosphatidylserine and the human brain. *Nutrition,* **31,** 781-786.

Gordon, N. (2000) Canavan disease: a review of recent developments. *European Journal of Paediatric Neurology,* **5,** 65-69.

Greer, J. M. and Lees, M. B. (2002) Myelin proteolipid protein--the first 50 years. *Int J Biochem Cell Biol,* **34,** 211-215.

Haas, D., Garbade, S. F., Vohwinkel, C., Muschol, N., Trefz, F. K., Penzien, J. M., Zschocke, J., Hoffmann, G. F. and Burgard, P. (2007) Effects of cholesterol and simvastatin treatment in patients with Smith-Lemli-Opitz syndrome (SLOS). *J Inherit Metab Dis,* **30,** 375-387.

Hajra, A. K. (1995) Glycerolipid biosynthesis in peroxisomes (microbodies). *Prog Lipid Res,* **34,** 343-364.

Hampton, D. W., Anderson, J., Pryce, G. et al. (2008) An experimental model of secondary progressive multiple sclerosis that shows regional variation in gliosis, remyelination, axonal and neuronal loss. *J Neuroimmunol,* **201-202,** 200-211.

Handel, A. E., Williamson, A. J., Disanto, G., Handunnetthi, L., Giovannoni, G. and Ramagopalan, S. V. (2010) An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. *PLoS One,* **5**.

Hovens, C. M. and Kaye, A. H. (2001) The tumour suppressor protein NF2/merlin: the puzzle continues. *J Clin Neurosci,* **8,** 4-7.

Huang, J. K., Phillips, G. R., Roth, A. D. et al. (2005) Glial membranes at the node of Ranvier prevent neurite outgrowth. *Science,* **310,** 1813-1817.

Hudson, L. D. (2004) *Proteolipid protein gene*: Myelin biology and disorders. Elsvier Academic Press, San Diego.

Irons, M., Elias, E. R., Abuelo, D. et al. (1997) Treatment of Smith-Lemli-Opitz syndrome: results of a multicenter trial. *Am J Med Genet,* **68,** 311-314.

Jaworski, D. M., Namboodiri, A. M. and Moffett, J. R. (2016) Acetate as a Metabolic and Epigenetic Modifier of Cancer Therapy. *J Cell Biochem,* **117,** 574-588.

Jung, J., Cai, W., Lee, H. K. et al. (2011) Actin polymerization is essential for myelin sheath fragmentation during Wallerian degeneration. *J Neurosci,* **31,** 2009-2015.

Kakalacheva, K., Munz, C. and Lunemann, J. D. (2011) Viral triggers of multiple sclerosis. *Biochim Biophys Acta,* **1812,** 132-140.

Kalyvas, A. and David, S. (2004) Cytosolic phospholipase A2 plays a key role in the pathogenesis of multiple sclerosis-like disease. *Neuron,* **41,** 323-335.

Kan, Q. C., Zhang, H. J., Zhang, Y. et al. (2016) Matrine Treatment Blocks NogoA-Induced Neural Inhibitory Signaling Pathway in Ongoing Experimental Autoimmune Encephalomyelitis. *Mol Neurobiol.*

Kennedy, E. P. (1986) *The biosynthesis of phospholipids*: Lipids and membranes: past, present, and future. Elsevier, Amsterdam.

Kihara, A. (2012) Very long-chain fatty acids: elongation, physiology and related disorders. *J Biochem,* **152,** 387-395.

Kim, H. Y., Huang, B. X. and Spector, A. A. (2014) Phosphatidylserine in the brain: metabolism and function. *Prog Lipid Res,* **56,** 1-18.

Kipp, M., Nyamoya, S., Hochstrasser, T. and Amor, S. (2017) Multiple sclerosis animal models: a clinical and histopathological perspective. *Brain Pathol,* **27,** 123-137.

Kirmani, B. F., Jacobowitz, D. M., Kallarakal, A. T. and Namboodiri, M. A. (2002) Aspartoacylase is restricted primarily to myelin synthesizing cells in the CNS: therapeutic implications for Canavan disease. *Brain Res Mol Brain Res,* **107,** 176-182.

Kirmani, B. F., Jacobowitz, D. M. and Namboodiri, M. A. (2003) Developmental increase of aspartoacylase in oligodendrocytes parallels CNS myelination. *Brain Res Dev Brain Res,* **140,** 105-115.

Kishimoto, Y., Radin, N. S., Tourtellotte, W. W., Parker, J. A. and Itabashi, H. H. (1967) Gangliosides and glycerophospholipids in multiple sclerosis white matter. *Arch Neurol,* **16,** 41-54.

Klepper, J., Engelbrecht, V., Scheffer, H., van der Knaap, M. S. and Fiedler, A. (2007) GLUT1 deficiency with delayed myelination responding to ketogenic diet. *Pediatr Neurol,* **37,** 130-133.

Knox, K. K., Brewer, J. H., Henry, J. M., Harrington, D. J. and Carrigan, D. R. (2000) Human herpesvirus 6 and multiple sclerosis: systemic active infections in patients with early disease. *Clin Infect Dis,* **31,** 894-903.

Kracun, I., Rosner, H., Drnovsek, V., Heffer-Lauc, M., Cosovic, C. and Lauc, G. (1991) Human brain gangliosides in development, aging and disease. *Int J Dev Biol,* **35,** 289-295.

Kumar, S., Mattan, N. S. and de Vellis, J. (2006) Canavan disease: a white matter disorder. *Ment Retard Dev Disabil Res Rev,* **12,** 157-165.

Lassmann, H. and van Horssen, J. (2011) The molecular basis of neurodegeneration in multiple sclerosis. *FEBS Lett,* **585,** 3715-3723.

Li, M., Li, Y., Liu, X., Gao, X. and Wang, Y. (2012) IL-33 blockade suppresses the development of experimental autoimmune encephalomyelitis in C57BL/6 mice. *J Neuroimmunol,* **247,** 25-31.

Lim, S. Y. and Constantinescu, C. S. (2010) Current and future disease-modifying therapies in multiple sclerosis. *Int J Clin Pract,* **64,** 637-650.

Liu, C. Y., Guo, S. D., Yu, J. Z. et al. (2015) Fasudil mediates cell therapy of EAE by immunomodulating encephalomyelitic T cells and macrophages. *Eur J Immunol,* **45,** 142-152.

Long, P. M., Tighe, S. W., Driscoll, H. E., Fortner, K. A., Viapiano, M. S. and Jaworski, D. M. (2015) Acetate supplementation as a means of inducing glioblastoma stem-like cell growth arrest. *J Cell Physiol,* **230,** 1929-1943.

Long, P. M., Tighe, S. W., Driscoll, H. E., Moffett, J. R., Namboodiri, A. M., Viapiano, M. S., Lawler, S. E. and Jaworski, D. M. (2013) Acetate supplementation induces growth arrest of NG2/PDGFRalpha-positive oligodendroglioma-derived tumor-initiating cells. *PLoS One,* **8,** e80714.

Lublin, F. D. and Reingold, S. C. (1996) Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology,* **46,** 907-911.

Lublin, F. D., Reingold, S. C., Cohen, J. A. et al. (2014) Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology,* **83,** 278-286.

Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M. and Lassmann, H. (2000) Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol,* **47,** 707-717.

Madhavarao, C. N., Arun, P., Anikster, Y., Mog, S. R., Staretz-Chacham, O., Moffett, J. R., Grunberg, N. E., Gahl, W. A. and Namboodiri, A. M. (2009) Glyceryl triacetate for Canavan disease: a low-dose trial in infants and evaluation of a higher dose for toxicity in the tremor rat model. *J Inherit Metab Dis,* **32,** 640-650.

Madhavarao, C. N., Hammer, J. A., Quarles, R. H. and Namboodiri, M. A. (2002) A radiometric assay for aspartoacylase activity in cultured oligodendrocytes. *Anal Biochem,* **308,** 314-319.

Maggio, B., Cumar, F. A. and Maccioni, H. J. (1972) Lipid content in brain and spinal cord during experimental allergic encephalomyelitis in rats. *J Neurochem,* **19,** 1031-1037.

Magnusson, C. D. and Haraldsson, G. G. (2011) Ether lipids. *Chem Phys Lipids,* **164,** 315-340.

Markoullis, K., Sargiannidou, I., Schiza, N., Hadjisavvas, A., Roncaroli, F., Reynolds, R. and Kleopa, K. A. (2012) Gap junction pathology in multiple sclerosis lesions and normal-appearing white matter. *Acta Neuropathol,* **123,** 873-886.

Martinez, M. (1996) Docosahexaenoic acid therapy in docosahexaenoic acid-deficient patients with disorders of peroxisomal biogenesis. *Lipids,* **31 Suppl,** S145-152.

Martinez, M. (2001) Restoring the DHA levels in the brains of Zellweger patients. *J Mol Neurosci,* **16,** 309-316; discussion 317-321.

Martinez, M. and Mougan, I. (1999) Fatty acid composition of brain glycerophospholipids in peroxisomal disorders. *Lipids,* **34,** 733-740.

Martinez, M., Pineda, M., Vidal, R., Conill, J. and Martin, B. (1993) Docosahexaenoic acid--a new therapeutic approach to peroxisomal-disorder patients: experience with two cases. *Neurology,* **43,** 1389-1397.

Martinez, M. and Vazquez, E. (1998) MRI evidence that docosahexaenoic acid ethyl ester improves myelination in generalized peroxisomal disorders. *Neurology,* **51,** 26-32.

Martinez, M., Vazquez, E., Garcia-Silva, M. T., Manzanares, J., Bertran, J. M., Castello, F. and Mougan, I. (2000) Therapeutic effects of docosahexaenoic acid ethyl ester in patients with generalized peroxisomal disorders. *Am J Clin Nutr,* **71,** 376S-385S.

Mata, S., Lolli, F., Soderstrom, M., Pinto, F. and Link, H. (1999) Multiple sclerosis is associated with enhanced B cell responses to the ganglioside GD1a. *Mult Scler,* **5,** 379-388.

Mathew, R., Arun, P., Madhavarao, C. N., Moffett, J. R. and Namboodiri, M. A. (2005) Progress toward acetate supplementation therapy for Canavan disease: glyceryl triacetate administration increases acetate, but not N-acetylaspartate, levels in brain. *J Pharmacol Exp Ther,* **315,** 297-303.

Mayatepek, E., Lehmann, W. D., Fauler, J., Tsikas, D., Frolich, J. C., Schutgens, R. B., Wanders, R. J. and Keppler, D. (1993) Impaired degradation of leukotrienes in patients with peroxisome deficiency disorders. *J Clin Invest,* **91,** 881-888.

Mayatepek, E. and Tiepelmann, B. (1996) Defective degradation of leukotrienes in peroxisomal-deficient human hepatocytes. *Biochem Biophys Res Commun,* **227,** 131-134.

McKerracher, L., David, S., Jackson, D. L., Kottis, V., Dunn, R. J. and Braun, P. E. (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron,* **13,** 805-811.

McQualter, J. L. and Bernard, C. C. (2007) Multiple sclerosis: a battle between destruction and repair. *J Neurochem,* **100,** 295-306.

Mehta, V. and Namboodiri, M. A. (1995) N-acetylaspartate as an acetyl source in the nervous system. *Brain Res Mol Brain Res,* **31,** 151-157.

Mikol, D. D., Rongnoparut, P., Allwardt, B. A., Marton, L. S. and Stefansson, K. (1993) The oligodendrocyte-myelin glycoprotein of mouse: primary structure and gene structure. *Genomics,* **17,** 604-610.

Mikol, D. D. and Stefansson, K. (1988) A phosphatidylinositol-linked peanut agglutinin-binding glycoprotein in central nervous system myelin and on oligodendrocytes. *J Cell Biol,* **106,** 1273-1279.

Minagar, A. (2013) Current and future therapies for multiple sclerosis. *Scientifica (Cairo),* **2013,** 249101.

Mitew, S., Hay, C. M., Peckham, H., Xiao, J., Koenning, M. and Emery, B. (2014) Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. *Neuroscience,* **276,** 29-47.

Moffett, J. R., Arun, P., Ariyannur, P. S., Garbern, J. Y., Jacobowitz, D. M. and Namboodiri, A. M. (2011) Extensive aspartoacylase expression in the rat central nervous system. *Glia,* **59,** 1414-1434.

Moffett, J. R., Arun, P., Ariyannur, P. S. and Namboodiri, A. M. (2013) N-Acetylaspartate reductions in brain injury: impact on post-injury neuroenergetics, lipid synthesis, and protein acetylation. *Front Neuroenergetics,* **5,** 11.

Morell, P. (1984) *Myelin*. Plenum Press, New York.

Morell, P. and Jurevics, H. (1996) Origin of cholesterol in myelin. *Neurochem Res,* **21,** 463-470.

Moser, A. B., Borel, J., Odone, A., Naidu, S., Cornblath, D., Sanders, D. B. and Moser, H. W. (1987) A new dietary therapy for adrenoleukodystrophy: biochemical and preliminary clinical results in 36 patients. *Ann Neurol,* **21,** 240-249.

Moser, A. B., Jones, D. S., Raymond, G. V. and Moser, H. W. (1999) Plasma and red blood cell fatty acids in peroxisomal disorders. *Neurochem Res,* **24,** 187-197.

Moser, H. W., Moser, A. B., Frayer, K. K., Chen, W., Schulman, J. D., O'Neill, B. P. and Kishimoto, Y. (1981) Adrenoleukodystrophy: increased plasma content of saturated very long chain fatty acids. *Neurology,* **31,** 1241-1249.

Moser, H. W., Moser, A. B., Hollandsworth, K., Brereton, N. H. and Raymond, G. V. (2007) "Lorenzo's oil" therapy for X-linked adrenoleukodystrophy: rationale and current assessment of efficacy. *J Mol Neurosci,* **33,** 105-113.

Mukhopadhyay, G., Doherty, P., Walsh, F. S., Crocker, P. R. and Filbin, M. T. (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron,* **13,** 757-767.

Murphy, A. C., Lalor, S. J., Lynch, M. A. and Mills, K. H. (2010) Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. *Brain Behav Immun,* **24,** 641-651.

Nakahara, J., Maeda, M., Aiso, S. and Suzuki, N. (2012) Current concepts in multiple sclerosis: autoimmunity versus oligodendrogliopathy. *Clin Rev Allergy Immunol,* **42,** 26-34.

Namboodiri, A. M., Moffett, J. R., Arun, P. et al. (2006a) Defective myelin lipid synthesis as a pathogenic mechanism of Canavan disease. *Adv Exp Med Biol,* **576,** 145-163; discussion 361-143.

Namboodiri, A. M., Peethambaran, A., Mathew, R., Sambhu, P. A., Hershfield, J., Moffett, J. R. and Madhavarao, C. N. (2006b) Canavan disease and the role of N-acetylaspartate in myelin synthesis. *Mol Cell Endocrinol,* **252,** 216-223.

Neuhaus, O., Wiendl, H., Kieseier, B. C., Archelos, J. J., Hemmer, B., Stuve, O. and Hartung, H. P. (2005) Multiple sclerosis: Mitoxantrone promotes differential effects on immunocompetent cells in vitro. *J Neuroimmunol,* **168,** 128-137.

Norton, W. T. and Poduslo, S. E. (1973) Myelination in rat brain: method of myelin isolation. *J Neurochem,* **21,** 749-757.

Oh, J. and O'Connor, P. W. (2015) Established disease-modifying treatments in relapsing-remitting multiple sclerosis. *Curr Opin Neurol,* **28,** 220-229.

Paintlia, A. S., Paintlia, M. K., Khan, M., Vollmer, T., Singh, A. K. and Singh, I. (2005) HMG-CoA reductase inhibitor augments survival and differentiation of oligodendrocyte progenitors in animal model of multiple sclerosis. *FASEB J,* **19,** 1407-1421.

Palumbo, S. and Bosetti, F. (2013) Alterations of brain eicosanoid synthetic pathway in multiple sclerosis and in animal models of demyelination: role of cyclooxygenase-2. *Prostaglandins Leukot Essent Fatty Acids,* **89,** 273-278.

Pan, B., Fromholt, S. E., Hess, E. J., Crawford, T. O., Griffin, J. W., Sheikh, K. A. and Schnaar, R. L. (2005) Myelin-associated glycoprotein and complementary axonal ligands, gangliosides, mediate axon stability in the CNS and PNS: neuropathology and behavioral deficits in single- and double-null mice. *Exp Neurol,* **195,** 208-217.

Pecina-Slaus, N. (2013) Merlin, the NF2 gene product. *Pathol Oncol Res,* **19,** 365-373.

Pham-Dinh, D., Dautigny, A. and Linington, C. (2004) *Myelin oligodendrocyte glycoprotein gene*: Myelin biology and disorders. Elsevier Academic Press, San Diego.

Poll-The, B. T. and Gartner, J. (2012) Clinical diagnosis, biochemical findings and MRI spectrum of peroxisomal disorders. *Biochim Biophys Acta,* **1822,** 1421-1429.

Polman, C. H., O'Connor, P. W., Havrdova, E. et al. (2006) A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med,* **354,** 899-910.

Procaccini, C., De Rosa, V., Pucino, V., Formisano, L. and Matarese, G. (2015) Animal models of Multiple Sclerosis. *Eur J Pharmacol,* **759,** 182-191.

Pu, H., Guo, Y., Zhang, W. et al. (2013) Omega-3 polyunsaturated fatty acid supplementation improves neurologic recovery and attenuates white matter injury after experimental traumatic brain injury. *J Cereb Blood Flow Metab,* **33,** 1474-1484.

Radin, N. S. (1981) Extraction of tissue lipids with a solvent of low toxicity. *Methods Enzymol,* **72,** 5-7.

Ramli, D., Aziz, I., Mohamad, M., Abdulahi, D. and Sanusi, J. (2017) The Changes in Rats with Sciatic Nerve Crush Injury Supplemented with Evening Primrose Oil: Behavioural, Morphologic, and Morphometric Analysis. *Evid Based Complement Alternat Med,* **2017,** 3476407.

Ransohoff, R. M. (2012) Animal models of multiple sclerosis: the good, the bad and the bottom line. *Nat Neurosci,* **15,** 1074-1077.

Reisenauer, C. J., Bhatt, D. P., Mitteness, D. J., Slanczka, E. R., Gienger, H. M., Watt, J. A. and Rosenberger, T. A. (2011) Acetate supplementation attenuates lipopolysaccharide-induced neuroinflammation. *J Neurochem,* **117,** 264-274.

Revina, E. S., Gromova, N. V. and Timoshina, T. E. (2011) Changes in phospholipid composition of the spinal cord in rabbits with allergic encephalomyelitis as an experimental model of multiple sclerosis. *Bull Exp Biol Med,* **152,** 224-227.

Rice, G. P., Hartung, H. P. and Calabresi, P. A. (2005) Anti-alpha4 integrin therapy for multiple sclerosis: mechanisms and rationale. *Neurology,* **64,** 1336-1342.

Ridge, S. C., Sloboda, A. E., McReynolds, R. A., Levine, S., Oronsky, A. L. and Kerwar, S. S. (1985) Suppression of experimental allergic encephalomyelitis by mitoxantrone. *Clin Immunol Immunopathol,* **35,** 35-42.

Rizzo, W. B., Phillips, M. W., Dammann, A. L., Leshner, R. T., Jennings, S. S., Avigan, J. and Proud, V. K. (1987) Adrenoleukodystrophy: dietary oleic acid lowers hexacosanoate levels. *Ann Neurol,* **21,** 232-239.

Robinson, A. P., Harp, C. T., Noronha, A. and Miller, S. D. (2014) The experimental autoimmune encephalomyelitis (EAE) model of MS: utility for understanding disease pathophysiology and treatment. *Handb Clin Neurol,* **122,** 173-189.

Rone, M. B., Cui, Q. L., Fang, J. et al. (2016) Oligodendrogliopathy in Multiple Sclerosis: Low Glycolytic Metabolic Rate Promotes Oligodendrocyte Survival. *J Neurosci,* **36,** 4698-4707.

Rosenberger, T. A., Oki, J., Purdon, A. D., Rapoport, S. I. and Murphy, E. J. (2002) Rapid synthesis and turnover of brain microsomal ether phospholipids in the adult rat. *J Lipid Res,* **43,** 59-68.

Rosner, H., Al-Aqtum, M. and Henke-Fahle, S. (1985) Developmental expression of GD3 and polysialogangliosides in embryonic chicken nervous tissue reacting with monoclonal antiganglioside antibodies. *Brain Res,* **350,** 85-95.

Roth, G. A., Monferran, C. G., Maggio, B. and Cumar, F. A. (1982) Central nervous system lipid alterations in rats with experimental allergic encephalomyelitis and its suppression by immunosuppressive drugs. *Life Sci,* **30,** 859-866.

Rouser, G., Siakotos, A. and Fleischer, S. (1969) Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. *Lipids,* **1,** 85-86.

Sadatipour, B. T., Greer, J. M. and Pender, M. P. (1998) Increased circulating antiganglioside antibodies in primary and secondary progressive multiple sclerosis. *Ann Neurol,* **44,** 980-983.

Saher, G., Brugger, B., Lappe-Siefke, C., Mobius, W., Tozawa, R., Wehr, M. C., Wieland, F., Ishibashi, S. and Nave, K. A. (2005) High cholesterol level is essential for myelin membrane growth. *Nat Neurosci,* **8,** 468-475.

Saher, G., Quintes, S. and Nave, K. A. (2011) Cholesterol: a novel regulatory role in myelin formation. *Neuroscientist,* **17,** 79-93.

Salvati, S., Attorri, L., Confaloni, A. and Di Biase, A. (1990) Lipid changes in central nervous system membranes in experimental allergic encephalomyelitis (EAE). *Neurochem Res,* **15,** 1051-1053.

Sango, K., McDonald, M. P., Crawley, J. N. et al. (1996) Mice lacking both subunits of lysosomal beta-hexosaminidase display gangliosidosis and mucopolysaccharidosis. *Nat Genet,* **14,** 348-352.

Sassa, T., Wakashima, T., Ohno, Y. and Kihara, A. (2014) Lorenzo's oil inhibits ELOVL1 and lowers the level of sphingomyelin with a saturated very long-chain fatty acid. *J Lipid Res,* **55,** 524-530.

Schachner, M. and Bartsch, U. (2000) Multiple functions of the myelin-associated glycoprotein MAG (siglec-4a) in formation and maintenance of myelin. *Glia,* **29,** 154-165.

Schiffmann, S., Ferreiros, N., Birod, K. et al. (2012) Ceramide synthase 6 plays a critical role in the development of experimental autoimmune encephalomyelitis. *J Immunol,* **188,** 5723-5733.

Schmitt, S., Castelvetri, L. C. and Simons, M. (2015) Metabolism and functions of lipids in myelin. *Biochim Biophys Acta,* **1851,** 999-1005.

Schnaar, R. L. (2010) Brain gangliosides in axon-myelin stability and axon regeneration. *FEBS Lett,* **584,** 1741-1747.

Schnaar, R. L., Gerardy-Schahn, R. and Hildebrandt, H. (2014) Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration. *Physiol Rev,* **94,** 461-518.

Segel, R., Anikster, Y., Zevin, S., Steinberg, A., Gahl, W. A., Fisher, D., Staretz-Chacham, O., Zimran, A. and Altarescu, G. (2011) A safety trial of high dose glyceryl triacetate for Canavan disease. *Mol Genet Metab,* **103,** 203-206.

Serafini, B., Rosicarelli, B., Franciotta, D. et al. (2007) Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. *J Exp Med,* **204,** 2899-2912.

Shimazu, T., Hirschey, M. D., Huang, J. Y., Ho, L. T. and Verdin, E. (2010) Acetate metabolism and aging: An emerging connection. *Mech Ageing Dev,* **131,** 511-516.

Shimizu, T. (2009) Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation. *Annu Rev Pharmacol Toxicol,* **49,** 123-150.

Simmons, S. B., Pierson, E. R., Lee, S. Y. and Goverman, J. M. (2013) Modeling the heterogeneity of multiple sclerosis in animals. *Trends Immunol,* **34,** 410-422.

Singh, S., Choudhury, A., Gusain, P., Parvez, S., Palit, G., Shukla, S. and Ganguly, S. (2016) Oral acetate supplementation attenuates N-methyl D-aspartate receptor hypofunction-induced behavioral phenotypes accompanied by restoration of acetyl-histone homeostasis. *Psychopharmacology (Berl),* **233,** 1257-1268.

Singhal, N. K., Huang, H., Li, S. et al. (2017) The neuronal metabolite NAA regulates histone H3 methylation in oligodendrocytes and myelin lipid composition. *Exp Brain Res,* **235,** 279-292.

Smerjac, S. M. and Bizzozero, O. A. (2008) Cytoskeletal protein carbonylation and degradation in experimental autoimmune encephalomyelitis. *J Neurochem,* **105,** 763-772.

Smith, M. D., Bhatt, D. P., Geiger, J. D. and Rosenberger, T. A. (2014) Acetate supplementation modulates brain adenosine metabolizing enzymes and adenosine A(2)A receptor levels in rats subjected to neuroinflammation. *J Neuroinflammation,* **11,** 99.

Smith, M. E. (1964) Lipid Biosynthesis in the Central Nervous System in Experimental Allergic Encephalomyelitis. *J Neurochem,* **11,** 29-37.

Soliman, M. L., Combs, C. K. and Rosenberger, T. A. (2013a) Modulation of inflammatory cytokines and mitogen-activated protein kinases by acetate in primary astrocytes. *J Neuroimmune Pharmacol,* **8,** 287-300.

Soliman, M. L., Ohm, J. E. and Rosenberger, T. A. (2013b) Acetate reduces PGE2 release and modulates phospholipase and cyclooxygenase levels in neuroglia stimulated with lipopolysaccharide. *Lipids,* **48,** 651-662.

Soliman, M. L., Puig, K. L., Combs, C. K. and Rosenberger, T. A. (2012a) Acetate reduces microglia inflammatory signaling in vitro. *J Neurochem,* **123,** 555-567.

Soliman, M. L. and Rosenberger, T. A. (2011) Acetate supplementation increases brain histone acetylation and inhibits histone deacetylase activity and expression. *Mol Cell Biochem,* **352,** 173-180.

Soliman, M. L., Smith, M. D., Houdek, H. M. and Rosenberger, T. A. (2012b) Acetate supplementation modulates brain histone acetylation and decreases interleukin-1beta expression in a rat model of neuroinflammation. *J Neuroinflammation,* **9,** 51.

Steinberg, S., Chen, L., Wei, L., Moser, A., Moser, H., Cutting, G. and Braverman, N. (2004) The PEX Gene Screen: molecular diagnosis of peroxisome biogenesis disorders in the Zellweger syndrome spectrum. *Mol Genet Metab,* **83,** 252-263.

Steinberg, S. J., Dodt, G., Raymond, G. V., Braverman, N. E., Moser, A. B. and Moser, H. W. (2006) Peroxisome biogenesis disorders. *Biochim Biophys Acta,* **1763,** 1733-1748.

Storoni, M. and Plant, G. T. (2015) The Therapeutic Potential of the Ketogenic Diet in Treating Progressive Multiple Sclerosis. *Mult Scler Int,* **2015,** 681289.

Suh, P. G., Park, J. I., Manzoli, L. et al. (2008) Multiple roles of phosphoinositide-specific phospholipase C isozymes. *BMB Rep,* **41,** 415-434.

Svennerholm, L. (1957) Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim Biophys Acta,* **24,** 604-611.

Sztriha, L. S., Nork, M. P., Abdulrazzaq, Y. M., al-Gazali, L. I. and Bakalinova, D. B. (1997) Abnormal myelination in peroxisomal isolated dihydroxyacetonephosphate acyltransferase deficiency. *Pediatr Neurol,* **16,** 232-236.

Taranova, N. P. (1985) Disturbance of lipid composition in spinal cord of rabbits with experimental allergic encephalomyelitis. *Neurochem Res,* **10,** 1483-1497.

Thacker, E. L., Mirzaei, F. and Ascherio, A. (2006) Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. *Ann Neurol,* **59,** 499-503.

Tilve, S., Difato, F. and Chieregatti, E. (2015) Cofilin 1 activation prevents the defects in axon elongation and guidance induced by extracellular alpha-synuclein. *Sci Rep,* **5,** 16524.

Tsen, A. R., Long, P. M., Driscoll, H. E. et al. (2014) Triacetin-based acetate supplementation as a chemotherapeutic adjuvant therapy in glioma. *Int J Cancer,* **134,** 1300-1310.

Turner, M. J., Pang, P. T., Chretien, N. et al. (2015) Reduction of inflammation and preservation of neurological function by anti-CD52 therapy in murine experimental autoimmune encephalomyelitis. *J Neuroimmunol,* **285,** 4-12.

Ueno, K., Ando, S. and Yu, R. K. (1978) Gangliosides of human, cat, and rabbit spinal cords and cord myelin. *J Lipid Res,* **19,** 863-871.

Virtanen, J. O. and Jacobson, S. (2012) Viruses and multiple sclerosis. *CNS Neurol Disord Drug Targets,* **11,** 528-544.

Vourc'h, P. and Andres, C. (2004) Oligodendrocyte myelin glycoprotein (OMgp): evolution, structure and function. *Brain Res Brain Res Rev,* **45,** 115-124.

Vyas, A. A., Patel, H. V., Fromholt, S. E., Heffer-Lauc, M., Vyas, K. A., Dang, J., Schachner, M. and Schnaar, R. L. (2002) Gangliosides are functional nerve cell ligands for myelin-associated glycoprotein (MAG), an inhibitor of nerve regeneration. *Proc Natl Acad Sci U S A,* **99,** 8412-8417.

Vyas, A. A. and Schnaar, R. L. (2001) Brain gangliosides: functional ligands for myelin stability and the control of nerve regeneration. *Biochimie,* **83,** 677-682.

Wanders, R. J., Ferdinandusse, S., Brites, P. and Kemp, S. (2010) Peroxisomes, lipid metabolism and lipotoxicity. *Biochim Biophys Acta,* **1801,** 272-280.

Wanders, R. J. and Waterham, H. R. (2006) Peroxisomal disorders: the single peroxisomal enzyme deficiencies. *Biochim Biophys Acta,* **1763,** 1707-1720.

Wang, K. C., Koprivica, V., Kim, J. A., Sivasankaran, R., Guo, Y., Neve, R. L. and He, Z. (2002) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature,* **417,** 941-944.

Warringa, R. A., Hoeben, R. C., Koper, J. W., Sykes, J. E., van Golde, L. M. and Lopes-Cardozo, M. (1987) Hydrocortisone stimulates the development of oligodendrocytes in primary glial cultures and affects glucose metabolism and lipid synthesis in these cultures. *Brain Res,* **431,** 79-86.

Weller, S., Gould, S. J. and Valle, D. (2003) Peroxisome biogenesis disorders. *Annu Rev Genomics Hum Genet,* **4,** 165-211.

Wergeland, S., Torkildsen, O., Myhr, K. M., Mork, S. J. and Bo, L. (2012) The cuprizone model: regional heterogeneity of pathology. *APMIS,* **120,** 648-657.

Wolswijk, G. (2000) Oligodendrocyte survival, loss and birth in lesions of chronic-stage multiple sclerosis. *Brain,* **123 ( Pt 1),** 105-115.

Woodruff, R. H., Tekki-Kessaris, N., Stiles, C. D., Rowitch, D. H. and Richardson, W. D. (2001) Oligodendrocyte development in the spinal cord and telencephalon: common themes and new perspectives. *Int J Dev Neurosci,* **19,** 379-385.

Yang, L. J., Zeller, C. B., Shaper, N. L., Kiso, M., Hasegawa, A., Shapiro, R. E. and Schnaar, R. L. (1996) Gangliosides are neuronal ligands for myelin-associated glycoprotein. *Proc Natl Acad Sci U S A,* **93,** 814-818.

Yednock, T. A., Cannon, C., Fritz, L. C., Sanchez-Madrid, F., Steinman, L. and Karin, N. (1992) Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature,* **356,** 63-66.

Yu, R. K., Tsai, Y. T., Ariga, T. and Yanagisawa, M. (2011) Structures, biosynthesis, and functions of gangliosides--an overview. *J Oleo Sci,* **60,** 537-544.

Zhang, J. and Liu, Q. (2015) Cholesterol metabolism and homeostasis in the brain. *Protein Cell,* **6,** 254-264.

Zindler, E. and Zipp, F. (2010) Neuronal injury in chronic CNS inflammation. *Best Pract Res Clin Anaesthesiol,* **24,** 551-562.