Defective carbohydrate metabolism in Multiple Sclerosis

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Dissertation to apply for a PhD degree in Neuroscience from the University of Valencia

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INFORMAN

Que Dña. Deepali Mathur, ha realizado bajo nuestra supervisión el trabajo de investigación recogido en la presente memoria, titulado "*Defective carbohydrate metabolism in Multiple Sclerosis"* de manera satisfactoria. Por ello, autorizan su presentación ante el tribunal correspondiente para que sea evaluada, y optar así al grado de Doctor por la Universidad de Valencia, dentro del programa ocial de Doctorado en Neurociencias.

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Maria Burgal-Marti Gerardo López Rodas Buenaventura Casanova

DEDICATION

I would like to dedicate my thesis to my beloved husband

Publications

Mathur D, López-Rodas G, Casanova B and Burgal-Marti M (2014) Perturbed glucose metabolism: insights into Multiple Sclerosis pathogenesis. *Frontiers in Neurology* **5** (250) 1-7. DOI: 10.3389/fneur.2014.00250.

Burgal-Marti M and **Mathur D** (2014) Molecular Shots. *Ann Neurosci.* **21** (3) 123. DOI: 10.5214/ans.0972.7531.210311.

Simó-Castelló M, Alcalá C, **Mathur D**, López-Rodas G, Burgal-Marti M and Casanova B (2015) Molecular Shots. *Ann Neurosci* **22** (1) 54. DOI: 10.5214/ans. 0972.7531.220213

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Abstract

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) in which repeated episodes of inflammation results in disruption of myelin sheath and neurodegeneration. The disease is a major cause of neurological disability and dysfunction in young adults that affects more than two million people worldwide. In Spain, the prevalence of the disease is approximately 102-160 per 100,000 population according to the current estimates. The disease typically manifests at 20-40 years of age when people are in their full employment and sometimes develops into an aggressive stage that alters the lives of patients and their families. Unfortunately the current treatments are only effective in preventing relapses and slowing down progression but not completely ceasing it and the development of more effective treatments has been hindered by our limited understanding of MS pathogenesis.

Axonal damage is widely accepted as the major cause of persistent functional disability in MS patients, although its origin is unknown. During the relapsing-remitting disease course the patient's brain itself is capable of repairing the damage, remyelinating the axon and recovering the neurological function. Cerebrospinal fluid (CSF) is in contact with the brain parenchyma and ventricles, which can be a site for deposition of cellular damage products that can influence the cellular physiology of neurons, oligodendrocyte progenitor cells (OPCs) and myelinating oligodendrocytes. It is a promising biofluid in the search for biomarkers and disease associated proteins in MS, both with respect to inflammatory and neurodegenerative processes. Factors released in CSF of MS patients including apoptotic factors, cytokines, proteolytic enzymes, oxidative products and free radicals are produced by glial and activated immune cells. These compounds are most likely to cause axonal damage. Accordingly, our hypothesis was to identify the

effect of CSF obtained from distinct clinical types of MS patients upon exposure to neurons on transcriptional grounds. Additionally, we were interested to determine the effect of CSF on OPCs, which could contain factors that damage OPCs during attempts at brain repair. Identification of these mechanisms involved in axonal degeneration-reconstruction may shed light in understanding MS progression and /or prognosis.

Furthermore, we were interested in identifying potential biomarkers using genomics based approach that could differentiate MS from another similar but totally distinct neurological disease known as Neuromyelitis optica (NMO). NMO shares many pathological similarities with MS and therefore it was previously considered as its variant. For this reason clinicians often used to encounter difficulty in distinguishing MS from NMO and hence similar treatment was provided to both the category of patients. However recent research shows that there are some NMO specific IgG antibodies present in the sera of NMO patients which differentiate both the diseases.

To study primary neuroaxonal damage independent of secondary damage resulting from demyelination, we used a cellular model with primary cultures of unmyelinated granular neurons (CGNs) from rat cerebellum. CGNs are small and the most numerous unmyelinated neurons. We employed these primary cultures and treated them with the CSF of distinct clinical forms of MS including oligoclonal bands of IgG + and IgM type – relapsing remitting MS, primary progressive MS, NMO and neurological controls. Previous data in our laboratory experienced variation in sodium channel gene(s) expression while normalizing them with commonly used housekeeping genes including *ActB* and *Gapdh*, therefore to further obtain accurate gene expression data it was first necessary to validate these reference genes for correct normalization of target mRNA transcripts. To do this,

we evaluated certain reference genes during the development of cerebellar granule neurons (CGNs) and when CGNs were exposed to the CSF from MS and NMO patients.

Unexpectedly, we found that *Gapdh* showed significant variation in gene expression when neurons were exposed to the CSF of MS and NMO patients. It is well known that GAPDH plays a crucial role in carbohydrate metabolism, therefore, we proposed to look for perturbance in expression of genes implicated in glucose metabolism in treated neurons. To do this, we performed microarray gene expression profiling in both neurons and proliferating OPCs exposed to the CSF from MS patients and looked at the genes involved particularly in glucose metabolism. Furthermore, the stability of reference genes in treated OPCs was validated using *GeNorm* and *NormFinder* algorithms. Although very limited research has been carried out so far that indicates a role of metabolic disturbances in the pathogenesis of axonal damage in MS there is evidence that shows an association of neurodegenerative disease etiology with metabolic impairment.

The present research shows a significant variation in gene expression catalyzing essential steps of carbohydrate metabolism in neurons and OPCs exposed to the CSF of different clinical forms of MS and NMO patients as compared to neurons exposed to the CSF of neurological controls. These data suggest that factors in CSF of MS and NMO patients cause a disturbance in metabolic gene(s) expression in neurons and oligodendrocytes; and demonstrates that MS seems to be associated with metabolic impairment. The results allow us to distinguish different clinical forms and aggressivity in MS patients and MS from NMO that is sometimes difficult by clinical criteria. These results are important to found biomarkers for NMO and MS diseases. Lastly, the significance of prior validation of reference housekeeping genes to accurately normalize the gene expression data is established.

TABLE OF CONTENTS

Tables Index

Figures Index

Abbreviations

ActB	Beta actin
ACN	Acetonitrile
AQP4	Aquaporin 4
APP	Amyeloid precursor protein
BBB	Blood brain barrier
BME	Basal Eagle Medium
B2m	Microglobulin beta-2
bFGF	Basic fibroblast growth factor
CFI	Cumulative metabolic flux index
CNS	Central nervous system
CSF	Cerebrospinal fluid
CGNs	Cerebellar granule neurons
CYC1	Cytochrome C1
CIS	Clinically isolated syndrome
DEPC	Deionized, diethylpyrocarbonate treated water
DCs	Dendritic cells
DAPI	4',6-diamidino-2-phenylindole dye
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded Disability Status Scale
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glucose 6-phosphate isomerase
GFAP	Glial Fibrillary Acidic Protein
Hprt	Hypoxanthine guanine phosphoribosyl-transferase

HKGs	Housekeeping genes
HK	Hexokinase
HMS	Hexose monophosphate shunt
Ig	Immunoglobulin
IEF	Isoelectric focusing
Iba1	Ionized calcium binding adapter molecule 1
LETM	Longitudinal extensive transverse myelitis
LDHA	Lactate dehydrogenaseA
MS	Multiple sclerosis
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte protein
MBP	Myelin basic protein
MRI	Magnetic resonance imaging
mtHsp70	Mitochondrial heat shock protein
MDH	Malate dehydrogenase
NIND	Non-inflammatory neurological diseases
NMO	Neuromyelitis Optica
NM10	Neuronal media 10
NMR	Nuclear magnetic resonance
ON	Optic neuritis
OCB	Oligoclonal bands
OCGB	Oligoclonal IgG bands
OCMB	Oligoclonal IgM bands
OPC	Oligodendrocyte progenitor cell
ODM	Oligodendrocyte chemically-defined media
OPCs	Oligodendrocytes
PFK-1	Phosphofructokinase-1

PKM2	Isozyme of pyruvate kinase
PI	Propidium iodide
PLP	Proteolipid protein
PPMS	Primary progressive multiple sclerosis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF-AA	Platelet-derived growth factor AA
qPCR	Quantitative real time PCR
RT	Reverse transcription
mRNA	Messanger RNA
Rpl19	Ribosomal protein L19
RRMS	Relapsing remitting multiple sclerosis
Rh-123	Rhodamine-123
SPMS	Secondary progressive multiple sclerosis
TR	Texas red dye
TPI	Triose phosphate isomerase
TFA	Trifluoroacetic acid
TFRC	Transferrin receptor

I- INTRODUCTION

1. Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a chronic, inflammatory and probably an autoimmune disease of the central nervous system (CNS) that causes damage to myelin sheath [Frohman *et al.,* 2006]. Autoimmunity means that body's natural immunological defense instead of destroying foreign cells destroys the body's native cells. In MS, the myelin sheath or white matter that surrounds and insulates nerve cells is destroyed. Without the myelin sheath, nerve cells lose their ability to conduct nerve impulse because of which the body loses its ability to perform the functions controlled by these cells. Demyelination leaves a scar tissue ("sclerosis") or lesions at numerous places in CNS ("multiple"). These scars, or lesions, consist mostly of dead nerve cells, whose axons have been denuded of the myelin sheaths. Depending on the location of the lesions and axonal injury, a variety of symptoms can be observed with muscular control the most visible symptom although many other brain functions are affected (Figure 1). Myelin loss may be a result of immune mediated processes that directly damages it or loss of oligodendrocytes function that are myelin-forming cells [Lassmann *et al.,* 2001; Barnett and Prineas, 2004].

The disease is a major cause of non-traumatic neurological disability afflicting more than two million people worldwide [Sadovnick, 1993]. With a typical age of onset at 20-40 years, the economic and social spheres of patients and their families are severely affected. In Europe and North America, the prevalence of MS is 1 in 800, with an annual incidence of 2 to 10 per 100,000 individuals. In Spain, the prevalence of the disease is approximately 102-160 with a mean prevalence of 120

per 100,000 with about 45,000 patients according to the current estimates [Fernandez *et al.,* 2012].

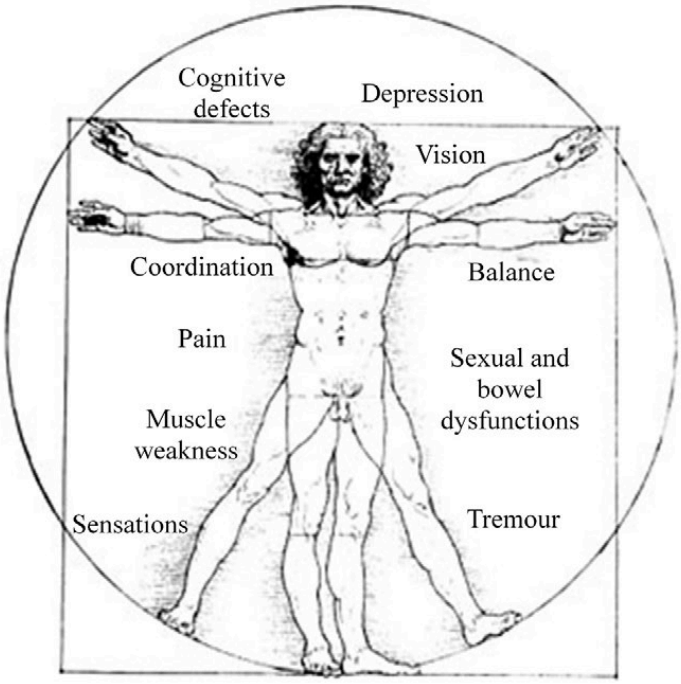


Figure 1: Common symptoms and functions affected in MS. Leonardo Da Vinci's "Vitruvian man" modified from a figure from Wikimedia commons.

Over the past decade, there has been a considerable amount of discussion about whether immune-mediated myelin damage is a primary or secondary event to axon degeneration in MS. Previous investigations have demonstrated that axonal loss is

a consequence of inflammatory demyelination in neurodegenerative diseases. However, recent *in vivo* imaging studies using Optical Coherence Tomography (OCT) have shown that neurons in the retina that lack myelin sheath degenerate as well, suggesting that neurodegeneration ensues in demyelinated areas of the CNS [Saidha *et al.,* 2011]. Reduced thickness of the retinal layer as observed by OCT in MS patients affected with optic neuritis further support the notion that damaged neurons in the retina contribute to the functional insufficiencies in MS [Tatrai *et al.,* 2012]. Optic neuritis is an inflammatory condition of the optic nerve that undergoes demyelination and neurodegeneration.

In this thesis, we wanted to study primary neuronal damage independent of secondary damage resulting from demyelination. Neuronal and non-neuronal cells of the brain including oligodendrocytes and astrocytes, all can be regarded as the cellular "victims" in multiple sclerosis. When oligodendrocytes (OPCs), which are the myelin forming cells, are damaged, astrocytes respond by forming a glial scar and axons degenerate. Since axonal damage has now been widely accepted as the major cause of persistent functional disability in patients with MS, we were mainly interested to study primary neuronal damage. Additionally, we were interested to determine the effect of CSF on OPCs, which could contain factors that damage OPCs during attempts at brain repair. During the relapsing-remitting disease course, the patient's brain itself is capable of repairing the damage, remyelinating the axon and recovering the neurological function. Furthermore, the cerebrospinal fluid (CSF) is a promising biofluid in the search for biomarkers and disease associated proteins in MS, both with respect to inflammatory and neurodegenerative processes.

In addition, we were interested to identify potential biomarkers that could differentiate MS from another similar but completely distinct neurological disease

known as Neuromyelitis Optica (NMO). Both diseases are similar with respect to their inflammatory, demyelinating and autoimmune characteristics they exhibit. NMO, also known as Devic's disease, is defined as a severe monophasic syndrome characterized by bilateral optic neuritis, acute transverse myelitis and inflammatory demyelination of the central nervous system (CNS) that selectively affects the spinal cord and optic nerves causing blindness and paralysis and spares the brain [Wingerchuk *et al.,* 1999]. Because of common features, clinicians often encountered difficulty in distinguishing MS from NMO. However, recent neuropathologic studies demonstrate that NMO is characterized by very distinct vasculocentric pathology with prominent immunoglobulin (Ig) deposition in sera and requires a different treatment approach. The identification of NMO-IgG antibodies found specifically in the sera of NMO patients distinguishes the disease from MS [Lennon *et al.,* 2004]. These antibodies have also been identified in the sera of two related neurologic conditions, bilateral optic neuritis (ON), and longitudinal extensive transverse myelitis (LETM) that are generally considered to lie within the NMO spectrum of diseases. About 75% of people with NMO have the circulating NMO IgG in their sera [Lennon *et al.,* 2005]. The antigenic target of NMO-IgG is aquaporin-4 (AQP4) protein expressed by astrocytes [Lennon *et al.,* 2005]. AQP4 acts as a channel that regulates water entry and exiting of the nerve cells in the CNS where it is preferentially localized within astrocytic end feet at the blood-brain barrier [Agre *et al.,* 2003; Nielsen *et al.,* 1997].

Previous data in our laboratory experienced variation in sodium channel gene(s) expression while normalizing them with commonly used housekeeping genes including *ActB* and *Gapdh*. Therefore, to further obtain accurate gene expression data, it was first necessary to validate these reference genes for correct normalisation of target mRNA transcripts (2010 Thesis of Eduardo Beltran, Directed by Dr. Maria Burgal, University of Valencia). Several recent studies have

documented that the expression of housekeeping genes may not necessarily be stable in all cells/tissues under all conditions. A gene may show stable expression in one condition whereas unstable in another. Invariable expression of these reference genes has been observed with cellular development [Maie Dawoud *et al.,* 2005] and under distinct experimental conditions [Deindl *et al.,* 2002; Glare *et al.,* 2002; Hamalainen *et al.,* 2001; Radonic *et al.,* 2004; Zhong *et al.,* 1999; Toegel *et al.,* 2007; Torres *et al.,* 2003; Gubern *et al.,* 2009]. Literature reveals that defects in metabolism may contribute to the pathogenesis of axonal injury in neurodegenerative diseases [Blum-Degen *et al.,* 1995]. Variation in expression of enzymes catalyzing essential steps in metabolic pathway may lead to mitochondrial dysfunction, apoptosis and cytotoxicity in neurons.

2. Historical overview of MS

2.1. Medical discovery

The French neurologist Jean-Martin Charcot (1825-1893) was the first person to recognize multiple sclerosis as a distinct disease in 1868 [Compston *et al.*, 1988]. Summarizing previous reports and adding his own clinical observations, Charcot called the disease "*sclerose en plaques*". The three signs of MS now known as Charcot's Triad are nystagmus, intention tremor and telegraphic speech, though these are not unique to MS. Charcot also observed cognition changes, describing his patients as having a "*marked enfeeblement of the memory*" and "*conceptions that formed slowly*" [Charcot *et al.*, 1868].

Prior to Charcot, Robert Carswell (1793–1857), a British professor of pathology, and Jean Cruveilhier (1791–1873), a French professor of pathologic anatomy, had described and illustrated many of the disease's clinical details, but did not identify

it as a separate disease [Compston *et al.*, 1988]. Specifically, Carswell described the injuries he found as "*a remarkable lesion of the spinal cord accompanied with atrophy*" [Compston *et al.*, 2008]. Under the microscope, Swiss pathologist Georg Eduard Rindfleisch (1836–1908) noted in 1863 that the inflammation-associated lesions were distributed around blood vessels [Lassmann *et al.,* 2005].

After Charcot's description, Eugene Devie (1858–1930), Jozsef Balo (1895–1979), Paul Ferdinand Schilder (1886–1940), and Otto Marburg (1874–1948) described special cases of the disease. During the 20th century, there was an important development on the theories about the cause and pathogenesis of MS while efficacious treatments began to appear in 1990 [Compston *et al.,* 2008].

2.2. Historical cases

There is surplus information about the history of people who lived before or shortly after the disease as described by Charcot. A young woman called Halldora, who lived in Iceland around 1200, suddenly lost her vision and mobility, but after praying to the saints, recovered them after seven days. Saint Lidwina of Schiedam (1380–1433), a Dutch nun, may be one of the first clearly identifiable MS patients. From the age of 16 until her death at 53, she suffered intermittent pain, weakness of the legs, and vision loss—symptoms typical of MS [Medaer *et al.*, 1979].

Augustus Frederick d'Este (1794–1848), son of Prince Augustus Frederick and the grandson of George III of the United Kingdom, almost certainly suffered from MS. D'Este left a detailed diary describing his 22 years living with the disease. His symptoms began at age 28 with a sudden transient visual loss. During the course of his disease, he developed weakness of the legs, clumsiness of the hands, numbness, dizziness, bladder disturbances, and erectile dysfunction. In 1844, he began to use a wheelchair. Another early account of MS was kept by the British

diarist W. N. P. Barbellion, nom-de-plume of Bruce Frederick Cummings (1889–1919), who maintained a detailed log of his diagnosis and struggle with MS [Pearce *et al.*, 2005].

3. Natural history of Multiple Sclerosis

The natural history of MS is unpredictable and has been discussed for several decades. Overall, the clinical course of the disease is composed of two distinct types of neurological episodes, exacerbations (relapse) and progression. An exacerbation is the reappearance or worsening of neurological symptoms lasting more than 24 hours with episodes seperated by at least one month. A relapse is usually followed by a remission although in some cases concludes with permanent neurological deficit. Progression is defined as a continuous neurological deterioration for a minimum of six months. The severity of MS is commonly measured on the Expanded Disability Status Scale (EDSS) from 0.0-10.0 [Kurtzke, 1983]. However, the EDSS score alone is not informative enough of the rate of progression. Multiple Sclerosis Severity Score (MSSS), which is based on the EDSS score but also takes into account the time from onset and can be used to predict the patient's approximate disability level after 30 years with MS [Roxburgh *et al.,* 2005].

In 1996, the US National Multiple Sclerosis Society (NMSS) Advisory Committee on Clinical Trials in Multiple Sclerosis defined the clinical subtypes of multiple sclerosis (MS) [Lublin, 1996]. The Committee provided standardized definitions for four MS clinical courses: relapsing remitting (RR), secondary progressive (SP), primary progressive (PP), and progressive relapsing (PR) [Lublin, 1996]. However, these clinical course descriptors were based on subjective views of MS experts and lacked imaging and biological correlates. Therefore, in 2011, the Committee and other

experts re-examined MS phenotypes, exploring clinical, imaging, and biomarker advances through working groups and literature searches. It was proposed that the core MS phenotype descriptions of relapsing and progressive disease should be retained with some modifications that include consideration of disease activity (based on clinical relapse rate and lesion activity detected by CNS imaging) and disease progression over a given time period. Evidence of disease activity and clinical progression, which by current understanding reflects ongoing inflammatory or neurodegenerative processes [Lassman, 2012], may impact prognosis, therapeutic decisions, and clinical trial designs and outcomes.

The following sections include detailed account of each MS clinical phenotype.

3.1. Clinically Isolated Syndrome (CIS)

Clinically isolated syndrome (CIS) was not included in the initial MS clinical descriptors. CIS is now recognized as the first clinical presentation of a disease that shows characteristics of inflammatory demyelination that could be MS, but has yet to fulfill criteria of dissemination in time [Miller *et al.,* 2005]. Use of the 2010 revisions to the McDonald MS diagnostic criteria allows some patients with a single clinical episode to be diagnosed with MS based on the single scan criterion for dissemination in time and space [Polman *et al.,* 2011], reducing the number of patients who will be categorized as CIS. In 1989, Miller *et al.* prospectively followed clinical status and MRI imaging in patients who presented with clinically isolated lesions of the brainstem or spinal cord. MS progression occurred in 13 brainstem syndrome patients (57%) and in 14 spinal cord syndrome patients (42%) after mean intervals of 15 and 16 months, respectively. Oligoclonal band (OCB) in the CSF increased progression risk in patients with both syndromes while the presense of disseminated brain lesions, increased risk only in those with a spinal cord syndrome

[Miller *et al.,* 1989; Thrower, 2007; Tintore *et al.,* 2015].

3.2. Relapsing remitting MS (RRMS)

In RRMS, patients experience an initial exacerbation followed by complete or incomplete recovery (RR, relapsing-remitting sequence). The 80-90% of patients with MS has a RR course in the following ten years after initial presentation. The disease begins with an initial attack (episode of symptom flare-ups) followed by a period of remission that could be as long as two years, a second attack is followed by either another period of remission or by progression, and this is termed secondary progressive MS (SPMS). Eventually, remissions are of shorter durations, relapses become longer and finally the patient enters a progressive stage [Noseworthy *et al.,* 2000]. Figure 2 shows the 1996 vs 2013 MS phenotype descriptions for relapsing disease. A patient with RRMS who had a new gadoliniumen enhancing lesion on a current MRI would be considered to be RR–active (Figure 2). Conversely, "not active" as a phenotype modifier could be used in the same way, to indicate a patient with a relapsing course but no relapses, gadolinium-enhancing activity, or new or unequivocally enlarging T2 lesions during the assessment period. Patients not assessed over a designated time frame would be considered "activity indeterminate." Inclusion of activity as a modifier of a basic clinical course phenotype allows elimination of the PRMS category.

3.3. Secondary progressive MS (SPMS)

Secondary progressive MS describes around 65% patients of those with progressive accumulation of disability after initial relapsing course, with or without occasional relapses and minor remissions [Compston *et al.,* 2008; Lublin *et al.,* 1996]. To date,

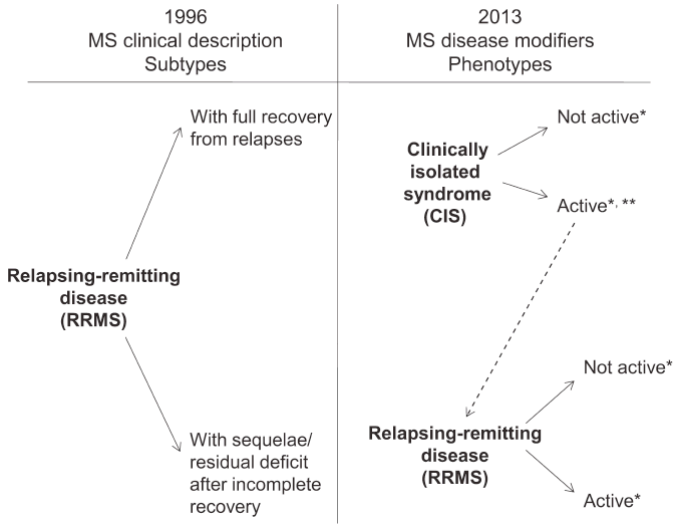


Figure 2: The 1996 vs 2013 multiple sclerosis phenotype descriptions for relapsing disease. *Activity determined by clinical relapses and/or MRI activity (contrast-enhancing lesions; new or unequivocally enlarging T2 lesions assessed at least annually); if assessments are not available, activity is "indeterminate." **CIS, if subsequently clinically active and fulfilling current multiple sclerosis (MS) diagnostic criteria, becomes relapsing-remitting MS (RRMS)

there are no clear clinical, imaging, immunologic, or pathologic criteria to determine the transition point when RRMS converts to SPMS; the transition is usually gradual. This has limited our ability to study the imaging and biomarker characteristics that may distinguish this course. Figure 3 shows the 1996 vs 2013

MS phenotype descriptions for relapsing disease. A patient with SPMS who has gradually worsened and has gadolinium-enhancing lesions on MRI would be classified as SPMS–active and progressing (Figure 3).

3.4. Primary progressive MS (PPMS)

The 10–15% of patients diagnosed with MS experience a progressive accumulation of disability from onset with or without temporary plateaus, minor remissions and improvements [Lublin *et al.,* 1996; Noseworthy *et al.,* 2000].. In 2013, the description of progressive disease was revised by incorporating disease activity and progression.

A patient with PPMS who has an acute attack (thus fulfilling prior criteria for PRMS) would be considered to be PP–active. On the other hand, a patient with PPMS with no acute attacks and no MRI activity would be considered to be PP–not active. Thus, a patient with PPMS who has not progressed over the past year would be classified as PPMS–not progressing.

4. Epidemiology of MS

4.1. Migration Studies

Geoffrey Dean performed one of the earliest and most infallible studies of migrants in South Africa. In 1967, he gave an idea of the annual incidence, prevalence and mortality statistics for MS in white South African-born individuals and immigrants to South Africa [Dean *et al.,* 1994]. He also addressed the issue of the marked difference in frequency of MS depending on age of arrival to South Africa in the English speaking white population. Migrants under the age of 15 showed lower

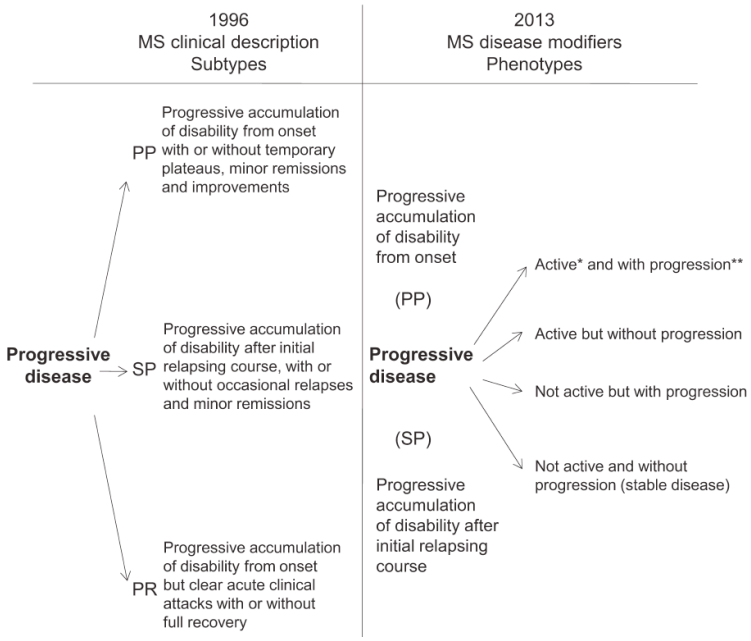


Figure 3: The 1996 vs 2013 multiple sclerosis phenotype descriptions for progressive disease

*Activity determined by clinical relapses assessed at least annually and/or MRI activity (contrast-enhancing lesions; new and unequivocally enlarging T2 lesions). **Progression measured by clinical evaluation, assessed at least annually. If assessments are not available, activity and progression are "indeterminate." MS = multiple sclerosis; PP = primary progressive; PR = progressive relapsing; SP = secondary progressive.

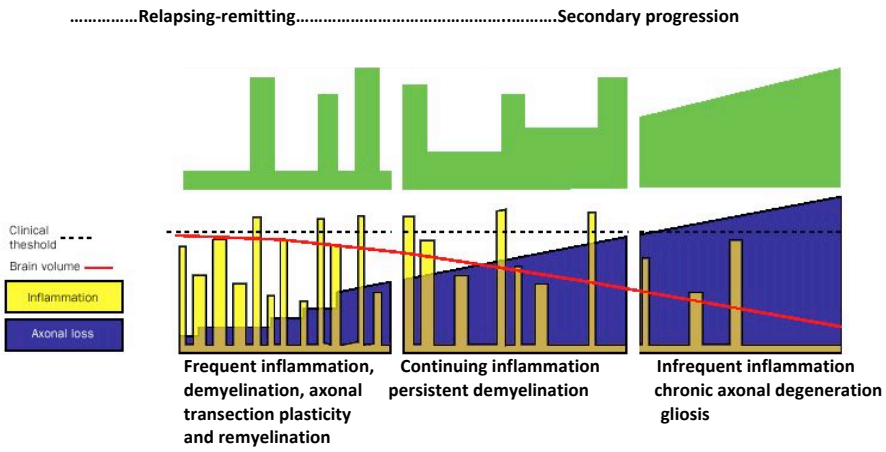


Figure 4: The course of multiple sclerosis. Initial acute inflammatory outbreaks can result in disability, which the patient may initially recover, but later becomes progressive. This correlates to a reduction of the inflammatory events and increased neuronal and axonal loss. This neuroaxonal loss is reflected in the loss of brain volume. The "*Relapsing remitting*" [1] and "*Secondary progressive MS*" [2] refers to clinical subtypes.

[1] The "RRMS" or "*Relapsing remitting MS*" corresponds to the initial clinical form characterized by the presence of outbreaks of relapsing and remitting events.

[2] The "SPMS" or "*Secondary progressive MS*" refers to a secondary progressive event. (Compston and Coles, 2002)

frequency as compared to the older immigrant population. African blacks had no MS whereas the rate was higher in the mixed race population (African and Caucasians). Since then numerous studies have been carried out on migrants in different countries. In UK in 1997, Dean and Elian studied 76 non-Caucasian immigrants from India, Pakistan and Bangladesh, who were born in the Indian subcontinent, East Africa, Fiji or Malaysia and had MS [Dean *et al.,* 1997]. A higher incidence than expected was observed in individuals arriving in the United Kingdom under the age of 15. Conversely, this did not apply to the 60 patients with MS born in the Caribbean.

In 1995 Gale *et al.* reviewed the literature on migrant studies, comparing rates of MS in migrant populations with those in the host country and in the country of origin. In their review they underscored the problems associated with interpreting and drawing conclusions from such studies. Migrating individuals rarely represent their original general population. They are often younger, healthier and of higher socioeconomic status. Therefore, the quality of the data produced by such studies may be poor and add little to our understanding of the genetics of MS but they focus on the importance of environmental factors [Gale *et al.,* 2004]. However, they supported studies that showed that individuals migrating from a high prevalence area to an area of low prevalence tend to adopt the low risk of new country. This is in contrast with those who migrate in the opposite direction who tend to retain the low risk of their country of origin [Marrie, 2004].

A correlation between the age of migration and the risk of MS has also been studied. Individuals migrating from a high risk area to a low risk area before the age of 15 acquire the risk of the new area, whereas individuals migrating after the age of 15 retain the risk of the area of their origin [Compston, 1997].

4.2. Racial factors

MS seldomly occurs among the indigenous black people of Africa. In 1987, the first account of a black patient with MS in South Africa was published. In 1994, seven cases of black patients in South Africa and five in Zimbabwe were diagnosed with MS [Dean *et al.,* 1994]. Six of the 12 patients had severe optic neuritis and subsequently became blind. Clinical features in these few black patients resembled those seen in oriental patients affected by MS rather than those seen in white South Africans or the black people of

North America or the Caribbean, indicating a real difference in the phenotype of the disease in the population [Dean *et al.,* 1994]. Approximately 6% of North American MS patients have an affected family member whereas in Asia and Chile, the figure is much lower. In 1979, Kurtzke *et al.* studied 5305 World War II and Korean conflict veterans who were diagnosed with MS and matched to controls on the basis of age and date of entry into military service. Their findings indicate that racial and genetic predispositions, as well as a geographically determined differential exposure to an environmental agent, are related to the risk of MS [Kurtzke *et al.,* 1979]. Analysis of Scandinavian co-affected sibling pairs carried out in 2004 by Oturai *et al.* on 136 Caucasian Scandinavian families suggest that disease course and age of onset are partly under genetic control. Furthermore, HLA-DR2 in probands of sibling pairs suggests importance for age of onset [Oturai *et al.,* 2004].

4.3. Age, gender and phenotype

MS affects mainly young adults with the peak age of onset of around 20-40 years. There is preponderance for female with a ratio of 2:1 irrespective of ethnicity, but this gender difference changes with the age at onset [Noseworthy *et al.,* 2000;

Compston *et al.,* 2002]. A very early onset of the disease has been described in children under the age of six with even higher female preponderance of 3F:M [Ruggieri *et al.,* 1999]. The youngest of them was a ten month old girl. For patients under thirty years of age, the disease is typically characterized by periods of inflammation (relapses) and variable periods of remission (complete or incomplete remission): a relapsing remitting pattern [Trojano *et al.,* 2002]. Fifteen to twenty five years after the first clinical onset, seventy percent of untreated patients enter the progressive phase known as secondary progressive MS [Noseworthy *et al.,* 2000]. In patients over fifty years of age, the disease often manifests as primary progresive MS, characterized by continuous progression of neurological disability from the initial onset of the disease, with only occasional plateau or temporary minor improvements [Koch *et al.,* 2007; Clark *et al.,* 1982].

4.4. Seasonal Variations

It has been suggested that environmental factors play an essential role in the aetiology and pathogenesis of MS. Several studies have demonstrated a relationship between seasons of the year and "outbreaks" of MS. Abella-Corral *et al.* followed up 31 patients over a period between 1997 and 2002 and calculated the monthly and quarterly rate of incidence of outbreaks [Abella-Corral *et al.,* 2005]. A higher incidence of outbreaks in the summer months (more in June) and a lower incidence in winter (less in December) were observed. This study indicated outbreaks of MS might be related to seasonal variations, with a higher number in the warmer months and fewer in the colder months. Eight hundred and thirty four patients with late onset MS residing in Washington and California were followed up over a 20 years period. It was found that disease worsen in patients exposed to hot climate [Clarck *et al.,* 1982]. Recent studies suggest that exposure to sunlight and a high level of Vitamin D in the serum reduces the risk of developing MS [Munger *et*

al., 2006]. Viral exposure, dietary fatty acids, solar ultraviolet radiation exposure, organic solvent exposure, and cigarette smoking are implicated in the pathogenesis of the disease. The risk of MS was 1.8-fold higher among tobacco smokers compared with those who had never smoked in one study [Riise *et al.,* 2003]. These data further highlight the necessity of advising patients with MS to quit smoking. In a small nested case-control study, presence of Epstein-Barr virus in plasma was associated with increased risk of MS [Wagner *et al.,* 2004]. Further studies are needed to explore the role of Epstein-Barr virus in MS susceptibility.

4.5. Genetic factors and prevalence of MS in Spain

Excess occurrence in North Europeans relative to indigenous populations from the same geographic location and familial aggregation (MS is 20-40 times more common in first-degree relatives, dropping off rapidly with the degree of relatedness) represent the factors supporting genetic effects. Monozygotic twin studies suggest that up to 25-30% of MS risk is genetically determined and the risk rapidly drops to 3-5% with dizygotic twins, supporting the complex susceptibility to MS [Ebers *et al.,* 2005].

Other than the well-defined human leukocyte antigen (HLA)-DRB1*1501-DQB1*0602 haplotype on chromosome 6p21, multiple genetic factors likely have small individual contributions to the etiology of MS [Sawcer S *et al.,* 2003]. Recent studies showed that HLA locus association is with HLA-DRB1 rather than the DO allele [Oksenberg *et al.,* 2004]. "There was also suggestive linkage with MS on chromosomes 5q33, 17q23, and 19p13 (Consortium IMSG 2005).

In Europe, the total estimated prevalence rate for the past three decades is 83 per 100 000 with higher rates in northern countries. In Spain the prevalence of the disease is approximately 70-80 per 100,000 with about 35,000 patients

according to the current estimates. Prevalence rates are higher for women with female: male ratio around 2.0. In northern part of Spain prevalence was 58 per 100.000 in 1997 [Tola *et al.,* 1999], 32 in 1996 in eastern Spain [Bufill *et al.,* 1995] and 43 in 1998 in central Spain [Benito-LeÅLon *et al.,* 1998]. The average annual incidence rate in Spain ranged from 2.2 per 100 000 in the period 1992–1996 [Modrego Pardo *et al.,* 1997] to 3.8 in the period 1994–1998 [Benito-LeÅLon *et al.,* 1998]. The estimated European mean annual MS incidence rate is 4.3 cases per 100 000.

5. Clinical Diagnosis

5.1. Imaging

Even though nuclear magnetic resonance (NMR) was developed as an imaging technique in the 1940s, imaging humans became possible in the 1970s with the use of large bore magnets. Shortly thereafter in 1981, the first inversion recovery scans were made of the brain of eight clinically definite MS patients. These scans demonstrated focal areas of abnormality around the ventricles. Since then and in the past 10-15 years, MRI scanning has played a pivotal part in the diagnosis and management of MS.

5.2. Cerebrospinal fluid

Cerebrospinal fluid (CSF) is the fluid that circulates around and within the brain and spinal cord. CSF provides a vehicle for removing waste products of cellular metabolism from the nervous system and is believed to be nutritive for both neurons and glial cells and to function as a transport system for biologically active substances such as releasing factors, hormones, neurotransmitters, and

metabolites. Sampling this fluid thus provides an index to substances active in the CNS and possibly those involved in MS pathology.

In 1925, Greenfield and Carmichael used CSF examination for the diagnosis of syphilis. It preceded neuroimaging and evoked potentials by decades as the first method of providing laboratory confirmation of a clinical diagnosis of MS. Since then, study of the CSF has played a vital role in the investigation of the aetiology and pathogenesis of MS, due to its direct contact with the CNS.

Immunoglobulins (Ig) are produced by plasma cells that arise from clones of B-lymphocytes. Kabat *et al.* in the 1950s showed that the proteins in the CSF of MS patients were different from those in the serum by using serum electophoresis techniques [Kabat *et al.,* 1950; Tiselius *et al.,* 1939]. Lowenthal *et al.* in the 1960s demonstrated the importance of the bands that are present in the Ig region of the CSF of MS patients in the diagnosis of MS [Lowenthal, 1960]. The elevated CSF IgG in comparison to the blood IgG is due to increased synthesis of IgG in the CNS, which is also disproportionately increased in comparison to the CSF albumin, this is highly characteristic of MS. In some patients IgM and IgA are also elevated. Oligoclonal bands (OCB) are found only in the CSF, and not in the serum, in 90% of patients diagnosed with MS [Freedman *et al.,* 2005]. Oligoclonal bands are produced by the overrepresentation of particular antibodies that can be visualized when CSF proteins are separated by gel electrophoresis where they appear as separate bands of protein on a gel matrix. Each of the bands contains a single type of antibody produced by a single clone of B cells. Oligoclonal bands are *typical* for the CSF of MS patients, but they are not *exclusive* to MS patients. For example, they are also found in the CSF of patients with other inflammatory status, such as viral brain infections. In MS, however, the particular antigens that elicit each antibody band are unknown [Thompson, 1995]. Oligoclonal IgM and IgA may also

be noted in CSF of MS patients, but to a lesser extent than IgG [Keir *et al.,* 1982; Grimaldi *et al.,* 1985].In our study, we have classified and named inflammatory MS into *"IgM+/-"* and *"IgM+/+ subtype".* Today the vast majority of laboratories determine the presence of OCB in the CSF, and they are a part of the diagnosis criteria of MS (McDonald *et al.,* 2001 and 2005), but as we will see late the presence of OCGB cannot be used as a prognosis factor, because nearly 95% of patients have these antibodies, and the only prognosis value is for the 5% of patients without OCGB that have a better prognosis. So, the fact that nearly all MS patients have OCGB is a useful tool for diagnosis but not for prognosis. At time the presence of OCGB inform us that the activation of the humoral system restricted to the CNS, that it is important since a pathogenic point of view.

On the first description of the presence of OCMB in the CSF by Sharief *et al.* in 1991, a worse prognosis in this subtype of patients is pointed in terms of conversion to clinical definitive MS (second relapses) since a clinically isolated syndrome. This observations have been confirmed by several authors, which have studied the main clinical prognosis factors of MS: time to EDSS of 4.0, time to SPMS diagnosis, time to a second relapses, and radiologic prognosis factors as basal T2 lesion volume, increases in T2LV and early brain atrophy, and at last patients which OCMB have a poor response to actual immunomodulatory drugs. For these reasons, at day we consider that relapsing-remitting MS in which OCGB plus OCMB comprise a subgroup of RRMS with a more aggressive course since the beginning of the diseases, it is of interest to characterize IgM+/+ subtype of MS.

The medullary MS form was defined as form of MS characterized by relapses and the rapid apparition of the secondary progression. The medullar MRI was affected in always cases, some of them with a longitudinal extensive transverse myelitis like in NMO patients, but other with lesions restricted to two cervical spinal cord

vertebral bodies, and with typical brain lesions of MS. In addition, the clinical relapses were not limited to the optic nerve and spinal cord a difference to NMO patients.

Normally, very few T lymphocytes are present in the brain, making it almost undetectable by immunohistochemical methods. In MS, these cells increase in number and are readily detectable in the brain parenchyma. All the CSF lymphocytes are ultimately derived from the peripheral circulation via transmigration across the BBB. However, the distribution of lymphocytes subsets in the CSF is different from that of peripheral blood. In the CSF, atleast 80% of T lymphocytes express CD3 compared with only 65% T lymphocytes in peripheral blood. The ratio of CD4+ (T helper) to CD8+ (T suppressor) remains at 2:1 as it exists in peripheral blood. CSF derived T lymphocytes demonstrate enhanced adhesion to vascular endothelial cells, they also express a greater number of interleukin-2 (IL-2) cell surface receptors and have increased mRNA and DNA synthesis, suggesting cellular activation.

6. Diagnostic criteria and treatment

The diagnosis of MS is based on both clinical parameters, such as medical history and neurological examination, and paraclinical parameters such as MRI, CSF oligoclonal banding, and evoked potentials. The general diagnostic criteria, established in 1965, state that a diagnosis of “clinically definite” MS requires clinical evidence of two or more white matter lesions on at least two occasions [Schumacher *et al.,* 1965]. In 1983, these criteria were expanded by Poser *et al.* to include the use of paraclinical parameters. The most recent criteria for MS diagnosis is McDonald's criteria [McDonald *et al.,* 2001, Polman *et al.,* 2005], which

incorporated direct detection of lesions through magnetic resonance imaging (MRI).

Disease-modifying treatments such as interferon beta-1a and 1b, natalizumab, glatiramer acetate, mitoxantrone, fingolimod and teriflunomide have been approved by regulatory agencies of different countries [US FDA approved 2012]. In a clinical trial, the relapse rate for patients using Aubagio (teriflunomide) was about 30 percent lower than the rate for those taking a placebo. Most of these drugs are approved only for the relapsing-remitting course. All of these medications are modestly effective at decreasing the number of attacks in RRMS while the capacity of interferons and glatiramer acetate is more controversial. Studies of their long-term effects are still inadequate. Comparisons between immunomodulators (all but mitoxantrone) show that the most effective is natalizumab, both in terms of relapse rate reduction and halting disability progression [Johnson, 2007]. Mitoxantrone may be the most effective of them all; however, it is generally not considered as a long-term therapy, as its use may result in severe secondary effects [Comi, 2009]. The earliest clinical presentation of RRMS is the clinically isolated syndrome (CIS). Treatment with interferons during an initial attack can decrease the chance that a person will develop clinical MS [Compston and Coles, 2008].

Treatment of progressive MS is cumbersome than relapsing-remitting MS. Mitoxantrone has shown positive effects in those with secondary progressive and progressive relapsing courses. It is moderately effective in reducing the progression of the disease and the frequency of relapses in short-term follow-up [Martinelli Boneschi *et al.,* 2005]. No treatment has been proven to modify the course of primary progressive MS [Leary and Thompson, 2005].

7. Pathogenesis of MS

7.1. Role of autoimmunity

Although there is no direct evidence, MS is commonly considered to be an autoimmune disease. It is believed that patient's own immune system attacks the nervous system, possibly as a result of exposure to a molecule with a similar structure to one of its own [Compston *et al.,* 2002]. This is called molecular mimicry. Myelin proteins (myelin basic protein (MBP), myelin oligodendrocyte protein (MOG), proteolipid protein (PLP)) are obvious candidates for autoantigens in MS, but their role is not yet understood.

7.2. Activation of immune response

Accumulating evidence suggests that only activated T cells can cross the normally impermeable blood brain barrier (BBB) and gain access to CNS [Hickey *et al.,* 1991]. However, the mechanism and location where the activation of autoreactive T cells takes place in MS patients is not apparent. It has been shown in mice that T cells can be activated in peripheral lymphoid organs by myelin presenting antigen-presenting cells (APC), specifically dendritic cells (DC), which are able to migrate out of the CNS [Karman *et al.,* 2004]. Another theory is that some viral or bacterial antigens with similarity to myelin antigens activate myelin-specific T cells [Wucherpfennig and Strominger, 1995]. However, due to lack of convincing evidence of association between MS and any specific pathogen, this molecular mimicry theory remains controversial [Libbey *et al.,* 2007]. Finally, although it seems to be the general view that T cells are activated prior to entry into CNS, it has been shown in experimental autoimmune encephalomyelitis (EAE), an animal

model of MS, that naïve lymphocytes are also able to penetrate the CNS if it is already inflamed [McMahon *et al.,* 2005].

Once lymphocytes have penetrated into the CNS, autoreative T cells are thought to become re-activated upon encountering endogenous myelin antigens presented to them by APCs. Re-activated T cells begin to produce cytokines and chemokines which activate CNS cells such as microglia and astrocytes, and promote recruitment of other immune cells from peripheral blood, including CD8+ T cells, B cells, mast cells, monocytes and macrophages [Sospedra and Martin, 2005] (Figure 3). These cells further produce various proinflammatory molecules, complement proteins, nitrogen and oxygen radicals, although the relevance of these molecules in MS pathogenesis is not clear in detail [Sospedra and Martin, 2005].

7.3. Demyelination

MS is defined as a demyelinating disease because the myelin sheaths and their parent cells, the oligodendrocytes, are major targets of immune-mediated damage but the direct cause of demyelination in MS is still not apparent [Conlon *et al.,* 1999; Noseworthy, 1999]. Activation of the complement system, which is a central mechanism for removing pathogens, may be responsible for myelin destruction as suggested by presense of complement proteins in demyelinated MS lesions [Compston *et al.,* 1989; Storch *et al.,* 1998; Ingram *et al.,* 2009]. Furthermore nitric oxide (NO) has been shown to be toxic to myelin producing cells, oligodendrocytes, *in vitro* [Mitrovic *et al.,* 1995]. Nevertheless, it has been proposed that as myelin gets damaged, additional myelin peptides become exposed and autoreactive T cells recognizing these particular epitopes (i.e. parts of antigens recognized by immune cells) are activated. This epitope spreading phenomena, where an initial response to one epitope results in tissue destruction and thereby in release of new epitopes

and activation of cells recognizing these epitopes, could be responsible for chronicity of the inflammatory reaction in MS [Tuohy and Kinkel, 2000].

7.4 Mechanism of axonal injury

The presence of axonal degeneration in MS was recognized in the early descriptions of this disease by Charcot [Charcot, 1868]. Axonal transection might be the structural basis for acquisition of permanent neurological deficits, making it an especially important part of the pathology of MS [Davie *et al.,* 1995; Lee *et al.,* 2000; Lee *et al.,* 2000]. In recent years it has become possible to measure axonal injury effectively. Using amyloid precursor protein (APP) as a marker for acute axonal injury a study demonstrated that massive axonal damage occurs during the stage of active demyelination in fresh lesions [Ferguson *et al.,* 1997]. Similar conclusions were obtained by another study that reported axonal transections in MS lesions [Trapp *et al.,* 1998]. In addition, these findings corroborated earlier observations that acute axonal injury was associated with the degree of macrophage infiltration in the lesions and that macrophages were closely attached to damaged axons [Ferguson *et al.,* 1997; Trapp *et al.,* 1998; Fraenkel *et al.,* 1913]. Kornek *et al.* later demonstrated the correlation between the quantity of acute axonal injury and lesional stages.

Two different phases of axonal damage have to be considered when we look at the molecular mechanisms of axonal disintegration: the triggers that induce axonal damage and the downstream pathways of axonal disintegration (Figure 6). Although the triggers of axonal injury seem to be specific for inflammatory conditions, such as MS, the downstream mechanisms of axonal disintegration appear similar in many different pathological conditions of the nervous system, including inflammation, stroke, or trauma.

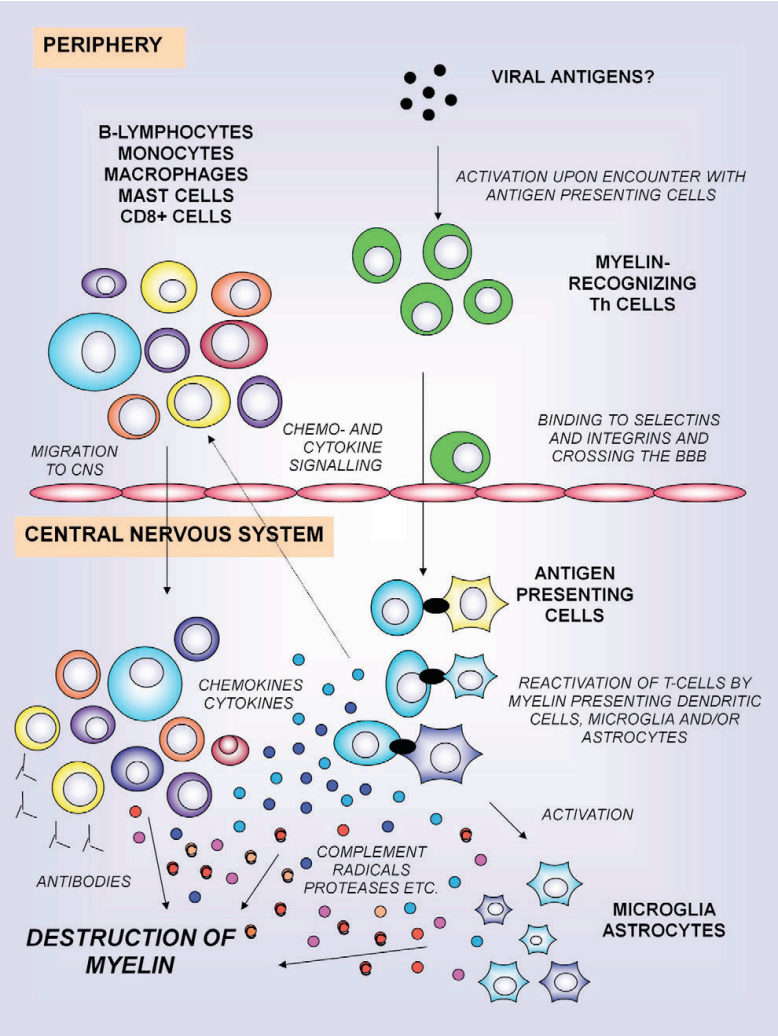
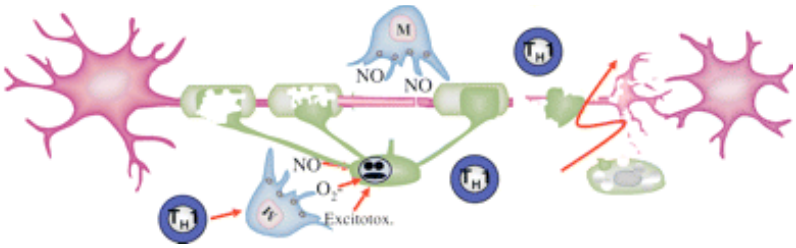


Figure 5: Hypothetic scenario of events leading to demyelination in MS. Based on text and figures in a review by Sospedra and Martin (2005)

It has been shown that T cell mediated cytotoxicity directly can induce axonal injury [Neumann *et al.,* 2002]. Axons can be transsected by class I MHC restricted T lymphocytes *in vitro* in an antigen dependent immunological reaction [Medana *et al.,* 2001]. Highly activated cytotoxic T cells are often seen attached to demyelinated axons in lesions of acute MS. In addition, activated macrophages or microglia cells are observed to interact with axons in the course of axonal injury. These cells are consistently found in close proximity with degenerating axons. Many of their toxic effector molecules may lead to axonal injury, although a direct detrimental effect has so far only been shown for proteases and reactive nitrogen species. In particular, nitric oxide (NO) intermediates are particularly attractive candidates. At low concentration they may slowdown the action potential and conduction ability of electrical impulses in the axons. At higher concentrations when axons are electrically active, NO derivatives may result in irreversible destruction of axons [Smith *et al.,* 2001]. This may in part be fulfilled by the blockade of mitochondrial function and the disturbance of energy metabolism, which can be induced by NO radicals [Bolanos *et al.,* 1997]. As nitric oxide intermediates can also contribute to oligodendrocyte damage, these molecules may be important candidates in the pathogenesis of tissue damage in MS [Smith *et al.,* 2002]. In addition to toxins produced by T cells and macrophages, specific antibodies may be involved in the initiation of axonal injury.

The trigger activates several downstream events, which results in the final dissolution of the axon. Axoplasmic membrane disturbance and energy failure leads to uncontrolled ion influx into the axoplasm. Accumulation of sodium ions inside axons is then counteracted by a reverse operation of the sodium/calcium exchanger (i.e. sodium efflux and calcium influx), resulting in excess intraaxonal Ca++. This activates Ca++ dependent proteases, which may degrade cytoskeletal proteins and, thus, lead to neuronal damage.



Triggers of axonal injury

- Cytotoxic T cells
  - Granzyme/perforin
  - Fas/FasL
- Macophages/microglia
  - Reactive oxygen/nitrogen species
  - Proteases
  - Excitotoxins

Axonal degeneration

- Memberane disturbance
- Sodium influx
- Energy failure
- Calcium influx
- Activation of proteases
- Dissolution of cytoskeleton

Figure 6: Triggers of axonal injury and axonal degeneration (Adapted from review by Gold *et al.,* 2006)

In nutshell, factors associated with axonal injury include cytokines, nitric oxide, proteases, antibodies, superoxides, $CD8^+$ T lymphocytes, and glutamate excitotoxicity [Werner *et al.,* 2001].

8. Remyelination and Repair

Regulatory T-lymphocytes (T_{reg}) may be involved in recovery from relapses in MS. The T_{reg} are specialized T lymphocytes which are able to compensate for inflammation. Evidence suggests that rearrangement of sodium channels may also participate in recovery stage of MS [Waxman, 2006].

Remyelination is the spontaneous repair process where new myelin sheaths are generated around demyelinated axons. 40% of MS lesions manifest some degree of remyelination [Barkhof, 2003]. It could result in partial restoration of the conductive properties of demyelinated axons [Smith, 1979; Jeffery, 1997]. The degree of remyelination depends on the mechanism and stage of progression of the lesion [Lucchinetti *et al.,* 2000], although remyelination has been seen in patients dying at an old age [Patrikios, 2006]. It also depends on whether the environment within the plaque favors remyelination [Franklin, 2002]. Also there is a positive correlation between remyelination and the number of surviving oligodendrocytes and a negative one with the presense of macrophages in the lesion [Lucchinetti *et al.,* 1999].

9. Neuromyelitis optica (NMO)

Neuromyelitis optica (NMO) was first described by Allbutt (1870) who reported a patient with a "*sympathetic disorder of the eye*" after an acute episode of myelitis. The disease was further characterized by Devic (1894), which is now also known as Devic's disease. NMO is described as an idiopathic, severe, inflammatory demyelinating disease that preferentially affects the optic nerves and spinal cord and typically spares the brain. Due to many common features, it was assumed for many years that NMO was simply a variant of MS. Infact, it was reported in Japan that an optic spinal variant of MS, which accounts for 15-40% of all MS cases shares many common features with NMO [Misu *et al.,* 2002; Kira *et al.,* 2003]. Therefore the disease was always mistaken for MS early in the disease course. However, recent evidence suggests that NMO is a totally distinct disease and requires a different treatment therapy. The identification of NMO-IgG antibodies found specifically in the sera of NMO patients clearly distinguishes the disease from MS. These antibodies have also been identified in the sera of two

related neurologic conditions, bilateral optic neuritis (ON), and longitudinal extensive transverse myelitis (LETM) that are generally considered to lie within the NMO spectrum of diseases. The antigenic target of NMO-IgG is aquaporin-4 (AQP4) protein expressed by astrocytes suggesting that NMO may be considered a novel autoimmune channelopathy. AQP4 acts as a channel that regulates water entry into and out of the nerve cells in the CNS where it is preferentially localized within astrocytic end feet at the blood-brain barrier. Knockout studies in mice demonstrate that AQP-4 is involved in maintaining the integrity of the blood brain barrier and loss of this protein disrupts the integrity of the blood brain barrier resulting in free translocation of molecules into the CNS [Zhou *et al.*, 2008]. Table 1. illustrates the key features exhibited by NMO and MS.

Wingerchuck *et al.* (2007) at the Mayo Clinic proposed a model that features the role of anti-AQP4 antibodies in the pathogenesis of NMO. They indicated that the pathogenic anti-AQP4 antibody binds to the extracellular component of the AQP4 protein and induces a reversible internalization of the AQP4-IgG complex. AQP4 expression is elevated in many conditions of brain inflammation and neurological diseases such as stroke, Alzheimer's, spongiform encephalopathy and others which do not have an autoimmune component. Perhaps the significance of AQP4 for regulating brain edema suggests that when its function is impaired and the blood brain barrier (BBB) is compromised, immunopathology ensues.

10. Overview of housekeeping genes

Housekeeping genes (HK) are endogenous controls that code for proteins required for the primary function of a cell, hence their expression should be constant under all conditions. However, recent research has reported that their expression may

Table 1. The key features of NMO and MS. Although MS and NMO are inflammatory demyelinating diseases of the CNS, comparison of the key features of both diseases reveals significant differences in their pathology and aetiology.

	Neuromyelitis óptica	**Multiple Sclerosis**
Definition	Optic Neuritis and transverse myelitis	Any white matter tract involvement
Course	Relapsing (~70%) Monophasic (~30%)	Relapsing (~85%)
Median age of onset	Forties	Twenties
Gender ratio (F:M)	5:1 (relapsing) 1:1 (monophasic)	2:1
MRI Brain	Usually normal	Periventricular white matter lesions
MRI Spinal Cord	Longitudinally extensive central lesions	Multiple small peripheral lesions
Oligoclonal Bands in CSF	Usually absent (~30%)	Usually present (~90%)
NMO IgG	Seropositive (>70%)	Seronegative

not necessarily be stable in all cells/tissues under all conditions. A gene may show stable expression in one condition whereas unstable in another. Invariable expression of the so-called housekeeping genes has been observed with cellular development [Maie Dawoud *et al.,* 2005] and under distinct experimental conditions [Deindl *et al.,* 2002; Glare *et al.,* 2002; Hamalainen *et al.,* 2001; Radonic *et al.,* 2004; Zhong *et al.,* 1999; Toegel *et al.,* 2007; Torres *et al.,* 2003; Gubern *et al.,* 2009; Tricarico *et al.,* 2002]. Therefore it is indispensible to pre-validate the expression stability of reference genes to accurately normalize the gene expression data. In addition, more than one stably expressed gene is recommended for precise normalization procedure [Ohl *et al.,* 2005; Zhong *et al.,* 1999; Hamalainen *et al.,* 2001; Vandesompele *et al.,* 2002].

10.1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

GAPDH has historically been viewed as a housekeeping cytosol protein and plays an important role in ATP production. Recent studies have enhanced our understanding of GAPDH function and distribution. Gapdh is found in several intracellular locations and has diverse activities independent of its traditional role in glycolysis. As a membrane protein, GAPDH functions in endocytosis; in the cytoplasm, it is involved in energy generation, polymerization of tubulin into microtubules, and the control of protein synthesis in the endoplasmic reticulum; whereas, in the nucleus, it is involved in nuclear tRNA export, DNA replication, and DNA repair.

Since both GAPDH and β-ACTIN are known to perform the basic functions of a cell, they are presumed to express at stable levels. Therefore, they are employed as common internal controls in most of the laboratories. However, several lines of evidence show that their rate of transcription is influenced by a variety of factors such as epidermal growth factor, transforming growth factor-β and platelet-derived growth factor while constitutively expressed [Leof *et al.,* 1986; Keski-Oja *et al.,* 1988; Elder *et al.,* 1984]. Therefore, their expression may not necessarily be constant in all conditions. Furthermore, Gapdh is implicated in non-metabolic processes independent of its metabolic function such as transcription activation, vesicle transport from endoplasmic reticulum to Golgi apparatus and polymerization of tubulin into microtubules [Tarze *et al.,* 2007; Zheng *et al.,* 2003; Hara *et al.,* 2005; Tisdale *et al.,* 2007].

The realization that these reference genes may not necessarily express in stable manner in cellular development or under all experimental conditions has led to

their pre-validation for their expression stability. Their evaluation prior to normalization is a critical step to obtain unbiased gene expression data.

11. Carbohydrate metabolism in CNS

The fate of dietary components after digestion and absorption constitutes metabolism—the metabolic pathways taken by individual biomolecules, their interrelationships, and the mechanisms that regulate the flow of metabolites through the pathways. Metabolic pathways are composed of three categories: (1) Anabolic pathways are those involved in the synthesis of compounds. Protein synthesis is such a pathway, as is the synthesis of fuel reserves of triacylglycerol and glycogen. Anabolic pathways are endergonic. (2) Catabolic pathways are involved in the breakdown of larger molecules, commonly involving oxidative reactions; they are exergonic, producing reducing equivalents and, mainly via the respiratory chain, ATP. (3) Amphibolic pathways act as link between the anabolic and catabolic pathways, eg, citric acid cycle.

The products of digestion of dietary carbohydrate, lipid, and protein mainly glucose, fatty acids and glycerol, and amino acids, are metabolized to a common product, acetyl-CoA, which is then oxidized by the citric acid cycle. In any tissue, glucose can follow various metabolic pathways; in the brain, glucose is almost entirely oxidized to CO_2 and water through its sequential processing by glycolysis, the tricarboxylic acid (TCA) cycle and the associated oxidative phosphorylation, which yield on a molecular basis, 38 ATP per glucose.

11.1. Glycolysis

Glycolysis is the pathway for the breakdown of glucose into pyruvate. All ten enzymes are in the cytosol, and all ten intermediates are phosphorylated

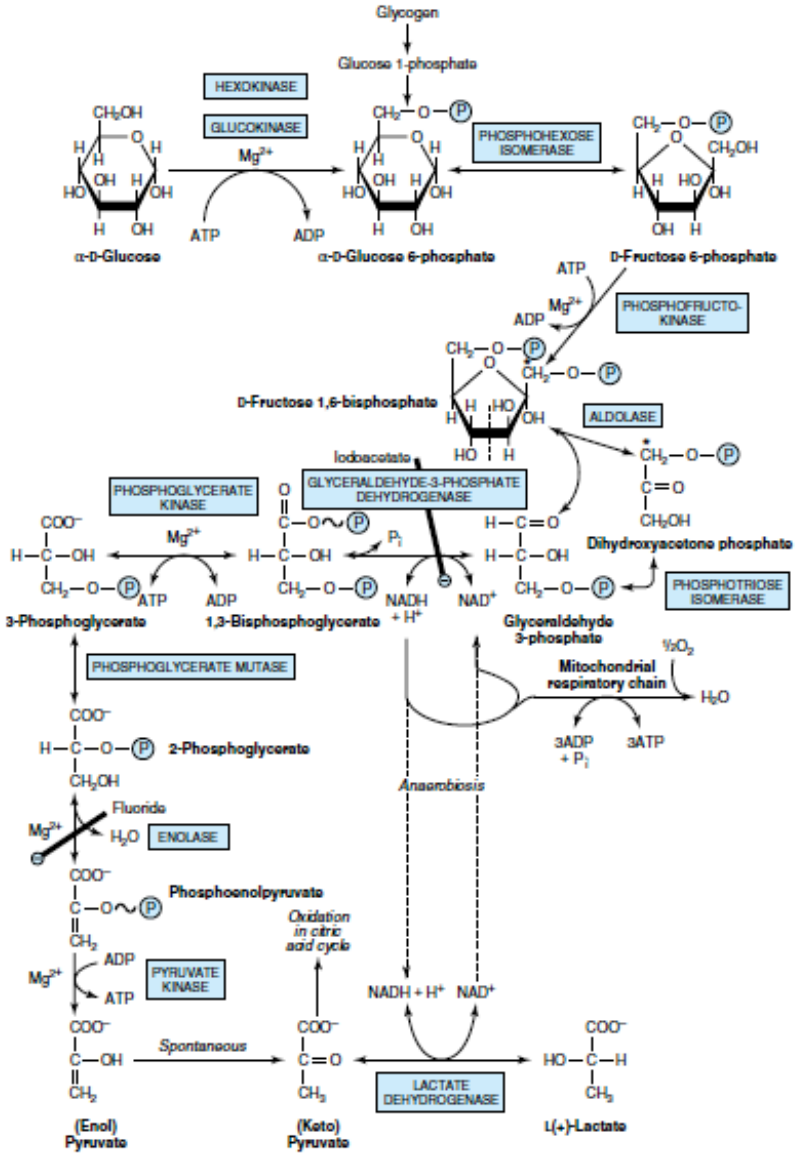
compounds of three or six carbons. In the preparatory phase of glycolysis, ATP is invested to convert glucose to fructose 1,6-biphosphate. The bond between C-3 and C-4 is then broken to yield two molecules of triose phosphate. In the payoff phase, each of the two molecules of glyceraldehyde 3-phosphate derived from glucose undergoes oxidation at C-1; the energy of this oxidation reaction is conserved in the formation of one NADH and two ATP per triose phosphate oxidized. The net equation for the overall process is

$$\text{Glucose} + 2NAD^+ + 2ADP + 2P_i \rightarrow 2 \text{ pyruvate} + 2NADH + 2H^+ + 2ATP + 2H_2O$$

Glycolysis is tightly regulated in coordination with other energy-yielding pathways to assure a steady supply of ATP. Hexokinase, PFK-1, and pyruvate kinase are all subject to allosteric regulation that controls the flow of carbon through the pathway and maintains constant levels of metabolic intermediates. The reactions of glycolysis and an interactive glycolytic pathway are shown in Figure 7.

11.1.1. Glycolytic enzymes

Metabolism is a vital process required for the existence of life. Cellular needs and conditions vary from cell to cell and change within individual cells over time. Therefore, the reactions of metabolism must be finely regulated to maintain a constant set of conditions within cells, a condition called homeostasis. Enzymes ultimately determine which chemical reactions a cell can carry out and the rate at which they can proceed. These chemical reactions determine a cell's function. A cell's functions are usually defined by its chemical reactions, so enzymes generally determine a cell's function as well. By lowering the activation energy of a chemical reaction, enzymes promote chemical reactions that are specific to the cell's function. Metabolic regulation also allows organisms to respond to signals and interact actively with their environments. Two closely linked concepts are



7: The pathway of glycolysis. (Adapted from textbook "*Biochemistry"* from Harper)

for understanding how metabolic pathways are controlled. Firstly, the regulation of an enzyme in a pathway is how its activity is increased and decreased in response to signals. Secondly, the control exerted by this enzyme is the effect that these changes in its activity have on the overall rate of the pathway (the flux through the pathway). For example, an enzyme may show large changes in activity (i.e. it is highly regulated) but if these changes have little effect on the flux of a metabolic pathway, then this enzyme is not involved in the control of the pathway.

Enzymes catalyzing metabolic reactions are regulated for the purpose of increased efficiency which is crucial for the evolution of living organisms. There are several ways by which metabolic flux can be regulated including allosteric regulation, changing enzyme or substrate concentration, compartmentalization, covalent modification, changing pH and specialization of organelles [Srere and Mathews, 1990; Mendes *et al.,* 1995; Saks *et al.,* 2008]. In addition, glycolytic enzymes form localized complexes and play an important role in allowing intricate regulatory control [Clarke *et al.,* 1975; Kurganov *et al.,* 1985]. Other than this, a glycolytic complex is involved in (i) increasing solvation capacity with the cytosol and (ii) channeling of substrates. The channeling promoted in a complex would allow a kinetic advantage [Cascante *et al.,* 1994] in addition to preserving the cytosol solvation capacity by limiting substrate diffusion.

This putative glycolytic complex, although in line with the cell's propensity for organization and consistent with considerable circumstantial evidence, has evaded direct experimental support [Boiteux and Hess, 1981; Srere, 1987] and is subject to controversial interpretations [Brooks and Storey, 1991]. It is seen that a larger number of subunits of glycolytic enzymes are associated with more intricate control in Saccharomyces cerevisiae [Boiteux and Hess, 1981]. That is, an increase in the number of subunits allows for greater regulatory control.

Glycolytic enzymes have been identified to be involved in a variety of non-glycolytic functions, even extracellularly [Gomez-Arreaza *et al.,* 2014]. GAPDH is a well-researched example [Zaffagnini *et al.,* 2013]. GAPDH has been implicated in regulation of gene expression [Sirover, 2005], apoptosis [Hara *et al.,* 2005; Sen *et al.,* 2008], and even playing a role as a cell surface receptor [Rawat *et al.,* 2012]. Furthermore, complexes may serve more than one function and that dysregulation at this level may be behind many pathologies [Sriram *et al.,* 2005; Jeffery, 2011]. It has been suggested that glycolytic enzymes have stabilizing effects on cytoskeletal elements such as actin and tubulin.

The fundamental importance of understanding enzymatic complexes, especially those involving metabolism, is widely recognized. Microcompartmentation, either due to membrane-restriction or by clusters of enzymes interacting with each other to localize their activity—such as in a complex—may even have further implications or incorporate functions that are currently unrecognized or not fully understood.

11.1.2. A new hypothesis on glycolytic enzyme complexes

Glycolytic complex was first proposed by Kurganov *et al.,* 1985 and its formation, excluding only hexokinase, was proposed to take place in three stages (Please see Menard *et al.,* 2014 for details). It was postulated that once PFK is bound to a support (e.g., F-actin), PK and GPI would bind; followed by aldolase (ALD), GAPDH, LDH; and finally, by PGK, PGM, ENO, TPI and glycerol-3-phosphate dehydrogenase (GPDH). However, support for "Kurganov's complex" gradually abraded in light of further studies [Harris and Winzor, 1989].

Table 2 shows the glycolytic enzymes from PFK to PK, indicating their reported

relationships with one another, noting by symbols whether there is recent (>1990) *in vitro, in vivo*, or *in silico* data suggesting direct interations between them or with F- actin. Reported propensities for self-aggregation have been omitted. Taking into consideration whether or not these interactions involve activation or deactivation of enzymatic activity, or stabilization, it is hypothesized that the complete, active enzyme complex consists of three subcomplexes. The summary table leaves out HXK and PGI due to a lack of data supporting interaction with other glycolytic enzymes. The table also excludes results from yeast. Both *in vitro* and *in silico* studies imply that not all glycolytic enzymes of S. cerevisiae (a widely used model) possess binding profiles comparable to those from mammalian systems. The case against PGI is not as strong as that against HXK, but is still substantial.

11.1.2.1. Glycolytic Subcomplex-I (GlySCx1): PFK, ALD, and GAPDH

The first of the three subcomplexes incorporates two well-studied glycolytic enzymes in complex formation: ALD and GAPDH. There have been multiple *in vitro* studies supporting GAPDH binding to actin [Woztera *et al.,* 2012; Schmitz and Bereiter, 2002] and ALD binding to actin and/or GAPDH [Parra and Pette, 1995; Woztera *et al.,* 2012; Melnikow *et al.,* 2013]. The general consensus is that ALD binds F-actin predominantly in an inactive state and close to its active site. Upon substrate binding, its conformation becomes unfavorable for actin binding and it dissociates [Wang *et al.,* 1996; Schindler *et al.,* 2001]. Similar to ALD, actin-bound GAPDH [Cao *et al.,* 1999] and tubulin-bound GAPDH [Holtgrawe *et al.,* 2005] has been suggested to be inactive. There is no indication whether or not activity levels of GAPDH are affected when GAPDH is bound to ALD.

The third enzyme of this first subcomplex is PFK, one of the most highly regulated

enzymes of the glycolytic pathway. Studies have suggested that PFK can bind GAPDH [Commichau *et al.,* 2009] and ALD [Srere and Mathews, 1990; Marcondes *et al.,* 2011; Rais *et al.,* 2000], although the evidence for this binding is not as strong as the evidence supporting the ALD-GAPDH interaction. Interestingly, PFK dimers have been found to be able to be bound and activated by ALD when PFK dimers would otherwise be inactive [Marcondes *et al.,* 2011]. This introduces the interesting possibility that, when involved in a complex, PFK may be in a dimer form, rather than a tetramer.

Table 2: Interactions of glycolytic enzymes from post-1990s literature, excluding S. cerevisiae. A green circle is indicative of modelling evidence, a blue square is indicative of *in vitro* studies and an orange diamond is indicative of *in vivo* bacterial studies [68]. Color intensity is indicative of the evidence supporting an interaction: light intensity, one to two published studies supporting an interaction; dark intensity, three or more published studies supporting an interaction. Self-interactions are not shown.

Ligands	PFK	ALD	TPI	GAPDH	PGK	PGM	ENO	PK	F-actin
PFK		●■	·	◆	·	■◆	■◆	·	■
ALD	●■	·	·	●■	·	·	■	·	●■
TPI		·	·	·	◆	·	·	·	·
GAPDH	◆	●■	·	·	◆	·	·	·	●■
PGK	·	·	◆	◆	·	·	◆	·	·
PGM	■◆	·	·	·	·	·	●■◆	·	·
ENO	■◆	■	·	·	◆	●■◆	·	■◆	·
PK		·	·	·	·	·	■◆	·	■

11.1.2.2. Glycolytic Subcomplex-2 (GlySCx2): TPI & PGK

The second glycolytic subcomplex (GlySCx2) is proposed to be composed of TPI and

PGK. Evidence for the binding between TPI and PGK comes from cross-linking studies in bacteria [Commichau *et al.,* 2009], in Drosophila flight muscles [Sullivan *et al.,* 2003] at the M band and Z disc. However, TPI is known not to bind F-actin from *in silico* modeling [Forlemu *et al.,* 2011; Lowe *et al.,* 2003] and *in vitro* studies [Waingeh *et al.,* 2006] and there is no evidence whether PGK does or does not bind actin.

The only support of TPI binding any other glycolytic enzyme is for PGK (Table 2). In the case of PGK, however, there appears equally weighted support for PGK binding TPI, GAPDH, and ENO.

11.1.2.3. Glycolytic Subcomplex-3 (GlySCx3): PGM, ENO and PK

The third proposed subcomplex is composed of PGM, ENO and PK and possesses some unique characteristics that differentiate this complex from the other two, with a greater emphasis being on tubulin or troponin binding over actin binding. There is little recent data suggestive of F-actin binding to any of GlySCx3 enzymes. There have been *in vitro* studies suggesting that ENO and PGM specifically do not bind actin [Waingeh *et al.,* 2006; Merkulova *et al.,* 1997] and limited recent studies of PK involving the binding of F-actin outside of S. cerevisiae [Puchulu-Campanella *et al.,* 2013]. Both PK and PGM have been found to bind ENO [Romagnoli *et al.,* 2010]. These three enzymes appear to bind to a common site in tubulin and in troponin [Lehotzky *et al.,* 1993; Mitchell *et al.,* 2005; Volker and Knull, 1997; Volker *et al.,* 1995; Keller *et al.,* 2007]. In the GlySCx3, ENO serves as the enzyme that links PGM and PK.

11.1.2.4. Formation of the Glycolytic Complex

These three putative subcomplexes appear feasible based on the studies that have been published; nevertheless, the subcomplexes may be interacting in ratios other than 1:1:1. In fact, this seems extremely likely. PFK first binds F-actin, possibly displacing an inactive form of ALD. After this, ALD binds PFK and GAPDH binds ALD. GAPDH is on the outskirts of this trifecta and there is the possibility of there being two GAPDH involved, either by its own natural capacity for self-association or by binding PFK [Commichau *et al.,* 2009].

Here, GlySCx1 is proposed to be responsible for binding and localization of GlySCx2 to the thin filament through GAPDH-PGK interaction. Interaction between PGK and GAPDH is supported by crosslinking studies [Commichau *et al.,* 2009]. Depending on the orientation of PGK binding GAPDH, the orientation could permit TPI, though not binding GAPDH directly, to have a localized benefit for the transfer of GAP to GAPDH. In such a case, it would follow that two GlySCx2 could bind two separate GAPDHs.

Figure 8 shows a two-dimensional interaction map of two proposed formats for a glycolytic enzyme complex consisting of the three proposed subcomplexes. In both possibilities, all three subcomplexes are in their proposed binding arrangements amongst themselves and PFK is anchored to F-actin. There is one GlySCx1 per every two GlySCx2s and two GlySCx3s in the final complex. In the first format (Figure 6a), PFK is additionally binding GAPDH and GlySCx3 is not directly bound to either GlySCx1 or 2 but is bound to either actin or troponin components of the thin filament by PK. In the second format (Figure 6b), ENO is linking GlySCx3 to PFK and both GAPDH enzymes are still linking two GlySCx2s to the GlySCx1. This second format is an example of how GlySCx3 may be bound to GlySCx1 by ENO, but this

connection, for instance, could also be formed with PGM. The positions of these enzymes promote channeling and an important note is the vicinity of PGK with PGM and TPI near ALD and GAPDH. TPI and PGK are not sequential in the glycolytic sequence but are shown binding each other here such that both of them are brought into close enough proximity for channeling to be possible.

The research to date has not been sufficiently consistent to establish, unequivocally, the structure of a glycolytic complex. Part of the problem is that the experiments have been subject to a wide variety of conditions. The lack of solid evidence highlights the need for progress in this area. Most published reports involving the interaction of the glycolytic enzymes with each other or bound to F-actin or troponin supports the idea that direct contact takes place among them, even if the reported evidence is not concrete. Not all of the glycolytic enzymes, however, have been researched to the same extent or have been reconfirmed in the same model organism. In muscle, many enzyme–enzyme interactions have not been examined or have been looked at only indirectly. Physical associations among glycolytic enzymes are generally not supported by protein interaction databases that include high-throughput experiments, such as MINT, and STRING. There is a dire need to examine the complex in an *in vivo* context to fully develop an accurate picture. Such an understanding is critical to the way we think about metabolic activity, regulation and its role in pathologies.

11.2. Citric Acid Cycle

Pyruvate from glycolysis enters into the citric acid cycle. This cycle occurs in the mitochondria of the cell in aerobic conditions. The pyruvate loses a carbon dioxide group, forming acetyl-CoA, the compound that forms a link for many other pathways and helps build other compounds. The citric acid cycle pathway consists

of eight reactions that process incoming molecules of Acetyl CoA. The carbon atoms leave the cycle in the form of molecules of carbon dioxide. The hydrogen atoms and electrons leave the cycle in the form of reduced coenzymes NADH and FADH2. The cycle is regulated by three allosteric enzymes in response to cellular levels of ATP. One Acetyl CoA molecule entering the citric acid cycle produces three molecules of NADH, one of FADH2, and one of GTP.

11.3. Oxidative Phosphorylation/Electron Transport Chain

The electron transport chain is the primary site for ATP synthesis, and occurs in the mitochondria. In this pathway, the electrons produced from reducing equivalents i.e. NADH and FADH, are transported through a series of transition molecules where they are moved from one transition molecule in its high-energy state to another molecule in its low energy state and finally to molecular oxygen. Each carrier in the series has an increasing affinity for electrons. Four of the carriers, known as cytochromes, contain iron, which accepts and then transfers the electrons. Cytochrome c1 (*Cyc1*) is one of the constituents of complex III, which forms the third proton pump in the mitochondrial electron transport chain (ETC). It is a subunit of the ETC protein Ubiquinol Cytochrome c Reductase (UQCR, Complex III or cytochrome bc1 complex). The general function of the complex is electron transfer between two mobile redox carriers, ubiquinol and cytochrome c; the electron transfer is coupled with proton translocation across the membrane, thus generating proton-motive form in the form of an electrochemical potential that can drive ATP synthesis. As NADH and FADH2 release their hydrogen atoms and electrons, NAD+ and FAD are regenerated for return to the citric acid cycle.

As these electron transfer reactions occur, energy is released that is used

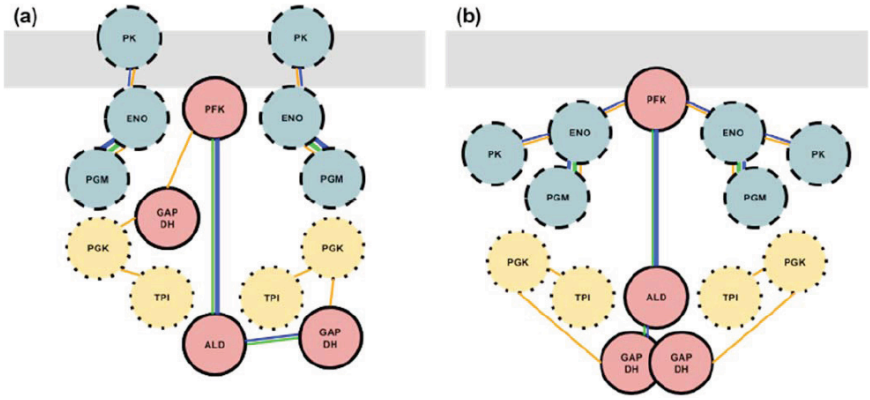


Figure 8. Two possibilities for how the three subcomplexes could form a full, active glycolytic complex. The members of the triose pathway are doubled in accordance with previous stoichiometric experimental studies[1]. Each circle represents an entire enzyme (i.e., all subunits). The grey bar represents a thin filament composed of F-actin, troponin and tropomyosin. The three subcomplexes are designated by their appropriate borders and colors[2]. Lines connecting the enzymes indicate a specific interaction. Physical contact of GAPDH represents self-binding.

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[1] Xu *et al.,* 1995

[2] GlySCx1 denoted by intact black borders and red interior with the second possible GAPDH in gray at the two possible positions; GlySCx2 with members denoted by dotted borders and yellow interior; GlySCx3 with members denoted with dashed borders and blue interior.

to pump the hydrogen ions across that membrane and into the area between the two mitochondrial membranes. This creates a concentration gradient that causes the hydrogen ions to pass back through the inner membrane and, specifically, through an enzyme called ATP synthase. This flow of hydrogen ions causes the ATP synthase molecule to rotate and this, in turn, converts ADP + P into ATP (a reaction called phosphorylation). So, what occurs in mitochondria involves electron transfer (or oxidation; the loss or transfer of an electron) and phosphorylation, or, in other words, oxidative phosphorylation. Oxidative phosphorylation is the process by which NADH and FADH2 are oxidized, with concomitant production of ATP. Two molecules of ATP are produced when FADH2 is oxidized, and 3 molecules of ATP are produced when NADH is oxidized. Oxidative phosphorylation produces lots of energy, but requires hydrogen (NADH and FADH2). The synthesis of ATP occurs because of a flow of protons across the inner mitochondrial membrane. The complete oxidation of one glucose molecule by the citric acid cycle and oxidative phosphorylation yields 36 molecules of ATP, vs. two molecules of ATP by glycolysis. A different enzyme controls each reaction in metabolic pathways. The failure of an enzyme to function may have serious and possibly fatal consequences.

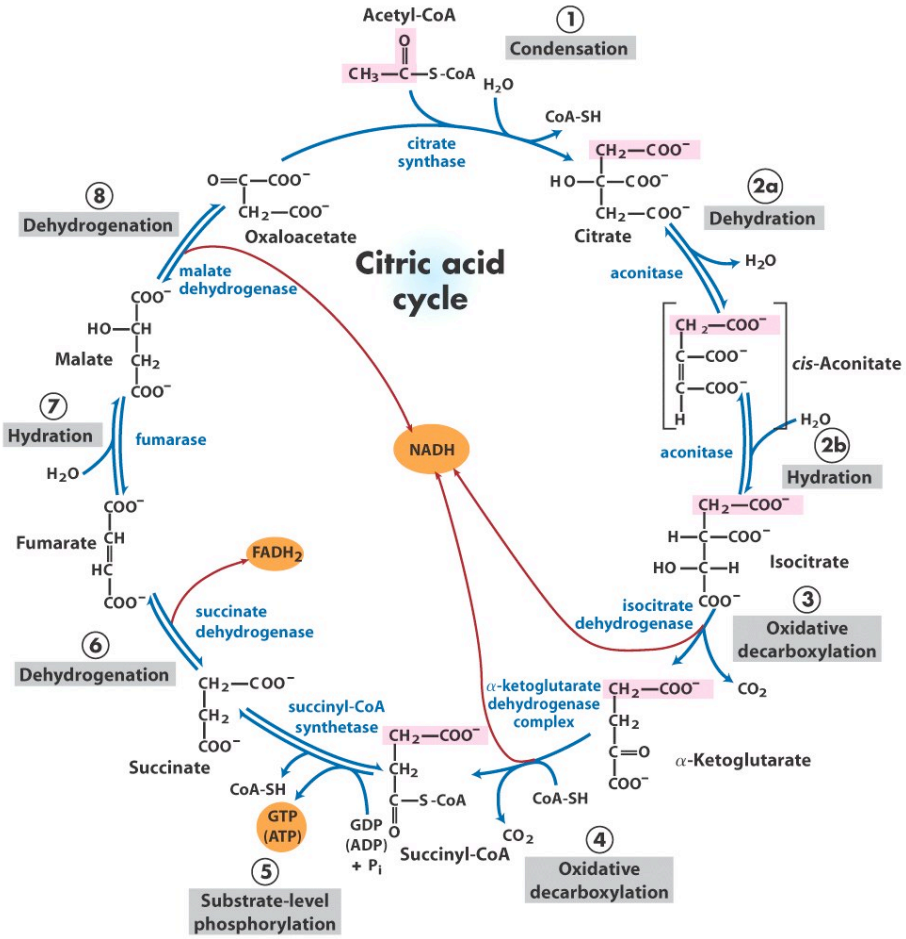


Figure 9: Reactions of the citric acid (Krebs) cycle. Adapted from textbook *"Lehninger Principles of Biochemistry"*

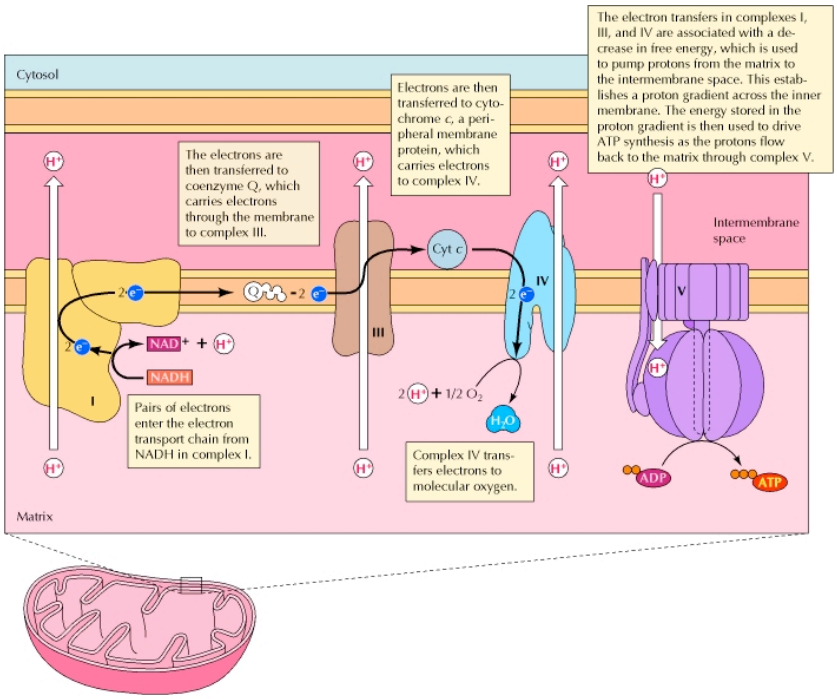


Figure 10: Mitochondrial electron transport chain. (Adapted from textbook "*The cell: A Molecular Approach" by GM Cooper*)

II- OBJECTIVES

OBJECTIVES

Aim of this work

The hypothesis of this work was to elucidate the mechanisms that are involved in axonal degeneration-regeneration in MS. Axonal damage is accepted as a major cause of persistent functional disability. We therefore wanted to study primary neuronal damage, independent of secondary amage, resulting from demyelination, using primary cultures of cerebellar granule neurons as a cellular model. It is believable that factors, hitherto unknown, present in the CSF of MS patients are able to regulate axonal destruction-repair, and make a stable remyelination and functional recovery possible. Furthermore, during the relapsing-remitting disease course the patient's brain alone is capable of repairing the damage, remyelinating the axon and recovering the neurological function. We therefore decided to treat neurons and oligodendrocytes from rat cerebellum with CSF derived from different clinical forms of MS and perform gene expression. In addition, we were interested to identify most stably expressed reference genes in treated neurons and oligodendrocytes to accurately normalize target mRNA transcripts. Finally, we were interested to identify biomarkers that can distinguish different clinical forms of MS and also MS from NMO.

The objectives proposed in this thesis were as follows:

1. To determine the effect of CSF on cerebellar granule neurons and oligodendrocytes

2. To establish a relation between neuronal affectation and aggressivity in different clinical forms of multiple sclerosis

3. To analyze the neuronal affectation in primary culture of CGNs and in OPCs by microarray profiling

4. To identify housekeeping genes during maturation of CGNs and when neurons and OPCs were exposed to the CSF from MS and NMO patients

5. To analyze variations in genes implicated in carbohydrate metabolism, energy production and consequently in the process of neuronal impairment by microarray

6. To look for biomarkers in Multiple Sclerosis and Neuromyelitis optica

III- MATERIALS AND METHODS

MATERIALS AND METHODS

Materials:

1. Culture plates (5ml and 10ml) and plastic materials were from Corning
2. Two laminar hoods (Vertical and horizontal) were from Telstar
3. CO_2 incubator was from Thermo Electron Corporation, model 371
4. Nanodrop 1000 spectrophotometer was from Thermo Scientific
5. PCR thermocycler was from Eppendorf
6. Microarray (One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling) was from Agilent Technologies, Inc

Chemicals:

1. Primers were from Sigma Aldrich
2. PCR Mastermix containing Taq DNA polymerase, dNTPs, and $MgCl_2$ was from Roche
3. SYBRGreen Mastermix was taken from Applied Biosystems
4. 100bp DNA ladder was from Invitrogen life technologies
5. Basal Eagle's médium (BME), fungizone (Amphotericin B), L-Glutamine, dispase and fetal bovine serum (FBS) were from GIBCO (Invitrogen)
6. Gentamycin was from Biowhittaker
7. DNaseI was from Roche

The other chemicals used are from Sigma Aldrich or Merck

1. Patient Cohort

1.1 Patient population

We have studied a total of 59 patients in all the different experiments and the CSF samples were obtained from the Department of Neurology, Hospital La Fe and Hospital Clinico (University of Valencia) where Doctors Dr. Bonaventura Casanova and Dr. Franscisco Coret are the clinicians of the study respectively. Out of 59 patients, 21 had inflammatory MS (11 IgM+/+ and 10 IgM +/-), 8 had medullary subtype, 11 had PPMS, 9 had NMO, and 10 were controls (NIND patients). In CSF, apart from factors related to MS or NMO, there are factors from other diseases, eg. headache, diabetes, genetic variation etc., that produce their action. This must be considered as "*background noise*" as average population. Mixing of 11 CSF samples from clinical forms such as IgM+/+ MS patients and others may sum up or potentiate the factors related to MS. Since we wanted to obtain clear results specifically from MS and NMO, we mixed total CSF samples in all forms.

MS patients were defined and grouped in different clinical courses, according to the current criteria [Lublin and Reingold, 1996, McDonald *et al.,* 2001; Polman *et al.,* 2005]. MS patients included in this study were diagnosed according to McDonald criteria, and they all met the following characteristics: oligoclonal IgG bands present, not in a phase of relapse, and have spent more than a month after the last dose of steroids. Wingerchuk criteria were used to diagnose patients with NMO disease [Wingerchuk *et al.,* 2006]. Patients suffered relapses of optic neuritis and myelitis, and 2 of the three criteria, normal MRI or that did not accomplish the Patty criteria for MRI diagnosis of MS. All studies were performed during the

diagnostic procedure, for which the patient signed an informed consent. Table 3 illustrates the clinical characteristics of the patients.

1.2. Patient Characteristics

1.2.1. Inflammatory MS (RRMS and SPMS forms)

According to the clinical classification, MS is categorized into 1) RRMS (inflammatory) that later develops secondary progressive stage (SPMS); and, 2) PPMS (primary progressive multiple sclerosis). Over 95% of patients with multiple sclerosis show oligoclonal bands of IgG in CSF (G+) [Kostulas *et al.,* 1987; McLean *et al.,* 1990; Villar *et al.,* 2005] and 40% show IgM oligoclonal bands in CSF (M+) related to a more aggressive course of disease [Sharief *et al.,* 1991; Villar *et al.,* 2002]. In our project we also classified and named inflammatory MS into *"IgM+/-"* and *"IgM+/+ subtype"* (see below) on the basis of aggressivity and prognosis which is more complete than just RRMS or PPMS and also includes better or worse prognosis. In addition we have studied separately a set of patients with MS but with a predominant affectation of the spinal cord, because these patients have some peculiarities, and we wanted to explore if they have some differences in light of our experiments. The most aggressive cases termed as *"medullary"* have more spinal injuries. The PPMS is the primary progressive clinical form of multiple sclerosis.

1.2.1.1. IgM+/- clinical form of MS

Patients named as *"IgM+/- subtype"* had IgG antibodies (+) but no IgM (-) oligoclonal antibodies detected in the CSF of brain.

Table 3. Clinical characteristics of the patients.

	G	Age	W.C.F.	C.F.	E.T.	A.E.	OCGB	OCMB	Ac-AQ4	T
Multiple Sclerosis patients										
1	F	23	RRMS (+/-)	RRMS	5	1.50	g+	m-	NA	IFN, FGM
2	F	21	RRMS (+/-)	SPMS	18	4.00	g+	m-	NA	IFN, MTZ
3	F	36	RRMS (+/-)	CIS	4	1.50	g+	m-	NA	Nt
4	M	22	RRMS (+/-)	RRMS	6	1.50	g+	m-	NA	IFN, CPX, NTZ, FGM
5	F	21	RRMS (+/-)	RRMS	3	3.00	g+	m-	NA	IFN
6	F	30	RRMS (+/-)	RRMS	22	4.00	g+	m-	NA	IFN, NTZ, FGM
7	F	29	RRMS (+/-)	RRMS	10	1.50	g+	m-	NA	No treatment
8	F	29	RRMS (+/-)	RRMS	7	1.50	g+	m-	NA	IFN, NTZ
9	F	28	RRMS (+/-)	RRMS	10	5.50	g+	m-	NA	MTZ, IFN
10	F	28	RRMS (+/-)	RRMS	4	1.00	g+	m-	NA	Nt
11	F	37	RRMS (+/+)	RRMS	7	3.50	g>	m+	NA	IFN, NTZ, CPX
12	M	32	RRMS (+/+)	RRMS	4	1.00	g+	m+	NA	IvIg
13	F	44	RRMS (+/+)	RRMS	5	2.00	g+	m+	NA	Nt
14	F	26	RRMS (+/+)	RRMS	5	2.00	g>	m>	NA	U
15	F	14	RRMS (+/+)	RRMS	18	3.50	g+	m+	NA	PE, IFN, NTZ, ASCT

16	M	25	RRMS (+/+)	RRMS	11	2.00	g+	m+	NA	IFN, FGM
17	F	21	RRMS (+/+)	SPMS	25	8.50	g>	m+	NA	IFN, AZA, MTZ, IvIg
18	F	17	RRMS (+/+)	RRMS	16	2.00	g+	m+	NA	U
19	F	23	RRMS (+/+)	SPMS	18	6.50	g+	m+	NA	IFN, IvIg, Cy
20	F	22	RRMS (+/+)	SPMS	5	4.00	g+	m+	NA	IFN, FGM, CPX
21	F	29	RRMS (+/+)	RRMS	5	2.50	g+	m+	NA	IFN, FGM, CPX
22	M	39	MedMS	SPMS	10	4.50	g+	m+	NA	IFN, Cy, FGM, NTZ
23	F	25	MedMS	SPMS	6	7.00	g+	m-	NA	IFN, MTZ, RTX
24	F	25	MedMS	SPMS	14	8.00	g+	m+	NA	IFN, Cy, RTX
25	M	34	MedMS	SPMS	9	6.00	g+	m-	NA	IFN, Cy
26	M	34	MedMS	SPMS	6	6.50	g+	na	NA	IFN, MTZ, IvIg
27	F	23	MedMS	RRMS	5	4.00	g-	m-	NA	IFN, NTZ, FGM, RTX
28	F	40	MedMS	SPMS	10	7.50	g-	m+	NA	AZA, IvIg, Cy, RTX
29	F	23	MedMS	SPMS	28	6.50	g+	m-	NA	IFN, MTZ, RTX
30	F	54	PPMS	PPMS	12	7.00	g+	m-	NA	No treatment

31	M	40	PPMS	PPMS	23	6.00	g+	m-	NA	MTZ, Cy, RTX
32	F	52	PPMS	PPMS	14	5.50	g>	m-	NA	AZA
33	F	38	PPMS	PPMS	11	5.50	g+	m-	NA	Nt
34	M	31	PPMS	PPMS	24	6.00	g+	m-	NA	Nt
35	F	47	PPMS	PPMS	14	5.50	g>	m-	NA	Nt
36	M	49	PPMS	PPMS	11	6.00	g-	m-	NA	FGM
37	F	26	PPMS	PPMS	13	6.50	g>	m-	NA	Nt
38	F	34	PPMS	PPMS	6	5.00	g>	m-	NA	Nt
39	F	39	PPMS	PPMS	8	8.50	g-	m-	NA	Cy
40	M	18	PPMS	PPMS	15	8.00	u	u	NA	U
Neuromyelitis optica patients										
41	F	39	NMO	NMO	5	9.00	g-	m-		IFN, MTZ, Cy, RTX
42	F	50	NMO	NMO	4	7.00	g-	m-	p	IFN, NTZ, RTX
43	M	15	NMO	NMO	17	4.00	g+	m+	n	IFN, IvIg
44	M	42	NMO	NMO	5	3.50	Na	na	p	IvIg
45	F	22	NMO	NMO	5	2.50	g+	m+	p	IvIg, IFN, CPX
46	F	27	NMO	NMO	5	2.00	g+	m-	n	IFN, AZA
47	M	9	NMO	NMO	14	1.00	g-	m-	n	IvIg, IFN, CPX
48	F	8	NMO	NMO	32	4.00	g+	m+	n	IFN, IvIg
49	M	19	NMO	NMO	20	8.50	g-	m-	n	IFN, MTZ, Cy, RTX
Non-inflammatory neurological diseases (control)										
50	M	23	C	C	n.a.	n.a.	g-	m-	n.a.	Nt
51	F	77	C	C	n.a.	n.a.	g-	m-	n.a.	Nt
52	F	33	C	C	n.a.	n.a.	g-	m-	n.a.	Nt
53	F	32	C	C	n.a.	n.a.	g-	m-	n.a.	Nt
54	M	59	C		n.a.	n.a.	g-	m-	n.a.	Nt
55	F	36	C	C	n.a.	n.a.	g-	m-	n.a.	Nt
56	F	57	C	C	n.a.	n.a.	g-	m-	n.a.	Nt
57	M	37	C	C	n.a.	n.a.	g-	m-	n.a.	Nt

58	F	21	C	C	n.a.	n.a.	g-	m-	n.a.	Nt
59	M	13	C	C	n.a.	n.a.	g-	m-	n.a.	Nt

Case = Patient number; Age = patient age in years; W.C.F = Working clinical form; C.F. = Clinical form; E.T. = evolution time in years; A.E. = Actual EDSS; EDSS = Expanded disability status scale (method of quantifying disability in MS); Gender = patient gender; T = treatment; F = Female; M = male; C = Control; OCGB=oligoclonal IgG bands in CSF; OCMB=oligoclonal IgM bands in CSF; Ac-AQ4 = Antiaquaporin 4 antibodies; RRMS = Relapsing-remitting multiple sclerosis; SPMS=Secondary progressive multiple sclerosis; CIS = Clinically isolated syndrome; MedMS = Medullary MS; PPMS = Primary progressive multiple sclerosis; NMO = Neuromyelitis optica patients; +/- = presense of IgG but no IgM antibodies in the CSF; +/+ = presense of both IgG and IgM antibodies in the CSF; OCGB=oligoclonal IgG bands in CSF; OCMB=oligoclonal IgM bands in CSF; n.a. = not applicable; n.t. = no treatment at the time of collection of the samples; IFN = interferon; FGM = fingolimod; MTZ = mitozantrone; u. = unavailable ; CPX = copaxone®; NTZ = natalizumab; IvIg = bi-monthly pulsed intravenous immunoglobulin; PE = plasma-exchange 2 months before lumbar puncture; ASCT = autologous stem cell transplant one year before LP; Cy = Cyclophosphamide; RTX=rituximab® five months before lumbar puncture.

1.2.1.2. IgM+/+ clinical form of MS

Patients named as *"IgM+/+ subtype"* had both IgG antibodies (+) and IgM (+) oligoclonal antibodies detected in the CSF of brain.

1.2.2. Medullary clinical form of MS

All these patients were positive for oligoclonal IgG bands (OCGB) and negative for oligoclonal IgM bands (OCMB) in the CSF; and negative for anti-NMO antibodies in serum.

1.2.3. Primary progressive MS (PPMS)

These patients are characterized by progressive decline in neurological disability with IgG antibodies but no IgM antibodies in CNS (G+M-).

1.2.4. Neuromyelitis Optica (NMO) patients

Individuals diagnosed with NMO met at least two of the following three features. 1) Long extensive transverse myelitis (>3 vestibule bodies); 2) Antibodies against Aquaporin 4; 3) Normal brain at the first event.

1.2.5. Controls (Non-inflammatory neurological diseases (NIND))

Individuals who were suspected to have MS but were not diagnosed with MS were classified as controls or non-disease individuals with no inflammation and no neurological disease.

1.3. Cerebrospinal fluid (CSF) samples of patients analyzed

CSF samples were obtained by lumbar puncture performed for diagnosis. Samples were centrifuged for 10 min at 700*xg* and aliquots were frozen at -80ºC until use. CSF samples were given a numerical code to perform all procedures in a blinded fashion related to the clinical characteristics of patients. No patient had received treatment with immunosuppressive drugs, immunomodulators or corticosteroids for at least one month prior to the extraction of CSF. Informed consent was obtained from all the patients and controls for this study and authorized by the Ethical Commitee for the project entitled "*Identification of neural autoantigens recognized by immunoglobulin M found in cerebrospinal fluid of multiple sclerosis patients. Axonal repair and remyelination*". Lead researcher: Mary Burgal Martí. Approved and funded by the Health Research Fund of the Institute of Health Carlos III. Sub- aid of Strategic Action for Health in the framework the National R + D + I 2009-2012. Expte: PS09/00976.

1.4. Cerebrospinal fluid (CSF) studies

1.4.1. Oligoclonal band studies

Paired CSF and serum samples were analysed to detect OCGB and OCMB by isoelectric focusing (IEF) and immunodetection. The clinicians used a commercial kit to determine OCGB (Helena BioScience IgG-IEF Kit) and the technique described by Villar *et al*. (2001) to detect OCMB. Serum samples were diluted in saline before the IEF in order to reach the same concentration range as that of CSF samples. All samples were incubated with 50 mmol/L dithiothreitol at pH 9.5 to reduce IgM.

Focusing was performed on a Multiphor II Electrophoresis System (GE Healthcare) at pH 5 to 8. Proteins were then transferred to a PVDF membrane and analysed by Western blot. Finally, immunodetection was performed by biotin-conjugate-goat anti-human IgM and streptavidin-alkaline phosphatase (Sigma-Aldrich).

1.4.2. Serum studies

Anti-AQP4 antibody in NMO has a high specificity so as to contribute to early diagnosis and optimized treatment of Devic disease.

Serum sample diluted 1:10 in PBS-Tween was used to detect the presense of NMO specific IgG antibodies. Indirect immunofluorescence (IFI) was performed in cells transfected by aquaporin 4 (EUROIMMUN Medizinische Labordiagnostika AG).

2. Animals

Wistar rats (Harlan Iberica) with weight between 200-250g were used. All animals were raised under controlled conditions with cycles of light/dark (12/12h), temperature of 23°C and humidity of 60%. Access to water and food (standard rodent feed supplied by Harlan, Teklad 2014 Global 14% Protein Rodent Maintenance Diet) was provided. To obtain offspring, pregnant females were separated and kept in isolated cages during gestation. The maintenance of the animals was performed in the lower animal facilities of Prince Felipe Research Center, Valencia, Spain.

3. Determination of cell viability

In order to determine whether factors in CSF of MS and NMO patients cause cell death in astrocytes or neurons or both we first incubated cerebellar granule

neurons with half concentration of cytosine arabinoside (0.5 μM) to allow the growth of astrocytes. To determine cell viability we used propidium iodide (PI) which is excluded by living cells. The membrane of these cells forms a selective permeability barrier between the intracellular content and the extracellular environment. Dead cells lose this property and therefore incorporate the dye, which intercalates between the bases of DNA and RNA in a ratio of 1 dye molecule every 4 or 5 base pairs. Once bound to nucleic acids, PI fluorescence is increased 20 to 30 times. Propidium iodide was added at a final concentration of 0.5 mg/ml in PBS and incubated for 30 minutes at room temperature in darkness. Living cells were incubated with rhodamine-123 cationic dye that accumulates within the mitochondria of living cells due to the negative potential difference inside. Rhodamine was used at a concentration of 10 mg/ml in PBS for 30 minutes at room temperature in darkness.

4. Primary culture of cerebellar granule neurons (CGNs)

All operations were performed under sterile conditions in vertical laminar flow chamber (Telstar AV-100 and Bio-II-A). All reagents and culture media were obtained from Gibco. The plastic material used was sterile, the petri dishes for culture from Nunc and Corning, the Corning tubes, syringes from BD and Millipore filters, the glass material used was sterile. All materials used were free of toxins. The cells were kept in an incubator at 37º C in a humidified atmosphere composed of 95% air and 5% CO_2 (CO2 incubator Thermo Form, model 371).

Primary cultures of cerebellar granule neurons (CGNs) were obtained according to previously described modified protocol [Minana *et al.,* 1998]. Cerebellum were collected from eight day old Wistar rats, mechanically dissociated

and cerebellum was dissected. Isolated cerebella were stripped of meninges, minced by mild trituration with a Pasteur pipette and treated with 3mg/ml dispase (grade II) for 30 min at 37º C in a 5% CO_2 humidified atmosphere. After half an hour, dispase was inactivated with 1mM EDTA. Granule cells were then resuspended in basal Eagle´s medium (BME, Gibco, ref. 41010) with 40µg/ml of DNaseI. The cell suspension was filtered through a mesh with a pore size of 90µm and centrifuged at 1500 rpm for 5 min and thereafter, cell suspension was washed three times with BME. Finally, the cells were resuspended in complete BME medium with Earle´s salts containing 10% heat inactivated FBS (fetal bovine serum, Gibco), 2 mM glutamine, 0.1 mg/ml gentamycin and 25mM KCl. The neuronal cells were counted (see below) and then the cell suspension was plated onto poly-L-lysine coated 6-well (35-mm) culture dishes (Fisher) at a density of $3x10^5$ cells/well and incubated at 37º C in a 5% CO_2/95% humidity atmosphere. After 20 min at 37º C, the medium was removed and fresh complete medium was added. 20µl of cytosine arabinoside (1 mM) was added to each culture plate after 18 to 24 hours to inhibit replication of non-neuronal cells. The cells were kept in an incubator at 37ºC in a humidified atmosphere composed of 95% air and 5% CO_2 (CO_2 incubator Thermo Form, model 371). Cells were fed every 3-4 days in culture with 5.6 mM glucose.

5. Treatment of cultured cells with CSF

Neuronal cell cultures from 14^{th} day were incubated with 10% v/v CSF from MS (IgM+/-, IgM+/+, medullary, PPMS) patients, NMO patients and controls for 24 hours.

6. Confocal Microscopy

The living cells were always kept at 37° C and 5% CO_2. Cells were analyzed on a Leica TCS SP2 confocal microscope AOBS (Leica Microsystems) inverted laser scanning confocal microscope using a 63°— Plan-Apochromat-Lambda Blue 1.4 N.A. oil objective lens. All confocal images were obtained under identical scan settings. Images of 1,024 °— 1,024 pixels, 8-bits were collected for each preparation. Best focus was based on highest pixel intensity. Imaging conditions were identical for all the images, and no images were saturated. Metamorph 7.0 (Molecular Devices, Downingtown, PA, USA) was used for image analysis on the images collected.

7. Total RNA isolation and Reverse Transcription (cDNA synthesis)

We isolated total RNA from neurons at different time points (day1, day4, day8 and day14) using Quick RNA MicroPrep Kit (Zymo Research Corp.) according to the manufacturer's instructions to study the development of unmyelinated cerebellar granule neurons. Secondly total RNA from 14^{th} day cell cultures exposed to the CSF of different experimental conditions (IgM+/-, IgM+/+, medullary, NMO, PPMS, Control) was isolated using the similar kit in order to study the primary damage caused by CSF exposure to CGNs. The RNA concentration was determined spectrophotometrically at 260 nm using the Nanodrop 1000 spectrophotometer (V3.7 software) and RNA purity was checked by means of the absorbance ratio at 260/280nm. Isolated RNA was stored at -80° and later reverse transcribed to cDNA. Equal amounts of RNA (1µg) were used for cDNA synthesis. The cDNA was synthesized and stored at –20°C. Primers for selected genes were designed using

Primer 3 software. The primer sequences, annealing temperature and the product size is listed in Table 4. PCR was performed in a thermocycler (BioRad) with cycling conditions (94° for 30s, 40 cycles at 59° for 30s and 72° for 30s). Each 25 µl reaction contained 12.5 µl Master Mix (Applied Biosystems), 1µl gene-specific forward and reverse primers (0.5 µM), 1µl undiluted cDNA and 10.5 µl DEPC (nuclease free) treated water. Negative controls with no template contained nuclease-free water instead.

8. Agarose gel electrophoresis

The electrophoresis was performed in 1.5% agarose gels, and they were run at 50 V, stained with ethidium bromide, photographed and evaluated with ImageJ software. A DNA ladder control (100bp, Invitrogen) was also used in the electrophoresis to evaluate DNA fragment size.

9. Gene microarray and data normalization

Firstly we performed gene expression profiling of granule neurons development and later the profiling of these cells exposed to the CSF from MS and NMO patients (experimental conditions). By doing this we could distinguish between neuronal cells that are developing and show how these cells react to a particular treatment.

We isolated total RNA during different developmental stages of CGNs (day1, 4, 8, 14) and handed over to genomics laboratory to perform one color microarray-based gene expression analysis (Agilent Technologies) by its respective staff. In experimental conditions, matured CGNs from day 14^{th} were incubated with the CSF of inflammatory MS (IgM+/- MS and IgM+/+ MS), medullary MS, PPMS, NMO and NIND patients and total RNA was isolated in our lab and handed over to genomics

laboratory to further subject the total RNA to one color microarray-based gene expression analysis (Agilent Technologies) by its respective staff.

The labeled cRNA was hybridized to the Agilent SurePrint G3 Rat GE 8x60K Microarray (GEO-GPL13521, in situ oligonucleotide) according to the manufacturer's protocol. Briefly, the mRNA was reverse transcribed in the presence of T7-oligo-dT primer to produce cDNA. cDNA was then *in vitro* transcribed with T7 RNA polymerase in the presence of Cy3-CTP to produce labeled cRNA. The labeled cRNA was hybridized to the Agilent SurePrint G3 Rat GE 8x60K Microarray according to the manufacturer's protocol. The arrays were washed, and scanned on an Agilent G2565CA microarray scanner at 100% PMT and 3 μm resolution. The intensity data was extracted using the Feature Extraction Software (Agilent).

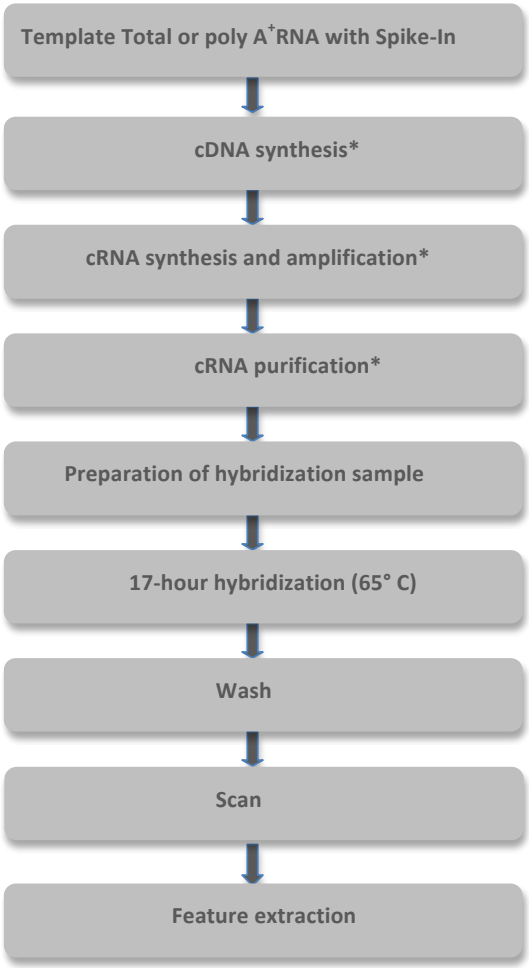
In order to account for technical variation between microarrays (i.e. amount of starting RNA, and differences in efficiencies of reverse transcription, labeling and hybridization), raw signal intensities were first normalized using the Percentile shift method available in GeneSpring 9.0 for one color microarray (Agilent, CA, USA). Since the 75th percentile is a more robust and representative intensity value of the overall microarray signal as compared to the median or the 50^{th} percentile signal, the default was set at 75^{th} percentile. Therefore, 75^{th} percentile signal value was used to normalize Agilent one-color microarray signals for inter-array comparisons. After normalization the data was filtered in order to exclude probesets with low expression and/or affected by differences between the laboratories. Differentially expressed genes were identified by comparing average expression levels in terms of fold change in cases and controls. All filtering steps and statistical analyses were conducted using GeneSpring 9.0.

During the maturation of cerebellar granule neurons (day1, 4, 8, 14), mRNA expression (in terms of fold change) from cultured neurons on day 4, 8 and 14 were

compared with day1. mRNA expression (in terms of absolute fold change) in neurons treated with CSF of MS, NMO, PPMS and NIND patients was compared with gene expression in neurons exposed only to culture medium. Fold change (FC) is a number describing how much a quantity changes from an initial to a final value. FC is calculated as the ratio of the final value to the initial value. In this study we have taken fold-change value that is less than 1 by the negative of its inverse. Fold change cut off was considered as 2. Figure 11 represents the workflow for sample preparation and array processing.

10. Analysis of gene-gene interaction networks using String v10 software

Next, we wanted to elucidate whether interaction of different metabolic genes related with a metabolic network (i.e. glycolysis, TCA cycle, and electron transport chain) affected the whole network included those genes we did not find too many changes in the whole carbohydrate network. As we discussed earlier the importance of glycolytic enzymes in forming localized complexes, which may play a crucial role in allowing intricate regulatory control of glycolytic pathway, we wanted to elucidate how interaction of genes affect the whole network in different experimental conditions. For this, we used *STRING v10* ("Search Tool for Retrieval of interacting genes and proteins" software) and correlated the gene interaction in different disease subtypes in neurons. Although not every gene related with a specific network is affected in a kind of MS disease, if we do a string analysis we may find that the physical interaction of enzymes related with a metabolic network (i.e. glycolysis, TCA cycle, and electron transport chain) exist and closely regulated in a complex ensures that the whole network is affected (increasing general intermediate metabolic flux within the network). Therefore now we define a



* Samples can be stored frozen at -80°C after these steps, if needed.

Figure 11: Workflow for sample preparation and array processing

parameter to “integrate” our data as “CUMULATIVE FLUX INDEX or CFI” within the network to compare our experimental MS conditions. The values means that the reducing on local flux due to the inhibition of an enzymatic activity in a specific gene affects synergicaly to the whole metabolic flux network. It means that as more genes are down regulated, the total flux are reducing as a multiplicative factor that we integrate as the CFI of the network. In the Discussion section we integrate in a draw the relative changes in the different MS and NMO patients by calculating the CFI of the carbohydrate metabolic networks in those patients.

11. Real-time polymerase chain reaction of selected housekeeping genes

Real-time polymerase chain reaction (Real-Time PCR), also called quantitative PCR, is a PCR method based on using either TaqMan® probes labeled with a fluorescent dye or SYBR® Green detector, which is a cyanine dye unselectively binding to all double-stranded DNA. The method differs from standard PCR in that the amplified product can be detected and quantified by measuring fluorescence at each amplification cycle. Because the level of fluorescence depends on the number of copies of the target sequence in the sample, the method can be used for estimating transcript and DNA copy numbers based on the amplification cycle at which a given fluorescence threshold is reached, i.e. the threshold cycle (Ct). In order to account for variation in the amount of total DNA or RNA in the reaction, the Ct values are corrected using an endogenous control gene such as *ActB*, encoding actin protein, in which there is assumed to be no quantitative variation between samples. The fold difference in the amount of target sequence copies between two samples can then be estimated using the comparative Ct method according to:

2^(Δ Ct(Sample1)- Δ Ct(Sample2)),

where Δ Ct is the Ct(target gene)-Ct(endogenous control gene).

This method assumes, however, that the amplification efficiency is 100%, i.e. that in each cycle the amount of amplified product doubles. A more accurate alternative to this method would therefore be to use standard curves to quantify the amount of target sequence in each sample.

We used real-time PCR for measuring transcript levels of commonly used housekeeping genes in which detection was based on SYBR green chemistry. Since recent evidence shows that some house keeping genes are not as refractory to experimental manipulations as previously thought, we validated 7 commonly used house keeping genes and have checked whether these genes remain stable during maturation (day1, day4, day8, day14) and experimental conditions. Candidate housekeeping genes were selected from those most commonly used in literature including beta-actin (*ActB*), hypoxanthine guanine phosphoribosyl-transferase (*Hprt*), ribosomal protein L19 (*Rpl19*), lactate dehydrogenaseA (*Ldha*), transferrin receptor (*Tfrc*), microglobulin beta-2 (*B2m*), and glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*). The function and references of the genes are listed in Table 4.

The primer sequences, annealing temperature and the product size is listed in Table 5. Real time PCR was performed in a 96-well plate (Roche) incubated in thermocycler (LC480, Roche) with cycling conditions (94° for 15s, 45 cycles at 60° for 30s and 72° for 30s). Each 10ml reaction contained 5 ml SYBR Green Master Mix (Applied Biosystems), 1 µl gene-specific forward and reverse primers (0.5 µM), 1 µl undiluted cDNA and 3 ml DEPC (nuclease free) treated water. Negative controls with no template contained nuclease-free water instead. All samples were run in duplicate and average values were calculated. Data was analyzed using 7300 Sequence Detection Software (SDS) Version 1.3 (Software Roche). Following qRT-

PCR, a dissociation curve was run to check the PCR product specificity.

12. Statistical Analysis

Data are mean ± standard error of the mean (SEM). A statistical test was applied to look for significant differences between experimental conditions for each candidate reference gene. A one-way analysis of variance (ANOVA) was conducted to determine the significantly variable genes. A p value < 0.05 was considered statistically significant.

12.1. Determination of reference gene expression stability

To determine the stability of these genes on the basis of their Cp values, we employed comparative ΔCT method. Data are plotted as fold change values which were calculated by $2^{\Delta(Ct\ exp-\ Ct\ control)}$. Cp value is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. The numerical value of the Cp is inversely related to the amount of amplicon in the reaction. Invariable genes were later assessed by publicly available software tools named GeNorm and NormFinder. GeNorm ranks the genes according to their average expression stability measure (highest M value) from the most stable (lowest M value) to the least stable (highest M value). An alternative program, NormFinder that was introduced later ranks the candidate reference genes based on the combined estimates of both intra- and intergroup variations.

Table 4. Panel of 7 candidate housekeeping genes selected for expression analysis

Gene Symbol	Gene name	mRNA accession number	Function	References
ActB	Beta Actin	NM_031144	Cytoskeletal structural protein	Stürzenbaum *et al.* 2001
Hprt	Hypoxanthine guanine phosphoribosyl transferase	NM_012583	Metabolic salvage of purines	Everaert *et al.* 2011
Rpl19	Ribosomal protein L19	NM_031103	Unclear	Zhou *et al.* 2010
Ldha	Lactate dehydrogenase A	NM_017025	NADH dependent enzyme that catalyzes reduction of pyruvate to lactate	-
Tfrc	Transferrin Receptor	NM_022712	Iron delivery from transferrin to cells	Gorzelniak *et al.* 2001
B2m	microglobulin-b-2	NM_012512	Major histocompatibility complex class I	Yurube *et al.* 2011
Gapdh	Glyceraldehyde-3-phosphate-dehydrogenase	NM_017008	NAD^+ dependent enzyme that catalyzes conversion of glyceraldehyde-3-phosphate to 1,3-bis phosphoglycerate	Gorzelniak *et al.* 2001; Fort *et al.* 1985; Medhurst *et al.* 2000; Harrison *et al.* 2000

Table 5: Primer sequences and amplification summary. F: forward primer; R: reverse primer

Gene	Primer Sequence (5´→3´)	Annealing temperature	Product size
Actb	F: ATTGAACACGGCATTGTCAC R: ACCCTCATAGATGGGCACAG	60°	294
Hprt	F: CCTCTCGAAGTGTTGGATACAG R: TCAAATCCCTGAAGTGCTCAT	60°	105
Rpl19	F: ACCTGGATGCGAAGGATGAG R: CCATGAGAATCCGCTTGTTT	60°	139
Ldha	F: AGGAGCAGTGGAAGGATGTG R: AGGATACATGGGACGCTGAG	60°	214
Tfrc	F: GTTGTTGAGGCAGACCTTCA R: ATGACTGAGATGGCGGAAAC	60°	112
B2m	F: GTCGTGCTTGCCATTCAGA R: ATTTGAGGTGGGTGGAACTG	60°	116
Gapdh	F: GGAAACCCATCACCATCTTC R: GTGGTTCACACCCATCACAA	60°	200

13. Primary cultures of OPCs from the neonatal rat brain

Cerebrospinal fluid was collected by lumbar puncture from MS patients of various diagnoses. We selected patients with definitive MS from our database according the 2010 McDonald criteria. For our study we selected two clinical forms of MS: 1) a set of PPMS patients, defined by a progressive onset for more than one year of neurological dysfunction with magnetic resonance Swanton criteria of dissemination in space (McDonald criteria) in which IgG oligoclonal bands were present in both the serum and the CSF, with more bands present only in the CSF, an OCB pattern that have been recognized as more specific of PPMS and 2) RRMS patient cohort defined by two or more relapses separated by one month; Swanton

criteria for dissemination in time and space, and also the OCB of the IgG type should be present in the CSF [Polman *et al.,* 2011; Villar *et al.,* 2009]. The spinal fluid was immediately placed on ice, aliquoted and snap frozen on dry ice.

OPCs were isolated from the cortex of postnatal day 1 rats and cultured according to a modified McCarthy and de Vellis procedure [McCarthy and de Vellis 1980]. The cells were grown in NM10 (high glucose DMEM supplemented with 10% fetal bovine serum), and cultured for one week at 37°C, 5% CO_2. After one week, loosely attached microglia were removed by a low-speed shaking (210 rpm) for 20 min on a rotary platform shaker. Media was removed and discarded and replaced with fresh NM10. The flasks were shaken overnight for 18-20 h at 220 rpm and cells were positively immunoselected on a Miltenyi MACS magnetic purification column using mouse anti-A2B5 antibody. Primary OPCs were plated on poly-Dlysine coated dishes at a density of 2000 cells per cm^2 and grown in oligodendrocyte chemically-defined media (ODM) containing 100 µg/mL bovine transferrin, 5.0 µg/mL yeast recombinant insulin, 100 µg/mL bovine serum albumin fraction V, 1 mg/mL biotin, 0.628 mg/mL progesterone, 0.3804 mg/mL sodium selenite, 16.1 µg/mL putrescine. For cell expansion, chemically-defined media was supplemented with 20 ng/mL basic fibroblast growth factor (bFGF) and 10 ng/mL platelet-derived growth factor AA (PDGF-AA) and cells were allowed to proliferate for 48 hours prior to treatment. OPC cultures were 99%+ pure cultures, with less than 1% detectable GFAP+ astrocytes, or Iba1+ microglia, as detected by immunocytochemistry.

14. Treatment of cultured OPCs with CSF and RNA extraction

OPCs were treated with CSF diluted at a 1:1 dilution in ODM supplemented with bFGF (20 ng/mL) and PDGF-AA (10 ng/mL). Cells were treated for 24 h with the

diluted CSF and RNA was extracted using the Qiagen RNeasy RNA extraction kit according to the manufacturer's instructions. RNA concentration and purity were determined using a NanoDrop machine. The quality of RNA was verified by capillary electrophoresis using 2100 Bioanalyzer instrument (Agilent).

15. Gene microarray and data normalization

The labeled cRNA was hybridized to the Agilent SurePrint G3 Rat GE 8x60K Microarray (GEO-GPL13521, in situ oligonucleotide) according to the manufacturer's protocol. Briefly, the mRNA was reverse transcribed in the presence of T7- oligo-dT primer to produce cDNA. cDNA was then in vitro transcribed with T7 RNA polymerase in the presence of Cy3-CTP to produce labeled cRNA. The labeled cRNA was hybridized to the Agilent SurePrint G3 Rat GE 8x60K Microarray according to the manufacturer's protocol. The arrays were washed, and scanned on an Agilent G2565CA microarray scanner at 100% PMT and 3 µm resolution. The intensity data was extracted using the Feature Extraction Software (Agilent).

IV- RESULTS

1. Clinical Diagnosis of Multiple Sclerosis and Neuromyelitis Optica patients

The first step for the study of the changes induced in brain cells by CSF of MS and NMO patients is the right classifying of the subtype and their level of the disease. The accurate clinical course (phenotypes) of MS are important for prognostication, design of clinical trials, and treatment decision and, in this work, to do the proper correlation of MS-type with changes in gene expression, cell behaviour, etc.

Therefore, we did first MS patients classification by clinical and imaging into RRMS, PPMS and MedMS groups. In the first group, we sub-grouped as patients with poor or worse prognosis based on the presense of oligoclonal bands in CSF. The NMO patients were identified on the presense of anti-AQP 4 antibodies in the sera (see Material and Methods for the final classified list of patients).

1.1. Detection of oligoclonal bands (OCGB and OCMB) in CSF of MS patients

The detection of oligoclonal bands in MS patients was performed by isoelectric focussing, followed by immunodetection to identify oligoclonal IgG bands (OCGB) and oligoclonal IgM bands (OCMB) in paired CSF and serum samples. The intrathecal IgG or IgM synthesis was confirmed when two or more bands appeared in CSF and not in serum.

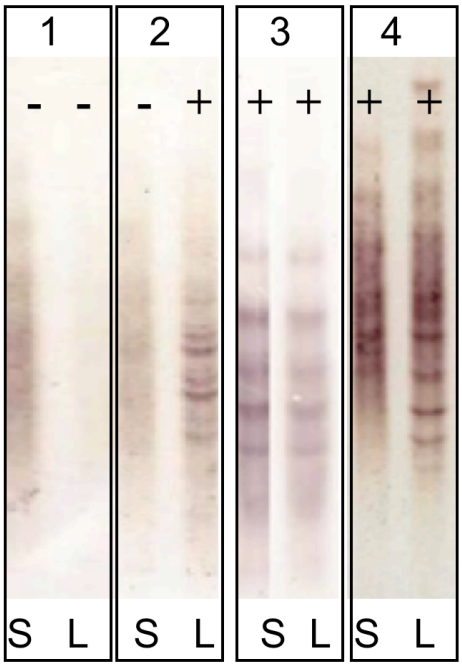


Figure 12: Immunodetection of oligoclonal bands (OCBs) in serum (S) and CSF (L). Pattern 1: No OCBs seen (negative, polyclonal): No oligoclonal bands in CSF or Serum. No intrathecal Ig synthesis; Pattern 2: OCBs in CSF only (positive): Oligoclonal bands present in CSF only. Intrathecal IgG synthesis as seen in MS; Pattern 3: Identical OCBs in both (mirror): Bands in serum mirror those in CSF. This suggests systemic Ig synthesis**;** Pattern 4: Identical OCBs in both with extra in CSF (more than): Identical bands in both serum and CSF with extra bands in CSF. Image demonstrates both intrathecal and systemic Ig synthesis. This is identical as it is seen in MS.

The figure 12 shows oligoclonal bands in CSF and in serum in some examples of the different types of RRMS/PPMS/control patients. The pattern 1 shows the absense of oligoclonal bands in CSF or serum, suggesting the absence of intrathecal IgG synthesis. The pattern 2 shows the presense of oligoclonal bands in CSF only, suggesting the synthesis of intrathecal IgG, as seen in MS. The pattern 3 shows the presense of identical OCBs in both CSF and serum suggesting systemic IgG synthesis. The pattern 4 shows identical OCBs in both CSF and serum, with extra bands in CSF. This demonstrates both intrathecal and systemic Ig synthesis.

1.2. Detection of anti-aquaporin 4 antibodies (Anti-AQP4, Anti-NMO, NMO-IgG) in sera of NMO patients

To diagnose NMO, we performed indirect immunofluorescence in cells transfected by aquaporin 4 (Euroimmun Medizinische Labordiagnostika AG) to detect the presense of NMO specific IgG antibodies in serum samples of the patients. Panel I of figure 13 shows as example a positive sample. NMO specific IgG antibodies were also observed in the serum. The panel II shows the case of an absense of IgG antibodies specific for NMO (NMO negative).

Therefore, the clinical form of MS we will use in this work are: 1) RRMS patients *"IgM+/- subtype"* had IgG antibodies (+) but no IgM (-) oligoclonal antibodies detected in the CSF. 2) RRMS patients *"IgM+/+ subtype"* had both IgG antibodies (+) and IgM (+) oligoclonal antibodies detected in the CSF. 3) MedMS patients are positive for oligoclonal IgG bands (OCGB) and negative for oligoclonal IgM bands (OCMB) in the CSF, as well as, negative for anti-NMO antibodies in

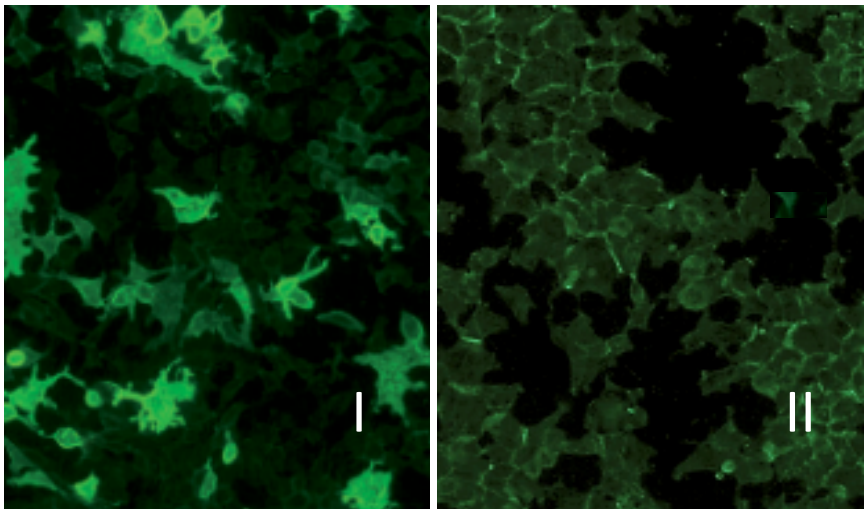


Figure 13: Indirect immunofluorescence in cells transfected by aquaporin 4 (EUROIMMUN Aquaporin-4 IIFT). Panel I: Anti-AQP4 antibodies observed in the serum of NMO patients (positive sample); Panel II: Absence of anti-AQP4 antibodies in serum sample (negative sample)

serum. 4) PPMS patients are characterized by progressive decline in neurological disability with IgG (+) antibodies but no IgM (-) antibodies in CSF. 5) NMO are positive with antibodies against Aquaporin 4, with long extensive transverse myelitis and/or normal brain at the first event. 6) Controls patients with no inflammation and no neurological disease diagnosed.

2. Demographic and clinical profiles of MS, NMO and NIND groups

According with the above classification, the population of patients we used in our study comprised of 59 patients out of which 21 had inflammatory RRMS (10 IgM+/- and 11 IgM +/+ subtypes), 8 had Medullary subtype, 11 had PPMS, 9 had NMO, and 10 were controls (NIND patients). The baseline characteristics of the patients population are described in Table 6.

The prevalence of MS, as known in the literature, was found more in women than in men (75% in women). The mean age of MS patients was 30.7±9.7 years old, whereas it was 25.6±15 years old for NMO patients. According to the clinical classification, the general characteristics of each MS patients are described in Table 7.

There were significant differences observed between the age at beginning of PPMS and the other two MS forms (p=0.003), between the EDSS (Expanded Disability Status Scale) of RRMS and the two other MS forms (<0.001), and the evolution time between PPMS and RRMS (p=0.043), after Bonferroni correction. The table 8 shows the characteristics of MS patients according to new proposal and working classification. After Bonferroni correction, the significance was due to differences between the age at beginning of the disease and the EDSS between medullary MS and PPMS with the inflammatory MS.

Table 6. General characteristics of series studied.

	Controls (n=10)	MS patients (n=40)	NMO patients (n=9)	P
% females (n)	60.0 (6)	75.0 (30)	55.6 (5)	0.40 (χ^2)
Age (mean, SD)	40.3 (19.5)	30.7 (9.7)	25.6 (15.0)	0.04 (Anova test)
EDSS	n.a.	4.5 (2.3)	4.6 (2.8)	0.94 (t-test)
Evolution time	n.a.	11.1 (6.6)	11.8 (9.7)	0.79 (t-test)

Table 7. Characteristics of MS patients according to clinical classification

	RRMS (n=18)	SPMS (n=11)	PPMS (n=11)	P
% females (n)	83.3 (15)	72.7 (8)	63.6 (7)	0.48 (χ^2)
Age (mean, SD)	27.3 (7.2)	27.9 (7.3)	38.9 (11.2)	0.003 (Anova test)
EDSS	2.4 (1.2)	6.2 (1.5)	6.3 (1.1)	<0.000 (Anova test)
Evolution time	8.1 (5.4)	13.5 (7.8)	13.8 (5.7)	0.043 (Anova test)

Also the significance between PPMS and the other two MS forms were due to the differences between the age at beginning, between the EDSS of RRMS and the two other MS forms, and the evolution time between PPMS and RRMS.

Our findings suggests that RRMS and SPMS cases shared similar age at disease

onset (mean=27.3 *versus* 27.9 years old; p=0.003) (Table 7), whereas significant differences were observed in the age at the beginning of the disease between PPMS and the other two MS forms (RRMS and SPMS) (p=0.003).

The people with PPMS are usually older at the time of diagnosis, with an estimated average age of 40 years old. Furthermore, the different subtypes of MS may help to predict disease severity and the response to treatments, hence their categorization is important in clinic.

In our study, we found significant differences between the EDSS of RRMS and the two other MS forms (RRMS and SPMS) (p<0.001) (Table 7). We may also say that although nerve injury always occurs, the pattern is specific for each individual with MS. The disease severity and disability increases from RRMS to SPMS course and in PPMS subtype, and the symptoms continually worsen from the time of diagnosis rather than having well-defined attacks and recovery. The PPMS usually results in disability earlier than RRMS.

Significant differences (p=0.043) were also found in the evolution time from the first to the second episode between RRMS and PPMS. In patients experiencing a progressive course, the evolution time was similar in SPMS cases and in cases that were progressive from onset (13.5 *versus* 13.8) (Table 7). According to the new proposal and working classification, the inflammatory MS subtypes shared similar age at disease onset (mean=26.7 *versus* 26.3 years old; p=0.005). Significant differences were found between the age at disease onset in medullary MS and PPMS with the inflammatory MS (p=0.005). The degree of disability, as measured by EDSS, was similar in MedMS and PPMS (6.2 *versus* 6.3) whereas significant differences were found between disability extent in MedMS and PPMS with the inflammatory MS (p<0.001). The IgM+/- group represents the less aggressive inflammatory subtype with OCGB in CSF with poor prognosis

Table 8. Characteristics of MS patients according to new proposal and working classification.

	Inflammatory MS (n=21)		Medullar MS (n=8)	PPMS (n=11)	p
	G+/M- (n=10)	G+/M+ (n=11)			
% females (n)	90 (9)	81.8 (9)	62.5 (5)	63.6 (7)	0.40 (χ^2)
Age (mean, SD)	26.7 (4.8)	26.3 (8.7)	31.4 (7.0)	38.9 (11.2)	0.005
EDSS	2.5 (1.5)	3.4 (2.2)	6.2 (1.4)	6.3 (1.1)	0.000
Evolution time	8.9 (6.3)	10.8 (5.9)	8.5 (3.1)	13.8 (5.7)	0.154

whereas IgM+/+ type signifies a more aggressive category with OCGB and OCMB in CSF with worse prognosis. On the contrary, MedMS represents the most aggressive subtype of MS, with increased neurological disability and dysfunction as compared to inflammatory subtypes. The disability in patients experiencing PPMS worsens over time with no relapses and remission.

3. CSF of MS patients causes cerebellar granule neuronal cell death

Since axonal damage has now been widely accepted as the major cause of persistent functional disability in patients with MS, we mainly focussed the first part of our study on primary neuronal damage, independent of secondary damage resulting from demyelination, through the study of possible factors present in the CSF of MS patients. Individuals with MS possess the ability to repair the damaged myelin during the relapsing remitting course of the disease, therefore, we wanted to delineate the mechanisms that cause primary damage in neurons in order to

ameliorate the disease from neurological deficit.

For this chapter, we isolated cerebellar granule neurons and exposed them to CSF of MS clinical subtypes. The cerebellar cortex is a well described structure that provides unique opportunities for studying neuronal properties and development [Altman and Bayer, 1997; Hatten and Heintz, 1995]. Of the cerebellar neuronal types (granule cells, Purkinje cells and inhibitory interneurons), the granule neurons are by far the most numerous and most abundant type of neurons in the mammalian brain. In rodents, cerebellar granule neurons are generated during the first two post-natal weeks from progenitor cells in the outermost layer of the cerebellar cortex, the external granule layer (EGL). Due to their postnatal generation and the feasibility of well-characterized primary *in vitro* cultures, cerebellar granule cells are a model of election for the study of cellular and molecular correlates of mechanisms of survival/apoptosis and neurodegeneration/neuroprotection.

Therefore, unmyelinated cerebellar granule neurons represent an excellent cellular model to study neurodegenerative diseases, and we employed these cells for our work and exposed them to CSF of MS patients to study neuronal damage.

Since all the cells of the brain, namely neuronal and non-neuronal cells, are affected in this disease, we first wanted to know if CSF causes any effect on non-neuronal cells. We determined the viability of cerebellar cultures by adding propidium iodide and rhodamine-123 to the primary cell cultures. The dead neuronal cells incorporated the dye propidium iodide (red fluorescence), however, living cells (astrocytes) retained their mitochondrial membrane potential by incorporating rhodamine-123 dye (green fluorescence). The healthy neurons and astrocytes were observed in control whereas dead neuronal cells were found in primary cerebellar cell cultures treated with CSF of MS patients (Figure 14). The

astrocytes were found viable whereas granule neurons underwent cell death (B, C and D panels of Figure 14). The results indicate that some factors present in CSF of MS patients may have caused cell death in cerebellar granule neurons but not in astrocytes.

4. Confocal images of cerebellar granule neuronal cell cultures

To exclude non-neuronal cells that might cause interference in the study, we first obtained pure cultures of CGNs to study primary neuronal damage. For this purpose, it was necessary to add a chemical to prevent the growth of non-neuronal cells. The presence of cytosine arabinoside (1 mM) in the culture during 18 to 24 hours, inhibit the replication of non-neuronal cells. To quantify the CGNs in the primary cultures of granule neurons isolated from cerebellum, we stained the cells with texas red and FITC dyes, and nuclei were stained with DAPI. The figure 15 showed the stained neurofilaments characteristic of neurons, indicating that the culture contains a pure population of CGNs devoid of any non-neuronal cells.

5. Treatment of primary cultures with CSF of MS, NMO patients and controls

The next chapter of our work was developed with the hypothesis that CSF, which may contain cellular damage products, including lipids [Vidaurre *et al.,* 2014], or cytokines [Rossi *et al.,* 2014; Rossi *et al.,* 2012], may influence the cellular physiology of neurons, oligodendrocyte progenitor cells (OPCs) and myelinating oligodendrocytes.

There are extensive studies in the literature, including proteomics [Komori *et al.,*

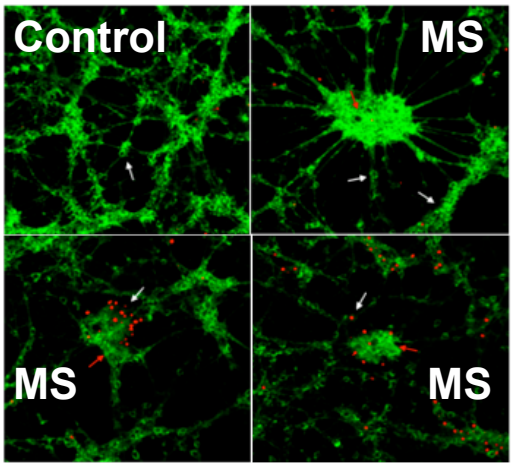


Figure 14: Viability and cell death in CGN (white arrow) and astrocytes (red arrow). 6 days culture of CGNs treated with CSF of MS patients**.** PI = red fluorescence and Rhodamine-123 = green fluorescence. Images from three independent experiments.

2012; Teunissen *et al.,* 2011; Ottervald *et al.,* 2010], metabolomics [Lutz *et al.,* 2007; Smolinska *et al.,* 2012], RNA expression in peripheral blood [Guo *et al.,* 2014] and microRNAs [Fenoglio *et al.,* 2013; Sondergaard *et al.,* 2013; Otaegui *et al.,* 2009], trying to identify the soluble factors that mediate nervous system damage and inhibit repair in distinct types of MS. For example, ceramides present in MS CSF promote axonal injury and neuronal damage through oxidative stress mechanisms and mitochondrial bioenergetic failure [Vidaurre *et al.,* 2014]. In

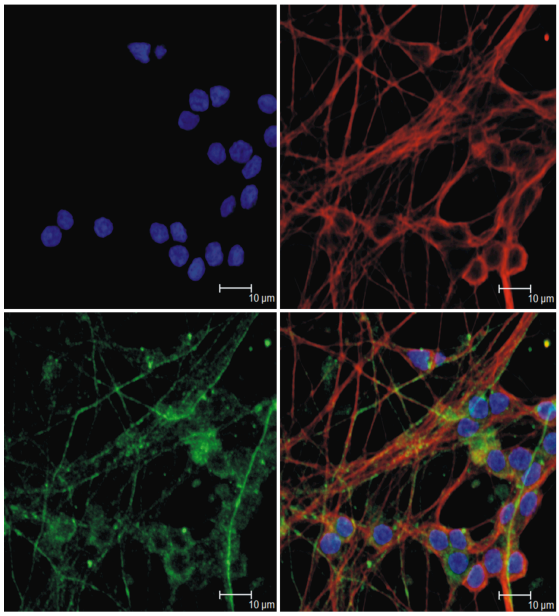


Figure 15: Immunofluorescence of primary cultures of CGNs. A) DAPI stained nuclei. Neurofilaments stained with B) Texas red and C) FITC dyes. D) Merged images

addition, microarray profiling of MS cortical gray matter revealed changes in some genes related with oxidative stress, and remyelination/repair [Fischer *et al.,* 2013; Haines *et al.,* 2015].

Therefore, in order to determine the effect of CSF in CGN culture, we perform experiments to identify global transcriptional changes by microarray analysis. For this purpose, we treated the neuronal cell cultures with 10% (v/v) CSF from MS

(IgM+/-, IgM+/+, medullary, PPMS) patients, NMO patients and controls for 24 hours in the next part of our experiments.

In some previous work from our laboratory [Beltran *et al.*, 2012] it was revealed that IgM-Ab from CSF of MS patients showed a pattern of reactivity to neuronal surface antigens when CGNs were treated with the CSF of MS patients We checked that in our conditions of work, with neuronal cell cultures from 14th days of growing in presence of 5.6 mM glucose, the same pattern of reactivity to neuronal surface antigens when CGNs were treated with the CSF of different MS subtype and NMO patients in comparison with control patients [Beltran *et al.*, 2012] (Figure 16). The investigators also found that the level of binding of anti-neuronal surface IgM-Ab to neurons in culture, measured as a percentage of average fluorescence intensity, was significantly higher for CSF from MS patients than for CSF from either the NIND group ($p=0.0004$) or NMO patients ($p=0.0003$), in which neuronal binding was detected at low levels, as also happened in our experiments (Figure 17).

6. Identification of stably expressed housekeeping genes for microarrays data normalization

One of the main problems in any microarray analysis, in order to obtain accurate gene expression data, is the correct normalization of the changes obtained of target mRNA transcripts using in most cases housekeeping genes as controls.

However, previous data from our laboratory experienced variation in sodium channel gene(s) expression while normalizing them with commonly used housekeeping genes including *ActB* and *Gapdh* (2010, Thesis of Dr. Beltran, University of Valencia). We asked ourselves in our experiments with CGNs treated

with the CSF of MS patients if we may use those common housekeeping genes to normalize the microarray data.

6.1. PCR of *Gapdh* and *β-actin*

We first quantified *ActB* and *Gapdh* genes by conducting conventional PCR in treated neuronal samples. We found that both *β-Actin* and *Gapdh* genes, which are presumed to express at constant levels showed, varying band intensity in cerebellar granule neurons when treated with the CSF of IgM+/+, IgM+/- (inflammatory MS), MedMS, PPMS, NMO and NIND patients. The figure 18 (panel A1 and B1) depicts unstable and fluctuated expression of *β-actin* and *Gapdh* in neurons treated with distinct clinical forms of MS and NMO. The panel A2 and B2 signifies the fold change *versus* control values of MS clinical forms, NMO, and control for both genes based on their band intensity analysed by ImageJ software.

The integrated density values of ß-Actin and Gapdh genes indicates that the band intensity of *ß-Actin* gene was highest in neurons treated with CSF of medullar patients as compared to control. IgM+/+ treated neurons exhibit the lowest band intensity as compared to control (Table 9). Similarly for *Gapdh* gene the band intensity was highest in neurons treated with CSF of medullar patients and lowest in IgM+/+ treated neurons as compared to control. Overall, it can be seen that the integrated density values of both commonly used reference genes, which are routinely used in majority of laboratories, are highly fluctuated in all the different experimental conditions we used.

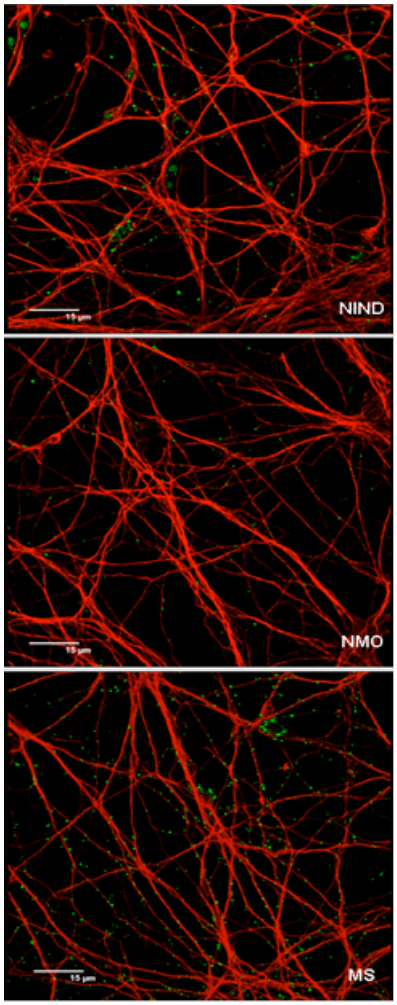


Figure 16: Immunofluorescent detection of neuroaxonal surface antigens recognized by IgMs present in the CSF of MS, NMO and NIND patients. Confocal images of rat cerebellar granule neurons, in primary culture, that have been exposed to 10% CSF for 2 h. IgM molecules in green (FITC) and β-tubulin from the microtubules of the cytoskeleton in red (Texas red).

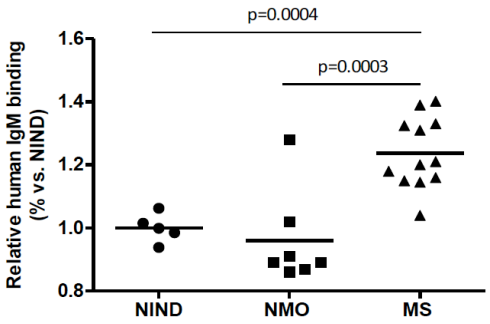


Figure 17: Graphical representation of human CSF IgM binding levels to neuroaxonal surface antigens. Human CSF IgM binding levels are expressed as a percentage of average fluorescence intensity relative to NIND. After incubation of CGN cultures with 10% CSF from MS, NMO or NIND controls.

From this data, we conclude that both *ActB* and *Gapdh* genes are not suitable to normalize gene transcipts in our experimental conditions.

6.2. Quantitative PCR of housekeeping genes

6.2.1. During the developmental stages of CGNs

The data provided in the previous chapter compiled to identify stably expressed reference genes suitable to be used in global gene expression analysis by microarrays.

With this aim, we performed real time PCR for a set of seven commonly used housekeeping genes (HKGs) chosen from literature as genes with invariable expression observed under distinct experimental conditions [Deindl *et al.,* 2002; Glare *et al.,* 2002; Hamalainen *et al.,* 2001; Radonic *et al.,* 2004; Zhong *et al.,* 1999;

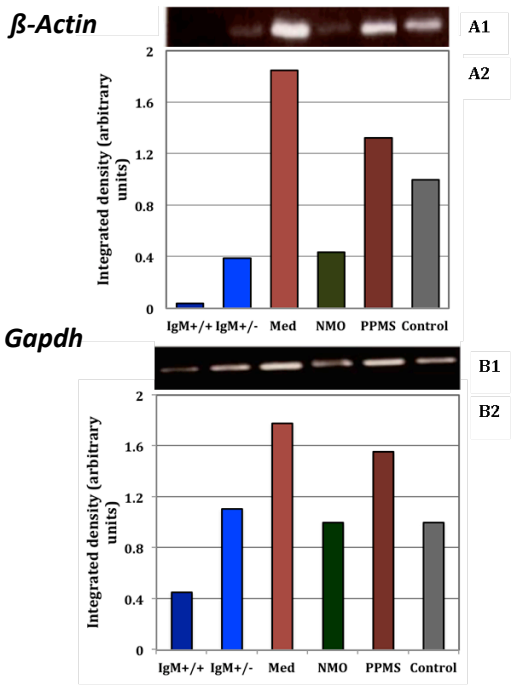


Figure 18: Analysis of *β-actin* (A) and *Gapdh* (B) expression by PCR: 1) Electrophoretic bands; 2) Integrated density obtained by Image J software quantification. The values correspond with the fold change *vs*. control values of the MS clinical forms, NMO, and control patients.

Table 9. Normalized integrated density values of *ß-Actin* and *Gapdh* genes in neurons treated with distinct clinical types of MS

MS/Control types	*ß-actin*	*Gapdh*
IgM+/+	574	5351
IgM+/-	5902	13124
Med	27890	21118
NMO	6580	11874
PP	19978	18432
Control	15076	11854

Toegel *et al.,* 2007; Torres *et al.,* 2003; Gubern *et al.,* 2009; Tricarico *et al.,* 2002] and recommended for precise normalization procedure [Ohl *et al.,* 2005; Zhong *et al.,* 1999; Hamalainen *et al.,* 2001; Vandesompele *et al.,* 2002]. The candidate HKGs selected were β-Actin (*ActB*), hypoxanthine guanine phosphoribosyl-transferase (*Hprt*), ribosomal protein L19 (*Rpl19*), lactate dehydrogenase A (*Ldha*), transferrin receptor (*Tfrc*), microglobulin beta-2 (*B2m*), and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*).

The cerebellar granule neurons were cultured for a period of 2 weeks and we determined expression of the HKGs selected during different time points of neuronal development (day 1, 5 8, 14). The ΔCT method and *GeNorm* and *NormFinder* algorithms were used to assess the expression stability of these reference genes. The *GeNorm* program defines the gene stability as the average pairwise variation of a particular gene with all other control genes and ranks the genes according to their average expression stability denoted by M [Vandesompele

et al., 2002]. The gene with minimum M value is considered to be highly stable whereas the gene with highest M value is least stable and can be excluded. The *NormFinder* algoritm ranks the candidate reference genes based on the combined estimates of both intra- and inter-group variations [Andersen *et al.,* 2004].

The *GeNorm* algorithm revealed that *Hprt, Gapdh* were the most stable genes followed by *Tfrc* during the maturation of CGNs (day 1, 5, 8, 14). For instance, average M value for *Hprt* and *Gapdh* genes was 0.06 and 0.08 for *Tfrc* gene. The *NormFinder* algoritm depicted that *Gapdh* and *Hprt* showed most invariable gene expression followed by *Tfrc* (average M value: 0.03 for *Gapdh* and *Hprt* genes and 0.036 for *Tfrc* gene). Therefore, both algoritms showed similar pattern of stably expressed genes hence they should be used as reference genes during the maturation of cerebellar granule neurons to accurately normalize the gene expression data.

On the contrary, *β-Actin*, a commonly used HKGs in most laboratories, showed the highest fluctuation. Therefore, the data suggests that *β-Actin* gene is not suitable for studies related to this one. The table 10 portrays the ranking of candidate HKGs during developmental stages of CGNs as per their expression stability.

The data indicates that the expression of beta actin (*ActB)* gene is two folds higher on day 5 and 0.4 times lower on day 8 as compared to day 1. There was 10 folds higher expression observed on day 14 as compared to day 1 during CGNs development. This is a marked fluctuation in *ActB* gene expression when neurons develop from day 1 to day 14. Both *GeNorm* and *NormFinder* algorithms identify *ActB* the highly variable gene during CGNs development. We conclude that this gene is not suitable to normalize gene transcripts during CGNs maturation.

The expression of hypoxanthine phosphoribosyltransferase (*Hprt)* gene was 0.2

folds on day 5 which was slightly lower as compared to day 1. Similary the expression was 0.13 folds lower on day 8 and 0.4 folds on day 14 as compared to day 1. The data suggests that there was slightest difference in gene expression during different time points of granule cell development. Additionally, *GeNorm* identifies *Hprt* as the most stable gene hence this gene may be used for normalization purpose in treated CGNs.

There was no difference in the expression of ribosomal protein L19 (*Rpl19)* gene during granule neuron development from day 1 to day 5. On day 8 and day 14, the expression level of this gene reduced to 0.6 folds as compared to control. The average expression stability of this gene was 0.214 as measured by *GeNorm* software.

The data suggests that the expression level of lactate dehydrogenase A (*Ldha)* gene dropped 0.2 folds from day 1 to day 5 during CGNs development and shoots up to 8.2 folds when granule neurons reached day 8 of its development. On day 14th the expression level again dropped to 0.5 folds as compared to day 1. We can see that there is a huge variation in *Ldha* gene expression during different developmental time points of granule neurons. To corroborate this data *GeNorm* and *NormFinder* softwares identifies this gene as one of the least stable genes during CGN development (Average expression stability value: 1.6 by GeNorm and 5.1 by NormFinder).

The expression of transferrin receptor (*Tfrc)* gene was reduced to 0.16 folds on day 5 as compared to day 1. Similarly when granule neurons reached day 8 of its development the expression remained same. The expression was further reduced to 0.36 folds when neurons reached day 14. This data indicates very slight difference in the expression level of this gene during different developmental stages of granule neurons. *GeNorm* confirms *Tfrc* as one of the most stable genes

with 0.08 as the average expression stability value.

The data indicates that beta-2 microglobulin (*B2m)* gene expression reduced to 0.17 folds from day 1 to day 5 and further 0.4 folds when the neurons reached day 8 of its development. On day 14th the expression remained almost same with 0.37 as the fold change compared to day 1.

The data indicates the expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh)* gene reduced to 0.34 folds from day 1 to day 5. There was negligible difference in its expression when the cells reached day 8 of the development. Furthermore the expression dropped to 0.4 folds when the neurons reached day 14th of its development. We can see that there was slightest difference in the expression of *Gapdh* gene during neuronal development. To corroborate this data, we used *GeNorm* and *NormFinder* softwares which further demonstrates *Gapdh* as the second most stable gene to be used in neuronal development (Average gene expression stability: 0.06 using *GeNorm* and 0.03 using *NormFinder*).

The figure 19 depicts the fold change values obtained from qPCR experiment for each reference gene tested during the development of cerebellar granule neurons (day 1, 5, 8, 14) normalized to the expression of day 1.

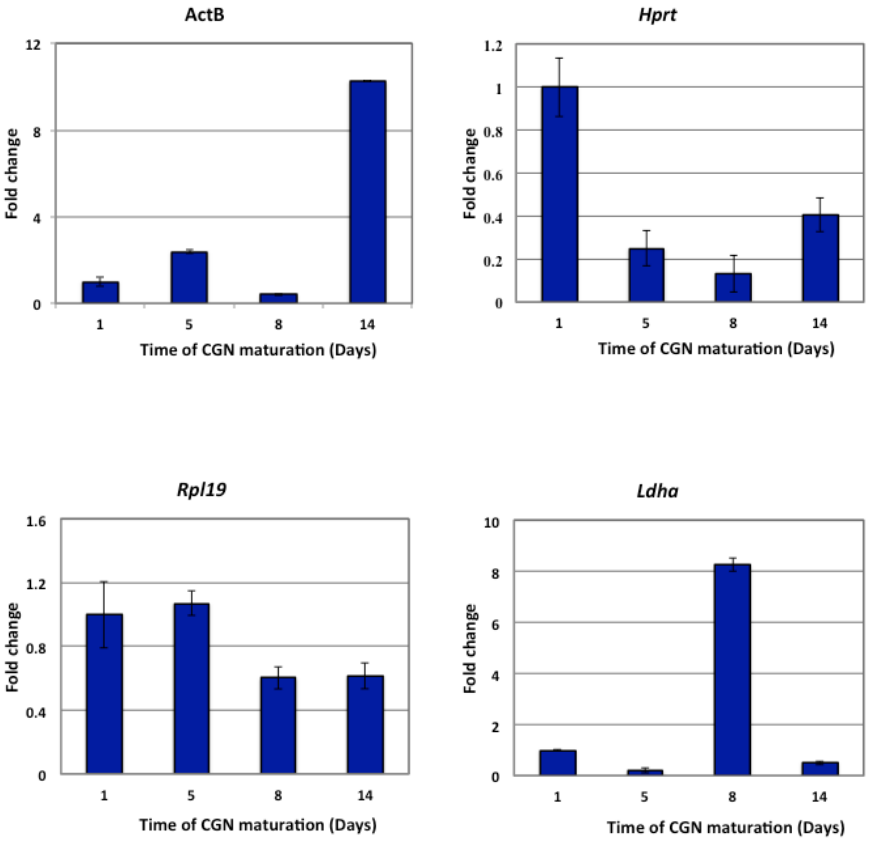
6.2.2. Most accurate experimental conditions established

From the data of the previous chapter we conclude from qPCR and using GeNorm and NormFinder algorithm that *Hprt Gapdh* are the most stable genes followed by *Tfrc* during CGNs development. On the other hand, *β–actin* showed highest fluctuation and therefore its use is strictly discouraged while normalizing gene expression data in studies related with global gene expression.

Once establish that we may used some HKGs to normalize data as constantly expressed, independently of changes in the time of growth for CGNs, we asked ourself if treatment of the cells with CSF of MS patients may induce additional changes in the expression. Changes in the HKGs depending on the type of MS disease would avoid the use them to normalize the global expression we wish to obtain in the microarray analysis.

The data of qPCR revealed that the expression of beta actin (*ActB)* gene dropped to 0.2 folds in neurons treated with IgM+/- MS CSF patients and increased to 1.4 folds in IgM+/+ treated neurons as compared to control. In neurons treated with medullary CSF the gene expression dropped to 0.04 folds and 0.2 folds in PPMS and increased to 1.78 folds in NMO patients compared to control. Although the variation in the expression level of this gene in all the different experimental conditions is not large, we employed GeNorm software to compare the expression stability of all the reference genes with each other (Table 11). The software GeNorm ranked *ActB* gene as the second last unstable gene as compared to the expression levels of other selected reference genes (M value: 2.92 using GeNorm). We conclude that this gene varies in our experimental conditions with respect to other selected reference genes. Hence it should not be used to normalize gene expression data in our experimental conditions.

The data obtained with hypoxanthine phosphoribosyltransferase *(Hprt)* indicates that the gene was 0.23 folds downregulated in neurons treated with IgM+/- CSF of MS patients as compared to control. The expression was up regulated 1.7 times in IgM+/+ treated neurons and down regulated by 0.1 folds, 0.27 folds and 0.4 folds in neurons treated with the CSF of medullary, PPMS and NMO patients respectively as compared to control. According to GeNorm program, *Hprt* was ranked as the third last unstable reference genes with respect to other reference genes (average


ActB
Fold change
12
8
4
0
1
5
8
14
Time of CGN maturation (Days)
Hprt
Fold change
1.2
1
0.8
0.6
0.4
0.2
0
1
5
8
14
Time of CGN maturation (Days)
Rpl19
Fold change
1.6
1.2
0.8
0.4
0
1
5
8
14
Time of CGN maturation (Days)
Ldha
Fold change
10
8
6
4
2
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1
5
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14
Time of CGN maturation (Days)

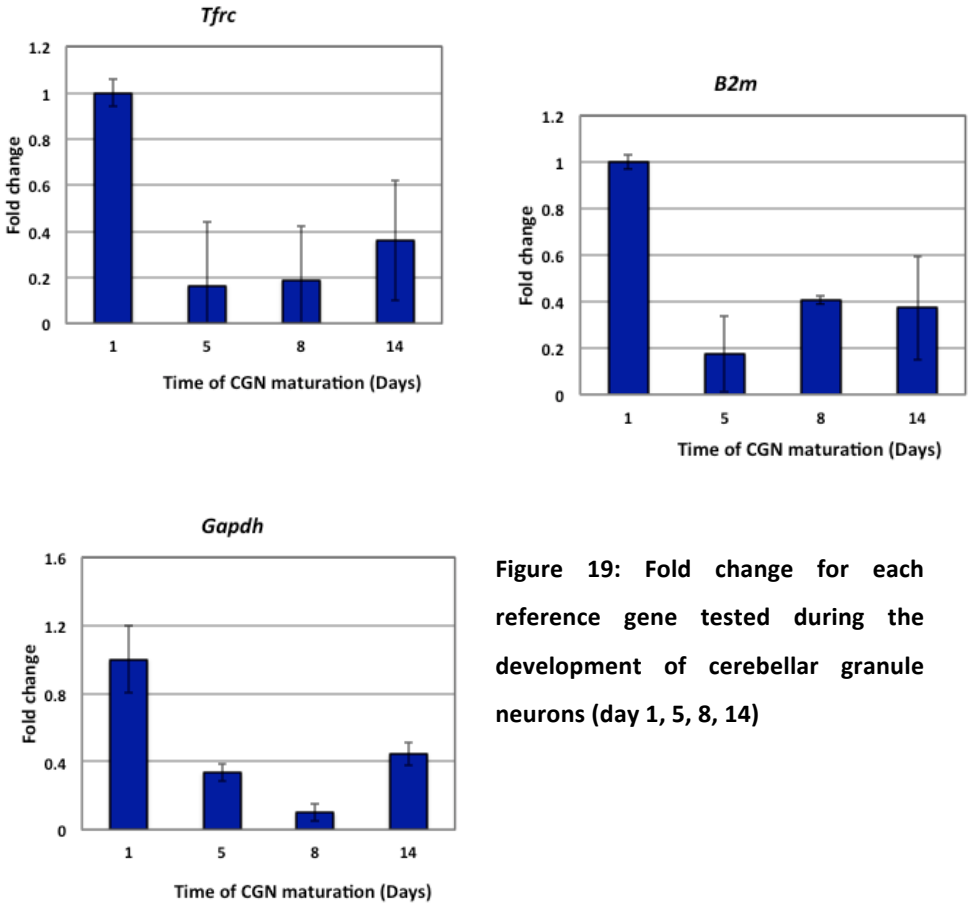


Figure 19: Fold change for each reference gene tested during the development of cerebellar granule neurons (day 1, 5, 8, 14)

Table 10. Candidate housekeeping genes ranked during the development of cerebellar granule neurons according to their expression stability by GeNorm and NormFinder methods. Average expression stability measure (M) of reference genes. Lower M value of average expression stability indicates more stable expression while the highest M value indicates the least expression.

GeNorm			NormFinder		
Ranking Order	**Gene Name**	**Average M value**	**Ranking Order**	**Gene name**	**Stability Value**
1	***Hprt***	**0.060**	**1**	***Gapdh***	**0.030**
1	***Gapdh***	**0.060**	**1**	***Hprt***	**0.030**
2	***Tfrc***	**0.088**	**2**	***Tfrc***	**0.036**
3	*B2m*	0.137	3	*B2m*	0.060
4	*Rpl19*	0.214	4	*Rpl19*	0.141
5	*Ldha*	1.689	5	*Ldha*	5.147
6	*ActB*	2.852	6	*ActB*	5.609

Average expression stability measure (M) of 7 reference genes with *Hprt, Gapdh and Ldha* the most stable genes. Lower M value of average expression stability indicates more stable expression while the highest M value indicates the least expression.

expression stability value: 1.3).

In relation with ribosomal protein L19 (*Rpl19)* gene, its expression was down regulated by 0.2 and 0.5 folds in IgM+/- and IgM+/+ treated neurons respectively as compared to control. There was only 0.1 folds decrease when neurons were treated with medullary, PPMS and NMO CSF patients as compared to control. We can see that there is a negligible variation in all the experimental conditions, as indicated by qPCR data, and in agreement with this data, GeNorm identifies this gene as the 3rd most stable gene (M value: 1.19).

The analysis with lactate dehydogenase A (*Ldha)* showed that there was 0.5 folds downregulation of gene in neurons treated with the CSF of IgM+/- patients with respect to control. The expression level increased up to 4 folds in neurons treated with IgM+/+ CSF treated neurons. In medullary, there was a 0.17 folds decrease in gene expression and we found 0.28 folds and 0.54 folds decrease gene expression in PPMS and NMO patients. The data signifies that the expression of this gene is not constant in all the experimental conditions. The average expression stability (M) value of this gene was 1.25 and thus was ranked as the 4^{th} stable gene according to GeNorm software.

In the transferrin receptor *(Tfrc)* study, the gene was up regulated by 1.2 folds in neurons treated with IgM+/- CSF treated neurons as compared to control. In the IgM+/+ CSF treated neurons, the expression level almost remained the same as compared to control. In medullary patients the expression was reduced by 0.2 folds and in PPMS CSF treated neurons the level was increased by only 1.1 folds which was almost similar as compared to control. There was a downregulation of this gene by 0.4 folds in neurons treated with the CSF of MS patients. Overall, we found that there was a negligible variation in the gene expression in different experimental conditions. Moreover, and according to the GeNorm algorithm, the average expression stability value was 1.092 and it was ranked the best reference gene with respect to others.

The data with beta-2 microglobulin (*B2m)* indicates that there was 1.2 folds up-regulation of gene in IgM+/- CSF treated neurons as compared to control. The expression was down regulated by 0.9 folds in IgM+/+ CSF treated neurons which was not a large variation as compared to control. It dropped to 0.2 folds in medullary CSF treated neurons and 0.4 folds in PPMS CSF treated neurons. The expression decreased by 0.8 folds in NMO treated neurons as compared to control.

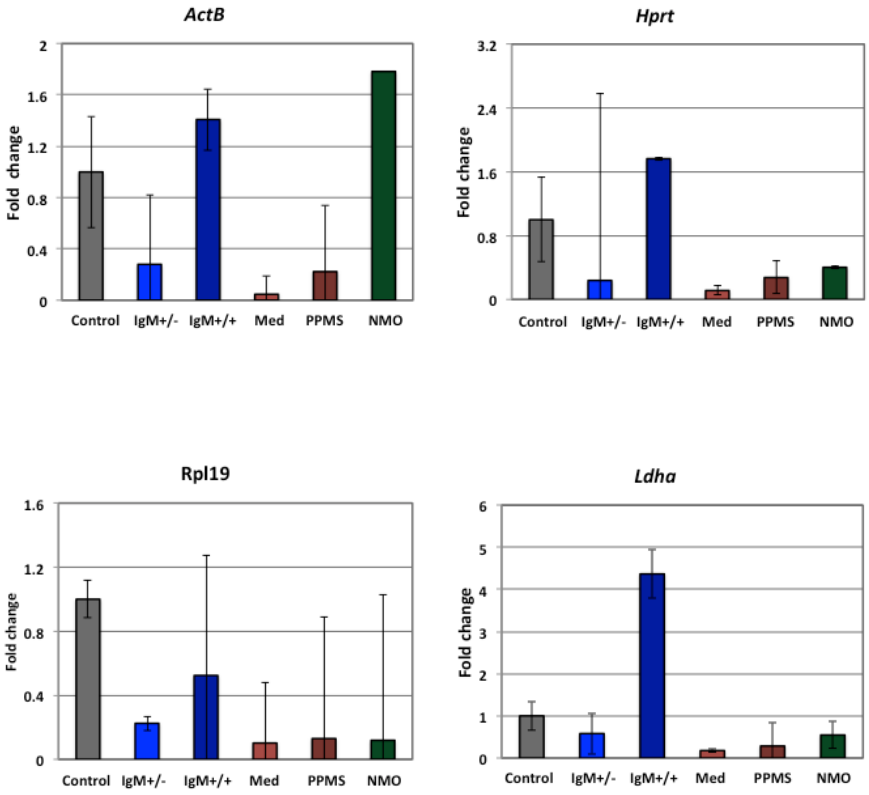
According to the GeNorm algorithm the average expression stability value was similar to *Tfrc* average expression stability (M: 1.092) and it was also ranked the best reference gene with respect to others.

The expression level of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh)* gene was 0.89 folds lower in IgM+/- treated neurons as compared to control. The expression level increased by 2 folds in IgM+/+ treated neuons as compared to control. In neurons treated with the CSF of medullary MS patients the gene downregulated by 0.02 folds and by 0.1 fold in neurons treated with the CSF of PPMS patients. Similarly the expression level declined by 0.38 folds in NMO treated patients. As we can see from the qPCR data that there is a huge fluctuation in *Gapdh* gene expression in our experimental conditions. Normally, *Gapdh* is used as a housekeeping gene but we find that it is not a housekeeping gene in our experimental conditions. GeNorm ranked this gene as the least stable gene with 4.2 as the average expression stability value.

The results of Figure 20 illustrates candidate HKGs genes in cerebellar granule neurons treated with CSF of MS/NMO patients according to their gene expression measured by qPCR. The gene expression values of all the experimental conditions were normalized to control.

Table 11 shows the candidate HKGs genes ranked according to their expression stability analyzed by GeNorm and NormFinder methods.

The data of this chapter allow us to conclude that both *Tfrc* and *B2m* with similar average expression stability values may be used to normalize global gene expression microarray data in the experimental conditions.


ActB
Fold change
2
1.6
1.2
0.8
0.4
0
Control
IgM+/-
IgM+/+
Med
PPMS
NMO
Hprt
Fold change
3.2
2.4
1.6
0.8
0
Control
IgM+/-
IgM+/+
Med
PPMS
NMO
Rpl19
Fold change
1.6
1.2
0.8
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Control
IgM+/-
IgM+/+
Med
PPMS
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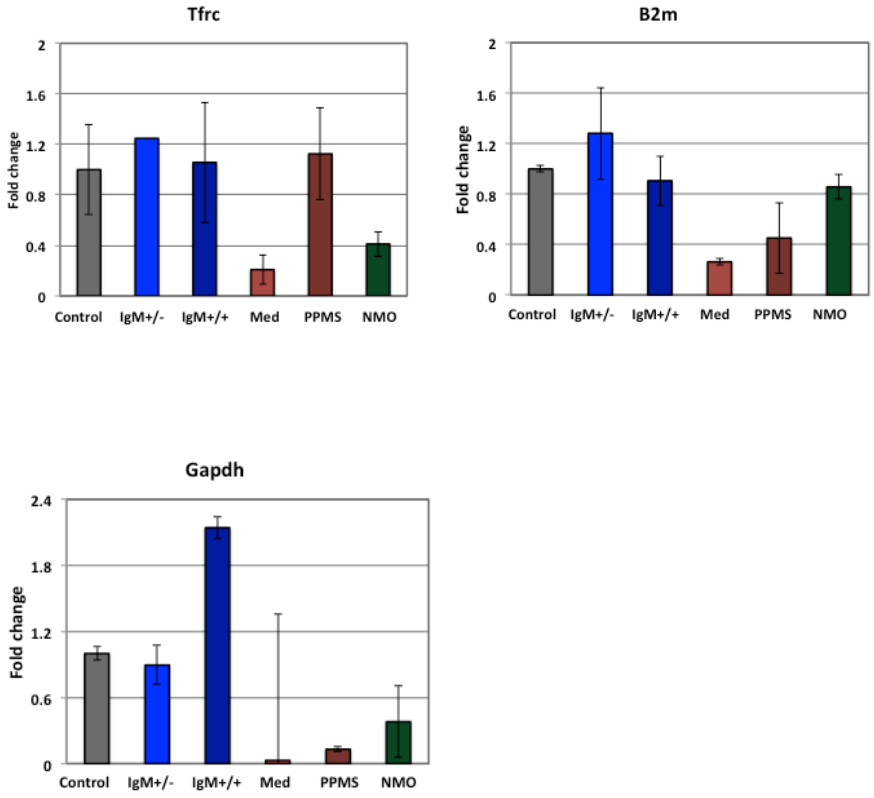


Figure 20: Fold change for each reference gene tested in distinct disease courses of multiple sclerosis. IgM+/+ and IgM+/-: Inflammatory forms of relapsing remitting multiple sclerosis; Med: Medullary form; NMO: Neuromyelitis Optica; Control: Other non-inflammatory neurological diseases (NIND).

Table 11. Candidate housekeeping genes ranked in cerebellar granule neurons treated with CSF of MS/NMO patients according to their expression stability by GeNorm and NormFinder methods. Lower M value of average expression stability indicates more stable expression while the highest M value indicates variable expression.

GeNorm			NormFinder		
Ranking Order	**Gene name**	**Average M value**	**Ranking order**	**Gene Name**	**Stability value**
1	***Tfrc***	**1.092**	**1**	***Tfrc***	**0.546**
1	***B2m***	**1.092**	**2**	***Ldha***	**0.589**
2	***Rpl19***	**1.198**	**3**	***Rpl19***	**0.972**
3	*Ldha*	1.253	4	*B2m*	1.102
4	*Hprt*	1.318	5	*Hprt*	1.379
5	*ActB*	2.929	6	*ActB*	6.099
6	*Gapdh*	4.201	7	*Gapdh*	6.953

7. Whole genome profiling in CGNs treated with the CSF of IgM+/-, IgM+/+, medullary, PPMS and NMO patients using microarray

The next step of our work was the study of the effect of CSF of different MS type patients, which could contain factors that damage neurons during attempts at brain repair, in global gene expression of CGNs cells by DNA microarrays.

To do this experiment, we isolated total RNA from different developmental stages of CGNs (day 1, 4, 8, 14) and handed for Agilent microarray-based gene expression analysis. Similarly we isolated total RNA from CGNs exposed to the CSF of inflammatory MS (IgM+/- MS and IgM+/+ MS), medullary MS, PPMS, NMO and

NIND patients and handed in parallel of simmilar microarray-based gene expression analysis. Both group of experiments were performed in genomics laboratory by its respective staff at centralized service of our center. The hybridization data was extracted using Agilent Feature Extraction software and, to normalize microarray signals, we used the 75th percentile as it is a more robust value in Gene Spring software (9.0 version).

From the different functional group of genes obtained, we found that genes related with carbohydrate metabolism and further pathways involved in ATP production, namely TCA cycle and electron transport chain, are overrepresented in our experimental conditions. We also we also found in the previous chapter that the widely used HKGs *ActB* did not express constitutively during CGNs cells development and therefore it was not regarded as a good reference gene to normalize gene expression in microarray data. On the contrary, other of the most widely used, HKG *Gapdh,* showed a constitutive expression during different time points of CGNs development (day 1, 5, 8, 14) but its expression fluctuated when CGNs were treated with the CSF of distinct clinical types of MS and NMO patients.

Having this data in mind, we redirect our research under the proposal that there should be some specific factors in the CSF of MS patients that should alter expression of genes specifically involved in carbohydrate metabolism and ATP production, and that these transcriptional changes may be related with the differential repairing capacity of neurons damage of the MS subtypes. These changes are not present in the normal CGNs development but they are deeply altered in the course of the MS disease.

7.1 Glycolytic pathway

The glycolysis is the most universal central pathway of carbohydrate metabolism to production of ATP and NADH in the cell. The metabolic pathway converts glucose into pyruvate and is a determined sequence of ten enzyme-catalyzed reactions.

The microarray data from the first step of glycolysis, the hexokinase (*Hk)* gene, show that its expression was downregulated showing around 30% of expression in neurons treated with RRMS IgM +/+ and medullary clinical forms of MS, as compared to neurons exposed to the CSF of non-inflammatory neurological controls. The other three conditions we analyze (RRMS IgM+/-, PPMS) have just negligible *Hk* gene expression differences in relation with the control, and a slight increasing of gene expression in NMO patients (Figure 21 A).

The data obtained of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh)* indicates that gene expression was downregulated to 33 %, 34 % and 35% of gene expression respectively in neurons treated with CSF of RRMS IgM+/+, IgM+/- and medullary form of MS as compared to neurological control. By contrary, PPMS and NMO show similar values of gene expression than the control (Figure 21 B).

The results obtained for the phosphogycerate kinase (*Pgk*) indicate that the enzyme is only affected by treatment of neurons with the CSF of IgM+/+ MS patients, but not with others treatments, as compared to the neurons treated with the CSF of control patients. The expression of *Pgk* gene was downregulated with a gene expression 35 % of the control (Figure 21 C).

The data indicates that phosphoglycerate mutase (*Pgm)* gene expression was downregulated up to around 30 % of gene expression in neurons treated with CSF of IgM+/-, IgM+/+, and PPMS clinical forms of MS clinical form of MS as compared to control. By contrary, CSF of medullary patients do not affect *Pgm* gene

expression, and produce a slight increasing of gene expression in treatment with CSF of NMO patients (Figure 21 D).

The data obtained with enolase *(Eno)* gene indicates that *Eno* gene was also downregulated up to 29 %, 20 % and 23 % of expression in neurons treated with IgM+/-, IgM+/+, and medullary clinical forms of MS clinical form of MS respectively as compared to neurons exposed to the CSF of neurological controls. Treatment with CSF of PPMS patients do not mainly affect *Eno* gene expression, and produce a slight increasing of gene expression, up to 150 % in treatment with CSF of NMO patients (Figure 21 E).

Finally, the expression of the pyruvate kinase isozyme 2 gene (*Pkm2*) was also downregulated up to 33% and 31% of the gene expression realted with controls in neurons treated with the CSF of IgM+/+ and medullary MS patients, with no significant changes in the other experimental conditions (Figure 21 F).

Figure 21 shows gene expression involved in carbohydrate metabolism in CGNs treated with CSF of MS and NMO patients related to neurons exposed to the CSF of neurological controls. We have plotted the percentage variation of genes in experimental conditions with respect to control calculated from the absolute fold change values from microarray data.

7.2 Krebs cycle pathway

The oxidative phosphorylation is the metabolic process that oxidizes several substrates through the Krebs (or tricarboxylic acid, TCA) cycle to produce reducing equivalents (NADH, $FADH_2$), which feed the respiratory chain to generate electrochemical gradient across the inner mitochondrial membrane that drives ATP synthesis through chemiosmotic coupling catalyzed by ATP synthase.

The first part of the oxidative phosphorylation, the TCA cycle, is connected with glycolysis by the reaction catalyzed by pyruvate decarboxylase (PDH). The *Pdh* gene was also downregulates to similar gene expression values, around 30 % of the expression of controls, during treatment of neurons with CSF of all clinical forms of MS, with the exception of CSF of NMO patients (Figure 22 A).

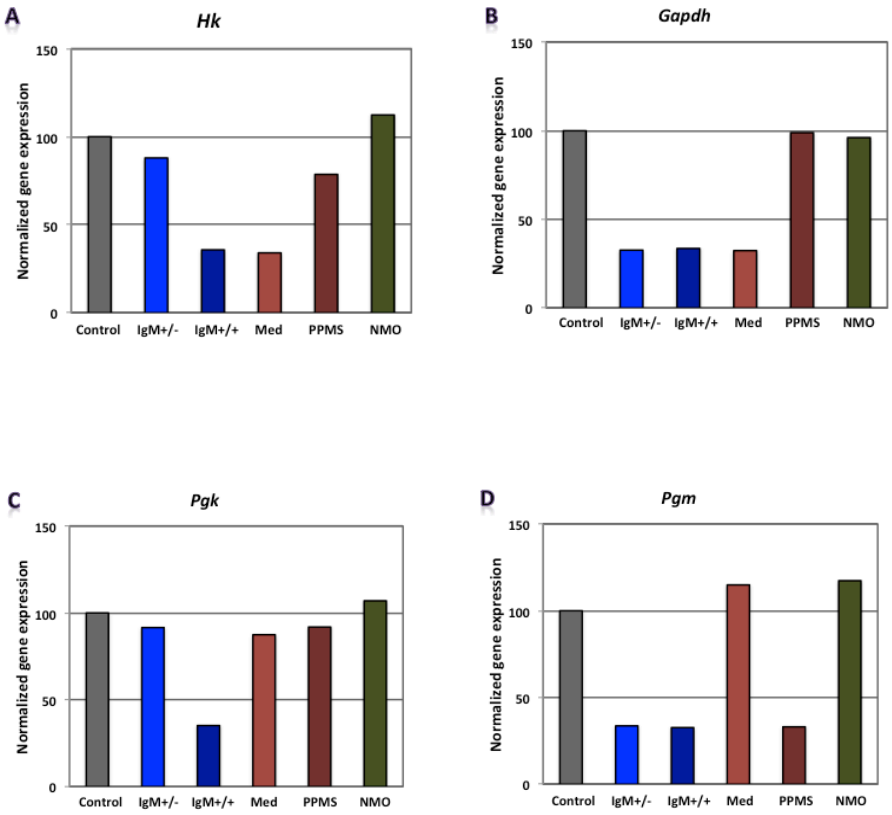
The unique gene of TCA cycle that is affected in neurons by CSF treatments was malate dehydrogenase (Mdh). The data indicates that *Mdh* gene was downregulated up to 23 % of gene expression in neurons treated with IgM+/+ clinical form of MS as compared to controls. Similarly, the expression was reduced to 27 % and 29 % in neurons treated with medullary and PPMS clinical forms of MS, and to 32 % and 35 % in neurons exposed to the CSF of RRMS IgM+/- and NMO patients, respectively in comparison with non-inflammatory neurological controls (Figure 22 B).

7.3. Mitochondrial genes involved in electron transport chain (ETC)

In the last part of the oxidative phosphorylation, the electron transport chain, two subunits of the ATP synthase complex are affected by CSF treatment of neurons, the α and β subunit.

The expression of ATP synthase subunit alpha (*Atp5A)* gene was downregulated up to around 35% of gene expression in neurons treated with CSF of Medullar and PPMS clinical form of MS as compared to controls, whereas just slight changes are observed in the other experimental conditions (Figure 23 A).

The other ATP synthase subunit beta affected, the gene (*Atp5B*), show that its expression is reduced up to around 30% of the control in all experimental


A
Hk
Normalized gene expression
150
100
50
0
Control
IgM+/-
IgM+/+
Med
PPMS
NMO
B
Gapdh
Normalized gene expression
150
100
50
0
Control
IgM+/-
IgM+/+
Med
PPMS
NMO
C
Pgk
Normalized gene expression
150
100
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0
Control
IgM+/-
IgM+/+
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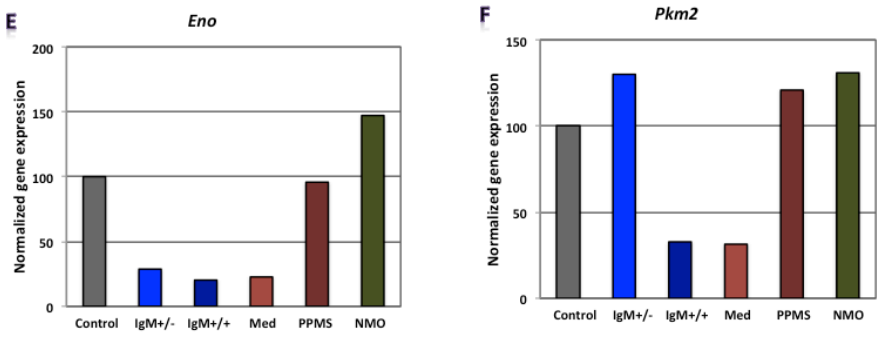


Figure 21: Normalized gene expression involved in glycolytic pathway in CGNs treated with CSF of MS and NMO patients normalized to gene expression in CGNs treated with CSF of non-inflammatory neurological controls. CGNs: cerebellar granule neurons; Blank: Neurons exposed only to culture medium; IgM+/- and IgM+/+: types of inflammatory MS; Med: Medullary MS; PP: Primary progressive multiple sclerosis; NMO: Neuromyelitis Optica

conditions treatment with the exception of NMO that show a slight increase of gene expression up to 129% in comparison with the neurological control (Figure 23 B).

Overall, the microarray findings demonstrate that the genes involved in carbohydrate metabolism were differentially expressed in cerebellar granule neurons treated with the CSF of MS and NMO patients, as compared to neurons exposed to the CSF of neurological controls. We conclude that CSF exposure to

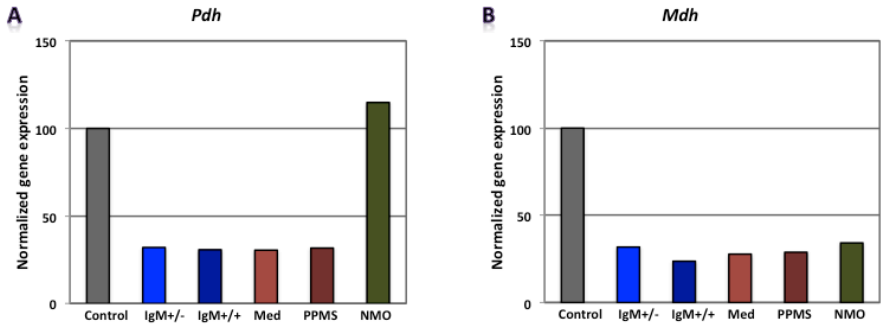


Figure 22: Normalized gene expression involved in TCA cycle pathway in CGNs treated with CSF of MS and NMO patients normalized to gene expression in CGNs treated with CSF of non-inflammatory neurological controls.

CGNs altered the carbohydrate metabolism in these neurons and may alter the capacity of these cells to repair axonal damage. In the discussion section we interprete the data for the different CSF MS type treatment with this reducing capacity of neuronal repairing.

8. Identification of stably expressed housekeeping genes in treated oligodendrocytes from microarray data

As commented in Introduction, the oligodendrocytes are the myelinating glial cells

of the CNS and the myelin sheath is crucial to neuronal function, enabling rapid propagation of nerve impulses and providing trophic support to the axon. Its loss in demyelinating diseases like MS has profound pathological consequences. The strategies to repair CNS myelin during the disease course of MS have gained significant momentum in order to find strategies for central nervous system repair. Many studies have been doing to identified molecular players involved in the blockade of oligodendrocyte progenitor cell (OPCs) differentiation, cells that are considered as the source for remyelination in the MS demyelinating disease. In addition to intrinsic factors, the characterization of extracellular molecules and the environmental milieu that OPCs experience in plaques is necessary in order to better understand repair strategies for MS. Since the CSF is in contact with the OPCs in the brain parenchyma and ventricles, some patient-dependent factors present in MS type disease can influence the cellular physiology of OPCs and neurons (Armati, 2010).

There are many research in the literature indicating that the type and amount of inflammation, the capacity of remyelination, and of tissue damage vary between different forms of MS and between different stages of the disease. These differences possibly reflecting different pathogenic mechanisms in the disease spectrum of MS types. In our work we are trying to identify aberrant transcriptional changes in carbohydrate metabolism genes that occur during brain repair by performing gene microarray profile, studiying the effect of CSF of MS and NMO patients to proliferating OPCs.

As happen in the microarray analysis of neurons, we need some housekeeping genes that do not vary in our experimental conditions in order to normalize gene expression data of target mRNA transcripts. Therefore, we wanted to identify the most stably expressed HKG genes out of a set of reference genes in treated OPCs.

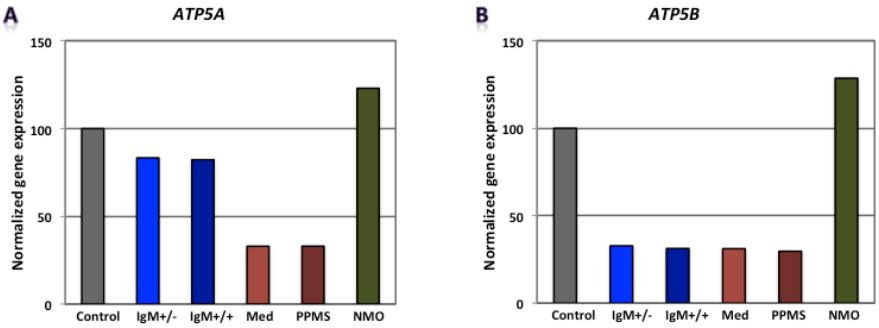


Figure 23: Normalized gene expression involved in oxidative phosphorylation in CGNs treated with CSF of MS and NMO patients related to gene expression in CGNs treated with CSF of non-inflammatory neurological controls. CGNs: cerebellar granule neurons; Blank: Neurons exposed only to culture medium; IgM+/- and IgM+/+: types of inflammatory MS; Med: Medullary MS; PP: Primary progressive multiple sclerosis; NMO: Neuromyelitis Optica

We selected the panel of reference genes studied with neurons analysis and we determine from the microarray data the changes of gene expression in the different MS and NMO patients, and determined their expression stability using *GeNorm* and *NormFinde*r algorithms. According to *GeNorm* algorithm *Mrpl19* and *Hprt1* were identified as the best housekeeping genes followed by *B2m* (average M value: 0.102 for *Mrpl19* and *Hprt1* genes and 0.147 for *B2m* gene). Surprisingly, *ActB* and *Gapdh* showed the most unstable gene expression in OPCs that were exposed to the CSF of our experimental conditions. *NormFinder* found an altogether different ranking of housekeeping genes. According to this software,

Tfrc and *B2m* were identified as the best housekeeping genes followed by *ActB* (average M value: 0.033 for *Tfrc* and *B2m* genes, and 0.087 for *ActB* gene). *Mrpl19 and Gapdh* showed the least stable gene expression in OPCs that were exposed to the CSF of our experimental conditions. We conclude from this data that *Mrpl19, Hprt*, *Tfrc* and *B2m* should be used to normalize the gene transcripts in experiments related to the current one and the use of *ActB* and *Gapdh* should be avoided.

The data indicates that the expression of beta actin (*ActB)* gene was downregulated significantly, showing 37% and 42% gene expression in OPCs exposed to the CSF of IgM+/+ and medullary MS as compared to control. The expression was downregulated by 37% in OPCs exposed to the CSF of NMO patients as compared to control (Figure 24A). We can see a marked fluctuation in *ActB* gene expression in OPCs exposed to the CSF of various experimental conditions. *GeNorm* identified *ActB* a highly variable gene in these experimental conditions. We conclude that this gene is not suitable to normalize gene transcripts in treated OPCs.

The expression of beta-2 microglobulin (*B2m)* gene was downregulated significantly, showing 50%, 48% and 58 % in OPCs exposed to the CSF of IgM+/-, IgM+/+ and medullary MS, as compared to control. Similarly significantly reduced expression with 43% in OPCs exposed to the CSF of NMO patients was observed as compared to control (Figure 24B). *GeNorm* identified B2m as the third most stable gene hence this gene can be used for normalization purpose in treated OPCs.

The expression of hypoxanthine phosphoribosyltransferase *(Hprt1)* gene was downregulated significantly by 49%, 38%, and 49 % in OPCs exposed to the CSF of IgM+/-, IgM+/+ and medullary MS as compared to control. Similarly the expression was around 52 % significantly reduced in OPCs exposed to the CSF of PPMS and NMO patients as compared to control (Figure 24C). *GeNorm* identified *Hprt1* as the

second most stable gene hence this gene can be used for normalization purpose in treated OPCs.

The expression of mitochondrial ribosomal protein L19 (*Mrpl19)* gene was downregulated significantly by 52%, 46% and 48 % in OPCs exposed to the CSF of IgM+/-, IgM+/+ and medullary MS as compared to control. Similarly the expression was 52 % and 45 % lower in OPCs exposed to the CSF of PPMS and NMO patients as compared to control (Figure 24D). *GeNorm* identified *Hprt1* as the second most stable gene hence this gene can be used for normalization purpose in treated OPCs.

As we can see from the data that there is a huge fluctuation in transferrin receptor (*Tfrc)* gene expression across whole experimental conditions (Figure 24E).

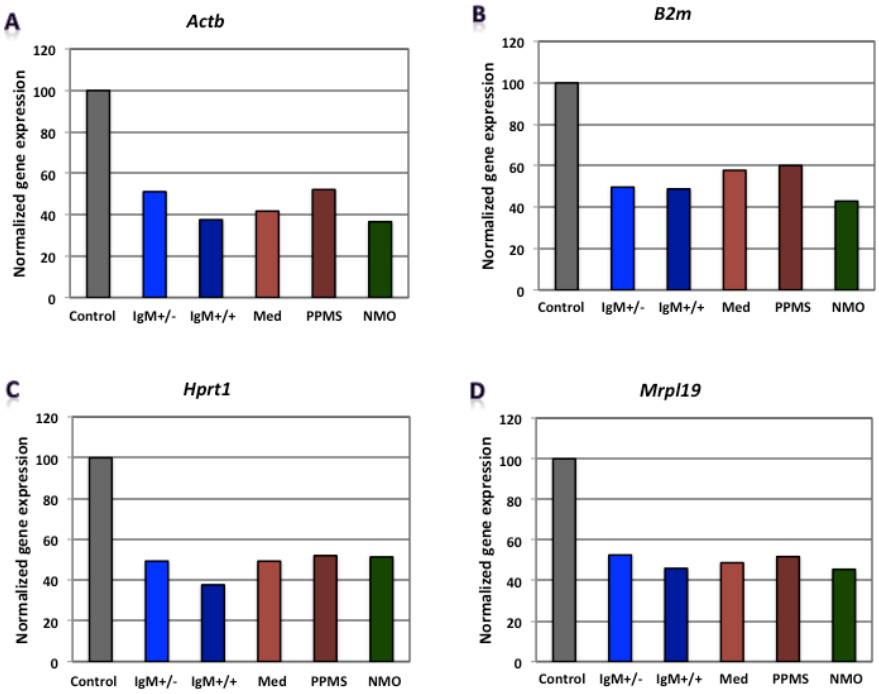
The data indicates that the expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh)* gene was downregulated by 44%, 54% and 49% in OPCs treated with IgM+/-, IgM+/+ and medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 55% and 44% in OPCs treated with the CSF of PPMS and NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 24F). According to *GeNorm* and *NormFinder* algorithms, *Gapdh* was ranked as an unstable gene for normalizing mRNA transcripts.

9. Whole genome profiling in oligodendrocytes (OPCs) treated with the CSF of IgM+/-, IgM+/+, medullary, PPMS and NMO patients using microarray

In the next group of experiments, we performed gene expression profiling in

oligodendrocytes treated with the CSF of IgM+/-, IgM+/+, medullary, PPMS and NMO patients using microarray technology. Oligodendrocytes (OPCs) are the myelinating glial cells of the CNS and the myelin sheath, generated by oligodendrocytes, is crucial to neuronal function, enabling rapid propagation of nerve impulses and providing trophic support to the axon. Its loss in demyelinating diseases like MS has profound pathological consequences. Strategies to repair CNS myelin during the disease course of MS have gained significant momentum in order to find strategies for central nervous system repair. Many studies have identified molecular players involved in the blockade of oligodendrocyte progenitor cell (OPC) differentiation. In addition to intrinsic factors, the characterization of extracellular molecules and the environmental milieu that OPCs experience in plaques is necessary in order to better understand repair strategies for MS. Cerebrospinal fluid (CSF) is in contact with the brain parenchyma and ventricles, which can be a site for deposition of cellular damage products, including lipids [Vidaurre *et al.,* 2014], cytokines [Rossi *et al.,* 2014; Rossi *et al.,* 2012] which can influence the cellular physiology of OPCs, myelinating oligodendrocytes and neurons. Numerous studies have attempted to identify soluble factors in the CSF of MS patients that account for the observed cellular effects. For example, ceramides present in MS CSF promote axonal injury and neuronal damage through oxidative stress mechanisms and mitochondrial bioenergetic failure [Vidaurre *et al.,* 2014]. In addition, microarray profiling of MS cortical gray matter revealed gene changes in oxidative stress, and remyelination/repair [Fischer *et al.,* 2013]. Therefore, in order to identify the transcriptional changes that occur to proliferating OPCs, we performed gene microarray profiling to determine the effect of CSF which could contain factors that damage OPCs during attempts at brain repair.

To conduct this experiment, we isolated mRNA from treated oligodendrocyte


A
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Normalized gene expression
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Control
IgM+/-
IgM+/+
Med
PPMS
NMO
B
B2m
Normalized gene expression
120
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60
40
20
0
Control
IgM+/-
IgM+/+
Med
PPMS
NMO
C
Hprt1
Normalized gene expression
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40
20
0
Control
IgM+/-
IgM+/+
Med
PPMS
NMO
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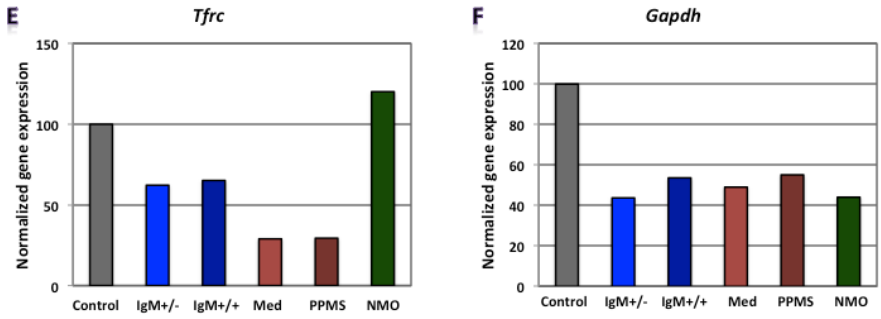


Figure 24: Normalized gene expression for each reference gene in OPCs tested in distinct disease courses of multiple sclerosis. IgM+/+ and IgM+/-: Inflammatory forms of relapsing remitting multiple sclerosis; Med: Medullary form; NMO: Neuromyelitis Optica; Control: Other non-inflammatory neurological diseases (NIND).

progenitor cells (OPLs) using the Qiagen RNeasy RNA extraction kit. Therafter, mRNA was reverse transcribed in the presence of T7- oligo-dT primer to produce cDNA. cDNA was then *in vitro* transcribed with T7 RNA polymerase in the presence of Cy3-CTP to produce labeled cRNA. The labeled cRNA was hybridized to the Agilent SurePrint G3 Rat GE 8x60K Microarray according to the manufacturer's protocol. The arrays were washed, and scanned on an Agilent G2565CA microarray scanner at 100% PMT and 3 μm resolution. The intensity data was extracted using the Feature Extraction Software (Agilent).

In the graphs shown below we have plotted the expression of genes in experimental conditions with respect to neurological control calculated from the

Table 12. Candidate housekeeping genes ranked in oligodendrocytes exposed to the CSF of RRMS and PPMS patients according to their expression stability by *GeNorm* and *NormFinder* methods. M: Average expression stability. Lower M value indicates more stable expression while the highest M value indicates variable expression.

GeNorm			***NormFinder***		
Ranking Order	**Gene name**	**Stability value**	**Ranking order**	**Gene name**	**Stability value**
1	***Mrpl19***	**0.102**	**1**	***Tfrc***	**0.033**
1	***Hprt1***	**0.102**	**1**	***B2m***	**0.033**
2	***B2m***	**0.147**	**2**	***ActB***	**0.087**
3	*Tfrc*	0.160	3	*Hprt1*	0.222
4	*ActB*	0.192	4	*Mrpl19*	0.260
5	*Gapdh*	0.272	5	*Gapdh*	0.426

raw Log2 values obtained from microarray data. Figure 25 shows normalized expression of genes involved in carbohydrate metabolism in OPLs treated with CSF of MS and NMO patients related to neurons exposed to the CSF of neurological controls.

The significance levels were obtained between medullary and control; PPMS and control; NMO and control; IgM+/+ and IgM+/-; IgM+/- and medullary; IgM+/+ and medullary; NMO and medullary; and PPMS and medullary.

9.1. Glycolytic pathway

Hexokinase *(Hk)* gene expression was found to be significantly reduced by 42% and

52% in OPCs treated with IgM+/- and IgM+/+ MS patients. The gene was 56% reduced in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 38% in OPCs treated with the CSF of PPMS patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 25 A).

The expression of glucose-6-phosphate isomerase (*Gpi)* gene was found to be downregulated by 56% and 40% in OPCs treated with IgM+/- and IgM+/+ MS patients. There was 46% down regulation in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 54% and 42% in OPCs treated with the CSF of PPMS and NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND). In addition, the gene expression in OPCs treated with the CSF of IgM+/- MS patients was significantly reduced by 15% as compared to OPCs treated with the CSF of IgM+/+ MS patients (Figure 25 B).

The data indicates that the expression of aldolase C (*Aldoc)* gene was downregulated showing 28% in OPCs treated with NMO as compared to OPCs exposed to the CSF of neurological controls. Furthermore, there was a significant difference found in neurons exposed to CSF from medullary MS with 64% expression and neurons exposed to CSF from NMO patients showing 28 % expression (Figure 25 C).

The data indicates that the expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh)* gene was downregulated showing 53% and 44% in OPCs treated with IgM+/- and IgM+/+ MS patients. There was 49% down regulation in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 55% and 44% in OPCs treated with the CSF of PPMS and NMO patients as compared to OPCs

exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 25 D).

The data indicates that the expression of triosephosphate isomerase (*Tpi)* gene was downregulated by 33% in OPCs treated with IgM+/+ MS patients. The gene was reduced by 40 % in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced with 37% in OPCs treated with the CSF of NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND). In addition, we found significant differences in the gene expression between OPCs treated with the CSF of IgM+/- MS patients with 33 % and the OPCs treated with the CSF of IgM+/+ MS patients with 60 % gene expression (Figure 25 E).

The expression of phosphoglycerate kinase (*Pgk1)* gene was significantly reduced by 57% and 39% in OPCs treated with IgM+/- and IgM+/+ patients. The gene was reduced by 45% in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced showing 46% in OPCs treated with the CSF of NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND). Furthermore, we found significant differences in the gene expression between OPCs treated with the CSF of IgM+/- MS patients and the OPCs treated with the CSF of IgM+/+ MS patients (Figure 25 F).

The data indicates that phosphoglycerate mutase (*Pgam1)* gene was upregulated several folds, around 2,882% in OPCs treated with IgM+/+ MS patients. In addition, there were significant differences in the expression of *Pgam1* gene between OPCs treated with the CSF of medullary clinical form of MS and OPCs exposed to the CSF of IgM+/- MS patients (Figure 25 G).

The data indicates that enolase (*Eno1)* gene expression was downregulated by 54% and 43% in OPCs treated with IgM+/- and IgM+/+ MS patients. There was 51%

down regulation in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 40% in OPCs treated with the CSF of NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 25 H).

The gene expression of another isoform of the same gene expressed in brain (*Eno2*) was downregulated by 41% and 44% in OPCs treated with IgM+/+ and medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced showing 5% and 44% in OPCs treated with the CSF of PPMS and NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND). In addition, we found significant differences in the gene expression between OPCs treated with the CSF of IgM+/- MS patients and the OPCs treated with the CSF of IgM+/+ MS patients (Figure 25 I).

The data indicates that the expression of pyruvate kinase (*Pk)* gene was downregulated by 50% and 42% in OPCs treated with IgM+/- and IgM+/+ MS patients. There was 49% decrease in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 58% and 37% in OPCs treated with the CSF of PPMS and NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 25 J).

The data indicates that the expression of *Pkm2* gene was downregulated by 44% and 47% in OPCs treated with IgM+/+ and medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 57% and 44% in OPCs treated with the CSF of PPMS and NMO patients as compared to OPCs exposed to the CSF of non-inflammatory

neurological controls (NIND). In addition, we found significant differences in the gene expression between OPCs treated with the CSF of IgM+/- MS patients and the OPCs treated with the CSF of IgM+/+ MS patients (Figure 25 K).

9.2. Mitochondrial genes involved in TCA cycle

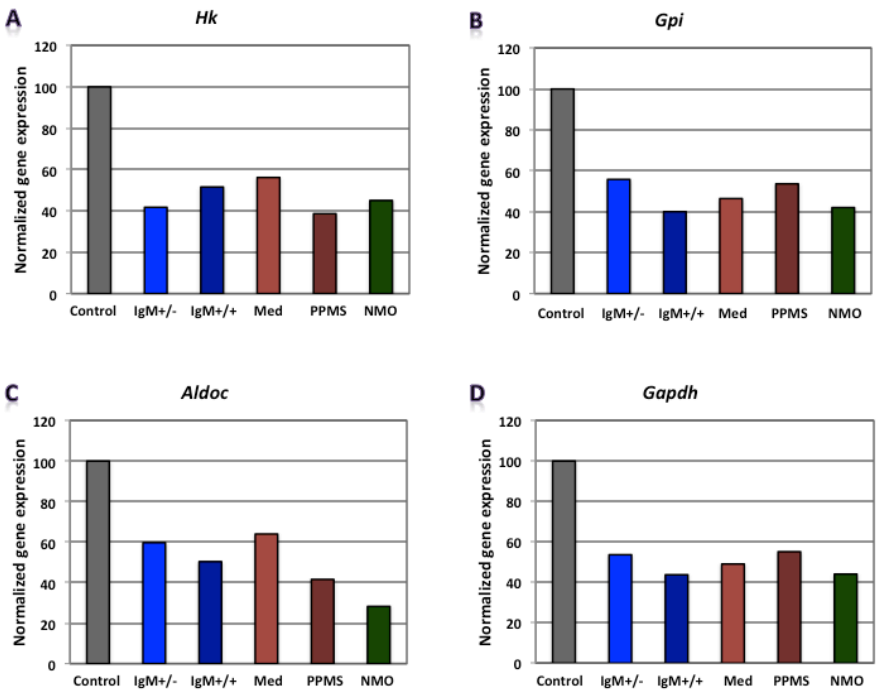
As occurs in neurons, genes implicated in the TCA cycle were affected by treatment of OPCs with CSF from MS patients.

The expression of pyruvate dehydrogenase (*Pdha1)* gene was significantly lowered by 56%, 28% and 41% in OPCs treated with the CSF of IgM+/-, IgM+/+ and medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 31% in OPCs treated with the CSF of NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 26 A).

The data indicates that the expression of mitochondrial aconitase *(Aco2)* gene was downregulated by 52% and 46% in OPCs treated with IgM+/- and IgM+/+ MS patients. The gene expression lowered by 50% in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the gene was down regulated with 54% and 38% expression in OPCs treated with the CSF of PPMS and NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 26 B).

The expression of isocitrate dehydrogenase (*Idh)* gene was significantly reduced by 39% and 48% in OPCs treated with IgM+/- and IgM+/+ MS patients. The gene was reduced by 52% in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls (Figure 26 C).

The data indicates that the expression of oxoglutarate (alpha-ketoglutarate)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Gpi
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Aldoc
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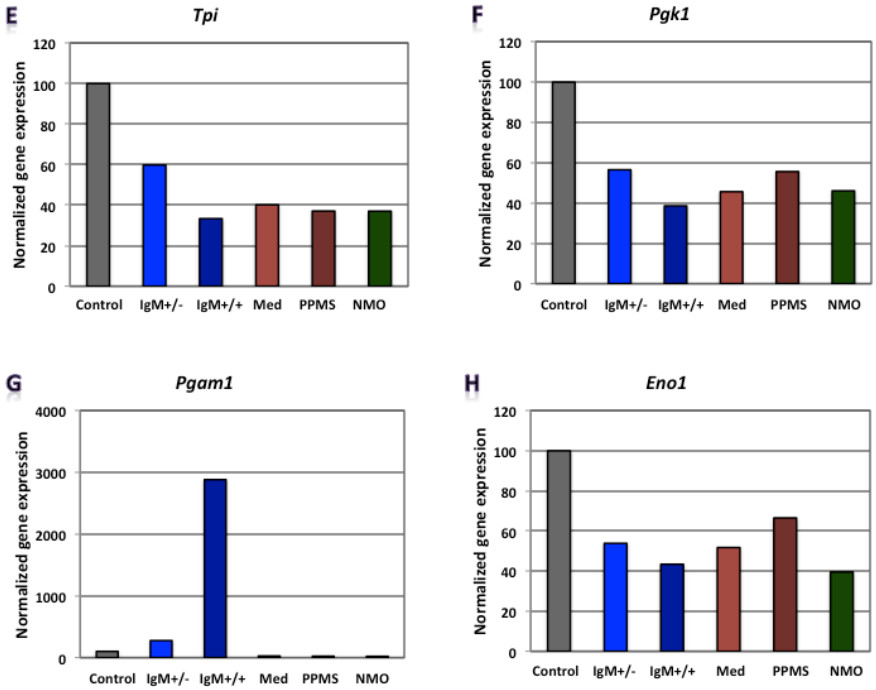
PPMS
NMO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Normalized gene expression
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Pgk1
Normalized gene expression
120
100
80
60
40
20
0
Control
IgM+/-
IgM+/+
Med
PPMS
NMO
G
Pgam1
Normalized gene expression
4000
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2000
1000
0
Control
IgM+/-
IgM+/+
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PPMS
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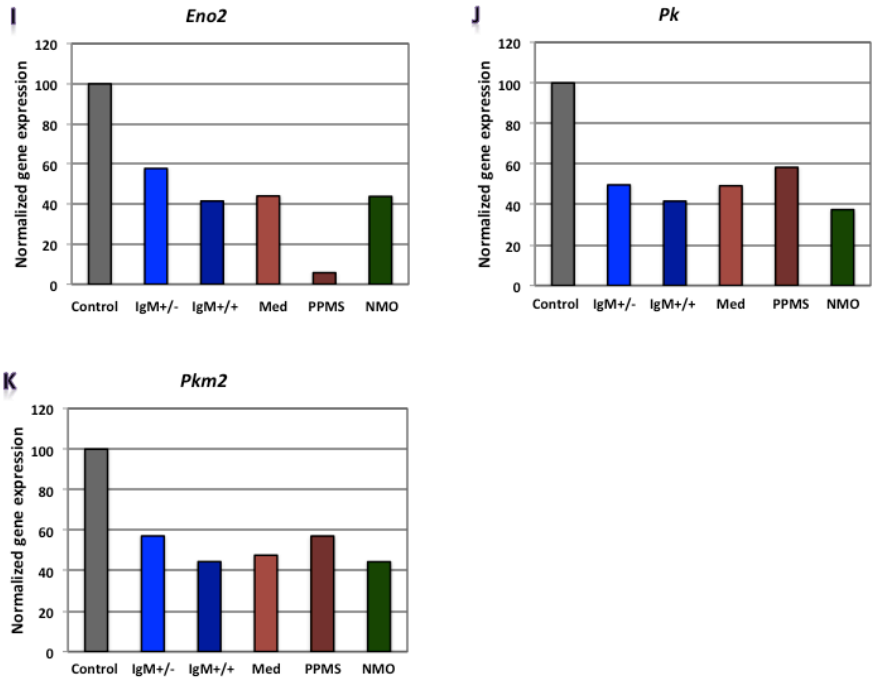


Figure 25: Normalized gene expression involved in glycolytic pathway in OPCs treated with CSF of MS and NMO patients related to gene expression in OPCs treated with CSF of non-inflammatory neurological controls. OPCs: oligodendrocytes; Control: Neurons treated with CSF of non-inflammatory neurological controls; IgM+/- and IgM+/+: types of inflammatory MS; Med: Medullary MS; PP: Primary progressive multiple sclerosis; NMO: Neuromyelitis Optica.

dehydrogenase (*Ogdh)* gene was downregulated showing 53%, 36% and 29% expression in OPCs treated with IgM+/-, IgM+/+ and medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced showing 38% in OPCs treated with the CSF of NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 26 D).

The expression of succinate dehydrogenase (*Sdh)* gene was found to be downregulated with 36% and 49% in OPCs treated with IgM+/+ and medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced showing 47% in OPCs treated with the CSF of NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND). In addition, we found significant differences in the gene expression between OPCs treated with the CSF of IgM+/- MS patients and the OPCs treated with the CSF of IgM+/+ MS patients (Figure 26 E).

The expression of mitochondrial malate dehydrogenase (*Mdh2)* gene was significantly reduced with 30% and 26% in OPCs treated with the CSF of IgM+/+ and NMO patients as compared to to OPCs exposed to the CSF from neurological controls (Figure 26 F).

9.3. Mitochondrial genes involved in electron transport chain (ETC)

We also analysed, as we did with neurons, the changes in the expression of the genes related with ATP production by the mitocondrial electronic chain and oxidative phosphorylation by treatment of OPC with CSF of MS patients.

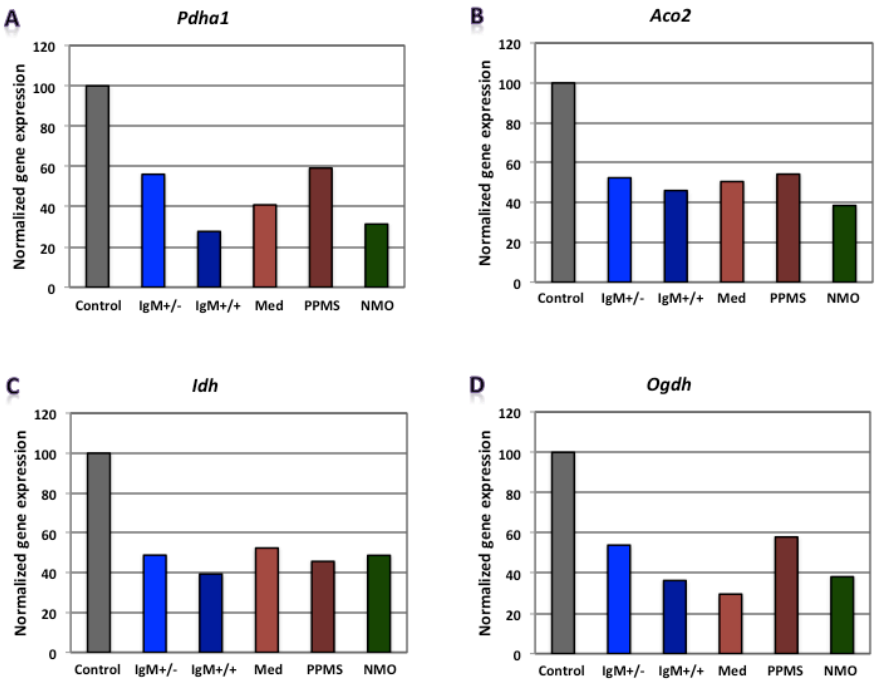
The expression of ATP synthase gene alpha subunit (*ATP5a1*) was found to be

downregulated by 46% and 41% in OPCs treated with IgM+/- and IgM+/+ MS patients. There was 48% reduction of gene expression in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 50% and 52% in OPCs treated with the CSF of PPMS and NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 27 A).

The expression of another gene that form the mitochondrial H^+/ATPase (the ß subunit, *ATP5b*) was found to be also downregulated by around 50% in OPCs treated with IgM+/+, and medullary clinical form of MS and around 54 % in IgM+/- as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 36% in OPCs treated with the CSF of NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 27 B).

The data also indicates that the expression of cytochrome c oxidase gene (*Cox*) was downregulated by around 47%, 46% and 56% in OPCs treated with IgM+/-, IgM+/+ and medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 28% and 42% in OPCs treated with the CSF of PPMS and NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 27 C).

The expression of NADH-ubiquinone oxidoredutase chain 2 (*MT-ND2*) gene was downregulated by 52% and 48% in OPCs treated with IgM+/- and IgM+/+ MS patients. The gene expression reduced by 52% in OPCs treated with medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 52% and 44% in OPCs treated with the CSF of PPMS and NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 27 D).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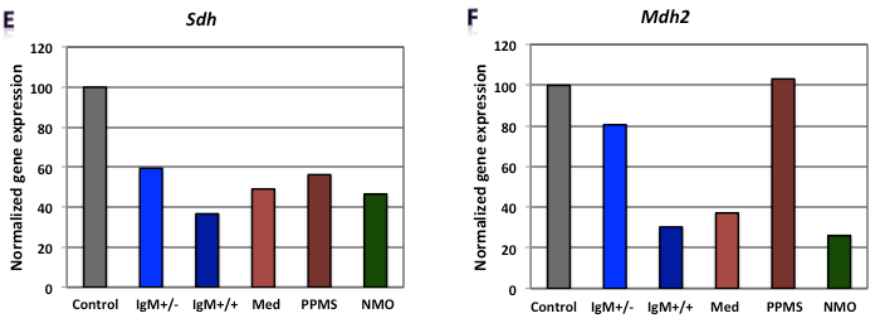


Figure 26: Normalized gene expression involved in TCA cycle in OPCs treated with CSF of MS and NMO patients related to gene expression in OPCs treated with CSF of non-inflammatory neurological controls. OPCs: oligodendrocytes; Control: Neurons treated with CSF of non-inflammatory neurological controls; IgM+/- and IgM+/+: types of inflammatory MS; Med: Medullary MS; PP: Primary progressive multiple sclerosis; NMO: Neuromyelitis Optica**.**

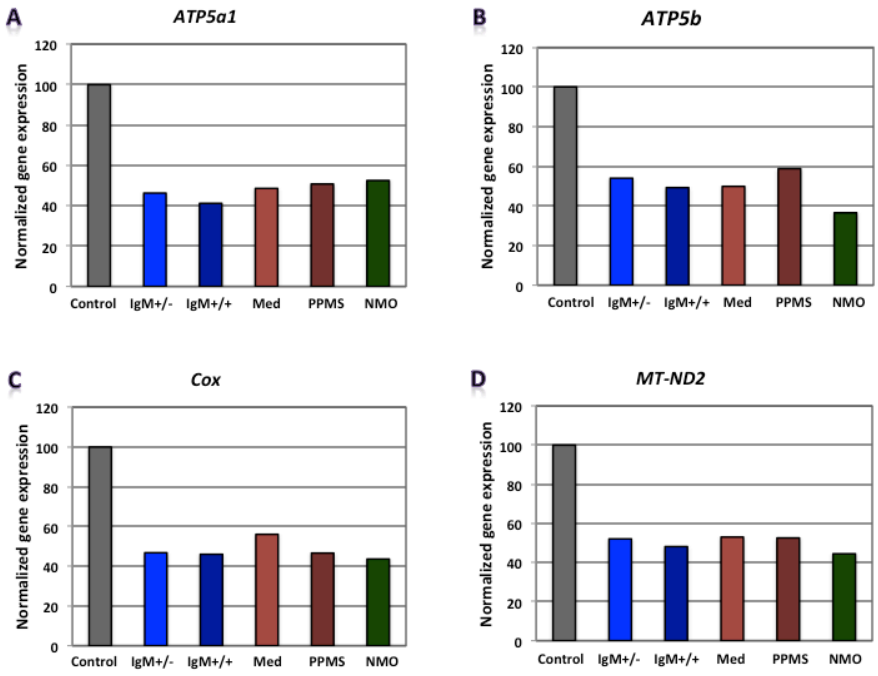
The expression of cytochrome c1 (*Cyc1)* gene was found to be downregulated by 50%, 39% and 47% in OPCs treated with IgM+/-, IgM+/+, and medullary clinical form of MS as compared to OPCs exposed to the CSF of neurological controls. Similarly, the expression was reduced by 40% in OPCs treated with the CSF of NMO patients as compared to OPCs exposed to the CSF of non-inflammatory neurological controls (NIND) (Figure 27 E).

In our work we found that the genes involved in glucose metabolism (including glycolysis, TCA cycle and oxidative phosphorylation) were differentially expressed

in OPC treated with the CSF of MS patients. This data demonstrates that the carbohydrate metabolism is altered in treated oligodendrocytes. In conclusion, MS patient-derived CSF alters carbohydrate metabolism in proliferating oligodendrocyte progenitors.

10. Analysis of gene-gene interaction networks using String v10 software

In the next section of the thesis, we were interested to know whether the interaction of different metabolic genes related with a metabolic network (i.e. glycolysis, TCA cycle, and electron transport chain) affected the whole network, included those genes we did not find too many changes in the whole carbohydrate network. As discussed in chapter 1 (Introduction) the importance of glycolytic enzymes in forming localized complexes may play a crucial role in allowing intricate regulatory control of glycolytic pathway. The formation of glycolytic complex is involved in increasing the solvation capacity with the cytosol and channeling of substrates. The channeling promoted in a complex would allow kinetic advantage of the reaction. The attraction of the enzyme molecule for the activated complex would lead to a decrease in its energy and hence to a decrease in the energy of activation of the reaction and to an increase in the rate of reaction. The fact that an enzyme-catalyzed reaction has a maximal velocity suggests the formation of enzyme substrate complex. Genes purely involved in glycolysis interact with each other or with other pathways and form complexes. Therefore, we wanted to elucidate how interaction of genes may affect the whole network in different experimental conditions. For this, we used *STRING v10* ("Search Tool for Retrieval of interacting genes and proteins" software) and correlated the gene interation in different disease subtypes both in neurons and in oligodendrocytes. The circles in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PPMS
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Normalized gene expression
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IgM+/-
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Med
PPMS
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Cox
Normalized gene expression
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IgM+/-
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PPMS
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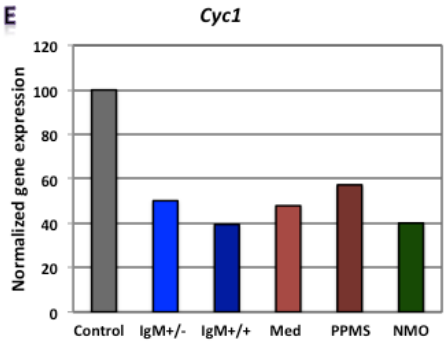

Figure 27: Normalized gene expression involved in oxidative phosphorylation in OPCs treated with CSF of MS and NMO patients related to gene expression in OPCs treated with CSF of non-inflammatory neurological controls. OPCs: oligodendrocytes; Control: Neurons treated with CSF of non-inflammatory neurological controls; IgM+/- and IgM+/+: types of inflammatory MS; Med: Medullary MS; PP: Primary progressive multiple sclerosis; NMO: Neuromyelitis Optica**.**

blue and in red correspond with the proteins that are downregulated and upregulated respectively in the different experimental MS or NMO conditions and connected with lines the known protein-protein interaction between the different proteins of the carbohydrate metabolism.

Although not every gene related with a specific network is affected in a kind of MS disease, if we do a string analysis we may find that the physical interaction of enzymes related with a metabolic network (i.e. glycolysis, TCA cycle, and electron transport chain) exist and closely regulated in a complex ensures that the whole

network is affected (increasing general intermediate metabolic flux within the network) and the synergy of the inhibition effects the whole network . Therefore now we define a parameter to "integrate" our data as "CUMULATIVE FLUX INDEX or CFI" within the network to compare our experimental MS conditions. The values means that the reducing on local flux due to the inhibition of an enzymatic activity in a specific gene affects synergicaly to the whole metabolic flux network. It means that as more genes are down regulated, the total flux are reducing as a multiplicative factor that we integrate as the CFI of the network. In the Discussion section we integrate in a draw the relative changes in the different MS and NMO patients by calculating the CFI of the carbohydrate metabolic networks in those patients.

With this calculation of CFI parameters, we may say that whole glycolytic flux decreased in neurons (CFI 0.0296) and OPCs (CFI 0.0192) when exposed to CSF derived from IgM+/- patients. The flux related to TCA cycle is also decreased in neurons (CFI 0.0960) and OPCs (CFI 0.0602) whereas it is down regulated in oxidative phosphorylation in neurons (CFI 0.3300) and OPCs (CFI 0.0119). Overall, carbohydrate flux is decreased in neurons and OPCs exposed to CSF from IgM+/- MS patients (Figure 28). Since the genes involved in glucose metabolism form complexes and interact among themselves and with others from related pathways, and they are closely regulated, we found that carbohydrate metabolism flux and ATP synthesis as a whole were slightly downregulated.

The results when neurons are treated with CSF from IgM+/+ patients showed that glycolytic flux is strongly decreased in neurons (CFI 0.0007) as well as in OPCs (CFI 0.0047). The flux related to TCA cycle is also decreased in neurons (CFI 0.0690) and strongly in OPCs (CFI 0.0024). In oxidative phosphorylation, flux is reduced in neurons (CFI 0.3000) and in OPCs (CFI 0.0173). Overall, carbohydrate metabolic flux

and ATP synthesis are profoundly decreased in neurons as well as in OPCs exposed to CSF from IgM+/+ MS patients (Figure 29). This values, lacking the needed energy resources to repair axonal damage, may be related with the pronounced severity of IgM+/+ MS in comparison with IgM+/- MS.

The CFI results in neurons treated with CSF from MS medullar patients indicated that glycolytic flux is strongly decreased both in neurons (CFI 0.0075) and OPCs (CFI 0.0019). The flux related to TCA cycle is also decreased in neurons (CFI 0.0810) and in OPCs (CFI 0.0124) as compared to control, and it is also decreased in the oxidative phosphorylation network in neurons (CFI 0.1050) and slightly decreased in OPCs (CFI 0.0328). Overall, carbohydrate flux and ATP synthesis are decreased in neurons and OPCs exposed to CSF from medullary MS patients to the similar magnitude to the IgM+/+ MS patients, and both are stronger reduced in comparison with IgM +/- MS (Figure 30). Those data are in close correlation with the severity and prognosis of medullary MS and IgM+/+ MS in contrast with the IgM+/- type of MS.

The results obtained with the neurons treated with CSF from PPMS patients indicated that glycolytic flux is slightly decreased in neurons (CFI 0.3000) and stronger decreased in OPCs (CFI 0.0033). The flux related to TCA cycle is decreased in both neurons (CFI 0.0870) and OPCs (CFI 0.5400), as well as decreased the oxidative phosphorylation in neurons (CFI 0.1050) and in OPCs (CFI 0.0728). Overall, we may say that the carbohydrate metablic flux and ATP production are slightly decreased in neurons and strongly decreased in OPCs as compared to control exposed to CSF from PPMS patients (Figure 31).

Finally, the CFI results with neurons treated with CSF from NMO patients showed that glycolytic flux is increased in neurons (CFI 1.9656) and decreased in OPCs (CFI

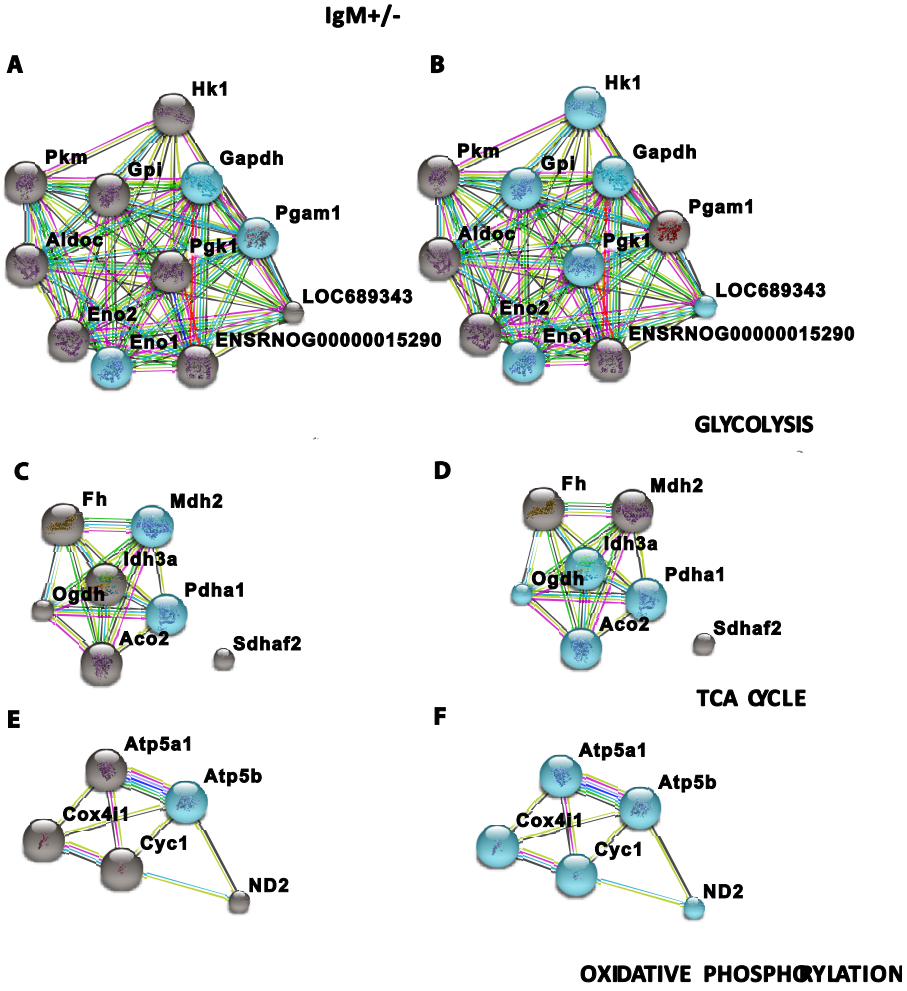

Figure 28: Visualization of gene interaction network generated by String v10 in neurons and oligodendrocytes exposed to CSF from IgM+/- MS. Different line colors represent the types of evidence for the association between the proteins. A, C, E) genes involved in glycolysis, TCA cycle and oxidative phosphorylation in neurons; B, D, F) genes involved in glycolysis, TCA cycle and oxidative phosphorylation in

oligodendrocytes; Red color signifies upregulated expression, blue color signifies down regulated expression and grey color signifies genes that we do not find variations in their expression in our experimental conditions. LOC689343 signifies *Pk* gene and ENSRNOG00000015290 signifies *Tpi* gene

0.0013) in comparison with the controls. The flux related to TCA cycle is slightly decreased in neurons (CFI 0.2174) and decreased in OPCs (CFI 0.0055) whereas it is increased in oxidative phosphorylation in neurons (CFI 1.5867) and decreased in OPCs (CFI 0.0149). Overall, data indicate that carbohydrate flux and ATP synthesis is almost not affected in neurons but decrease intensively in OPCs exposed to CSF from NMO patients (Figure 32).

The variation of the metabolic flux, estimated with their values of CFI, in the different treatments with CSF of MS and NMO patients were integrated by designing a draw of the carbohydrate metabolism and ATP production. These figures will allow us to compare how CFI changes may give some explanation on the diferent severity of pathology depending on the type of MS (see section of Discussion).

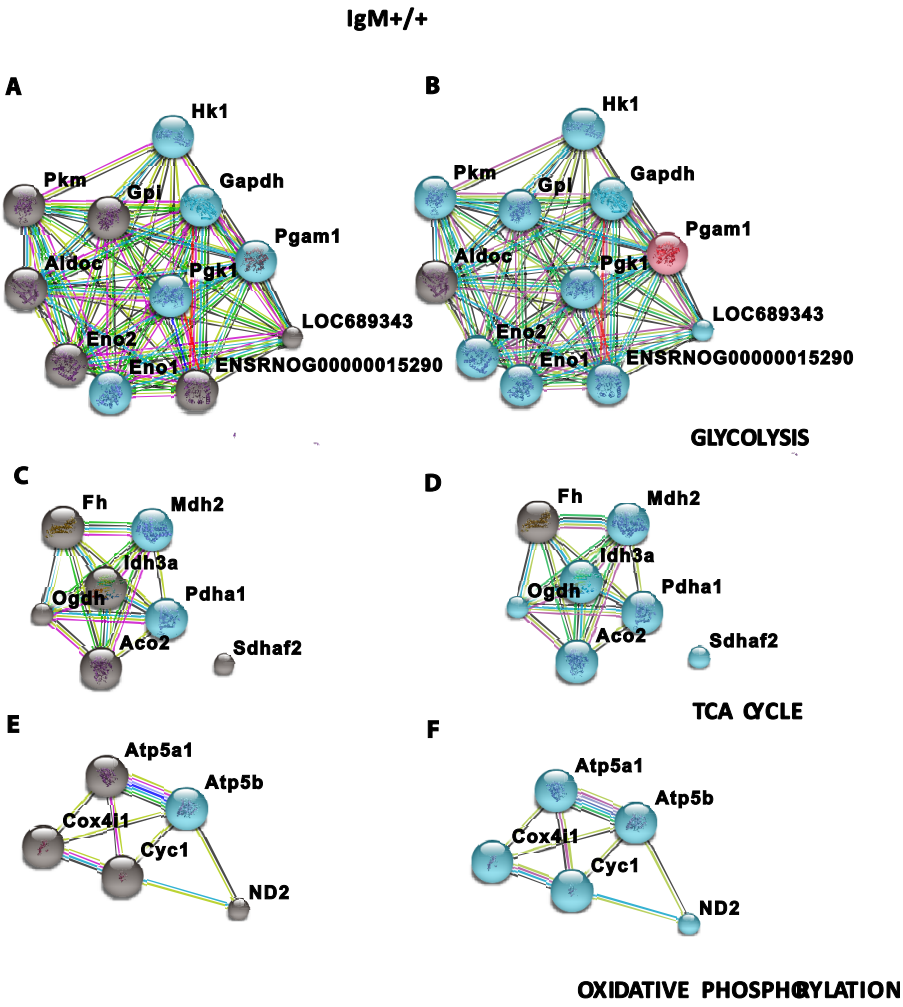

Figure 29: Visualization of gene interaction network generated by String v10 in neurons and oligodendrocytes exposed to CSF from IgM+/+ MS. Different line colors represent the types of evidence for the association between the proteins. A, C, E) genes involved in glycolysis, TCA cycle and oxidative phosphorylation in neurons; B, D, F) genes involved in glycolysis, TCA cycle and

oxidative phosphorylation in oligodendrocytes; Red color signifies upregulated expression, blue color signifies down regulated expression and grey color signifies genes that we do not find variations in their expression in our experimental conditions. LOC689343 signifies *Pk* gene and ENSRNOG00000015290 signifies *Tpi* gene

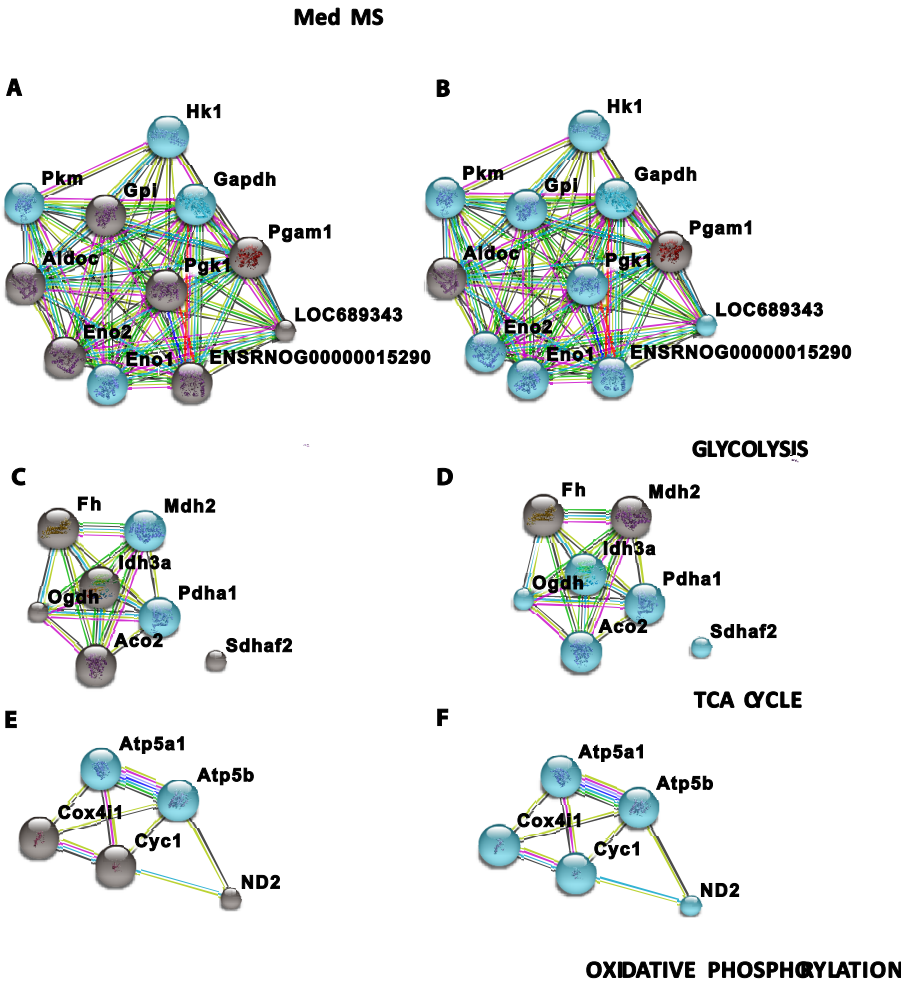

Figure 30: Visualization of gene interaction network generated by String v10 in neurons and oligodendrocytes exposed to CSF from medullary MS. Different line colors represent the types of evidence for the association between the proteins. A, C, E) genes

involved in glycolysis, TCA cycle and oxidative phosphorylation in neurons; B, D, F) genes involved in glycolysis, TCA cycle and oxidative phosphorylation in oligodendrocytes; Red color signifies upregulated expression, blue color signifies down regulated expression and grey color signifies genes that we do not find variations in their expression in our experimental conditions. LOC689343 signifies *Pk* gene and ENSRNOG00000015290 signifies *Tpi* gene

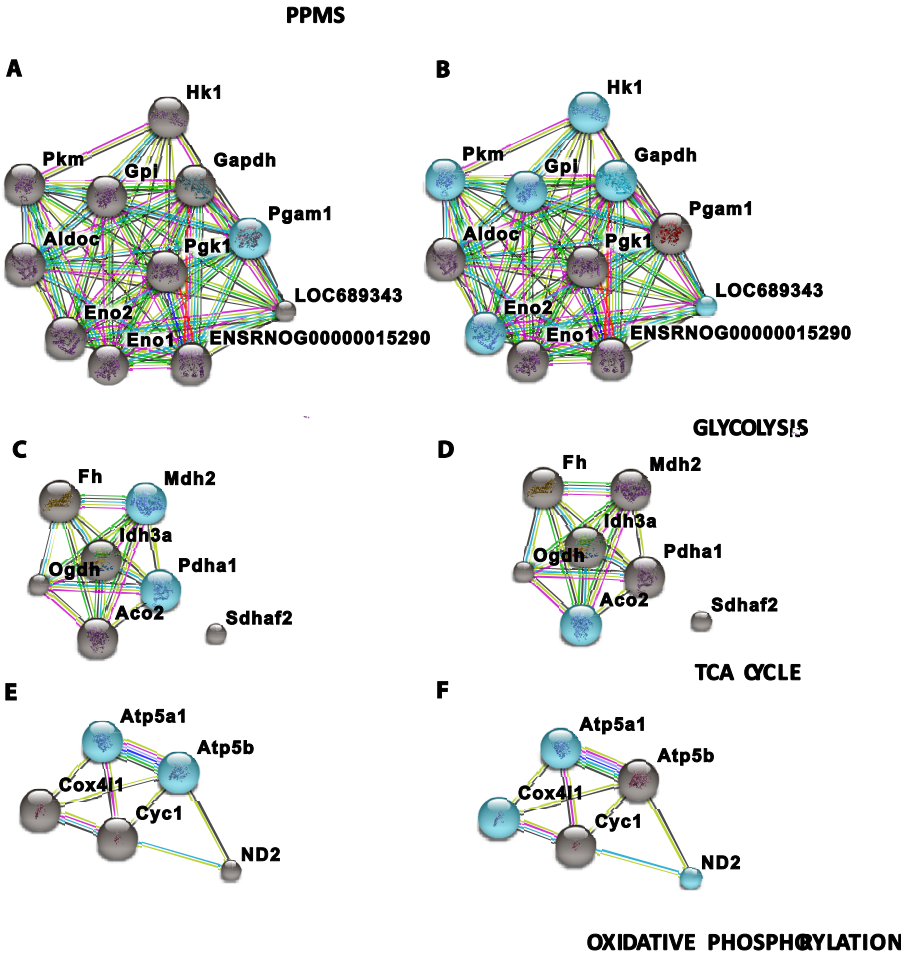

Figure 31: Visualization of gene interaction network generated by String v10 in neurons and oligodendrocytes exposed to CSF from PPMS. Different line colors represent the types of evidence for the association between the proteins. A, C, E) genes involved in glycolysis, TCA cycle and oxidative phosphorylation in neurons; B, D, F) genes involved in glycolysis, TCA cycle and

oxidative phosphorylation in oligodendrocytes; Red color signifies upregulated expression, blue color signifies down regulated expression and grey color signifies genes that we do not find variations in their expression in our experimental conditions. LOC689343 signifies *Pk* gene and ENSRNOG00000015290 signifies *Tpi* gene

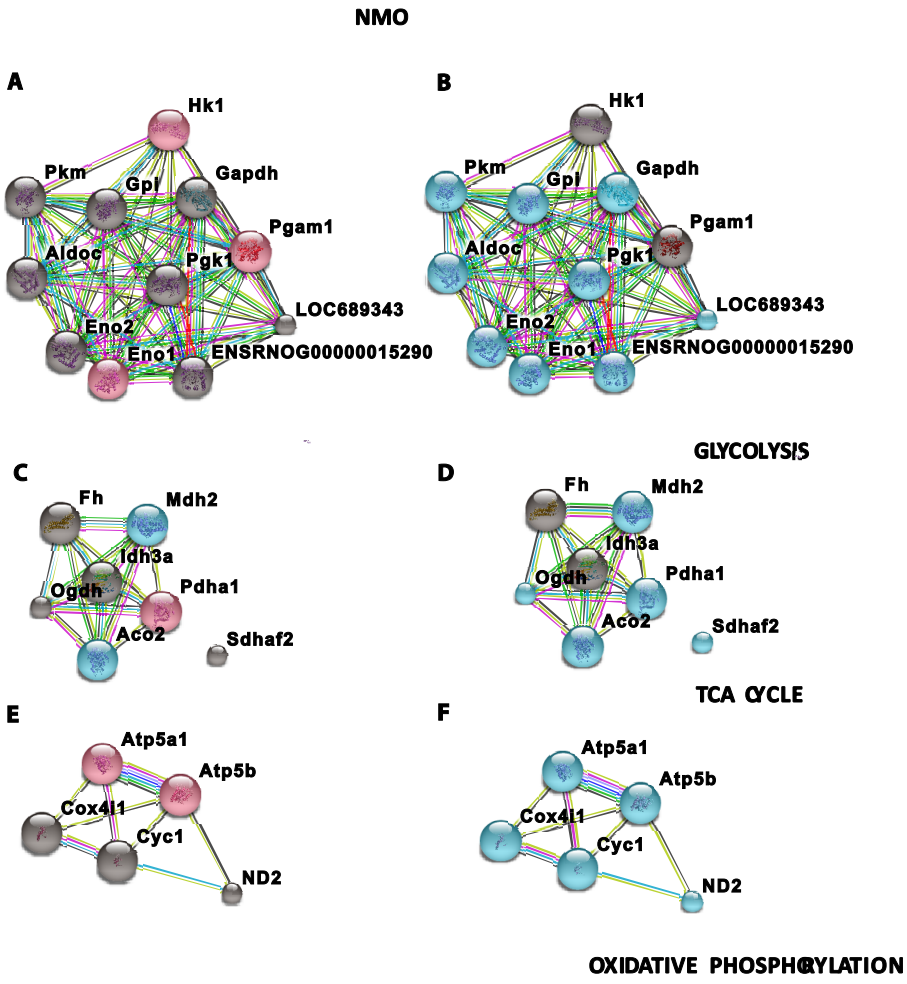

Figure 32: Visualization of gene interaction network generated by String v10 in neurons and oligodendrocytes exposed to CSF from NMO. Different line colors represent the types of evidence for the association between the proteins. A, C, E) genes involved in glycolysis, TCA cycle and oxidative phosphorylation in neurons; B, D, F) genes involved in glycolysis, TCA cycle and

oxidative phosphorylation in oligodendrocytes; Red color signifies upregulated expression and blue color signifies down regulated expression. LOC689343 signifies *Pk* gene and ENSRNOG00000015290 signifies *Tpi* gene

V- DISCUSSION

DISCUSSION

Multiple Sclerosis (MS) is an unpredictable and a potentially debilitating disease of the central nervous system that mainly affects young people. The disease often manifests at the age of 20-40 years when people are in their full employment resulting in socioeconomic burden on caregivers. The disease could be regarded as a heterogenous condition due to diffferences observed with respect to pathological symptoms and treatment responses in different subtypes. These differences led to its classification into into relapsing–remitting (RRMS), secondary progressive (SPMS) or primary progressive MS (PPMS). The majority of patients commence with a RRMS disease course characterized by periodic attacks (relapses) followed by partial or complete recovery (remissions). Inspite of receiving anti-inflammatory drugs to prevent relapse the same patients eventually enter the secondary progressive phase (SPMS) with irreversible neurologic worsening. Patients with primary progressive disease course (PPMS) undergo continuous deterioration of neurological symptoms suggesting that the pathophysiology of progression is not solely inflammatory in nature (McDonald, 2000; Confavreux *et al.,* 2000). A deeper understanding and the identification of mechanisms underpinning the progression of the disease may assist in the development of therapeutic strategies for treatment.

Although all cells of the CNS including oligodendrocytes, myelin they form and astrocytes that undergo astrogliosis can be regarded as the cellular “victims” in MS, neuronal damage is widely accepted as the major cause of persistent functional disability in these patients, the origin of which is unclear. One of the overarching hypotheses is that axonal damage in demyelinated axons results from the increased energetic demand consequent to the redistribution of Na+/K+ ATPase before increased intracellular Ca2+ levels (Waxman, 2006b). Axonal damage has

also been attributed to defective metabolic or impaired trophic support, due to damaged oligodendrocytes (Funfschilling *et al.,* 2012; Lee *et al.,* 2012). However, it is believable that diffusible factors present in the CSF could also impact the ability of neurons to respond with adequate energy production, especially in the pathogenesis of cortical lesions. Decreased ability to meet energetic demand can be observed in the setting of mitochondrial impairment, as suggested by studies in cultured neurons (Kim *et al.,* 2010), preclinical animal models of multiple sclerosis (Nikic *et al.,* 2011) and in multiple sclerosis samples (Dutta *et al.,* 2006; Fischer *et al.,* 2013).

Furthermore, during the RRMS disease course the patient's bodies alone are capable of repairing the damage, remyelinating the axon and recovering the neurological function. Cerebrospinal fluid (CSF) which is in contact with the brain parenchyma and ventricles, is a site of tissue damage products and hence a promising biofluid in the search for biomarkers in MS, both with respect to inflammatory and neurodegenerative processes. The main objective of the thesis was to study the effect of diffusible factors present in CSF on bioenergetics profile of neurons, which are able to regulate axonal destruction-repair, and make a stable remyelination and functional recovery possible.

Additionally we were interested to explore the effect of CSF from PPMS and RRMS patients upon exposure to proliferating oligodendrocyte progenitor cells (OPCs) on bioenergetics profile. In order to study primary neuroaxonal damage independent of secondary damage resulting from demyelination, we used a cellular model with primary cultures of unmyelinated granular neurons from rat cerebellum, and treated them with the CSF from MS and NMO patients. Previous studies in similar xenogeneic models revealed that exposure to human CSF resulted in neurotoxicity in culture, although the molecular mechanisms remained elusive (Xiao *et al.,* 1996;

Alcazar *et al.,* 2000). A potential explanation was the presence of pro-inflammatory cytokines in the CSF, leading to cytokine induced synaptic hyperexcitability and consequent glutamate dependent neurotoxicity (Rossi *et al.,* 2012, 2013). This interpretation was supported by elevated glutamate levels detected in acute lesions and normal-appearing white matter in patients with multiple sclerosis (Srinivasan *et al.,* 2005) and by studies reporting elevated levels of TNF-α, INF-γ, IL-1b, IL-6, IL-12 in patients with multiple sclerosis.

To determine whether CSF causes cell death in neurons, or astrocytes or both, we added cytosine arabinoside to the cell cultures to allow the growth of astrocytes. Specific amount of cytosine arabinoside (1 mM) prevents the replication of glial cells whereas a little lower with half concentration allows the growth of astrocytes. The results demonstrate that neurons underwent cell death whereas astrocytes were found to remain viable with CSF exposure as measured by propidium iodide and Rhodium-123 dyes (Refer Figure 10).

Since the study was centered on understanding the mechanisms of primary neuronal damage, we used a higher concentration (1 mM) of cytosine arabinoside to prevent the replication of glial cells. Previous data in our laboratory experienced variation in sodium channel genes while normalizing the target mRNA expression. Therefore we first identified the most stably expressed reference genes in our experimental set up using quantitative PCR in order to obtain accurate gene expression data.

Quantitative RT-PCR has recently become the most widely accepted method of quantification for its sensitive, accurate and reliable determination of gene expression levels in cells and tissues. To avoid sample-to-sample variation, normalization of gene transcripts is required. The conventional way to perform normalization is to select a housekeeping gene whose expression is believed to

remain stable in all cell types/tissues, during cellular development and under various experimental conditions then relate the expression of gene of interest to that of a housekeeping gene. For many years it has been assumed by molecular biologists that the genes such as *ActB* and *Gapdh* express constitutively in all cells and tissues.

Beta actin (*ActB*) is a cytoskeletal protein that maintains the structure and integrity of cells. *Gapdh*, on the other hand, is a key glycolytic enzyme involved mainly in the production of energy. Since both ACTB and GAPDH are known to perform the basic functions of a cell, they are presumed to express at stable levels. Therefore, they are employed as common internal controls in most of the laboratories. However, several lines of evidence show that their rate of transcription is influenced by a variety of factors such as EGF factor, TGF-β and PDG factor while constitutively expressed [Leof *et al.*, 1986; Keski-Oja *et al.,* 1988; Elder *et al.,* 1984]. Therefore, their expression may not necessarily be constant in all conditions. Furthermore, GAPDH is implicated in non-metabolic processes independent of its metabolic function such as transcription activation, vesicle transport from endoplasmic reticulum to Golgi apparatus and polymerization of tubulin into microtubules [Zheng *et al.,* 2003; Tisdale *et al.,* 2007; Kumagai *et al.,* 1983; Durrieu *et al.,* 1987; Muronetz *et al.,* 1994]. Previous literature reveals that neuronal apoptosis is associated with suppressed glycolytic activity of GAPDH [Dastoor *et al.,* 2001; Burke *et al.,* 1983; Makhina *et al.,* 2009]. It has been observed that GAPDH interacts with other proteins which results in reduced glycolytic activity [Hara, 2005]. This process may lead to neuroaxonal damage in neurodegenerative diseases such as Huntington's, Parkinson's and Alzheimer's disease [Ve´csei *et al.,* 1993; Senatorov *et al.,* 2003; Tsuchiya *et al.,* 2005; Mazzola *et al.,* 2003; Li *et al.,* 2004]. The realization that these reference genes may not necessarily express in stable manner in cellular development or under all experimental conditions has led to their pre-validation for their expression stability.

Their evaluation prior to normalization is a critical step to obtain unbiased gene expression data.

Our findings indicate *Hprt* and *Gapdh* (M: 0.06) the most stable genes followed by *Tfrc* (M: 0.08) during the maturation of cerebellar granule neurons using *GeNorm* algorithm. Therefore, these genes should be used to normalize gene transcripts. On the contrary, β-actin (M: 2.8) showed the highest fluctuation hence it is not suitable for normalization purpose during neuronal development. In experimental conditions when cerebellar granule neurons were exposed to CSF from different MS subtypes, *Tfrc* and *B2m* (M: 1.09) were found as best stably expressed genes followed *by Rpl19* (M: 1.19) as indicated by *geNorm* algorithm whereas *Gapdh* (4.2) and *β-actin* (2.9) showed highest fluctuation. In another experiment when OPCs were treated with diseased CSF, *Mrpl19* and *Hprt* (M: 0.1) showed most stable expression followed by *B2m* (M: 0.14) whereas *β-actin* (M: 0.19) and *Gapdh* (M: 0.27) were least stably expressed genes as indicated by *geNorm* algorithm.

After evaluating best stably expressed reference genes, the next step was to perform global gene expression profiling using microarray. In parallel to this, we were interested to search for biomarkers in distinct clinical courses of multiple sclerosis and another similar but distinct neurological disease, neuromyelitis optica (NMO). In MS, the pattern of nerves damaged, severity of relapses, extent of recovery, and time between relapses all differ extensively from individual to individual. Our results reveal significant differences in the age at beginning between PPMS and the other two MS forms (RRMS and SPMS) ($p<0.003$). People with primary progressive MS are usually older at the time of diagnosis with an average age of 40.

Different subtypes of MS help predict disease severity and response to treatment hence their categorization is important. In our study, we found significant differences between the Expanded Disability Status Scale (EDSS) of RRMS and the

two other MS forms (RRMS and SPMS) ($p<0.001$). Although nerve injury always occurs, the pattern is specific for each individual with MS. Disease severity and disability increases from RRMS to SPMS course and in PPMS subtype, symptoms continually worsen from the time of diagnosis rather than having well-defined attacks and recovery. Primary progressive MS usually results in disability earlier than RRMS. Significant differences were found in the evolution time from the first to the second episode between RRMS and PPMS ($p<0.043$). In patients experiencing a progressive course, evolution time was similar in SPMS cases and in cases who were progressive from onset (13.5 *versus* 13.8).

According to the new proposal and working classification, inflammatory MS subtypes shared similar age at disease onset (mean=26.7 *versus* 26.3 years; $p=0.005$). Significant differences were found between the age at disease onset in medullary MS and PPMS with the inflammatory MS ($p<0.005$). The degree of disability as measured by EDSS was similar in medullary MS and PPMS (6.2 *versus 6.3*) whereas significant differences were found between disability extent in medullary MS and PPMS with the inflammatory MS ($p<0.001$). IgM+/- represents the less aggressive inflammatory subtype with OCGB in CSF with poor prognosis whereas IgM+/+ signifies a more aggressive category with OCGB and OCMB in CSF with worse prognosis. On the contrary, medullary MS represents the most aggressive subtype of MS with increased neurological disability and dysfunction as compared to inflammatory subtypes. Disability in patients experiencing primary progressive MS worsens over time with no relapses and remission.

The presence of lipid-specific oligoclonal IgM bands (OCMB) and increased IgM index have been associated with poor prognosis in MS and a more rapid progression to irreversible disability (Villar *et al.,* 2005; Thangarajh *et al.,* 2008). These OCMB usually persist in MS patients throughout the course of the disease

(Walsh and Tourtellotte, 1986), and their persistence indicates that they are not elements of a primary immune response. Persistent IgM responses are usually produced by the long-lived, self-renewing B1 subset of B cells, responsible for the secretion of the so-called natural antibodies that appear in the absence of apparent antigenic stimulation (Hamilton *et al.,* 1994). More frequently, naturally occurring autoantibodies are IgMs rather than IgGs (Rodriguez *et al.,* 2009).

Literature reveals that there is a possible role of impaired energy metabolism in the pathogenesis of MS [see Figure 44]. Initial studies describing the possibility of defective pyruvate metabolism in MS were performed by Jones *et al*. in 1950s [Jones *et al.,* 1950]. The group observed elevated blood pyruvate level in both fasting and postprandial times in MS patients with relapse. Similarly, other investigators also reported increased fasting pyruvate level in this disease [Ervenich 1952; Ervenich 1953; Sercl and Johnova 1956]. However, there were conflicting reports demonstrating normal fasting lactate level, or increase in only a small number of patients [Bauer 1956; Henneman *et al.,* 1954; Jeanes and Cumings 1958]. On the other hand, Jeanes and Cumings (1958) found an abnormal rise in the blood pyruvate level after glucose intake. These reports hint to a possible abnormality of pyruvate metabolism in MS patients.

A report from Regenold´s group describe the role of CNS energy metabolism in MS disease progression. The group measured the levels of lactate, sorbitol and fructose, all metabolites of extra-mitochondrial glucose metabolism, in the CSF of RRMS and SPMS patients [Regenold *et al.,* 2008]. Sorbitol and fructose are the metabolites of polyol pathway that run parallel to glycolysis and lactate is the metabolite of anaerobic pathway. The finding demonstrated elevated levels of all three metabolites in the CSF of SPMS patients and to a lesser extent to RRMS patients. There are two assumptions set forth as an explanation for increased lactate in MS patients. First ascribes to the accumulation of leukocytes in inflamed

active MS lesions in which glycolytic pathway is activated. In addition, a strong correlation of the number of inflammatory plaques with lactate deposition further strengthened the hypothesis [Lutz *et al.,* 2007]. Second explanation is deduced from the relationship between the extra-mitochondrial pathways and mitochondrial dysfunction. Under anaerobic conditions, pyruvate is converted into lactate by neurons and astrocytes to meet the energy requirements. In mitochondrial disorders, oxidative phosphorylation is impaired leading to lactate accumulation. Taken together, the finding supports a link between increased activity of extra-mitochondrial pathways of glucose metabolism and MS disease progression. Neuroimaging and molecular studies have shown that both pathways are dysregulated in mitochondrial disorders [Frackowiak *et al.,* 1998; Danielson *et al.,* 2005; Rango *et al.,* 2001]. These alterations in energy metabolism may contribute to mitochondrial dysfunction and neuroaxonal degeneration underlying MS progression.

Furthermore, mitochondrial dysfunction is implicated in various pathological conditions like diabetes, cardiovascular disease, anxiety disorders; neurodegenerative diseases like Alzheimer's, Huntington's and Parkinson's disease; cancer and fatigue. It is only recently that mitochondrial aberrations have been studied in MS. Unlike nuclear DNA, mitochondrial DNA (mtDNA) is not surrounded by histones, proteins that shield nuclear DNA from free radicals. Therefore it is quite prone to damage. The number of mitochondria in a cell is determined by its energy requirement. For instance, there may be up to 200 to 2000 mitochondria present in a single somatic cell whereas the number is fixed to 16 in spermatozoa germ cells and 100,000 in oocytes. Metabolically active cells like skeletal muscle, cardiac muscle and brain contain largest number of mitochondria. It has been seen that the number of mitochondria and their activity is increased in MS plaques [Mahad *et al.,* 2009; Witte *et al.,* 2009]. In demyelinating diseases particularly MS

where demyelination is distinct with axonal degeneration, it is likely that cells need ample energy to survive. Hence, metabolic activity of biomolecules in mitochondria increases with concomitant impairment of Krebs cycle and/or neuronal oxidative phosphorylation within the CNS.

A body of evidence indicates a link between disturbed metabolic function and the progression of neurodegenerative diseases like Alzheimer's disease, Huntington's disease and Parkinson's disease. A short comparison between disturbed glucose metabolism in MS and other neurodegenerative diseases is illustrated in Table 13. A report documented that GAPDH glycolytic function is impaired in subcellular fractions of fibroblasts from Alzheimer and Huntington patients whereas the gene expression remained unchanged [Mazzola and Sirover 2001]. This might have occured due to post-translational modification of the GAPDH protein. In a transgenic model of Huntington's disease, *Gapdh* is seen to overexpress in specific neuronal populations of several brain regions, such as the neocortex and caudate putamen neurons. This study also revealed translocation of GAPDH into the nucleus and the subsequent cell loss in the neocortex and caudate putamen region of the brain [Senatorov *et al.,* 2003]. Similar finding was observed by Byoung-Il Bae *et al*. who demonstrated that GAPDH facilitates nuclear translocation of mutant Huntingtin protein (mHtt) and causes neurotoxicity [Bae *et al.,* 2006]. In Aβ (amyloid beta) resistant cells of Alzheimer's brain, glycolytic pathway was upregulated and hexose monophosphate shunt (HMS) was activated. The activity of Gapdh, hexokinase and pyruvate kinase was increased in both glycolysis and HMS [Soucek *et al.,* 2003].

Moreover, there are studies that have investigated the metabolic disorders in alcoholic brain. Gapdh protein known to exhibit diverse functionality is reported to increase significantly in the brains of human subjects with alcoholism [Alexander-

Kaufman *et al.,* 2006]. This study used proteomics based approach and found variation in protein expression in alkoholic brain. Most of the proteins identified through this study were metabolic enzymes including Gapdh, creatine kinase, NADH2, ubiquinone, fructose biphosphate aldolase C, transketolase and others. This demonstrates that metabolic disturbances play an important role in alkohol related brain cell damage. Another recent study showed that nuclear GAPDH was elevated in response to ethanol treatment in brain-derived cell lines [Ou *et al.,* 2009].

To give a more global picture about the altered genes in different clinical courses of MS and NMO, we incorporated the whole data in a general metabolic pathway design in this work. This also allowed us to better interrelate the changes in the neurons and OPCs, with the aim to find correlations in the whole process of axonal degeneration-reconstruction in different MS types. Figure 33 depicts an overall carbohydrate metabolic network that includes glycolytic pathway, tricarboxylic acid cycle and electron transport chain yielding 38 ATP molecules per glucose.

Increased activity of metabolic enzymes including enolase, pyruvate kinase, lactate dehydrogenase and aldolase in the CSF of patients with disseminated sclerosis make them a sensitive indicator of active demyelination [Royds *et al.,* 1981]. Kolln *et al.,* 2006 demonstrated that B cells and antibodies reactive with *Tpi* and *Gapdh* are produced intrathecally in CSF and lesions of multiple sclerosis. Both TPI and GAPDH are essential metabolic enzymes involved in ATP production. Other functions of GAPDH include endocytosis [Jeffery 1999], polymerization of tubulin into microtubules [Kumagai and Sakai 1983; Durrieu *et al.,* 1987; VI *et al.,* 1994], curbing protein synthesis in the endoplasmic reticulum [Schekman and Orci 1996], DNA replication, DNA repair and the export of tRNA out of the nucleus [Nagy *et al.,* 1995]. Another investigation by the same group showed that these antibodies bind

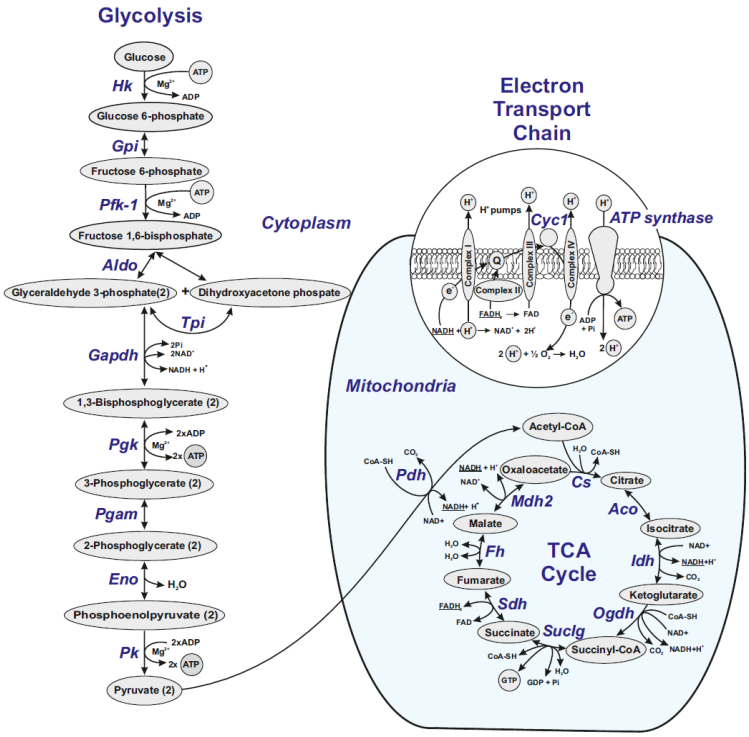

Figure 33: A schematic diagram of a general metabolic network including glycolysis, tricarboxylic acid (TCA) cycle and electron transport chain (ETC)

with *Tpi* and *Gapdh* and inhibit the glycolytic activity of GAPDH but not TPI in MS patients [Ko¨lln *et al.,* 2010]. This inhibitory effect of antibodies on GAPDH was not visualized when anti-GAPDH IgG was exhausted from the CSF demonstrating the role of anti-GAPDH antibodies in impeding the GAPDH enzyme activity in brain leading to neuronal apoptosis and cytotoxicity.

In systemic lupus, an autoimmune rheumatoid disorder, autoantibodies were found reactive to GAPDH [Takasaki *et al.,* 2004]. Accumulating evidence indicates that inhibition of GAPDH activity in glycolytic pathway is associated with neuronal apoptosis. It has been purported that GAPDH enzyme activity suppresses when it reacts with other proteins in the CNS [Burke 1983; Hara *et al.,* 2005]. Single chain variable fragment antibodies (scFv-abs) obtained from clonally expanded B cells binds specifically with GAPDH and TPI in active MS lesions [Zhang *et al.,* 2005; Zhang *et al.,* 2005]. In addition, interaction of GAPDH with ß-amyloid protein and Huntingtin protein well demonstrates the role of GAPDH in neurodegenerative disorders [Schulze *et al.,* 1993].

RRMS IgM+/-

Our findings revealed that glycolytic genes including *Gapdh*, *Pgam,* and *Eno* showed down regulated expression in neurons exposed to the CSF derived from IgM+/- clinical form of MS (Figure 21). The data indicated that *Gapdh* gene expression was downregulated up to around 34 % of gene expression in neurons treated with CSF of IgM+/- clinical form of MS as compared to control. Similarly, *Pgam* and *Eno* genes were down regulated up to 30 % as compared to control. Genes involved in TCA cycle including *Pdh* and *Mdh2* were also down regulated up to around 30 % and 32 % in these neurons (Figure 22).

Smith and Lassman revealed a reduction in ATP synthase expression in MS lesions

[Smith and Lassmann 2002]. Mitochondrial proteins are expressed in greater amounts in both active and inactive lesions. Activity of complex IV found on mitochondrial membrane is increased dramatically in MS lesions and it is found that, mitochondrial heat shock protein (mtHsp70) is highly immune-reactive in axons and astrocytes of MS lesions. mtHsp70 is a chaperone protein the level of which is abnormally increased during oxidative stress [Mitsumoto *et al.,* 2002]. This controls the formation of reactive oxygen species and protects mitochondria from oxidative stress. *In vitro* studies reveal that mtHsp upregulation protects astrocytes from ischemic insult [Volobouéva *et al.,* 2008]. This shows that oxidative stress generated in response to defective mitochondrial metabolism may contribute to the formation of free radicals and subsequent tissue damage. Lu *et al*. revealed defects in complex I component of electron transport chain in white matter lesions [Lu *et al.,* 2000]. Furthermore, reduced functional activity of complex I and complex III and a decrease in gene expression of complex I, complex III, complex IV, and ATP synthase has been observed in nonlesional motor cortex [Dutta *et al.,* 2006].

Our results indicated that *ATP5b,* gene involved in oxidative phosphorylation and ATP generation, showed 30% decreased expression as compared to neurological controls (Figure 23). In another experiment when OPCs were exposed to IgM+/- CSF, expression of many glycolytic genes including *Hk, Gpi, Gapdh, Tpi, Pgk1, Eno1, Eno2, Pk,* and *Pkm2* was decreased (Figure 25). Furthermore, genes implicated in TCA cycle including *Pdha1, Aco2, Idh,* and *Ogdh* were downregulated (Figure 26). Similarly genes involved in oxidative phosphorylation, namely *ATP5a1, ATP5b, MT-ND2,* and *Cyc1,* showed decreased expression as compared to neurological controls (Figure 27).

The results obtained when neurons and OPCs were treated with CSF from +/- IgM RRMS patients (Figures 34 and 35) showed that the cumulative flux index related to

glycolytic pathway decreases significantly in neurons (CFI 0.0296) and in the OPC (CFI 0.0192). The cumulative flux index associated with the TCA cycle is also reduced in neurons (CFI 0.0960) and OPCs (CFI 0.0602). In oxidative phosphorylation, the flux is also reduced in neurons (CFI 0.3300) and in the OPCs (CFI 0.0119). In general we can say that the metabolic flux index of carbohydrates and ATP synthesis are reduced significantly both in neurons (CFI 0.00094), and the OPCs (CFI 0.000014) when exposed to the CSF from patients with MS type +/- IgM RRMS.

Neuronal cells undergoing tissue damage require more energy in form of ATP to survive, but in these patients metabolic genes are inhibited leading to a reduction in the overall metabolic flux. This causes a decrease in ATP production and overall ability of these cells to repair the damaged healthy cells, which can be related to the development of pathology in these patients.

RRMS IgM+/+

The results obtained when neurons were exposed to CSF from patients with IgM+/+ RRMS demonstrated that genes catalyzing crucial steps of glucose metabolic pathway and ATP generation such as *Hk, Gapdh, Pgk, Pgam, Eno, Pkm2, Pdh, Mdh2,* and *ATP synthase subunit beta* had reduced expression compared to control (Figure 21-23). There was around 30% expression showed by these genes in these neurons with respect to control. When OPCs were exposed to CSF of IgM+/+ RRMS patients, it was found that most of the enzymes involved in glycolysis (*Hk* (52%), *Gpi* (40%), *Gapdh* (44%), *Tpi (33%), Pgk1 (39%), Pgam (2,882%), Eno1 (43%), and Pk (42%)*), the related Kreb's cycle enzymes (*Pdha1, Aco2, Idh, Ogdh, Sdh,* and *Mdh2*) and the mitochondrial electron chain enzymes (*MT-ND2, Cyc1, Cox, ATP5a1*

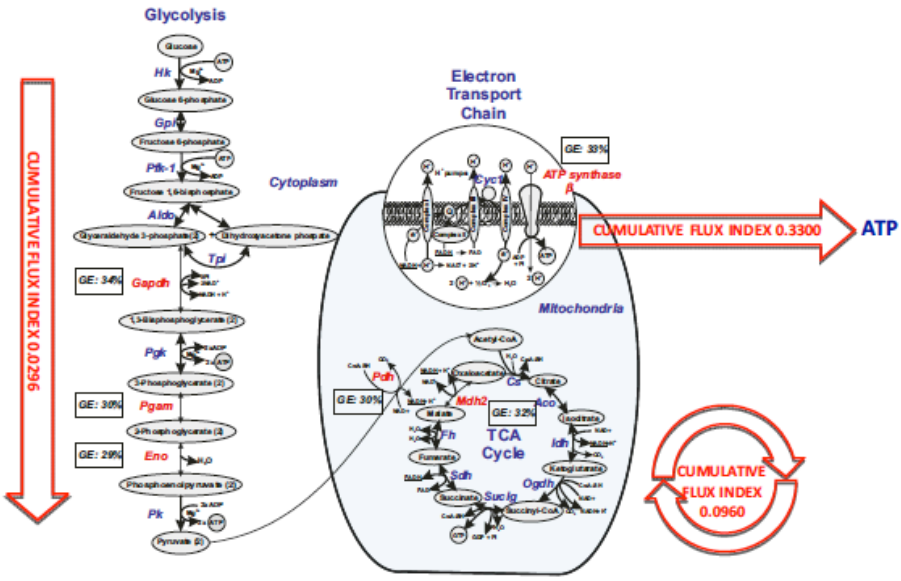

Figure 34: Metabolic network showing differential expression of metabolic genes in neurons treated with the CSF of MS patients presenting with IgM+/- antibodies; GE: Gene expression in CGNs treated with IgM+/- CSF related to CGNs exposed only to culture (untreated neurons); thick black arrow represents elevated expression whereas a thin arrow represents a relative lower expression. Genes shown in red signifies differential expression.

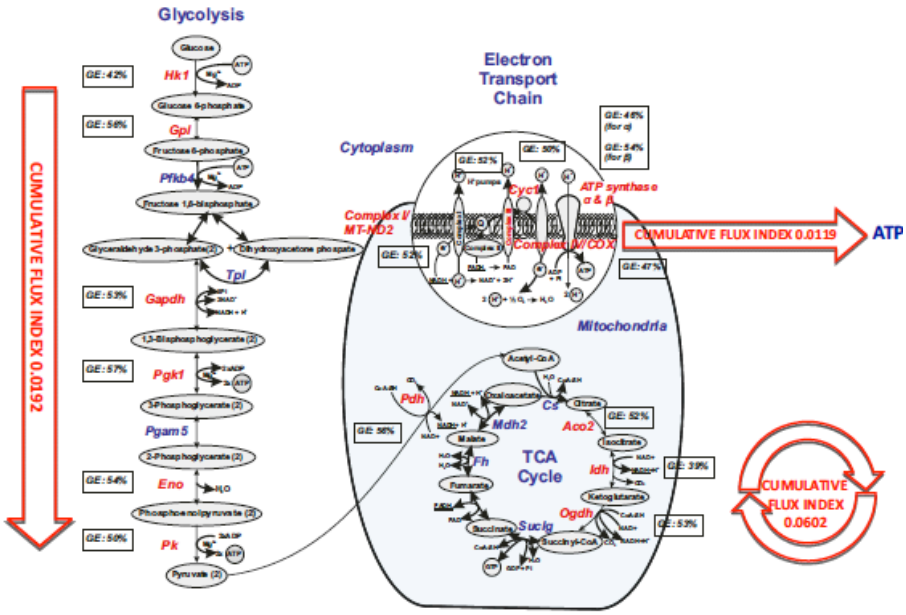

Figure 35: Metabolic network showing differential expression of metabolic genes in OPCs treated with the CSF of IgM+/- MS patients; GE: Gene expression in OPCs treated with the CSF of IgM+/- patients related to OPCs exposed to CSF from neurological controls

and *ATP5b*) were strongly reduced in gene expression as compared to neurological controls (Figure 25-27).

The overall results show that cumulative flux index of glycolytic pathway decreased in IgM+/+ derived CSF exposed neurons (CFI 0.0007) as well as in OPCs (CFI 0.0047). The overall flux index related to TCA cycle was also reduced in neurons (CFI 0.0690) and forcefully in OPCs (CFI 0.0024). In oxidative phosphorylation, flux index was reduced both in neurons (CFI 0.3000) and in OPCs (CFI 0.0173). Altogether, total cumulative flux index in carbohydrate metabolism was found decreased in neurons (CFI 1.42016E-05) and in OPCs (CFI 1.95969E-07) when exposed to CSF from IgM+/+ patients (Figure 36 and 37).

Comparing the results obtained with subtypes IgM +/- and IgM +/+ RRMS, it may be indicated that genes involved in metabolism of carbohydrates and ATP synthesis are most affected in the second type in both neurons and OPCs. IgM+/- RRMS is a more benign and less aggressive, inflammatory clinical form of MS, with plenty of IgG antibodies but not IgM in CSF. Thus the energy in the form of ATP required to repair damaged neurons degenerated in MS lesions can be much lower than the aggressive IgM +/+ RRMS.

To combat oxidative stress generated in neurological diseases, the cells need to produce large quantities of reducing equivalents for energy production. However, if there is insufficient energy production, such as in how aggressive form severely impairs the ability to repair nerve damage. The same happens in the OPCs cells, where lower energy level which can block the repair process occurs by the OPCs. We conclude that the differential expression of metabolic genes influences the bioenergetic profile of neurons and also block the repair processes by OPCs which could be related to poor prognosis in patients with RRMS type EM IgM +/+ versus IgM type +/- RRMS.

Another important result we highlight is that the CSF treated OPCs reveals significant differences in gene expression of *Eno2, Gpi, Tpi, Pdha1, Pgk1, Pkm2, Aldoc,* and *Sdhaf2* between IgM+/- and IgM+/+ RRMS patients. Similarly, differences were found in *Pgam1* and *Cox6b2* gene expression between OPCs exposed to CSF from IgM+/- and OPCs exposed to CSF from medullary patients (see below). These genes could serve as potential biomarkers to distinguish between different kinds of inflammatory MS patients (the IgM +/- vs IgM +/+ RRMS and +/- IgM vs med MS).

Medullary MS

The results obtained when neurons were exposed to the CSF of medullary clinical form of MS revealed that glycolytic genes, including *Hk1* (30 % expression), *Gapdh* (35 % expression), *Eno* (23 % expression), and *Pkm2* (31 % expression), showed down regulated expression in them as compared to neurons exposed to the CSF from neurological controls. In amyotrophic lateral sclerosis (ALS), abnormalities in electron transport chain have been reported. The study found decreased mRNA expression levels of electron transport chain proteins, specifically FAD synthetase, riboflavin kinase (RFK), cytochrome C1 (CYC1), and succinate dehydrogenase complex subunit B (SDHB) [Lin *et al.,* 2009]. We found that genes implicated in TCA cycle including *Pdh* (30 % expression) and *Mdh2* (27 % expression) and *ATP synthase* gene associated with electron transport chain both alpha (35 % expression) and beta (30 % expression) subunits were reduced in expression in treated neurons (Figure 21-23). Overall we found decreased cumulative flux index (CFI) of 0.0075 in neurons exposed to CSF from medullary patients. CFI related to TCA cycle was 0.0810 while that for oxidative phosphorylation was 0.1050. Overall, the accumulated total flux rate of glucose metabolism and ATP synthesis was found to be as low as 6.36727E-05 in these neurons (Figure 38).

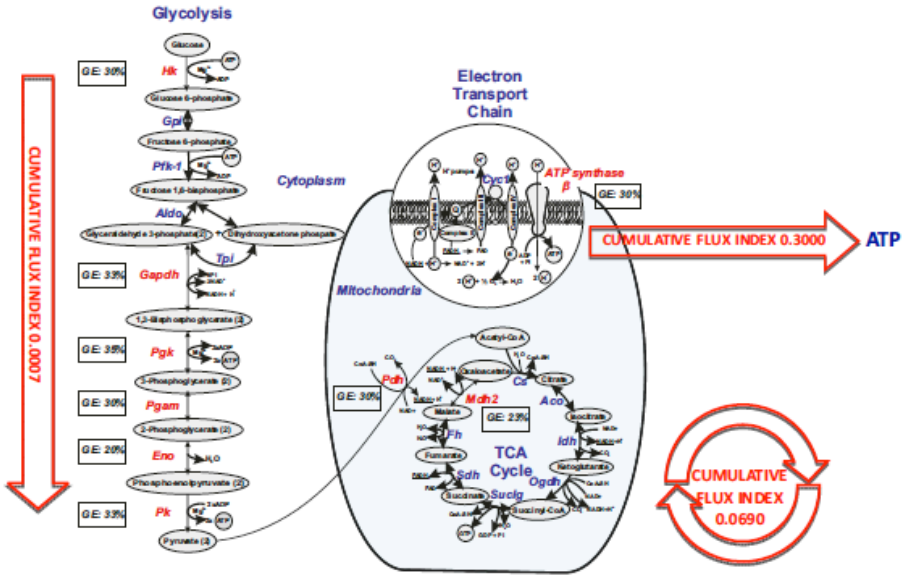

Figure 36: Metabolic network showing differential expression of metabolic genes in neurons treated with the CSF of MS patients presenting with IgM+/+ antibodies; GE: Gene expression in CGNs treated with IgM+/+ CSF related to CGNs exposed only to culture (untreated neurons); thick black arrow represents elevated expression whereas a thin arrow represents lower expression. Genes shown in red signifies differential expression.

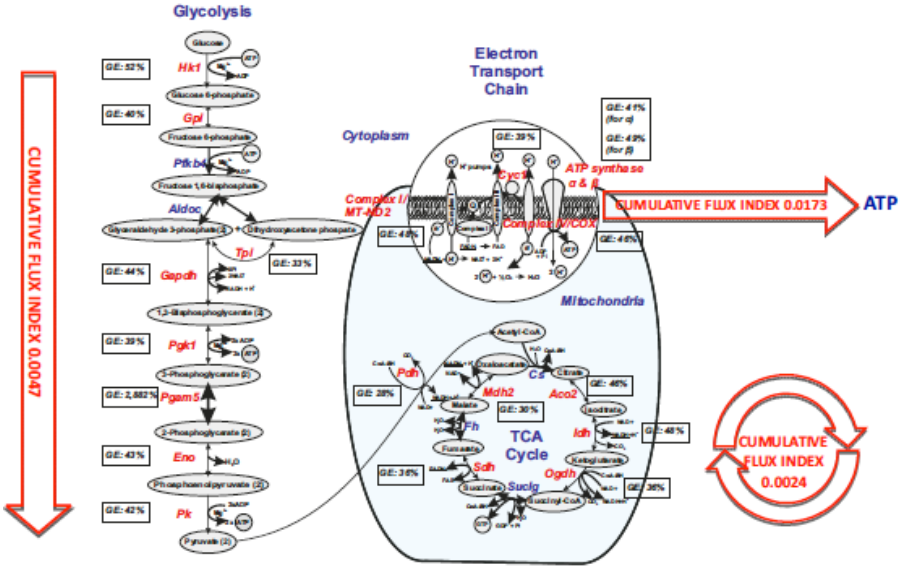

Figure 37: Metabolic network showing differential expression of metabolic genes in OPCs treated with the CSF of IgM+/+ MS patients; GE: Gene expression in OPCs treated with the CSF of IgM+/+ patients related to OPCs exposed to CSF from neurological controls

In experiments when OPCs were exposed to CSF from medullary patients, we found that most of the glycolytic genes including *Hk1, Gpi, Gapdh, Tpi, Pgk1, Eno,* and *Pkm2* (see figure 25) were reduced in expression. These genes may serve as biomarker in medullary MS patients.

Previous reports demonstrated increase in levels of Krebs cycle acids like alpha-ketoglutarate in fasting and citrate after glucose intake in MS patients [Henneman *et al.,* 1954]. McArdle *et al.* in 1960 found elevated levels of pyruvate and α-keto glutarate in MS [McArdle *et al.,* 1960]. Our findings revealed down regulation of genes implicated in TCA cycle including *Pdh, Aco2, Idh, Ogdh,* and *Sdh* in treated OPCs (Figure 26). A recent finding by Pedro *et al.* demonstrated changes in mitochondrial complex enzyme activities and cytochrome c expression in platelets of MS patients. Krebs cycle enzyme aconitase activity was higher in patients without fatigue and all respiratory complex enzyme activities (complex I, II, III, IV, V) were higher in MS patients compared to controls. Complex II activity increased significantly in MS group between patients with and without fatigue [Iñarrea *et al.,* 2013]. Safavizadeh *et al.,* (2013) observed a significant reduction in the gene expression of cytochrome c oxidase 5B subunit (COX5B) in MS patients. This suggests that there is a down regulation of genes associated with mitochondrial electron transport chain. Analysis of a number of respiratory chain proteins reveals functionally important defects of mitochondrial proteins [cytochrome c oxidase (COX) and its catalytic component COX-1] in complex III in MS [Mahad *et al.,* 2008]. Different expression of mitochondrial proteins namely cytochrome c oxidase subunit 5b (COX5b), hemoglobin β, creatine kinase and myelin basic protein (MBP) was found in the brain of multiple sclerosis [Broadwater *et al.,* 2011]. Taken together, these studies show that mitochondrial abnormalities and energy failure may cause functional disturbance in the surviving demyelinated axons in MS resulting in neurological dysfunction. Our findings suggest that genes of ETC

including ComplexI/NADH:ubiquinone oxidoreductase, Cyc1, Complex IV/COX and *ATP synthase* (both alpha & beta subunits) showed down regulated gene expression in these treated OPCs (Figure 27).

Our results revealed a significant decrease in metabolic flux rates accumulated in the glycolytic pathway (CFI 0.0019) Krebs cycle (CFI 0.0124) and electron transport chain and oxidative phosphorylation (CFI 0.0328) in the OPCs treated with CSF of these patients (Figure 39). Compared with non-MS patients (controls), total cumulative flux index fell in all three metabolic pathways to a very low value (CFI 7.94269E-07), similar to what occurs in IgM +/+ RRMS (Figure 39).

The spinal form of MS is a subtype of MS with damaged neurons found mostly in the region of the bone and is one of the most aggressive subtype of MS, comparable to more aggressive CNS IgM+/+ RRMS form, showing a similar gene expression pattern. The drastic reduction of metabolic genes expression in both types of MS would result in a bioenergetic failure, eventually causing oligodendrocytes to block the repair of important axonal damage in neurons exposed to energy depletion of these aggressive types of MS correlated with worse prognosis in contrast to the IgM type +/- RRMS.

As the data indicates that *Idh2,* gene showed differential expression in OPCs exposed to the CSF of medullary patients. The gene expression was not altered in any other patient which suggests that the gene may serve as a biomarker to distinguish between medullary and other types of diseases (IgM+/- vs. medullary, IgM+/+ vs. medullary, PPMS vs. medullary and NMO vs. medullary patients).

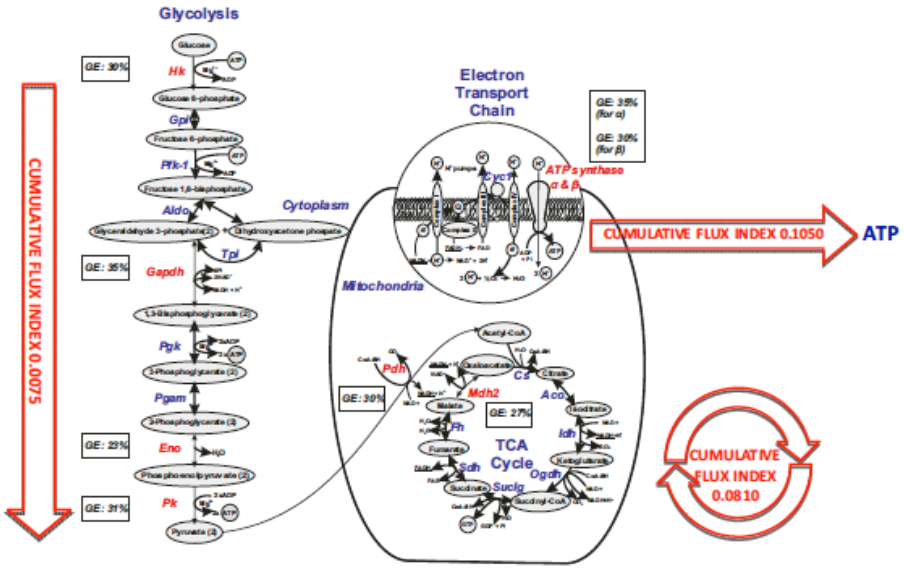

Figure 38: Metabolic network showing differential expression of metabolic genes in neurons treated with the CSF of medullary subtype of MS patients; GE: Gene expression in CGNs treated with medullary CSF related to CGNs exposed only to culture(untreated neurons); thick black arrow represents elevated expression whereas a thin arrow represents lower expression. Genes shown in red signifies differential expression.

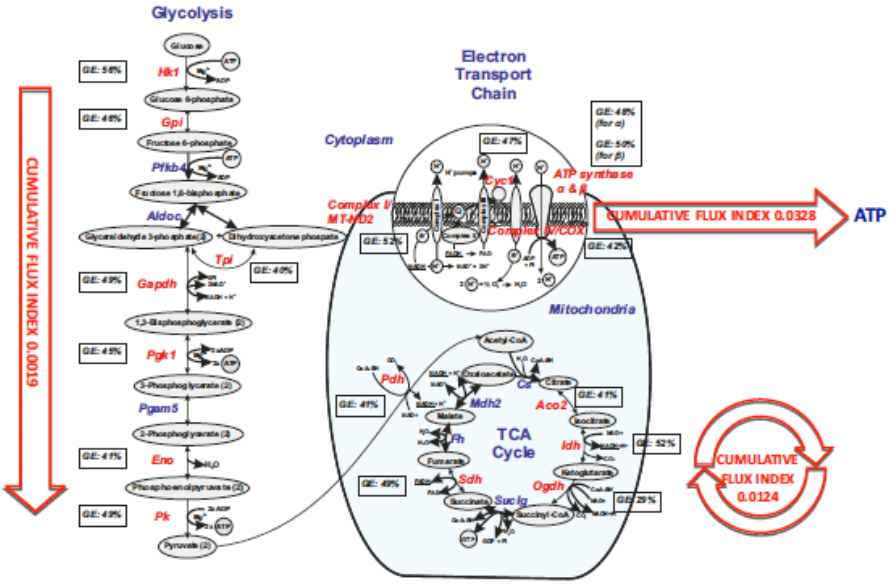

Figure 39: Metabolic network showing differential expression of metabolic genes in OPCs treated with the CSF of medullary subtype of MS patients; GE: Gene expression in OPCs treated with medullary CSF related to OPCs exposed only to culture (untreated OPCs); thick black arrow represents elevated expression whereas a thin arrow represents lower expression. Genes shown in red signifies differential expression.

PPMS

In experiment when neurons were treated with CSF from PPMS patients, we found reduction in gene expression of *Pgam* gene of glycolytic pathway (Figure 21). Among the genes involved in TCA cycle, *Pdh* and *Mdh2* showed down regulated expression in these neurons (Figure 22). Similarly, both *alpha* and *beta* subunits of *ATP synthase* gene which is involved in ATP production showed decreased expression (Figure 23). Conversely, several of the genes of glycolytic pathway including *Hk1, Gpi, Pfkb4, Aldoa, Gapdh, Eno2, Pk* were down regulated in OPCs treated with the CSF from PPMS (Figure 25). These genes may serve as biomarker in PPMS patients. As we can see from the data that *Hk3* gene showed differential expression in OPCs exposed to the CSF of PPMS patients. However, the gene expression was not altered in NMO group of patients which suggests that it may serve as a biomarker to distinguish between PPMS and NMO (NMO vs. PPMS patients). Genes involved in TCA cycle including *Aco2,* was also down regulated in these OPCs (Figure 26). Similarly, ComplexI/MT-ND2, Complex IV/COX and *ATP synthase* (alpha subunit) which is involved in ATP production showed decreased expression (Figure 27).

The results obtained with treatment with CSF of patients with PPMS indicated that glycolytic flux slightly decreased in neurons (CFI 0.3000) (Figure 40) and more heavily on the OPC (CFI 0.0033) (Figure 41) . The flux associated with the TCA cycle is decreased in both neurons (CFI 0.0870) (Figure 40) and the OPC (CFI 0.5400) (Figure 41) and in oxidative phosphorylation decreased in neurons (CFI 0.1050) (Figure 40) and the OPC (CFI 0.0728) (Figure 41) .

In general, we can say that the cumulative flux index in carbohydrates and ATP production in neurons decreases slightly (CFI 0.00274) and stronger in the OPC (CFI

0.00013) in patients with PPMS compared to controls. These results would be consistent with the fact that these patients have a subtype of MS less aggressive, slower and progressive course and better prognosis than RRMS subtypes IgM +/+ or med MS.

NMO

Another inflammatory, demyelinating and autoimmune disease of the CNS but totally distinct from MS known as "Neuromyelitis optica" showed an altogether different pattern of gene expression in neurons exposed to CSF of these patients than MS types studied above. We found that the expression of genes that catalyze reactions in glycolytic pathway, including *Hk1, Pgam,* and *Eno* are overexpressed in neurons exposed to CSF derived from NMO patients compared to neurons treated with CSF from neurological controls (Figure 21). Conversely the *Mdh2* and *Aco* genes of TCA cycle expressed in significantly lower quantities (Figure 22). Furthermore, chain genes TCA cycle and electron transport chain including *Pdh* (Figure 22) and the alpha and beta subunits of ATP synthase showed increased expression in treated neurons (Figure 23).

In general, an increase in the values of CFI on two metabolic pathways, the glycolytic pathway (CFI 1.9656) and the electron transport chain and oxidative phosphorylation (CFI 1.5867), and a slight decrease in Kreb cycle enzymes was observed (CFI 0.2174) (Figure 42). This causes the cumulative flux index in all three metabolic pathways be virtually unchanged (0.67787 IFC).

Unlike MS NMO it is not progressive. In fact less than 2% of patients NMO has a progression to disability relapsing. So this clinical difference may be because the gene pattern that leads to the synthesis of cellular ATP this slightly altered in patients with NMO.

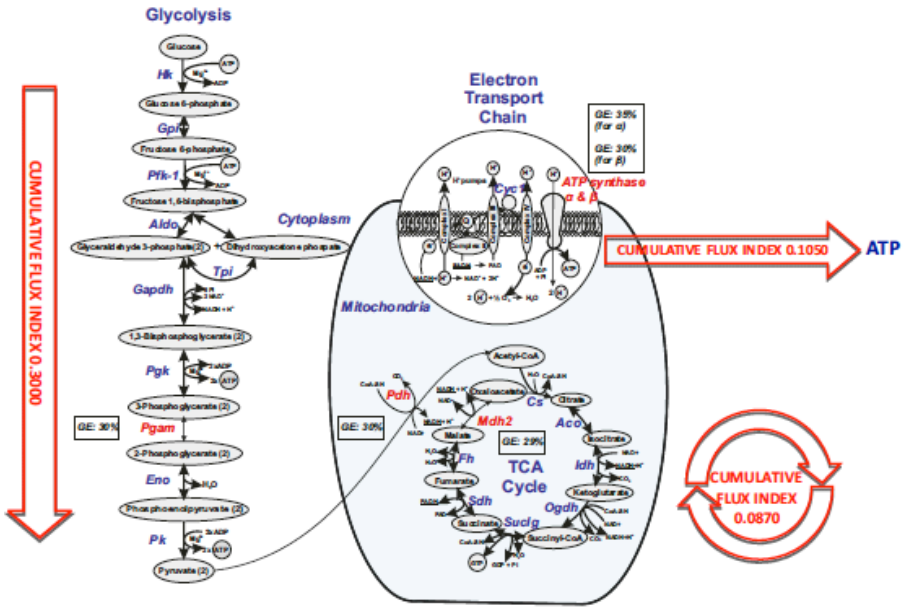

Figure 40: Metabolic network showing differential expression of metabolic genes in neurons treated with the CSF of patients with primary progressive MS (PPMS); GE: Gene expression in CGNs treated with the CSF of PPMS patients related to CGNs exposed only to culture (untreated neurons); thick black arrow represents elevated expression whereas a thin arrow represents lower expression. Genes shown in red signifies differential expression.

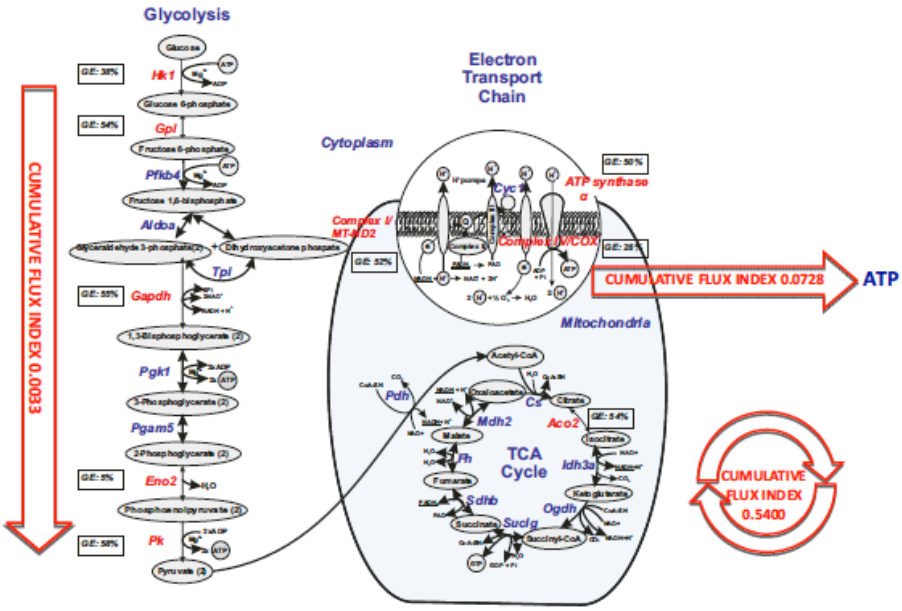

Figure 41: Metabolic network showing differential expression of metabolic genes in OPCs treated with the CSF of PPMS subtype of MS patients; GE: Gene expression in OPCs treated with PPMS CSF related to OPCs exposed only to culture (untreated neurons); thick black arrow represents elevated expression whereas a thin arrow represents lower expression. Genes shown in red signifies differential expression.

Furthermore, in OPCs exposed to CSF of patients with NMO, expression of genes that catalyze reactions via glycolysis including *Gpi, Aldo, Gapdh, Tpi, Pgk, Eno* and *Pk,* showed reduced expression of the genes compared to controls (Figure 25). Furthermore, the TCA cycle genes, including *Pdh, Aco2, Igdh Ogdh, Sdh* and *Mdh2* showed decreased expression in treated OPCs (Figure 26). Similarly, the genes of the electron transport chain and oxidative phosphorylation, such as CI, Cyc1, CIV / Cox and the alpha and beta subunits of ATP synthase also showed the reduction in gene expression (Figure 27). SInce IgG anti-NMO do not have a direct effect over OPCs, and it has high affinity for the astroglial receptors [Marignier *et al.,* 2010]. Despite purified cultures of OPCs were used, the 1% presence of astrocytes could explain the downregulation of metabolic genes. Another possible explanation (not tested), is that some NMO patients could presented anti-MOG, a recent antibody against myelin associated oligodendrocyte, that in cases of NMO patients cause a profound oligodendropathy non-associated to astrocyte damage [Ikeda *et al.,* 2015]. Becouse anti-MOG is present in 40% of seronegative-NMO, and in our series we have four NMO patients with no anti-NMO antibodies, we can rule out the possibility that some patients presented anti-MOG.

Our results revealed a decrease in the cumulative flux index accumulated in glycolysis (CFI 0.0013), Krebs cycle (CFI 0.0055), and oxidative phosphorylation (CFI 0.0149) in OPCs treated with CSF from NMO patient compared to non-MS (controls) (Figure 43). The results indicate that the flux indexes accumulated in neurons exposed to CSF of NMO patients suffer a very slight decrease (IFC 0.67787), while the OPCs decreased significantly (IFC 1.10046E-07).

Altogether, results obtained from oligodendrocyte data are consistent with neuronal data except for NMO where we found upregulated expression in treated

neurons compared to downregulated expression in treated OPCs. Moreover, metabolic genes were downregulated in all forms of MS in neurons and OPCs.

Our findings indicate an up regulated expression of enolase gene in neurons exposed to the CSF from NMO patients. However, the gene was down regulated in neurons exposed to the CSF from inflammatory MS (IgM+/- and IgM+/+), and medullary MS. Enolase may serve as a potential biomarker to distinguish NMO from MS. At the same time, we did not find any differential expression of this gene in neurons exposed to CSF from PPMS patients. Therefore enolase differential expression in NMO can be used to differentiate it from PPMS patients. The findings of our other experimental conditions reported down regulated expression of enolase (*Eno2)* gene in oligodendrocytes (OPCs) exposed to the CSF from medullary, PPMS and NMO patients as compared to neurological controls. Furthermore, we found significant differences in the gene expression between OPCs treated with the CSF of IgM+/- MS patients and the OPCs treated with the CSF of IgM+/+ MS patients. Another gene aldolase showed a differential expression in OPCs exposed to the CSF from medullary MS and NMO patients. We found that the gene was down regulated in OPCs treated with the CSF from medullary and NMO as compared to OPCs treated with the CSF from neurological controls. There was a marked difference in aldolase gene expression between medullary and NMO treated OPCs. This data suggests that aldolase may serve as a biomarker of MS and NMO which can clearly differentiate medullary MS from NMO.

Overall the data demonstrates a marked difference in the gene expression between MS and NMO. Nearly most of the genes from all the three pathways of glucose metabolism were down regulated in CGNs treated with CSF of MS compared to CGNs treated with CSF of NMO where the genes showed an up regulated expression. The differentially expressed genes may serve as biomarkers to

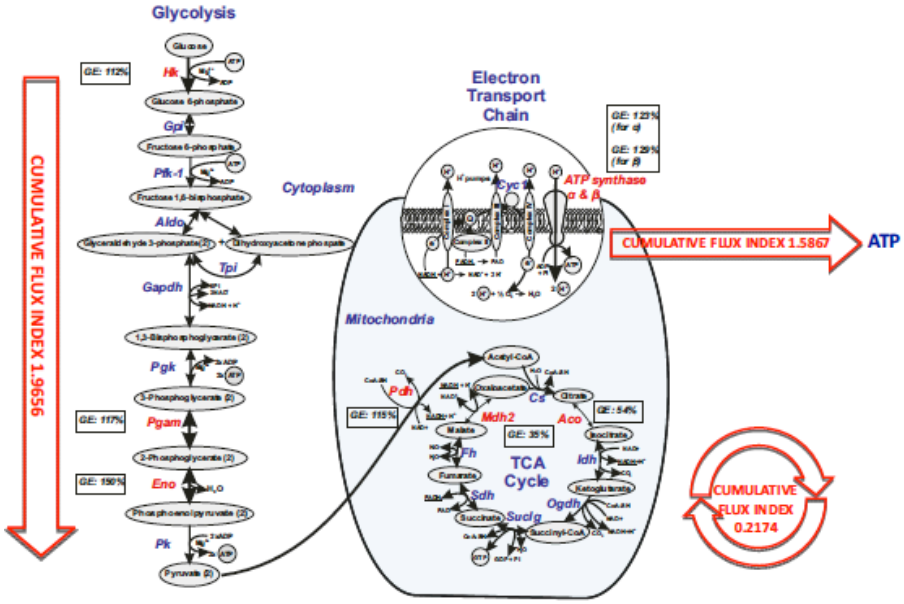

Figure 42: Metabolic network showing differential expression of metabolic genes in neurons treated with the CSF of NMO patients; GE: Gene expression in CGNs treated with the CSF of NMO patients related to CGNs exposed only to culture (untreated neurons); thick black arrow represents elevated expression whereas a thin arrow represents lower expression. Genes shown in red signifies differential expression.

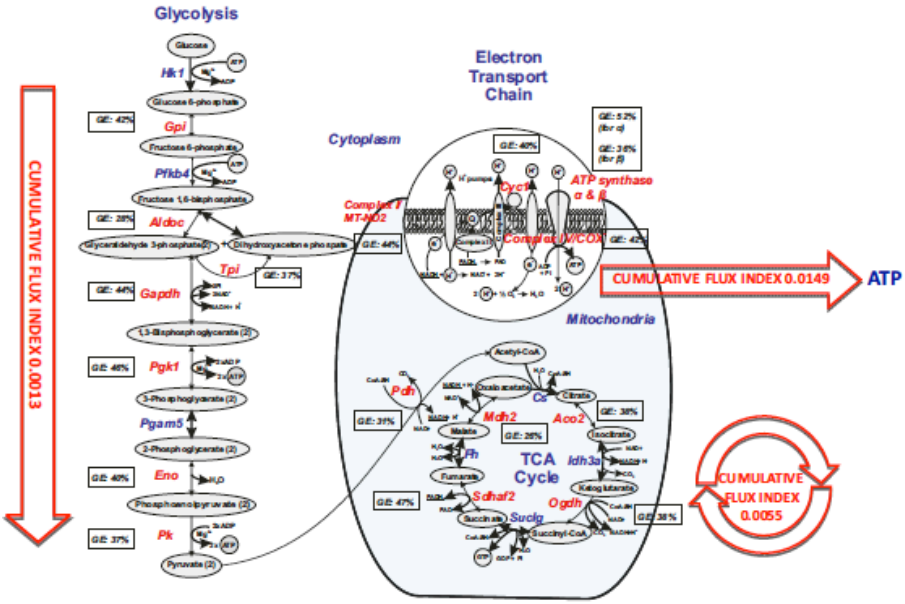

Figure 43: Metabolic network showing differential expression of metabolic genes in OPCs treated with the CSF of NMO patients; GE: Gene expression in OPCs treated with NMO CSF related to OPCs exposed only to culture (untreated neurons); thick black arrow represents elevated expression whereas a thin arrow represents lower expression. Genes shown in red signifies differential expression.

distinguish MS from NMO. In oligodendrocytes, most of the genes were down regulated in MS and NMO.

The findings of this thesis demonstrate a defective glucose metabolism in cultured cerebellar granule neurons and oligodendrocytes treated with the CSF derived from MS and NMO subjects. Since CSF is in contact with brain parenchyma and ventricles, any cellular damage product deposited in the fluid can influence the cellular physiology of neurons, oligodendrocyte progenitor cells (OPCs) and myelinating oligodendrocytes. Therefore, the exposure of CGNs to diseased CSF represents an excellent cellular model to study the primary neuronal damage. Oligodendrocytes which repair the damaged myelin during the relapsing remitting phase of MS also showed an altered carbohydrate metabolism upon CSF exposure from MS patients. Our results indicate that the factors secreted in the CSF of MS and NMO patients cause a disturbance in the metabolic pathways by altering the expression of genes catalyzing essential steps.

The results indicate that factors present in the CSF, in our model, perturb the metabolism of neurons and clearly differentiate more benign forms from the most aggressive forms in MS. The study also differentiates NMO from MS, which is sometimes difficult to distinguish by the clinicians. However, whether these alterations in metabolic gene expression cause MS and NMO or are simply a mere consequence of the disease is still elusive.

In addition to identifying a disturbed metabolic function associated with the cellular model of MS, we found a fluctuated expression of *Gapdh* and *beta actin* in our experimental conditions. These genes are commonly used as housekeeping genes in most of the laboratories. It is now demonstrated that expression of housekeeping genes which are used as endogenous controls for normalizing mRNA transcripts may not be necessarily stable in all cells/tissues under all conditions. A

gene may show stable expression in one condition whereas unstable in another. Invariable expression of reference genes has been observed with cellular development and under distinct experimental conditions. Therefore it is indispensible to pre-validate the expression stability of reference genes to accurately normalize the gene expression data. In this thesis, we have validated best housekeeping genes to be used in our experimental set up and we found *Gapdh* showed the most fluctuated expression.

There are some limitations of the study. First, we did not perform kinematic assays to measure the activity of proteins. There is a lot of processing and modification that occurs in cells, and these determine which protein is functionally active. Secondly, the proteomics analysis in neurons and oligodendrocytes exposed to the CSF from MS patients was not performed.

The identification of altered expression of genes catalyzing pivotal steps in glucose metabolism pathway in CGNs and OPCs treated with the CSF of distinct clinical forms of MS and NMO open new avenues of study and allow the development of therapeutic agents targeted to restore the metabolic function and hence repair and/or prevent axonal damage responsible for functional disability in the patient. A greater understanding of these impaired metabolic pathways may offer new insights into more efficacious treatments for MS and NMO. In addition our data suggests that it is desirable to determine the suitability of any common HK genes and their validation should be a routine step for any experimental system in a laboratory.

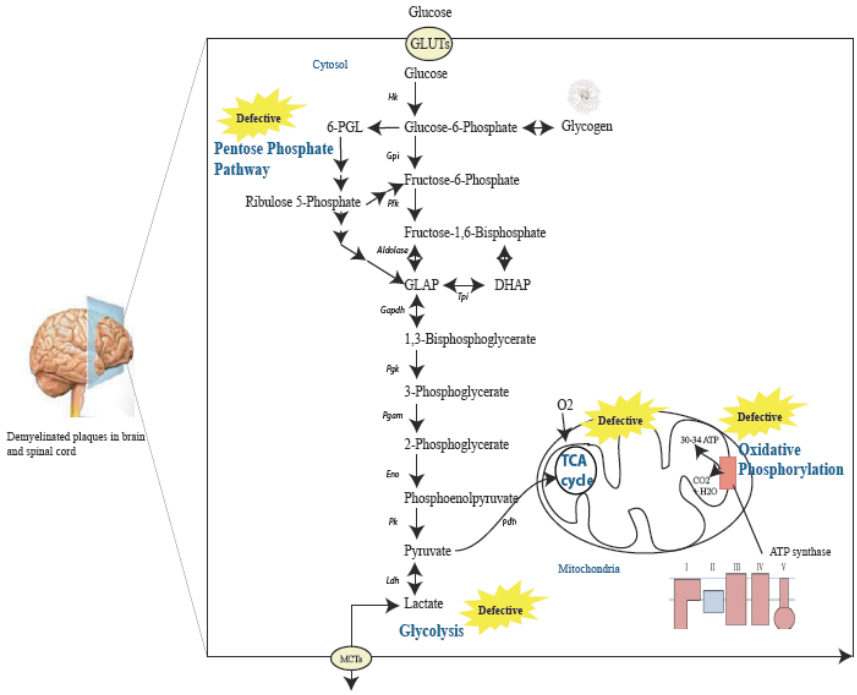

Figure 44. A schematic representation of glucose metabolism in MS brain. Glucose enters cells through glucose transporters (GLUTs) and is phosphorylated by hexokinase to produce glucose 6-phosphate. Glucose 6-phosphate can be processed into three main coordinated metabolic pathways. First, it can be metabolized through glycolysis giving rise to two molecules of pyruvate and producing ATP and NADH. Pyruvate can then enter mitochondria, where it is metabolized through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, producing ATP and CO2. Alternatively, pyruvate can be reduced to lactate by lactate dehydrogenase (Ldh). This lactate can be released into

the extracellular space through monocarboxylate transporters (MCTs). The complete oxidation of glucose produces 30-34 ATP molecules in the mitochondria. Alternatively, glucose 6-phosphate can be processed through the pentose phosphate pathway (PPP) leading to the production of reducing equivalent in the form of NADPH. Note that the PPP and glycolysis are linked at the level of glyceraldehyde-3-phosphate (GLAP) and fructose 6-phosphate. Finally, glucose 6-phosphate can be converted to glycogen through the process of glycogenesis in astrocytes. Abbreviations are as follows: Hk: Hexokinase; Gpi: glucose-6-phosphate isomerase; Pfk: phosphofructokinase-1; DHAP: dihydroxyacetone phosphate; Tpi: triose phosphate isomerase; GAPDH: glucose-6-phosphate dehydrogenase; Pgk: phosphoglycerate kinase; Pgam: phosphoglycerate mutase; Eno: enolase; Pk: pyruvate kinase; Ldh: lactate dehydrogenase; 6-PGL: 6-phosphoglucono-d-lactone. (Reproduced from review by Mathur *et al.,* 2014)

Table 13. Comparison of disturbed glucose metabolism in MS and other neurodegenerative disorders

Glucose metabolism	Multiple Sclerosis (MS)	Other neurodegenerative disorders
Glycolysis	Elevated blood **pyruvate level** was observed in both fasting and postprandial times in MS patients with relapse (Jones HH *et al.*, 1950) **Pyruvate levels** were found to be increased in MS (McArdle *et al.*, 1960) The activity of metabolic enzymes including **enolase and pyruvate kinase** was found to be increased in the CSF of MS patients (Royds *et al.*, 1981) Antibodies reactive with **triose phosphate**	Impaired **GAPDH** function was observed in subcellular fractions of fibroblasts from Alzheimer and Huntington patients (Mazzola and Sirover, 2001) **GAPDH** was found to be overexpressed in the neocortex and caudate putamen neurons in a transgenic model of Huntington's disease (Senatorov *et al.*, 2003) The activity of **GAPDH, hexokinase and pyruvate kinase** was increased in

	isomerase (TPI) and GAPDH, bind with them and inhibit the glycolytic activity of GAPDH in MS patients (Kölln *et al.*, 2010) The levels of **enolase, pyruvate kinase, lactate dehydogenase and aldolase,** all metabolic enzymes were increased in MS [Royds *et al.,* 1981]	alzheimer's disease (Soucek *et al.*, 2003)
TCA cycle	Krebs cycle proteins like **α-ketoglutarate levels** in fasting and **citrate levels** after glucose intake were found to be increased in MS patients (Henneman *et al.*, 1954) **α-ketoglutarate levels** were found to be increased in MS (McArdle *et al.*, 1960) Krebs cycle enzyme	Mitochondrial **aconitase**, **succinyl-CoA synthetase ß**, **fumarase** and **malate dehydrogenase** showed decreased gene expression in hippocampal samples from autopsy alzheimer's disease (AD) brains in two independent studies (Blalock *et al.,* 2004, Brooks *et al.,* 2007) Mitochondrial **oxoglutarate**

	aconitase activity was found to be higher in MS patients without fatigue (Iñarrea *et al.*, 2013)	**dehydrogenase**, showed increased and decreased gene expression in moderate and severe AD patients (Blalock *et al.,* 2004)

Oxidative phosphorylation	A reduction in the expression of **ATP synthase** gene was observed in MS lesions (Smith and Lassmann, 2002) Activity of mitochondrial electron transport chain **complex IV** was increased dramatically in MS lesions (Regenold *et al.*, 2008) Defects in **complex I** component of mitochondrial electron transport chain were observed in white matter	Decreased mRNA expression levels of electron transport chain proteins, specifically **FAD synthetase, riboflavin kinase (RFK), cytochrome C1 (CYC1),** and **succinate dehydrogenase** complex subunit B were reported in amyotrophic lateral sclerosis (Lin *et al.*, 2009) Decreased mRNA levels of the mitochondrial-encoded **cytochrome oxidase (COX) subunits I, II and III** were observed in brains

	lesions (Lu *et al.*, 2000) Furthermore, reduced functional activity of **complex I and complex III** and a decrease in gene expression of **complex I, complex III, complex IV, and ATP synthase** has been observed in nonlesional motor cortex (Dutta *et al.*, 2006). In contrast, enzyme activities of **complex I, II, III, IV and V** were found to be higher in MS patients compared to controls. **Complex II** activity increased significantly in MS group between patients with and without fatigue (Iñarrea *et al.*, 2013 A significant reduction in the gene expression of **cytochrome c oxidase 5B subunit**	of AD patients (Brooks *et al.,* 2007, Chandrasekaran *et al.,* 1994, Simonian et al., 1994) Reduced expression of nuclear encoded subunits of mitochondrial enzymes of oxidative phosphorylation including **subunit IV of COX** and the **beta-subunit of the F0F1-ATP synthase** was also observed in vulnerable areas of AD brain (Brooks *et al.,* 2007, Chandrasekaran *et al.,* 1994)

	(COX5B) was observed in MS patients (Safavizadeh *et al.*, 2013) Analysis of a number of respiratory chain proteins reveals functionally important defects of mitochondrial proteins [**cytochrome c oxidase (COX) and** its catalytic component, **COX-1**] in complex III in MS (Mahad *et al.*, 2008) Different expression of mitochondrial proteins namely **cytochrome c oxidase subunit 5b (COX5b), hemoglobin b, creatine kinase and myelin basic protein (MBP)** was found in the brain of multiple sclerosis (Broadwater *et al.*, 2011).	

VI- CONCLUSIONS

CONCLUSIONS AND FUTURE PROSPECTS

Despite extensive research being carried out for a decade the underlying cause of MS still remains elusive. Perturbed glucose metabolism is implicated in neurodegenerative disorders like Alzheimer's, Parkinson's and Huntington's. However, little is known about its role in MS pathology. The findings reported in this thesis indicate that factors present in the CSF, in our model, are affecting the metabolism of neurons and oligodendrocytes and clearly differentiate more benign forms from the most aggressive forms in MS. The study also differentiates NMO from MS, which is sometimes difficult to distinguish by the clinicians.

1. CSF factors from multiple sclerosis cause death in neurons but not astrocytes.
2. The housekeeping gene *Gapdh* variates in CGNs treated with CSF from MS and NMO patients. The best ones are *Tfrc, B2m* and *Rpl19.*
3. CSF factors from MS induce an alteration in glucose metabolism and ATP production both related with neuronal death.
4. CSF factors altered glucose metabolism in oligodendrocytes exposed to the CSF from MS and NMO patients.
5. The housekeeping gene *Gapdh* variates in treated oligodendrocytes.
6. The CSF effect is different in MS aggressivity of RRMS and PPMS clinical form (IgM+/-, IgM+/+, Med, PP).
7. IgM+/+ and medullary derived CSF treated neurons and OPCs are strongest affected by reducing carbohydrate metabolism as evidenced by

down regulation of most of the genes which is suggestive of least ATP synthesis. This indicates more damage to neurons and blockage of myelin repair by OPCs and correlated with worst prognosis.

8. NMO derived CSF exposed neurons reveals increased carbohydrate metabolism as indicated by increased expression of genes whereas neurons and OPCs treated with CSF from IgM+/- MS patients demonstrates slightly reduced carbohydrate metabolism with less neuronal damage correlated with poor prognosis.

9. The results allow us to differentiate different clinical forms and aggressivity in MS.

10. The results allow us to differentiate MS from NMO.

These results are important to establish biomarkers of NMO and MS to target and monitor the effects of future therapies aimed at preventing MS disease progression. These observations open new perspectives for the understanding of metabolic dynamics in multiple sclerosis yet many puzzling aspects and critical questions need to be addressed. For instance, how does defect in metabolic pathway contributes to demyelination? Does metabolic pathway alters in other cell types in MS? The changes in a cell type, may affect directly, through some unknown factor, or indirectly, through global changes in metabolic intermediates levels in CSF, to other cell types? Is disturbance in metabolic pathway a mere cause or a consequence of MS? Can those genes be used as a pharmacological target to alleviate MS pathology? Much more research is required to fully unravel the disease mechanism, and a proper understanding of the disease could eventually lead to new treatments.

VII- SPANISH SUMMARY/RESUMEN EN CASTELLANO

SPANISH SUMMARY/RESUMEN EN CASTELLANO

1. INTRODUCCIÓN

La esclerosis múltiple (EM) es una enfermedad crónica del sistema nervioso central (SNC) en el que episodios repetidos de inflamación (bortes), dan lugar a inflamación que conduce a la interrupción de la vaina de mielina por daños producidos en la misma. Junto a este fenómeno de inflación focal, existe una inflamación difusa en el SNC, que unida a la anterior, dará lugar a que aparezca un proceso de neurodegeneración, que será el responsable último de la afectación axonal y neuronal difusa que es la que va a condicionar la discapacidad en los pacientes afectos de EM. La enfermedad es una causa importante de discapacidad neurológica y de disfunción neurológica en adultos jóvenes que afecta a más de dos millones de personas en todo el mundo. En España, la prevalencia de la enfermedad es de aproximadamente 70-80 casos por cada 100.000 habitantes según las estimaciones actuales. La enfermedad se manifiesta típicamente hacia los 20 a 40 años de edad, cuando las personas están en su plena capacidad profesional y, a veces se convierte en un proceso agresivo que altera la vida de los pacientes y sus familias.

La EM es una enfermedad heterogénea, tanto en su forma de presentación, el curso evolutivo, la anatomía patológica (donde se han descrito hasta 4 patrones distintos de afectación patológica) y la respuesta a los tratamientos. Estas diferencias llevaron a la clasificación de los tipos de EM en remitente-recurrente (EMRR), EM secundaria progresiva (EMSP) o EM primaria progresiva (EMPP). La mayoría de los pacientes comienzan con un curso de la enfermedad del tipo EMRR que se caracteriza por ataques periódicos (brotes), seguidos de su recuperación

parcial o completa (remisiones). A pesar de recibir medicamentos para prevenir la recaída de los pacientes, que tienen un efecto antiinflamatorio pero sobre todo inmunomodulador, los pacientes entran finalmente en la fase secundaria progresiva (EMSP) con un empeoramiento neurológico irreversible. Los pacientes que cursan con una enfermedad progresiva primaria (PPMS) sufren un deterioro continuo de los síntomas neurológicos, lo que sugieren que la fisiopatología de la progresión no es únicamente de naturaleza inflamatoria.

El desarrollo de tratamientos eficaces se ha visto obstaculizado por nuestro aún limitado entendimiento de la patogénesis de la EM. Es evidente que una comprensión más profunda de los mecanismos que sustentan la progresión de la enfermedad pueden ayudar en el desarrollo de estrategias terapéuticas para el tratamiento de esta patología.

Aunque todas las células del SNC, incluyendo oligodendrocitos y astrocitos, pueden considerarse como "víctimas" celulares en la EM, se acepta ampliamente que es el daño neuronal la causa principal de la discapacidad funcional persistente en estos pacientes, aunque su origen no está aún del todo aclarado. Una de las hipótesis generales es que el daño axonal en los axones desmielinizados es el resultado del aumento de la demanda energética consecuente con la redistribución de la bomba Na^+/K^+ ATPasa anterior al aumento de los niveles intracelulares de Ca^{2+}. El daño axonal también se ha atribuido a defectos metabólicos defectuosa o a un soporte trófico deteriorado a causa de daños en los oligodendrocitos. Sin embargo, también se cree que ciertos factores difusibles presentes en el líquido cefalorraquídeo (LCR) podrían también afectar a la capacidad de las neuronas para responder al daño axonal con una producción de energía adecuada, especialmente en la patogénesis de las lesiones corticales. La disminución de la capacidad para satisfacer la demanda energética se ha observado durante el deterioro

mitocondrial en estudios con neuronas cultivadas, modelos preclínicos de EM en animales, y en muestras de EM humana.

El LCR se encuentra en contacto con el parénquima y los ventrículos del cerebro y puede ser un sitio para la deposición de los productos del daño celular que pueden influir en la fisiología celular de las neuronas, las células progenitoras de los oligodendrocitos (OPCs) y los propios oligodendrocitos mielinizantes. El LCR es por tanto un biofluido prometedor para la búsqueda de biomarcadores y de proteínas asociadas al desarrollo de la EM, tanto con respecto al proceso inflamatorio como al neurodegenerativo. Los factores liberados en el LCR de pacientes con EM incluyen factores apoptóticos, citoquinas, enzimas proteolíticas, productos oxidativos y radicales libres que son producidos por las células gliales y las células inmunes activadas. Estos compuestos serían probablemente los más propensos a causar el daño axonal durante el desarrollo de la enfermedad.

La hipótesis de trabajo sobre la que se articula el trabajo desarrollado es que la identificación del efecto a nivel transcripcional de células neuronales incubadas con LCR, obtenido de distintos tipos clínicos de pacientes con EM, puedan tal vez explicar ese daño neuronal. Por otra parte, el estudio de los posibles patrones genéticos diferenciales sobre las OPCs incubados con el LCR, el cual podría contener factores que dañan éstas células durante los intentos de reparación de las neuronas, podría facilitar la progresión de la EM. La identificación de los mecanismos implicados en la degeneración-reparación del daño axonal puede arrojar luz en la comprensión de la progresión de la enfermedad y/o su pronóstico dependiente del tipo clínico de la EM.

2. OBJETIVOS

En base a esta hipótesis, el objetivo principal fue intentar determinar los mecanismos que están implicados en la degeneración axonal-regeneración en la EM. Para ello se centró el estudio en el daño neuronal primario, independiente del daño secundario como resultado de la desmielinización, utilizando cultivos primarios de neuronas granulares del cerebelo (CGCs) como modelo celular. Es verosímil que factores hasta ahora desconocidos presentes en el LCR de pacientes con EM son capaces de regular la destrucción a la reparación axonal, y hacer una remielinización estable o no y que la recuperación funcional pueda ser posible o permanecer en el curso de la enfermedad. Además, durante las fases de la enfermedad remitente-recurrente el cerebro del paciente por sí solo es capaz de reparar el daño, remielinizar el axón y recuperar la función neurológica. Por lo tanto, decidimos tratar a las CGC y OPCs con LCR derivado de diferentes formas clínicas de la EM y llevar a cabo estudios de expresión génica global. Por otro lado nos planteamos también como objetivo identificar los genes de referencia más estables expresadas en CGC y OPCs tratados para poder normalizar con precisión las niveles de mRNA en los perfiles de expresión. Por último, nos planteamos intentar identificar posibles biomarcadores que ayuden a distinguir las diferentes formas clínicas de la EM y permitan explorar las diferencias y similitudes con la neuromielitis óptica (NMO).

Por todo ello, los objetivos concretos que se proponen en esta tesis son los siguientes:

1. Establecer una clasificación de pacientes con EM y NMO en base a criterios clínicos y la presencia de bandas oligoclonales de IgG e IgM en el LCR y aquaporina en suero.

2. Determinar el efecto de la LCR en la viabilidad y el daño celular de las neuronas granulares del cerebelo y en oligodendrocitos.

3. Establecer una relación entre la afectación neuronal y la agresividad en diferentes formas clínicas de EM y NMO.

4. Identificar genes constitutivos durante la maduración de CGCs y en neuronas y OPC expuestos a la LCR de pacientes con EM y NMO.

5. Analizar mediante ensayos con micromatrices de DNA la afectación en la expresión génica del tratamiento con el LCR de los cultivos primarios de CGCs y en OPC.

6. Analizar las variaciones en los genes implicados en el metabolismo de los hidratos de carbono y en la producción de energía y correlacionarlos con el deterioro neuronal y la prognosis en los diversos tipos de EM y NMO.

7. Buscar potenciales biomarcadores para diferenciar los diversos tipos de EM y NMO y en su prognosis.

3. MATERIALES Y METODOS

3.1. Clasificación de pacientes

Se han estudiado un total de 59 pacientes en los diferentes experimentos usando las muestras de LCR obtenidas en el Departamento de Neurología del Hospital La Fe y en el Hospital Clínico (Universidad de Valencia).

Estos pacientes fueron analizados mediante electroforesis para determinar la presencia en el LCR de bandas oligoclonales de IgG e IgM, en pacientes con EM, y de aquaporina en el suero de pacientes con NMO.

Los pacientes con EM se definieron y se agrupan en diferentes cursos clínicos, de acuerdo con los criterios de Lublin. Adicionalmente, los pacientes con EMRR fueron reclasificamos en función de la presencia de bandas oligoclonales de tipo IgM, manteniendo un subgrupo con predominio de afectación medular, que se clasificó como tal. Los pacientes con EM incluidos en este estudio fueron diagnosticados según los criterios de McDonald 2010, y todos ellos cumplen las siguientes características: bandas IgG oligoclonales presentes, habiéndose realizado la punción lumbar fuera de un brote y que hayan pasado al menos un mes después de la última dosis de corticoides. Se utilizaron los criterios Wingerchuk para diagnosticar pacientes con enfermedad NMO. Los pacientes sufrieron recaídas de neuritis óptica y mielitis, y 2 de los tres criterios, la RM normal o que no cumplen los criterios de Patty para MRI de diagnóstico de la EM. Se realizaron todos los estudios durante el procedimiento de diagnóstico, para lo cual el paciente firmó un consentimiento informado.

En base a estos criterios clínicos y bioquímicos se pudo establecer que de los 59 pacientes, 21 tenían EM de tipo inflamatoria (11 EMRR subtipo IgM +/+ y 10 EMRR subtipo IgM +/-), 8 tenían EM de subtipo medular (EMMed), 11 tenían EM primaria progresiva (EMPP), 9 tenían neuromielitis óptica (NMO), y 10 eran controles (pacientes NIMD). La tabla 3 muestra la clasificación final y las características clínicas de los pacientes usados en este estudio.

Estos pacientes fueron analizados mediante electroforesis para determinar la presencia en el LCR de bandas oligoclonales de IgG e IgM, en pacientes con sospecha de EM, y de aquaporina en el suero de pacientes con sospecha de NMO.

3.2. Análisis de LCR y suero en pacientes con EM y NMO

Para determinar los subtipos de EM y NMO señalados arriba, se analizaron muestras de LCR y suero pareadas para detectar las bandas oligoclonales de IgG y IgM mediante isoelectroenfoque (IEF) e inmunodetección. La técnica consiste, usando un kit comercial (Helena BioScience IgG-IEF) y el método descrito por Villar et al. (2001), consiste en la dilución de las de las muestras de LCR hasta concentraciones similares de proteínas, y la incubación con ditiotreitol para reducir IgM. El electroenfoque enfoque se realizó en un sistema de electroforesis Multiphor II (GE Healthcare) a un pH entre de 5 a 8. Las proteínas se transfirien a una membrana de PVDF y se analizan mediante Western blot usando en la inmunodetección anticuerpo anti-IgM humano conjugado con biotina y estreptavidina-fosfatasa alcalina para la detección. En los estudios con suero de pacientes con NMO se usó el anticuerpo anti-AQP4 para la inmunodetección. Se usó además la para detectar la presense de NMO anticuerpos IgG específicos. La inmunofluorescencia indirecta (IFI) se realizó además en las células transfectadas por acuaporina 4 usando el mismo anticuerpo (EUROIMMUN Medizinische Labordiagnostika AG).

3.3. Material biológico

3.3.1. Animales

Para los diversos experimentos con cultivos celulares primarios se utilizaron ratas Wistar (Harlan Iberica) con peso entre 200-250 g. El mantenimiento de los animales se realizó en las instalaciones de los animales inferiores del Príncipe Centro de Investigación, Felipe, Valencia, España.

3.3.2. Cultivos primarios de neuronas granulares de cerebelo (CGCs)

Los cultivos primarios de neuronas granulares del cerebelo (CGCs) se obtuvieron de acuerdo con el protocolo modificado descrito previamente [Minana et al., 1998]. Los extrajeron los cerebelos de ratas Wistar de 8 días y, tras eliminar las meninges, se disgregaron con pipeta Pasteur, se trataron con 3 mg/ml de dispasa (grado II) durante 30 min a 37ºC en atmósfera humidificada de CO_2 5% y se inactivó la enzima con 1 mM EDTA. Las células granulares se resuspendieron en medio basal de Eagle (BME, Gibco) con 40µg/ml de DNAasa I. La suspensión celular se filtró a través de una malla con un tamaño de poro de 90µm, se centrifugó a 1.500 rpm durante 5 min y se lavó la suspensión de células con BME. Finalmente, las células se resuspendieron en medio completo BME con sales de Earle con 10% de suero bovino fetal, glutamina 2 mM, 0.1 mg/ml de gentamicina y 25 mM KCl. Se contaron las células neuronales, se sembró la suspensión en placas de cultivo revestidas de poli-L-lisina a una densidad de $3x10^5$ células/pocillo y se incubaron a 37º C. Para la obtención de cultivos enriquecidos en neuronas, se incubaron las células con arabinósido de citosina 1 mM entre de 18 a 24 horas, lo que inhibe la replicación de células no neuronales.

3.3.3. Cultivos primarios de células precursoras de oligodendrocitos (OPCs)

Las OPCs se aislaron a partir de la corteza de ratas postnatales el día 1 y se cultivaron de acuerdo con un procedimiento de McCarthy y De Vellis modificado [McCarthy y De Vellis 1.980]. Las células fueron cultivadas en medio NM10 (DMEM de alta glucosa suplementado con SBF al 10%), y se cultivaron durante una semana

a 37 ºC en 5% CO_2. Después de una semana, las células de microglía poco adheridas se eliminaron mediante una agitación de baja velocidad (210 rpm) durante 20 min en un agitador de plataforma rotatoria. El medio se retira y se desecha y se reemplaza con medio fresco NM10. Los matraces se agitaron durante la noche durante 18-20 horas a 220 rpm y las células se inmunoseleccionaron en una columna de purificación magnética Miltenyi MACS usando anticuerpo de ratón anti-A2B5. Las OPCs primarias se sembraron en placas revestidas de poli-lysine a una densidad de 2.000 células por cm^2 y se cultivaron en medio de definición química de oligodendrocitos (ODM) que contiene 100 mg/ml de transferrina bovina, 5.0 mg/ml de insulina recombinante de levadura, 100 mg/ml de BSA, 1 mg/ml de biotina, 0.628 mg/ml de progesterona, 0,38 mg/ml de selenito de sodio, 16.1 mg/ml de putrescina.

Para la expansión de los OPCs, el medio ODM se suplementó con 20 ng/ml del factor de crecimiento de fibroblastos (bFGF) y 10 ng/ml del factor de crecimiento AA derivado de plaquetas (PDGF-AA), y las células se dejaron proliferar durante 48 horas antes del tratamiento con los LCR . Los cultivos de OPCs, tal como se detectó por inmunocitoquímica, eran 99%+ cultivos OPCs puros, con <1% de astrocitos detectables GFAP+ y <1% de microglia detectables Iba1 +.

3.3.4. Tratamiento de CGCs y OPCs con LCR de pacientes con MS y NMO

Los cultivos de CGCs de día 14º se incubaron con 10% (v/v) de los LCF de pacientes con MS (IgM +/-, IgM +/+, medular, PPMS y controles) y NMO durante 24 h. Los cultivos de OPCs fueron tratados con LCR diluido 1:1 en medio ODM suplementado con bFGF (20 ng/ml) y PDGF-AA (10 ng / ml) durante 24 h.

La viabilidad celular de las CGCs y OPCs durante los cultivos de LCR se utilizó yoduro de propidio (PI), que está excluido en las células vivas, a una concentración final de 0.5 mmg/ml en PBS y se incubó durante 30 min a temperatura ambiente en oscuridad. Las células se incubaron además con rodamina-123, colorante catiónico que se acumula dentro de la mitocondria de las células debido a la diferencia de potencial negativa interna, a una concentración de 10 mg/ml en PBS durante 30 min a temperatura ambiente en oscuridad. Las células se visualizaron mediante microscopía confocal en microscopio Leica TCS SP2 AOB de escaneo láser confocal invertido. El análisis de las imágenes obtenidas se realizó usando el programa *Metamorph 7.0* (Molecular Devices).

3.4. Análisis de expresión global con micromatrices de DNA y normalización de datos.

Los análisis de expresión de genes de células de CGCs y OPCs en las diversas condiciones experimentales señaladas en resultados se llevaron a cabo tras la extracción de RNA con el kit *Quick RNA Microprep* (Zymo Research Corp.) de acuerdo con las instrucciones del fabricante. Concentración de ARN y la pureza se determinaron utilizando una máquina de NanoDrop. La calidad de ARN

La concentración y pureza de los RNA se determinó espectrofotométricamente utilizando el espectrofotómetro Nanodrop 1.000 y la calidad de los RNA pureza se verificó por electroforesis capilar utilizando un Bioanalyzer 2.100 (Agilent) y se retro-trascribieron a cDNA.

Estos RNA se usaron tanto en los ensayos con micromatrices de DNA como para los análisis por qPCR de genes individuales, incluidos los genes constitutivos de referencia (tabla 4), en un aparato Applied Biosystems 7.300, y los datos fueron analizados utilizando el software de detección (SDS) versión 1.3 (Software Roche).

Por otra parte, los genes constitutivos de referencia invariables fueron evaluados por herramientas de software *GeNorm* y *NormFinder* disponibles públicamente. El programa *GeNorm* clasifica los genes de acuerdo a su medida promedio de estabilidad de la expresión, desde los más estables (valor de M más bajo) a los menos estables (el más alto valor de M). Un programa alternativo, *NormFinder,* que se introdujo posteriormente clasifica los genes candidatos de referencia en base a las estimaciones combinadas de variaciones intra e intergrupales.

Los RNA purificados se usaron para el análisis de expresión génica basado en micromatrices de DNA de un color (Agilent Technologies), mediante hibridación con micromatrices *Agilent SurePrint G3 Rata GE 8x60K Microarray* (GEO-GPL13521) de acuerdo con el protocolo del fabricante. Con el fin de dar cuenta de la variación técnica entre micromatrices (es decir, la cantidad de RNA de partida, y las diferencias en la eficiencia de la transcripción inversa, el etiquetado y la hibridación, etc.), la intensidad de la señal en bruto se normalizó utilizando el método de "desplazamiento de percentil", fijado en la intensidad más robusta del percentil 75, disponible en *GeneSpring 9.0* para micromatrices de un color (Agilent). Después de la normalización, los datos se filtraron con el fin de excluir los "*probesets*" con baja expresión y/o los afectados por las diferencias entre los laboratorios. Los genes expresados diferencialmente se identificaron mediante la comparación de los niveles de expresión promedio en término de veces de inducción en las muestras de MS y NMO respecto a los controles. La figura 11 representa el flujo de trabajo para la preparación de las muestras y el procesamiento de las micromatrices.

3.5. Análisis de redes de interacción proteína-proteína utilizando el software STRING v10

Para dilucidar si la interacción de diferentes enzimas metabólicos relacionados con una ruta metabólica (en nuestro caso la glucólisis, el ciclo de Krebs y la cadena de transporte de electrones) puede condicionar la respuesta de toda la ruta a cambios locales en la concentración de las enzimas hemos utilizado el software STRINGv10.

Aunque no todos los genes relacionados con una red específica puedan ser localmente afectados en algún tipo de EM o NMO, el análisis STRING mostraría la interacción física de enzimas relacionadas en una ruta y existir un complejo regulador estrechamente relacionado que modifique el flujo metabólico en esa ruta concreta.

Para expresar los cambios en el flujo metabólico global en una ruta concreta resultado de cambios locales en alguna de las enzimas de la misma, hemos definido el parámetro "índice del flujo metabólico acumulado" (CFI) que indica el cambio sinérgico en el flujo global como resultado de la disminución de la actividad de uno o varios enzimas de la ruta analizada. Estos valores nos permitirán hacer comparaciones en los cambios de los flujos metabólicos de las rutas del metabolismo de carbohidratos y de obtención de energía en los distintos tipos de EM y NMO.

3.6. Análisis estadístico

Los datos obtenidos, resultado de al menos tres experimentos independientes, se expresan como el valor medio $\pm$ su error estándar. Una prueba estadística se aplicó para buscar diferencias significativas entre las condiciones experimentales

para cada gen candidato. Se realizó un análisis unidireccional de la varianza (ANOVA) para determinar los genes con variaciones significativas. Un valor de $p<0.05$ fue considerado estadísticamente significativo.

4. RESULTADOS Y DISCUSION

4.1. Diagnóstico clínico y clasificación de pacientes con EM y NMO

El primer paso para el estudio de los cambios inducidos en las células cerebrales por el tratamiento con LCR de pacientes con EM y NMO es la clasificación correcta del subtipo y del nivel de la enfermedad. El curso clínico preciso de los pacientes con EM (fenotipos) es importante para el pronóstico, el diseño de los ensayos clínicos y la toma adecuada de los tratamientos. En este trabajo es además importante su conocimiento para hacer la adecuada correlación del tipo de MS y NMO con los cambios en la expresión génica o el comportamiento celular en los tratamientos.

Por ellos, se realizó la clasificación de los pacientes en base a la clínica y la imagen MRI en grupos EMRR, EMPP y MedMS. En el primer grupo EMRR se clasificó en subgrupos con pacientes con menor o peor pronóstico en base a la presencia de bandas oligoclonales IgG e IgM en el LCR (Figura 12). Los pacientes con NMO se identificaron mediante inmunofluorescencia indirecta en células transfectadas con acuaporina 4 que detecta la presencia por la presencia de anticuerpos IgG específicos de NMO en muestras de suero de los pacientes (Figura 13).

Por lo tanto, las formas clínicas de la EM que utilizaremos en este trabajo son: 1) Pacientes con EMRR subtipo IgM +/- que poseen anticuerpos oligoclonales IgG

(+) pero no IgM (-) detectables en el LCR. 2) Pacientes con EMRR subtipo IgM +/+ que poseen tanto anticuerpos oligoclonales IgG (+) como IgM (+) detectables en el LCR. 3) Pacientes EMMed que son positivos para bandas oligoclonales de IgG (+) y negativo para bandas oligoclonales de IgM (-) en el LCR, así como, son negativos para anticuerpos anti-NMO en el suero. 4) Pacientes EMPP que se caracterizan por disminución progresiva de la capacidad neurológica, con anticuerpos IgG (+) pero no anticuerpos IgM (-) en el LCR. 5) Pacientes con NMO que son positivos con anticuerpos contra acuaporina 4, con una mielitis transversa con gran extensión y/o cerebro normal en el primer evento. 6) Controles con pacientes sin inflamación y sin enfermedad neurológica diagnosticada (NIND).

La prevalencia de EM observada en estos pacientes, como se conoce en la literatura, es mayor en las mujeres que en los hombres (75% en mujeres). La edad media de los pacientes con EM fue de 30,7 ± 9,7 años, mientras que fue de 25,6 ± 15 años para los pacientes NMO. De acuerdo con la clasificación clínica, las características generales de cada MS pacientes se describen en la Tabla 7.

Se encontraron diferencias significativas entre la edad al inicio de la EMPP y las otras dos formas de EM ($p<0.003$), entre la EDSS (Escala Ampliada del Estado de Discapacidad) de la EMRR y las otras dos formas de EM ($p <0.001$), y el tiempo de evolución entre la EMPP y EMRR ($p<0.043$), después de la corrección de Bonferroni. La tabla 8 muestra las características de los pacientes con EM de acuerdo con la nueva propuesta y la clasificación de trabajo. Después de la corrección de Bonferroni se encontraron diferencias significativas importantes entre la edad al inicio de la enfermedad y la EDSS entre las formas EMMed y EMPP respecto a las formas inflamatorias de MS.

Los diferentes subtipos de EM ayudan a predecir la severidad de la enfermedad y la respuesta al tratamiento, por tanto, su clasificación es importante. En nuestro estudio, encontramos diferencias significativas entre la EDSS de EMRR y

las otras dos formas de EM (EMSP y EMPP) (p <0,001). Aunque la lesión del nervio se produce siempre, el patrón es específico para cada individuo con EM. La gravedad y discapacidad de la enfermedad aumenta en el curso de la EMRR a EMSP y en el subtipo PPMS ~~subtipo~~, dado que los síntomas empeoran continuamente desde el momento del diagnóstico en lugar de tener ataques y recuperación bien definidos. En nuestros estudios se encontraron diferencias significativas en el tiempo de evolución entre la EMRR y las formas progresivas (p=0.043), así como en el grado de discapcidad medido por la EDSS (p<0.001). En los pacientes que experimentan un curso progresivo, el tiempo de evolución fue similar en los casos de EMSP y en los casos que eran progresivas desde el inicio (13,5 *versus* 13,8).

4.2. LCR de pacientes con EM provoca muerte celular en CGCs

Como el daño axonal ahora ha sido ampliamente aceptado como la causa principal de discapacidad funcional persistente en pacientes con EM, nos centramos en la primera parte de nuestro estudio en el daño neuronal primario, independientemente del daño secundario resultante de la desmielinización. Los individuos con MS poseen la capacidad, y los factores necesarios adecuados, para reparar la mielina dañada durante el curso remitente-recidivante de la enfermedad.

Debido a su generación posnatal y la viabilidad de los cultivos primarios bien caracterizados *in vitro*, las células granulares del cerebelo son un modelo de elección idóneo para el estudio de los eventos celulares y moleculares implicados en los mecanismos de supervivencia/apoptosis y la neurodegeneración/neuroprotección durante la EM.

Puesto que potencialmente todas las células del cerebro, neuronales y no neuronales, pueden verse afectadas por esta enfermedad, se determinó el efecto de los LCR de pacientes en la viabilidad celular mediante la adición de yoduro de propidio y rodamina-123 a los cultivos celulares primarios de cerebro de rata (Figura 14). Los resultados muestran que los astrocitos se encontraron viables mientras que las neuronas granulares sufrían muerte celular al tratar los cultivos primarios con LCR de pacientes con EM (paneles B, C y D de la Figura 14). Los resultados indican que algunos de los factores presentes en el LCR de pacientes con EM pueden causar la muerte celular por apoptosis específica en las neuronas granulares del cerebelo pero no en astrocitos.

Dado que no se puede excluir que las células no neuronales pueden causar interferencias en los estudios del daño neuronal primario, se debe obtener cultivos puros de CGCs. Para este propósito, fue necesario añadir arabinósido de citosina para inhibir la replicación y crecimiento de las células no neuronales. Para estimar la pureza de las CGCs en los cultivos primarios aislados de cerebelo tras el tratamiento, las células se tiñeron con colorantes Texas-Red y FITC, los núcleos se tiñeron con DAPI, y se visualizaron por microscopía confocal. La figura 15 muestra la característica tinción de los neurofilamentos de las neuronas, lo que indica que el cultivo contiene una población pura de CGCs, y que se encuentra desprovisto de cualquier célula no neuronal.

4.3. Identificación de genes constitutivos estables para normalización de datos de micromatrices de DNA

Uno de los principales problemas en cualquier análisis de micromatrices de DNA, con el fin de obtener datos de expresión génica precisos (objetivo del

siguiente apartado), es la normalización correcta de los cambios obtenidos de las transcripciones de mRNA diana utilizando en la mayoría de los casos genes constitutivos invariables como controles.

Sin embargo, datos anteriores de nuestro laboratorio en donde se analizaba la variación del gen del canal de sodio de expresión en células neuronales mostraban que los genes *ActB* y *Gadph* usados habitualmente para normalizar los datos se alteraban en los tratamientos usados (2010, Tesis Doctoral del Dr. Beltrán, Universitat de Valencia). En base a estos datos el apartado siguiente de esta tesis fue el encontrar genes constitutivos adecuados para normalizar los datos de micromatrices de expresión.

Los datos iniciales con los genes *ActB* y *Gadph*, mediante la realización de PCR convencional en muestras neuronales tratadas con LCR de pacientes con EM (de IgM+/+, IgM+/- (MS inflamatoria), EMMed, EMPP, NMO y NIND), mostraban diferencias significativas en su valores relativos de expresión génica (Figura 18). Se puede concluir que tanto *ActB* como *Gapdh* no son adecuados para normalizar la transcripción de genes de interés en nuestras condiciones experimentales.

Para encontrar genes adecuados para la normalización, se realizaron qPCR de un conjunto de siete genes constitutivos de uso general (HKGs). Estos genes fueron elegidos de la literatura como genes con expresión invariable observadas en condiciones experimentales distintas. Los HKGs candidatos seleccionados fueron ba-actina (*ActB*), hipoxantina guanina fosforribosil transferasa (*Hprt*), proteína ribosomal L19 (*Rpl19*), lactato deshidrogenasa A (*LdhA*), receptor de transferrina (*Tfrc*), microglobulina beta-2 (B*2m*), y gliceraldehído 3-fosfato deshidrogenasa (*Gapdh*).

Por otra parte, los datos de qPCR y de las micromatrices de DNA fueron evaluados con los softwares *GeNorm*y *NormFinder* de acuerdo a su medida promedio de estabilidad de la expresión.

Los resultados de la Figura 20 ilustra los resultados de los HKGs genes candidatos en las neuronas granulares del cerebelo tratados con CSF de pacientes de MS / NMO en función de su expresión génica. Los datos de este apartado (Figuras 19 y Tabla 10) nos permiten concluir que *Hprt* y *Gadph* son los genes más estables transcripcionalmente seguidos por *Tfrc* durante la maduración de las neuronas granulares de cerebelo. Por lo tanto, estos genes se pueden utilizar para normalizar los valores de expresión génica. Por el contrario, *ActB* mostró la mayor fluctuación, por lo tanto, no es adecuado para el propósito de la normalización de la expresión durante el desarrollo neuronal.

En condiciones experimentales cuando las neuronas granulares de cerebelo fueron expuestos a los LCR de los diferentes subtipos de EM (Figura 20 y Tabla 11) se observó que *Tfcr* y *B2m* eran expresados de forma estable constitutivamente, seguido de *Rpl19* mientras *Gapdh* y bActB mostraron la más alta fluctuación.

En los experimentos en lo que las OPCs fueron tratados con LCR de pacientes con EM y NMO (Figura 24 y Tabla 12), se encontró que los genes *Mrpl19* y *Hprt* mostraron la expresión más estable seguida de *B2m*, mientras que *ActB* y *Gadph* fueron los genes menos estables en su expresión en las condiciones experimentales usadas.

4.4. Perfiles de expresión génica global de CGCs y OLPs tratadas con LCR de pacientes con EM y NMO.

Los datos disponibles en la literatura revelan que hay un posible papel de la alteración del metabolismo energético alterado en la patogénesis de la EM [véase

la figura 44]. Los estudios iniciales aportados por Jones en los años 50 describen la posibilidad de alteraciones del metabolismo del piruvato en la EM [Jones et al., 1.950].

Un informe del grupo Regenold (2008) describe un posible papel del metabolismo energético del SNC en la progresión de la EM. Estos investigadores midieron los niveles de lactato, sorbitol y fructosa, y todos los metabolitos del metabolismo mitocondrial extra-glucososídico, en el LCR de EMRR y pacientes EMSP. Los datos demostraron niveles elevados de los tres metabolitos en el LCR de pacientes EMSP y en menor grado en pacientes con EMRR.

En el siguiente grupo de experimentos, se estudiaron los perfiles de expresión génica en neuronas granulares del cerebelo (Figuras 21-23) y en células precursoras de oligodendrocitos (Figuras 25-27) tratadas con el LCR de pacientes con EMRR IgM+/-, EMRR IgM +/+, EM medular, EMPP y NMO utilizando la tecnología de micromatrices de expresión. Nuestros estudios se centraron en la red del metabolismo de carbohidratos, que incluye la vía glicolítica y el ciclo del ácido tricarboxílico, y la cadena de transporte de electrones y fosforilación oxidativa como rutas de obtención de energía que podrían estar incidiendo en el curso de la EM y NMO (Figura 34).

Para dar una imagen más global acerca de los genes alterados en estas rutas metabólicas en los diferentes cursos clínicos de la EM y NMO, hemos incorporamos toda la información en un diseño de vía metabólica general desarrollado en este trabajo (Figura 35). Esta integración también nos permitió interrelacionar mejor los cambios en las neuronas y los OPC, con el objetivo de encontrar correlaciones en todo el proceso de degeneración axonal-reconstrucción en los diferentes tipos de EM y NMO.

Para poder "integrar" nuestros datos en las rutas del metabolismo, hemos definido un parámetro denominado "ÍNDICE DEL FLUJO METABÓLICO ACUMULADO o CFI" dentro de la red para comparar las diversas condiciones experimentales de EM y NMO. Los valores de CFI quieren significar que la reducción en el flujo local debido a la inhibición de una o varias actividades enzimáticas afectan sinérgicamente sobre el flujo metabólico de toda la red. Por tanto, a medida que más genes están regulados negativamente, disminuyendo su actividad enzimática, el flujo total están siendo reduciendo como un factor multiplicativo que integramos como el valor CFI de esa ruta metabólica. En la sección de Discusión integramos los cambios relativos del metabolismo energético en los diferentes pacientes con EM y NMO calculando el CFI de las rutas metabólicas en esos pacientes.

4.4.1. EMRR IgM +/-

Nuestros resultados revelaron que los genes glicolíticos incluyendo *Gapdh*, *Pgam* y *Eno* mostraron una menor expresión en las neuronas expuestas al LCR de la CSF derivado de IgM +/- forma clínica de la EM (Figura 21). Los genes que participan en el ciclo TCA incluyendo *Pdh* y *Mdh2* and gene que participan en la fosforilación oxidativa incluyendo *ATP5b* también fueron inhibidos en estas neuronas (Figura 22 y Figura 23). En los experimentos en los que las OPCs se expusieron al LCR de pacientes con EMRR tipo IgM +/- se observó la disminución en la expresión de muchos genes glicolíticos incluyendo *Hk, Gpi, Gadph, Tpi, Pgk1, Eno1, Eno2, Pk,* y *Pkm2* (Figura 25). Por otra parte, se reduce la expresión de los genes implicados en el ciclo TCA incluyendo *Pdha1, Aco2, Idh,* and *Ogdh*. (Figura 26). Del mismo modo los genes implicados en la fosforilación oxidativa, a saber *Atp5a1, ATP5b, MT-ND2, Cox,* y *Cyc1*, mostraron asimismo disminución de la expresión en comparación con los controles neurológicos (Figura 27).

Los resultados obtenidos cuando las neuronas se tratan con LCR de pacientes EMRR IgM+/- (Figuras 34 y 35) mostraron que el flujo glicolítico disminuye significativamente en las neuronas (CFI 0,0296), así como en los OPC (CFI 0,0192). El flujo relacionado con el ciclo del TCA también se reduce en las neuronas (CFI 0,0960) y en OPCs (CFI 0,0602). En la fosforilación oxidativa, el flujo se reduce asimismo en las neuronas (CFI 0,3300) y en los OPC (CFI 0,0119). En general podemos señalar que el flujo metabólico de carbohidratos y de la síntesis de ATP se redujeron sensiblemente tanto en las neuronas (CFI 0,00094), como en las OPCs (CFI 0,000014) cuando son expuestas al LCR de pacientes con EM tipo EMRR IgM +/-.

Las células neuronales sometidas a daños en los tejidos requieren más energía en forma de ATP para sobrevivir, pero en estos pacientes los genes metabólicos están inhibidos lo que lleva a una reducción en el flujo metabólico general. Esto provoca la disminución en la producción de ATP y en general la capacidad de estas células para reparar el daño que las células sanas, lo que puede estar relacionados con el desarrollo de la patología en estos pacientes.

4.4.2. EMRR IgM +/+

Los resultados obtenidos cuando las neuronas se expusieron al LCR de pacientes con EMRR IgM +/+ demostraron que los genes que catalizan pasos cruciales de la vía metabólica de la glucosa y la generación de ATP, tales como *Hk, Gadph, Pgk, Pgam, Eno, Pkm2, Pdh, Mdh2, y ATP sintasa* tenían una expresión reducida en comparación con el control (Figura 21-23).

En el tratamiento de las OPCs al LCR de estos pacientes se encontró que casi todas las enzimas implicadas en la glicolisis (*Hk1, Gpi, Tpi, Gadph, Pgk1, Pgam5, Eno* y *Pk*), las relacionadas con el ciclo de Krebs (*Pdh, Aco2, Idh, Ogdh, Sdh, y Mdh2*) así como las de la cadena electrónica mitocondrial y fosforilación oxidativa

(complejos CI, Cyc1, CIV/Cox, ATPasaA y ATPasaB) se encuentran fuertemente reducidas en su expresión génica.

Los resultados globales muestran que cuando las neuronas se tratan con LCR de pacientes EMRR IgM +/+ pacientes el flujo glucolítico disminuye fuertemente (CFI 0,0007) así como en los OPCs (CFI 0,0047). El flujo global relacionado con el ciclo del TCA también se reduce en las neuronas (CFI 0,0690) y con fuerza en OPCs (CFI 0,0024). En la fosforilación oxidativa, el flujo se reduce tanto en las neuronas (CFI 0,3000) como en los OPC (CFI 0,0173). En general, el flujo metabólico de las rutas de metabolismo de los carbohidratos y de la síntesis de ATP se redujeron profundamente tanto en las neuronas (CFI 1.4201E-05), como en los OPCs (CFI 1.9596E-07) cuando se exponen a los LCR de pacientes EMRR IgM +/+.

Si comparamos los resultados obtenidos con los subtipos de EMRR IgM+/- y IgM+/+, se puede indicar que los genes implicados en el metabolismo de los hidratos de carbono y la síntesis de ATP están más afectados en el segundo tipo que el primero tanto en neuronas como en OPC. La EMRR IgM +/- es una forma clínica inflamatoria más benigna y menos agresiva de MS, con abundante IgG pero sin anticuerpos IgM en el LCR. Por lo tanto la energía en forma de ATP se requiere para reparar las neuronas dañadas y degenerado en lesiones de la EM puede ser mucho más baja que la forma agresiva EMRR IgM +/+.

Para combatir el estrés oxidativo generado en las enfermedades neurológicas, las células necesitan para producir grandes cantidades de equivalentes reductores para la producción de energía. Sin embargo, si no hay suficiente producción de energía, como ocurre en la forma agresiva se perjudica severamente la capacidad de reparación del daño neuronal. Del mismo modo ocurre en las células OPCs, en donde se produce menor nivel de energía lo cual puede bloquear el proceso de reparación por las OPCs. Llegamos a la conclusión de que la expresión diferencial de genes metabólicos influye en el perfil de la bioenergética de las neuronas y,

bloquear asimismo los procesos de reparación por los OPC lo que podría estar relacionado con el mal pronóstico en los pacientes con EM del tipo EMRR IgM +/+ en comparación con el tipo EMRR IgM +/-.

Otro resultado importante que podemos destacar es que en las OPCs tratadas con LCR se observó una diferencia significativa en la expresión génica de *Eno2, Gpi, Tpi, PDHA1, Pgk1, Pkm2, AldoC,* y *Sdhaf2* entre los pacientes de EMRR IgM+/- e IgM +/+. Del mismo modo, no se encontraron diferencias en la expresión de los genes *Pgam1* y *Cox6b2* entre los OPC expuestas al LCR de IgM +/- y las OPC expuestas a LCR de pacientes medulares (ver más abajo). Estos genes podrían servir como potenciales biomarcadores para distinguir entre las diferentes clases inflamatorias de los pacientes con EM (las EMRR IgM +/- *vs* EMRR IgM +/+ y las EMRR IgM +/- *vs* EMMed).

4.4.3. EMMed

Los resultados que se obtienen en los tratamientos las neuronas expuestas a la LCR de la forma clínica medular de EM (EMMed) (Figuras 21-23) muestran que los genes glicolíticos, incluyendo *Hk1, Gapdh, Eno* y *Pkm2,* disminuyen su expresión génica en comparación con los controles no neurológicos. Por otra parte, los genes implicados en el ciclo de Krebs, incluyendo *Pdh* y *Mdh2,* y los genes de la *ATP sintasa alfa* y *beta* muestran asimismo una importante disminución en su expresión génica. Los valores de los índices de flujo acumulativo fueron de 0,0075, de 0,0810 en las enzimas del ciclo del Krebs, y de 0,1050 en las enzimas de la cadena de transporte y de fosforilación oxidativa al exponer a las neuronas al LCR de pacientes medulares (Figura 38). En total, el índice de flujo total acumulada del metabolismo de la glucosa y de la síntesis de ATP se encontró que era tan bajo como 6.36727E-05 en estas neuronas.

En los experimentos con OPCs tratados con el LCR de estos pacientes se encontró que muchos de los genes de la ruta glicolítica, como *Hk1, Gpi, Gapdh, Tpi, Pgk1, Eno y Pkm2* (Figura 25), del ciclo de Krebs, como *Pdh, Aco2, Idh, Ogdh* y *Sdh* (Figura 26), y de los complejos de la cadena de transporte electrónica y la fosforilación oxidativa, como *CI, CycC1, CIV/Cox, ATPasa A* y *ATPasa B* (Figura 27), se encuentran profundamente reprimidos.

Nuestros resultados revelaron una importante disminución de los índices de flujo metabólico acumulado en la ruta glicolítica (CFI 0,0019), del ciclo de Krebs (CFI 0,0124) y de la cadena electrónica y de la fosforilación oxidativa (CFI 0,0328) en las OPC tratadas con el LCR de estos pacientes (Figura 39). En comparación con los pacientes no-MS (controles), el índice total del flujo acumulado se redujo en el conjunto de las tres rutas metabólicas hasta un valor muy bajo (CFI 7.94269E-07), similar al que ocurre en la EMRR IgM+/+.

La forma medular de la EM es un subtipo de EM con neuronas dañadas que se encuentran sobre todo en la región de la médula y que constituye uno de los subtipo más agresivo de MS, comparable al más agresivo del SNC de EMRR IgM +/+, con el que muestra un patrón de expresión génica similar. La drástica reducción en ambos tipos de EM de la expresión de los genes metabólicos daría lugar a un fracaso bioenergético lo que hace que los oligodendrocitos no sean capaces de reparar el importante daño axonal en las neuronas con depleción energética expuestas al LCR de estos tipos agresivos y de peor pronóstico de EM en contraste con el tipo EMRR IgM +/-.

4.4.4. EMPP

En los experimentos de tratamiento de las neuronas con el LCR de pacientes con EM primaria progresiva (EMPP) se encontró tan solo reducción en la expresión del gen *Pgam* en la ruta glicolítica (Figura 21). Entre los genes que participan en el

ciclo TCA los genes *Pdh* y *Mdh2* mostraron reducción en su expresión (Figura 22), mientras que las subunidades alfa y beta de la ATP sintasa implicadas en la producción de ATP mostraron asimismo disminución de su expresión (Figura 23).

Por el contrario, varios de los genes de la ruta glicolítica se ven afectados a la baja en las OPCs tratadas con LCR de esto pacientes, incluidos *Hk1, Gpi, Gapdh, Eno2* y *Pk* (Figura 25). Algunos de estos genes pueden servir como biomarcadores en pacientes con EMPP. Los genes que participan en el ciclo TCA, incluyendo Aco2, también disminuyen su expresión en comparación con los controles (Figura 26). Del mismo modo, los complejos *CI, CIV/Cox* y *ATP sintasa (subunidad alfa)* muestran asimismo reducción en su expresión (Figura 27).

Los resultados obtenidos con el tratamiento con LCR de pacientes con EMPP indicaron que el flujo glucolítico disminuyó ligeramente en las neuronas (CFI 0,3000) (Figura 40) y más fuertemente en los OPC (CFI 0,0033) (Figura 41). El flujo relacionado con el ciclo TCA se disminuye en ambos neuronas (CFI 0,0870) (Figura 40) y los OPC (CFI 0,5400) (Figura 41), así como la disminución de la fosforilación oxidativa en las neuronas (CFI 0,1050) (Figura 40) y en los OPC (CFI 0,0728) (Figura 41).

En general, podemos decir que el flujo metabólico en las rutas de hidratos de carbono y de producción de ATP disminuye ligeramente en las neuronas (CFI 0,00274) y más fuerte en los OPC (CFI 0,00013) en pacientes con EMPP en comparación con los controles. Estos resultados serían compatibles con el hecho de que estos pacientes tengan un subtipo de EM menos agresiva, de curso más lento y progresivo y de mejor pronóstico que los subtipos EMRR IgM+/+ o EMMed.

4.4.5. NMO

La neuromielitis óptica (NMO) es otra enfermedad del SNC de tipo inflamatoria, desmielinizante y autoinmune pero totalmente distinta de la EM. La NMO tiene un patrón de expresión génica completamente diferente en las neuronas expuestas a LCR de estos pacientes que la de los tipos de EM estudiados más arriba. Se encontró que la expresión de los genes que catalizan reacciones en vía glicolítica, incluyendo *Hk1, Pgam, y Eno,* estan sobreexpresados en las neuronas expuestas a la LCR de pacientes NMO en comparación con las neuronas tratadas con LCR de controles neurológicos. Sólo los genes *Gadph* y *Mdh2* mostraron una ligera disminución de su expresión (Figura 21). Además, los genes de cadena del ciclo TCA y transporte de electrones incluyendo *Pdh* (Figura 22) y las subunidades alfa y beta de la *ATP sintasa* mostraron una mayor expresión en las neuronas tratadas (Figura 23). Por el contrario el gen *Mdh2* del ciclo del TCA se expresó en cantidades considerablemente más bajos (Figura 22).

En general, se observó una aumento de los valores de CFI en dos rutas metabólicas, la vía glicolítica (CFI 1,9656) y la cadena de transporte y fosforilación oxidativa (CFI 1,5867), y una ligera disminución de las enzimas del ciclo de Krebs (CFI 0,2174) (Figura 42). Esto hace que el índice de flujo acumulativo en el conjunto de las tres rutas metabólicas se encuentre prácticamente inalterado (CFI 0,67787).

A diferencia de la EM la NMO no es una enfermedad progresiva. De hecho menos de 2% de los pacientes NMO tiene una progresión hacia la discapacidad con recaídas. Así que esta diferencia clínica puede ser la causa de que el patrón génico que conduce a la síntesis de ATP celular este poco alterado en pacientes con NMO.

Por otro lado, en OPCs expuestas al LCR de pacientes con NMO, la expresión de genes que catalizan reacciones en vía glucolítica, incluyendo *Gpi, Aldo, Gadph,*

Tpi, Pgk, Eno y *Pk,* muestran reducción en la expresión de los genes en comparación con los controles (Figura 25). Por otra parte, los genes del ciclo TCA, incluyendo *Pdh, Sdh, Aco, Ogdh* y *Mdh2,* mostraron disminución de la expresión en las OPCs tratadas (Figura 26). Del mismo modo, los genes de la cadena de transporte y la fosforilación oxidativa, como *CI, CycC1, CIV/Cox* y las *subnidades alfa y beta* de al *ATP sintasa* muestran también reducción en su expresión génica (Figura 27).

Nuestros resultados revelaron una disminución en el índice de flujo acumulado en la glicolisis (CFI 0,0013), ciclo de Krebs (CFI 0,0055), y la fosforilación oxidativa (CFI 0,0149) en las OPCs tratadas con LCR de paciente NMO en comparación con los no-MS (controles) (Figura 43).

Los resultados señalan que de los índices de flujo acumulado en las neuronas expuestas al LCR de pacientes NMO sufren una muy ligera disminución (CFI 0,67787), mientras que en las OPC disminuyó sensiblemente (CFI 1.10046E-07).

Las conclusiones de esta tesis demuestran un metabolismo defectuoso de la glucosa y de la síntesis de ATP en las neuronas granulares de cerebelo cultivadas y oligodendrocitos tratados con el LCR derivado de los sujetos con EM y NMO. Dado que el LCR está en contacto con el parénquima cerebral y los ventrículos, cualquier producto en el fluido puede influir en la fisiología celular de las neuronas, las células progenitoras de oligodendrocitos y los oligodendrocitos mielinizantes. Los datos aportados en este último capítulo nos indican que los potenciales factores presentes en el LCR pueden estar perturbando el metabolismo energético de las neuronas y las OPCs. El estudio también diferencia la NMO de la EM, lo que a veces es difícil distinguir por los clínicos, por poseer patrones de expresión génica diferencial, y claramente diferencia las formas más benignas de las formas más agresivas en la EM. Sin embargo, conocer con precisión si estas alteraciones en la

expresión génica es la causa metabólica de la EM y la NMO o son simplemente una mera consecuencia de la enfermedad sigue siendo difícil de alcanzar.

5. CONCLUSIONES Y PERSPECTIVAS FUTURAS

A pesar de la amplia investigación que se ha llevado a cabo durante la última década, la causa subyacente de la EM sigue siendo difícil de conocer. El metabolismo perturbado de la glucosa está implicado en diversas enfermedades neurodegenerativas como el Alzheimer, el Parkinson y el Huntington. Sin embargo, se sabe poco sobre su papel en la patología de la EM. Los resultados que se resumen más adelante muestran que los potenciales factores presentes en el LCR, podrían estar afectando el metabolismo de las neuronas y oligodendrocitos y, en función de su presencia o no en los diversos subtipos de EM y NMO, diferenciar el curso de las formas más benignas de las formas más agresivas en la EM. El estudio también diferencia la NMO de la EM en base a su diferente patrón de expresión génica, que a veces es difícil distinguir por los clínicos.

Las conclusiones que se derivan de esta tesis se pueden resumir en las siguientes:

1. Se clasificaron los pacientes en base a la clínica, la existencia de bandas oligoclonales IgG e IgM en el LCR, de anticuerpos anti-NMO en suero y la imagen MRI en grupos EMRR, EMPP, MedMS y NMO. Se encontraron diferencias significativas entre la edad al inicio de la EMPP y las otras dos formas de EM ($p<0.003$), entre la EDSS de la EMRR y las otras dos formas de EM ($p <0.001$), y en el tiempo de evolución entre la EMPP y EMRR ($p<0.043$).

2. Los estudios de viabilidad celular muestran que las neuronas granulares, no así los astrocitos, sufren muerte celular al tratar los cultivos primarios de cerebro de rata con LCR de pacientes con EM. La obtención de cultivos puros de CGCs

requiere el tratamiento de los cultivos con arabinósido de citosina para inhibir la replicación y crecimiento de las células no neuronales.

3. En condiciones experimentales cuando las neuronas granulares de cerebelo fueron expuestas a los LCR de los diferentes subtipos de EM se observó que los genes Tfcr y B2m eran expresados de forma estable constitutivamente, seguido de Rpl19, mientras Gapdh y bActB mostraron la más alta fluctuación. En los experimentos con las OPCs se encontró que los genes Mrpl19 y Hprt mostraron la expresión más estable seguida de B2m, mientras que ActB y Gadph fueron los genes menos estables en su expresión.

4. En los experimentos con micromatrices de expresión se determinaron los cambios de expresión de las enzimas implicadas en el metabolismo de carbohidratos [Glicolisis (GLI), Ciclo de Krebs (TCA) y cadena de transporte electrónica mitocondrial y fosforilación oxidativa (CTE/PO)] cuando CGCs y OPCs son tratadas con LCR de pacientes con EM y NMO. Los datos indican que las formas más agresivas y de peor pronóstico de EM, EMRR IgM+/+ y EMMed poseen los índices de flujo metabólico acumulado (CFI) más bajos en estas rutas metabólicas, con valores de CFI globales en EMRR IgM+/+ de 1.4201E-05 en CGCs y de 1.9596E-07 en OPCs; y en EMMed de 6.36727E-05 en CGCs y de 7.94269E-07 en OPCs.

5. Por el contrario, en la forma más benigna de EMRR IgM+/- , los valores de CFI globales son sensiblemente superiores, de 0,00094 en CGCs y de 0,000014 en OPCs, lo que puede reflejar la disponibilidad de mejores recursos energéticos en estas células para reparar el daño neuronal en el curso de la enfermedad.

6. En la forma intermedia de agresividad y pronóstico y de curso más lento y progresivo, la EMPP, los valores de CFI globales son asimismo más elevados, de 0,00274 en CGCs y de 0,00013 en OPCs, y posiblemente las enzimas

directamente afectadas directamente pueden influir en su pronóstico y agresividad.

7. La NMO como un tipo de enfermedad inflamatoria, desmielinizante y autoinmune del SNC distinta de la EM tiene un patrón de expresión génica completamente diferente. Se observó un aumento de los CFI en la ruta GLI (CFI 1,9656) y en la CTE/PO (CFI 1,5867), y una ligera disminución en la TCA (CFI 0,2174), lo que da un CFI global prácticamente inalterado (CFI 0,67787), en neuronas. Por el contrario, en las OPC disminuyó sensiblemente (CFI 1.10046E-07). Estas diferencias pueden ayudar diferenciar los tipos de EM de la NMO.

8. Aunque no todas las enzimas de una ruta metabólica están afectados por cambios en la expresión, el análisis STRING señala que la formación de complejos multiproteicos por interacción proteína-proteína puede coordinar la respuesta global conjunta de la totalidad de la ruta metabólica.

9. Se han podido encontrar algunos cambios en la expresión diferencial de genes concretos en los distintos tipos de EM y NMO que usarse como biomarcadores que podrían ayudar a diferenciar clínicamente los diversos subtipos de EM y NMO.

Estas observaciones abren nuevas perspectivas para la comprensión de la dinámica del metabolismo en la EM con todavía muchos aspectos desconcertantes y preguntas críticas que deben ser abordados. Por ejemplo: ¿Cómo el defecto en una ruta metabólica contribuye a la desmielinización? ¿Las vías metabólicas en otros tipos de células se encuentran asimismo alteradas en la EM? ¿Los cambios en un tipo de célula, pueden afectar directamente a través de algún factor desconocido, o indirectamente por cambios globales en los niveles de los intermedios metabólicos en el LCR a otros tipos de células? ¿Esta perturbación en

las rutas metabólica es causa o consecuencia de la EM? ¿Pueden esos genes ser usados como una diana farmacológica para aliviar la patología MS?.

Es evidente que se requiere mucha más investigación para desentrañar plenamente el mecanismo de la enfermedad si bien una comprensión adecuada de la enfermedad y de sus mecanismos podría conducir a nuevos tratamientos de esta grave e incapacitante patología.

VIII- REFERENCES

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Perturbed glucose metabolism: insights into multiple sclerosis pathogenesis

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Multiple sclerosis (MS) is a complex debilitating disease of the central nervous system (CNS) perceived to result from the autoimmune effect of T cells in damaging myelin sheath. However, the exact pathogenesis of the disease remains elusive. Initial studies describing the possibility of defective pyruvate metabolism in MS were performed in 1950s. The group observed elevated blood pyruvate level in both fasting and postprandial times in MS patients with relapse. Similarly, other investigators also reported increased fasting pyruvate level in this disease. These reports hint to a possible abnormality of pyruvate metabolism in MS patients. In addition, increase in levels of Krebs cycle acids like alpha-ketoglutarate in fasting and citrate after glucose intake in MS patients further strengthened the connection of disturbed pyruvate metabolism with MS progression. These studies led the investigators to explore the role of disturbed glucose metabolism in pathophysiological brain function. Under normal circumstances, complex molecules are metabolized into simpler molecules through their respective pathways. Differential expression of genes encoding enzymes of the glucose metabolic pathway in CNS may result in neurological deficits. In this review article, we discuss the studies related to disturbed carbohydrate metabolism in MS and other neurodegenerative diseases. These observations open new perspectives for the understanding of metabolic dynamics in MS yet many puzzling aspects and critical questions need to be addressed. Much more research is required to fully unravel the disease mechanism, and a proper understanding of the disease could eventually lead to new treatments.

Keywords: brain glucose metabolism, cell-specific mechanisms, mitochondrial defects, multiple sclerosis, neurodegenerative diseases

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of probably immune origin affecting more than two million people worldwide. Demyelinated plaque, inflammatory infiltrates, accumulation of antibodies, and complement proteins are the pathological hallmarks of MS (1). It is believed that immune cells particularly T cells penetrate the brain and mistakenly recognize myelin tissue as foreign and damage it. The inflammatory cells including microglia, macrophages; antibodies, cytokines, complement system, and others enhance the damaging effect. The process of demyelination is associated with axonal degeneration that underlies neurological deficits in MS (2). The lesions formed as a consequence of neuroaxonal injury are found in both white and gray matter of the central nervous system (CNS) (3). Although research underscores the role of immunological, genetic, and environmental pathologic influences, the exact mechanisms underlying MS pathology are yet uncertain.

The disease course often begins with clinically isolated syndrome involving optic nerve, brain stem or spinal cord. The 70–80% of these patients experience relapsing-remitting events, which at later stage are transformed into a secondary progressive stage that causes irreversible neurologic worsening. This suggests that the pathophysiology of progression is not solely inflammatory in nature (4). To prevent disease progression investigators have explored a possible role of energy metabolism in the CNS. Metabolic disturbances have been implicated in neurodegenerative disorders including Alzheimer's, Huntington's, and Parkinson's diseases. In demyelinating diseases particularly MS, investigations on the contribution of disturbed glucose metabolism in MS pathology are limited. However, the existing literature hints to a connection between disturbed glucose metabolism and MS pathogenesis. In this context, this article will first focus on some basic aspects of brain's energy balance, its regulation at cellular level, and its role in normal and diseased conditions with a special focus on MS. Before we discuss the cell-specific mechanisms underlying brain energy metabolism, it seems pertinent to briefly review the metabolic pathways, which include glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway (PPP). These metabolic pathways are similar in brain and other tissues.

Abbreviations: CNS, central nervous system; ETC, electron transport chain; FAD, flavin adenine dinucleotide; MS, multiple sclerosis; NAD^+, nicotinamide adenine dinucleotide; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; TCA cycle, tricarboxylic acid cycle.

GLYCOLYSIS

Glycolysis (Embden–Meyerhof pathway) is the metabolism of glucose to pyruvate. Four ATP molecules are produced in the processing of glucose to pyruvate, where two ATP molecules are consumed in the pathway resulting in a net production of two ATP molecules per glucose molecule. In the absence of oxygen (anaerobic conditions), pyruvate is converted into lactate, allowing the regeneration of nicotinamide adenine dinucleotide (NAD^+), which is necessary to maintain a continued glycolytic flux. Without the regeneration of NAD^+ the pathway could not have continued beyond glyceraldehyde 3-phosphate.

TRICARBOXYLIC ACID CYCLE

Under aerobic conditions, pyruvate undergoes oxidative decarboxylation to yield acetyl-CoA in a reaction catalyzed by pyruvate dehydrogenase. Acetyl-CoA in the presence of citrate synthase condenses with oxaloacetate and forms citrate. This is the first step of the TCA cycle in which three molecules of NADH are formed from NAD + and one molecule of FADH2 is formed from flavin adenine dinucleotide (FAD) through four oxidation–reduction steps. The reducing equivalents NADH and FADH2 transfer their electrons to molecular oxygen via mitochondrial electron transport chain (ETC). There are five enzyme complexes, denoted as I–V that forms the ETC in mitochondria. Electron transfer from complexes I and II to complex III and from complex III to complex IV is accomplished by co-enzymes ubiquinone and cytochrome c. During this process, protons are transported across the inner mitochondrial membrane to the intermembrane space to generate an electrochemical gradient. The enzyme ATP synthase utilizes this energy and produces ATP.

PENTOSE PHOSPHATE PATHWAY

Overall, glucose is completely oxidized to carbon dioxide and water via three synchronized pathways namely glycolysis, the TCA cycle, and the ETC, and finally produces energy in the form of ATP. However, there are conditions when extra metabolic energy is required in the form of reducing power in addition to ATP. These are the situations when precursors are in a more oxidized state than the products. In PPP, glucose 6-phosphate is converted into ribulose 5-phosphate utilizing two molecules of NAD+. Thus, when ample amount of energy is required, the level of NADPH falls down and the pathway is activated to generate more reducing equivalents.

GLUCOSE SERVING AS THE MAIN FUEL IN BRAIN

The human brain represents only 2% of the body weight, yet 25% of total body glucose is utilized by it. Energy requirement is highest in neurons of adult brain (5). Therefore, a continuous supply of glucose is needed from bloodstream into the brain. With a few exceptions, glucose is the main source of energy in mammalian brain (6). Nevertheless, there are certain circumstances when brain uses ketone bodies as energy source including starvation, during strenuous exercise and development (7). Metabolism of glucose yields energy in the form of ATP that is used for neuronal and non-neuronal cell survival and generation of neurotransmitters. Therefore, tight regulation of glucose metabolism is necessary for normal brain physiology and perturbation in any step of its regulation pathway may form the pathophysiological nexus for many brain disorders.

CELL-SPECIFIC MECHANISMS UNDERPINNING BRAIN ENERGY METABOLISM

GLIA AND VASCULAR ENDOTHELIAL CELLS – ROLE IN BRAIN ENERGY METABOLISM

Neurons are usually regarded the most important cells of the CNS taking part in energy metabolism. However, other cells like glial and vascular endothelial cells also play a critical role in the distribution of energy substrates to neurons. Glial cells constitute nearly half of the brain volume. Out of many different cell types in the brain, neurons represent only a small proportion for glucose utilization. It has been seen that the presence of specialized end-feet processes makes astrocytes the first cellular barrier that glucose entering the brain parenchyma come across and makes them a probable site of glucose uptake and energy substrate distribution. Besides possessing end-feet processes, astrocytes contain processes that ensheathe synaptic contacts. The receptors and uptake sites present on astrocytes allow neurotransmitters to communicate with them. These structural and functional characteristics exhibited by astrocytes makes them perfectly suitable to couple local changes in neuronal activity with coordinated adaptations in energy metabolism.

GLUCOSE METABOLISM IS TIGHTLY REGULATED IN ALL CELL TYPES OF THE BRAIN – NEURONAL AND NON-NEURONAL

Due to enormous degree of cellular heterogeneity in brain, it is quite cumbersome to understand the relative role of each cell type in energy substrate flux. However, usage of primary cultures *in vitro* enriched in neurons, astrocytes or vascular endothelial cells have proved very beneficial in localizing the cellular sites for glucose uptake and its consequent metabolic fate particularly as regards glycolysis and oxidative phosphorylation. As it is well understood that *in situ* cellular preparation does not exhibit all the properties as observed in whole brain tissues many other aspects of brain energy metabolism can be studied using cultures *in vitro*.

Under basal conditions, glucose uptake and its utilization occur in all cell types of the brain with a high specificity. This is due to the presence of unique glucose transporters (GLUTs) on different cell types. Due to low lipid solubility and lack of specific transport carriers in the luminal membrane of the capillary endothelial cell entry of neuroactive compounds such as glutamate, aspartate, and glycine into the blood–brain barrier is limited. Glucose being an obligatory fuel enters through facilitated transport mechanism mediated by specific transporters. Six genes and one pseudogene encoding glucose transporter proteins have been identified so far. These are designated as GLUT1 to GLUT7 (8). In brain, GLUT1, 3, and 5 are preponderantly expressed in cell-specific manner (9). Based on the degree of glycosylation, GLUT1 is expressed in two forms in the brain with molecular weight 55 and 45 kDa (10, 11). The 55 kDa form of GLUT1 is expressed in choroid plexus, ependymal cells, and vascular endothelial cells. The other form with 45 kDa molecular mass is localized on astrocytes (12). Neurons possess GLUT3 transporter on their membrane (13) whereas microglial cells, the resident macrophages of the brain, are found to have GLUT5 form of the transporter (9). So, it is apparent that glucose enters the brain via 55 kDa GLUT1 receptor present on endothelial cells. The uptake by astrocytes is mediated by 45 kDa

form of GLUT1 while GLUT3 receptors mediate this process in neurons. Ultimately, microglial cells uptake glucose from the surrounding medium through GLUT5 receptor.

GLUCOSE METABOLISM AND MULTIPLE SCLEROSIS

Studies show that there is a possible role of impaired energy metabolism in the CNS of MS patients (**Figure 1**). Initial studies describing the possibility of defective pyruvate metabolism in MS were performed by Jones et al. (14). The group observed elevated blood pyruvate level in both fasting and postprandial times in MS patients with relapse. Similarly, other investigators also reported increased fasting pyruvate level in this disease (15). However, there were conflicting reports demonstrating normal fasting lactate level, or increase in only a small number of patients (16–18). On the other hand, Jeanes and Cumings (16) found an abnormal rise in the blood pyruvate level after glucose intake. These reports hint to a possible abnormality of pyruvate metabolism in MS patients. In addition, increase in levels of Krebs cycle acids like alpha-ketoglutarate in fasting and citrate after glucose intake in MS patients further strengthened the connection of disturbed pyruvate metabolism with MS progression (18). McArdle et al. (19) found elevated levels of pyruvate and α-ketoglutarate

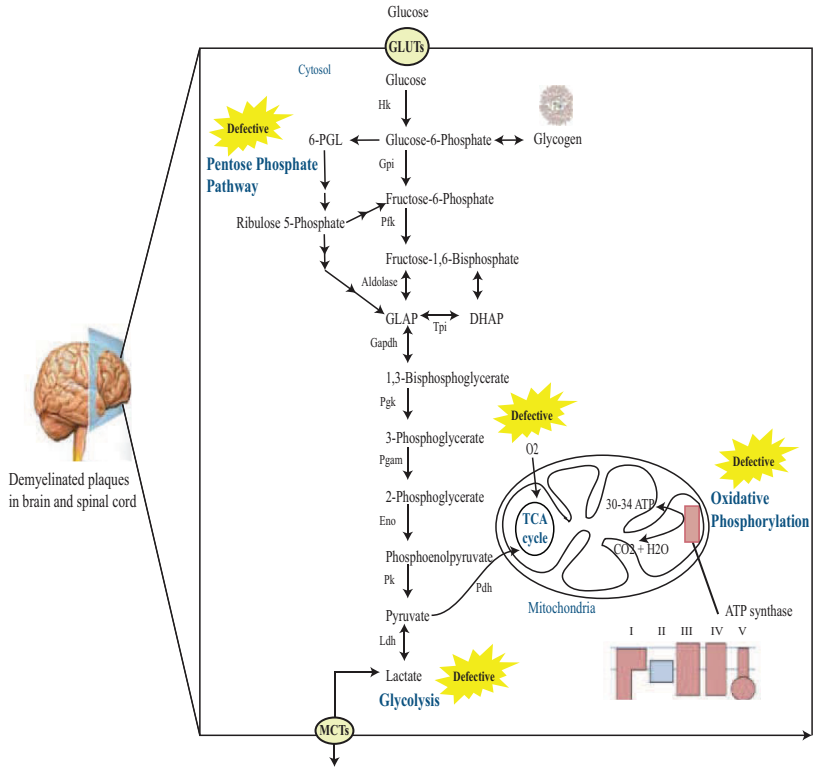

FIGURE 1 | Schematic representation of glucose metabolism in MS brain. Glucose enters cells through GLUTs and is phosphorylated by hexokinase to produce glucose 6-phosphate. Glucose 6-phosphate can be processed into three main coordinated metabolic pathways. First, it can be metabolized through glycolysis giving rise to two molecules of pyruvate and producing ATP and NADH. Pyruvate can then enter mitochondria, where it is metabolized through the TCA cycle and oxidative phosphorylation, producing ATP and CO_2. Alternatively, pyruvate can be reduced to lactate by Ldh. This lactate can be released into the extracellular space through monocarboxylate transporters (MCTs). The complete oxidation of glucose produces 30–34 ATP molecules in the mitochondria. Alternatively, glucose 6-phosphate can be processed through the PPP leading to the production of reducing equivalent in the form of NADPH. Note that the PPP and glycolysis are linked at the level of glyceraldehyde-3-phosphate (GLAP) and fructose 6-phosphate. Finally, glucose 6-phosphate can be converted to glycogen through the process of glycogenesis in astrocytes. Hk, hexokinase; Gpi, glucose-6-phosphate isomerase; Pfk, phosphofructokinase-1; DHAP, dihydroxyacetone phosphate; Tpi, triose phosphate isomerase; GAPDH, glucose-6-phosphate dehydrogenase; Pgk, phosphoglycerate kinase; Pgam, phosphoglycerate mutase; Eno, enolase; Pk, pyruvate kinase; Ldh, lactate dehydrogenase; 6-PGL, 6-phosphoglucono-d-lactone.

in MS. Increased activity of metabolic enzymes including enolase, pyruvate kinase, lactate dehydrogenase (Ldh), and aldolase in the CSF of patients with disseminated sclerosis make them a sensitive indicator of active demyelination (20).

B cells and antibodies reactive with triose phosphate isomerase (TPI) and GAPDH are produced intrathecally in CSF and lesions of MS (21). Both TPI and GAPDH are essential metabolic enzymes involved in ATP production. Another investigation by the same group showed that these antibodies bind with TPI and GAPDH and inhibit the glycolytic activity of GAPDH but not TPI in MS patients (22). This inhibitory effect of antibodies on GAPDH was not visualized when anti-GAPDH IgG was exhausted from the CSF demonstrating the role of anti-GAPDH antibodies in impeding the GAPDH enzyme activity in brain leading to neuronal apoptosis and cytotoxicity. In systemic lupus, an autoimmune rheumatoid disorder, autoantibodies were found reactive to GAPDH (23). Accumulating evidence indicates that inhibition of GAPDH activity in glycolytic pathway is associated with neuronal apoptosis. It has been purported that GAPDH enzyme activity suppresses when it reacts with other proteins in the CNS (24). Single chain variable fragment antibodies (scFv-abs) obtained from clonally expanded B cells binds specifically with GAPDH and TPI in active MS lesions (25). In addition, interaction of GAPDH with ß-amyloid protein and Huntingtin protein well demonstrates the role of GAPDH in neurodegenerative disorders (26).

A recent study demonstrated that a ligand-activated transcription factor known as peroxisome proliferator-activated receptor gamma (PPARγ) playing a pivotal role in regulation of glucose and lipid metabolism is markedly increased in CSF of MS patients (27). Furthermore, elevated expression of PPARγ has been reported within the spinal cord of EAE mice (28) and in an *in vitro* model of antigen induced demyelination (29). These findings may contribute to our understanding about the role of PPARγ in the pathogenesis of MS. William et al. studied the role of CNS energy metabolism in MS disease progression. The group measured the levels of lactate, sorbitol, and fructose, all metabolites of extra-mitochondrial glucose metabolism, in the CSF of relapsing remitting and secondary progressive MS patients (30). Sorbitol and fructose are the metabolites of polyol pathway that run parallel to glycolysis and lactate is the metabolite of anaerobic pathway. The finding demonstrated elevated levels of all three metabolites in the CSF of SPMS patients and to a lesser extent to RRMS patients (Figure S1 in Supplementary Material). These alterations in energy metabolism may contribute to mitochondrial dysfunction and neuroaxonal degeneration underlying MS progression. Taken together, the finding supports a link between increased activity of extra-mitochondrial pathways of glucose metabolism and MS disease progression. Other studies found that the activity of enolase, pyruvate kinase, Ldh, and aldolase, all metabolic enzymes was increased in disseminated sclerosis (20).

A recent investigation demonstrated differences in the gene expression levels of various NADPH subunits between initial MS lesions and control white matter brain. The group performed whole genome profiling of MS brain tissue and observed significant up regulation of nicotinamide dinucleotide phosphate oxidase I and nicotinamide dinucleotide phosphate oxidase organizer I in active MS lesions (31). NADPH oxidase is a multi-subunit enzyme complex that is activated under pathological conditions in microglia and catalyzes the production of superoxide from O_2. Other studies have identified defects in mitochondrial electron transport gene expression and function in post-mortem MS cortex (32, 33). These studies have found transcriptional changes in important mitochondrial genes; however, translational or post-translational changes in several other proteins may also influence mitochondrial function and energy production.

MITOCHONDRIAL DEFECTS IN MS

Mitochondrial dysfunction is implicated in various pathological conditions like diabetes, anxiety disorders, neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's disease, and cancer and fatigue (see for instance **Table 1**). It is only recently that mitochondrial aberrations have been studied in MS. Unlike nuclear DNA, mitochondrial DNA (mtDNA) is not surrounded by histones, proteins that shield nuclear DNA from free radicals. Therefore it is quite prone to damage. The number of mitochondria in a cell is determined by its energy requirement. For instance, there may be up to 200–2000 mitochondria present in a single somatic cell whereas the number is fixed to 16 in spermatozoa germ cells and 100,000 in oocytes. Metabolically active cells like skeletal muscle, cardiac muscle, and brain contain largest number of mitochondria. It has been seen that the number of mitochondria and their activity is increased in MS plaques (34). In demyelinating diseases particularly MS, it is likely that cells need ample energy to survive. Hence, metabolic activity of biomolecules in mitochondria increases with concomitant impairment of Krebs cycle and/or neuronal oxidative phosphorylation within the CNS. Another study revealed a reduction in ATP synthase expression in MS lesions (35). Mitochondrial proteins are expressed in greater amounts in both active and inactive lesions. Activity of complex IV found on mitochondrial membrane is increased dramatically in MS lesions (30). Lu et al. (36) revealed defects in complex I component of ETC in white matter lesions. Furthermore, reduced functional activity of complex I and complex III and a decrease in gene expression of complex I, complex III, complex IV, and ATP synthase has been observed in non-lesional motor cortex (33).

A recent finding demonstrated changes in mitochondrial complex enzyme activities and cytochrome c expression in platelets of MS patients. Krebs cycle enzyme aconitase activity was higher in patients without fatigue and all respiratory complex enzyme activities (complex I, II, III, IV, and V) were higher in MS patients compared to controls. Complex II activity increased significantly in MS group between patients with and without fatigue (42). Interestingly, a significant reduction in the gene expression of cytochrome c oxidase 5B subunit (COX5B) was observed in MS patients (46). This data suggest that there is a down regulation of genes associated with mitochondrial ETC. Analysis of a number of respiratory chain proteins reveals functionally important defects of mitochondrial proteins [cytochrome c oxidase (COX) and its catalytic component, COX-1] in complex III in MS (47). Different expressions of mitochondrial proteins namely cytochrome c oxidase subunit 5b (COX5b), hemoglobin ß, creatine kinase, and myelin basic protein (MBP) were found in the brain of MS (48). Taken together, these

Table 1 | Comparison of disturbed glucose metabolism in MS and other neurodegenerative disorders.

Glucose metabolism	Multiple sclerosis	Other neurodegenerative disorders
Glycolysis	Elevated blood **pyruvate level** was observed in both fasting and postprandial times in MS patients with relapse (14) **Pyruvate levels** were found to be increased in MS (19) The activity of metabolic enzymes including **enolase and pyruvate kinase** was found to be increased in the CSF of MS patients (20) Antibodies reactive with **triose phosphate isomerase (TPI) and GAPDH**, bind with them and inhibit the glycolytic activity of GAPDH in MS patients (22) The levels of **enolase, pyruvate kinase, Ldh, and aldolase**, all metabolic enzymes were increased in MS (20)	Impaired **GAPDH** function was observed in subcellular fractions of fibroblasts from Alzheimer and Huntington patients (37) **GAPDH** was found to be overexpressed in the neocortex and caudate putamen neurons in a transgenic model of Huntington's disease (38) The activity of **GAPDH, hexokinase and pyruvate kinase** was increased in Alzheimer's disease (AD) (39)
TCA cycle	Krebs cycle proteins like **α-ketoglutarate levels** in fasting and **citrate levels** after glucose intake were found to be increased in MS patients (18) **α-Ketoglutarate levels** were found to be increased in MS (19) Krebs cycle enzyme **aconitase** activity was found to be higher in MS patients without fatigue (42)	Mitochondrial **aconitase, succinyl-CoA synthetase β, fumarase** and **malate dehydrogenase** showed decreased gene expression in hippocampal samples from autopsy AD brains in two independent studies (40, 41) Mitochondrial **oxoglutarate dehydrogenase**, showed increased and decreased gene expression in moderate and severe AD patients (40)
Oxidative phosphorylation	A reduction in the expression of **ATP synthase** gene was observed in MS lesions (35) Activity of mitochondrial ETC **complex IV** was increased dramatically in MS lesions (30) Defects in **complex I** component of mitochondrial ETC were observed in white matter lesions (36) Furthermore, reduced functional activity of **complex I and complex III** and a decrease in gene expression of **complex I, complex III, complex IV, and ATP synthase** has been observed in non-lesional motor cortex (33). In contrast, enzyme activities of **complex I, II, III, IV, and V** were found to be higher in MS patients compared to controls. **Complex II** activity increased significantly in MS group between patients with and without fatigue (42) A significant reduction in the gene expression of **cytochrome c oxidase 5B subunit (COX5B)** was observed in MS patients (46) Analysis of a number of respiratory chain proteins reveals functionally important defects of mitochondrial proteins [**cytochrome c oxidase (COX)** and its catalytic component, **COX-1**] in complex III in MS (47) Different expression of mitochondrial proteins namely **cytochrome c oxidase subunit 5b (COX5b), hemoglobin β, creatine kinase, and myelin basic protein (MBP)** was found in the brain of MS (48)	Decreased mRNA expression levels of ETC proteins, specifically **FAD synthetase, riboflavin kinase (RFK), cytochrome C1 (CYC1),** and **succinate dehydrogenase** complex subunit B were reported in amyotrophic lateral sclerosis (43) Decreased mRNA levels of the mitochondrial-encoded **cytochrome oxidase (COX) subunits I, II, and III** were observed in brains of AD patients (41, 44, 45) Reduced expression of nuclear encoded subunits of mitochondrial enzymes of oxidative phosphorylation including **subunit IV of COX** and the **beta-subunit of the F0F1-ATP synthase** was also observed in vulnerable areas of AD brains (41, 44)

studies show mitochondrial abnormalities that may cause functional disturbance in the surviving demyelinated axons in MS and may result in neurological dysfunction.

GLUCOSE METABOLISM AND NEURODEGENERATIVE DISORDERS

A body of evidence indicates a link between disturbed metabolic function and the progression of neurodegenerative diseases like AD, Huntington's disease and Parkinson's disease. A report documented that GAPDH glycolytic function is impaired in subcellular fractions of fibroblasts from Alzheimer and Huntington patients whereas the gene expression remained unchanged (37). This might have occurred due to post-translational modification of the GAPDH protein. In a transgenic model of Huntington's disease, GAPDH is seen to be overexpressed in specific neuronal populations of several brain regions, such as the neocortex and caudate putamen neurons. This study also revealed translocation of GAPDH into the nucleus and the subsequent cell loss in the neocortex and caudate putamen region of the brain (38). Similar finding was observed by Bae et al. (49), who demonstrated that GAPDH facilitates nuclear translocation of mutant Huntingtin protein (mHtt) and causes neurotoxicity. In Aβ (amyloid

beta) resistant cells of Alzheimer's brain, glycolytic pathway was upregulated and hexose monophosphate shunt (HMS) was activated. The activity of GAPDH, hexokinase, and pyruvate kinase was increased in both glycolysis and HMS (39). In amyotrophic lateral sclerosis (ALS), abnormalities in ETC have been reported. The study found decreased mRNA expression levels of ETC proteins, specifically FAD synthetase, riboflavin kinase (RFK), cytochrome C1 (CYC1), and succinate dehydrogenase complex subunit B (SDHB) (43).

CONCLUSION

Despite extensive research being carried out for a decade the underlying cause of MS still remains elusive. Perturbed glucose metabolism is implicated in neurodegenerative disorders like Alzheimer's, Parkinson's, and Huntington's. However, little is known about its role in MS pathology. The observations reviewed in this article, especially those referred with mitochondrial aberrations and impaired glucose metabolism in MS, pointed to a relationship between glucose metabolism and MS disease pathogenesis. Although traditionally considered as an autoimmune, inflammatory, and demyelinating disease of the CNS, the scenario of MS pathogenesis associated with metabolic abnormalities is speculated.

These observations open new perspectives for the understanding of metabolic dynamics in MS yet many puzzling aspects and critical questions need to be addressed. For instance, how does defect in metabolic pathway contributes to demyelination? Does metabolic pathway alter in other cell types in MS? The changes in a cell type, may affect directly, through some unknown factor, or indirectly, through global changes in metabolic intermediates levels in CSF, to other cell types? Is disturbance in metabolic pathway a mere cause or a consequence of MS? Could those genes be used as a pharmacological target to alleviate MS pathology? Much more research is required to fully unravel the disease mechanism, and a proper understanding of the disease could eventually lead to new treatments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fneur.2014.00250/abstract

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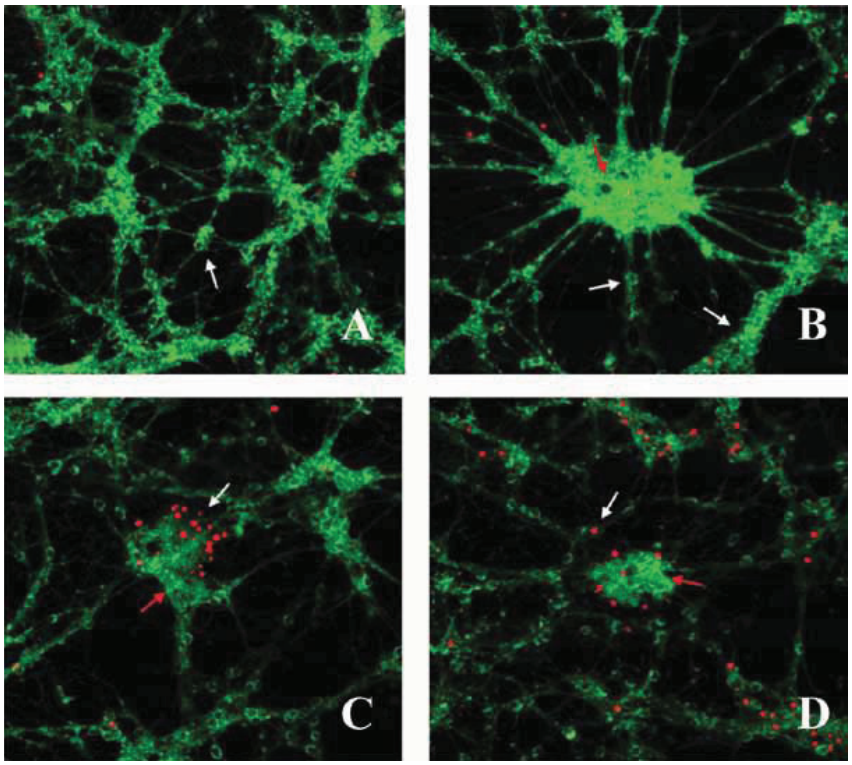

Viability and cell death in cerebellar granule neurons (CGNs) (white arrow) and astrocytes (red arrow).

Immunostaining pictures of rat cerebellar granule neurons treated with cerebrospinal fluid of multiple sclerosis patients. Apoptotic cell death occurred in cerebellar granule neurons but not astrocytes. 6 days culture of rat cerebellar granule neurons A) in control B,C,D) treated with cerebrospinal fluid of multiple sclerosis patients. Rhodamine-123 that fluoresces green was used to identify viable cells and propidium iodide that fluoresces red was used to identify dead cells.

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Molecular Shots

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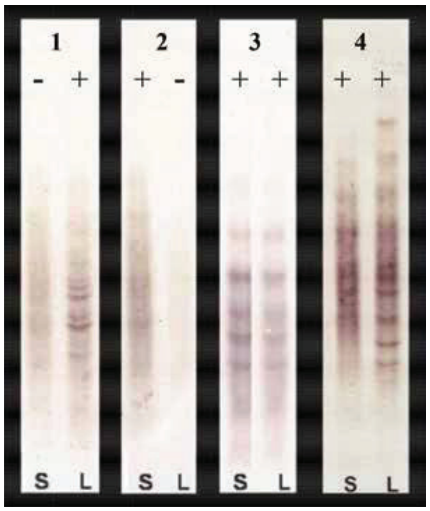

Figure 1: Immunodetection of oligoclonal bands in serum and CSF of Multiple Sclerosis patients. Pattern 1: OCBs in CSF only (positive): Oligoclonal bands present in CSF only. Intrathecal IgG synthesis as seen in MS; Pattern 2: No OCBs seen (negative, policlonal): No oligoclonal bands in CSF or Serum. No intrathecal Ig synthesis; Pattern 3: Identical OCBs in both (mirror): Bands in serum mirror those in CSF. This suggests systemic Ig synthesis; Pattern 4: Identical OCBs in both with extra in CSF (more than): Identical bands in both serum and CSF with extra bands in CSF. Image demonstrates both intrathecal and systemic Ig synthesis. This is identical as it is seen in MS.

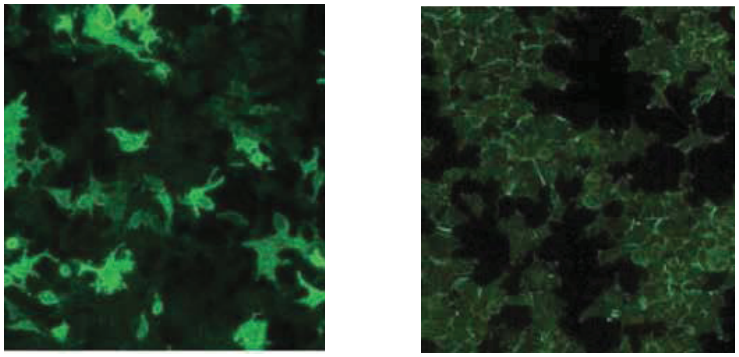

Figure 2: Indirect immunofluorescence in cells transfected by aquaporin4 (EUROIMMUN Aquaporin-4 IIFT). Panel A: Anti-AQP4 antibodies observed in the serum of NMO patients (positive sample); Panel B: Absence of anti-AQP4 antibodies in serum sample (negative sample)

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Bypassing hazard of housekeeping genes: their evaluation in rat granule neurons treated with cerebrospinal fluid of multiple sclerosis subjects

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Gene expression studies employing real-time PCR has become an intrinsic part of biomedical research. Appropriate normalization of target gene transcript(s) based on stably expressed housekeeping genes is crucial in individual experimental conditions to obtain accurate results. In multiple sclerosis (MS), several gene expression studies have been undertaken, however, the suitability of housekeeping genes to express stably in this disease is not yet explored. Recent research suggests that their expression level may vary under different experimental conditions. Hence it is indispensible to evaluate their expression stability to accurately normalize target gene transcripts. The present study aims to evaluate the expression stability of seven housekeeping genes in rat granule neurons treated with cerebrospinal fluid of MS patients. The selected reference genes were quantified by real time PCR and their expression stability was assessed using *GeNorm* and *NormFinder* algorithms. *GeNorm* identified transferrin receptor (*Tfrc*) and microglobulin beta-2 (*B2m*) the most stable genes followed by ribosomal protein L19 (*Rpl19*) whereas β-actin (*ActB*) and glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*) the most fluctuated ones in these neurons. *NormFinder* identified *Tfrc* as the best invariable gene followed by *B2m* and *Rpl19*. *ActB* and *Gapdh* were the least stable genes as analyzed by *NormFinder* algorithm. Both methods reported *Tfrc* and *B2m* the most stably expressed genes and *Gapdh* the least stable one. Altogether our data demonstrate the significance of pre-validation of housekeeping genes for accurate normalization and indicates *Tfrc* and *B2m* as best endogenous controls in MS. *ActB* and *Gapdh* are not recommended in gene expression studies related to current one.

Keywords: housekeeping genes, multiple sclerosis, normalization, *GeNorm*, *NormFinder*

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Introduction

Techniques employed for calibrating gene expression are paramount in studies directed toward accurate analysis of transcriptomic profiles. Quantitative real time PCR (qRT-PCR) has gained significant momentum over the past decade to quantify gene expression profiles. Considering the utmost sensitivity and reliability of qRT-PCR, a careful selection of a constitutively expressed gene

is required to account for variation in the amount and quality of starting RNA and cDNA synthesis efficiency. In general, the expression of target gene transcripts is normalized with an internal control, often referred to as a housekeeping gene. Housekeeping (HK) genes are endogenous controls that are required for the primary function of a cell hence their expression should be constant in all conditions. However, recent research has indicated that their expression may not necessarily be stable in all cells/tissues. A gene showing consistent expression in one condition may show unstable expression in another. Invariable expression of the so-called housekeeping genes has been observed during cellular development (Al-Bader and Al-Sarraf, 2005) and under distinct experimental conditions (Zhong and Simons, 1999; Hamalainen et al., 2001; Deindl et al., 2002; Glare et al., 2002; Torres et al., 2003; Radonic et al., 2004; Toegel et al., 2007; Gubern et al., 2009). Therefore it is essential to pre-validate the expression stability of reference genes to accurately normalize the gene expression data. It is recommended that more than one stably expressed gene should be used for precise normalization procedure (Zhong and Simons, 1999; Tricarico et al., 2002; Vandesompele et al., 2002; Ohl et al., 2005).

In this context, we aimed to evaluate the expression stability of seven commonly used housekeeping genes in cerebellar granule neurons (CGNs) treated with cerebrospinal fluid (CSF) from multiple sclerosis (MS) and neuromyelitis optica (NMO) patients. Axonal damage is widely accepted as a major cause of persistent functional disability in MS. Therefore to study primary neuronal damage independent of secondary damage, resulting from demyelination, we used primary cultures of unmyelinated CGNs as a cellular model and exposed them to CSF derived from MS patients. Prior to comprehending mechanisms involved in axonal degeneration–regeneration, it was first necessary to identify best stably expressed housekeeping genes that can be used to normalize target mRNA transcripts in our experimental system. We therefore used a xenogeneic system comprising of primary rat CGN cultures incubated with CSF from patients with MS or controls and investigated the stability of reference genes in these rat neuronal cells. Previous studies in similar xenogeneic models showed that treatment with human CSF resulted in neurotoxicity in culture, although the molecular mechanisms remained unknown (Xiao et al., 1996; Alcazar et al., 2000). Recently, Vidaurre et al. (2014) reported that ceramides present in CSF from patients with MS disturb neuronal bioenergetics in rat neuronal cultures.

Primary cultures of rat CGNs represent an excellent model to study almost every aspect of neurobiology. While neuronal cell lines have been very useful in the study of neuronal cell cultures, there are certain drawbacks they exhibit. These cell lines are derived from neuronal tumors and hence will show many important physiological differences with the cell type from which they were derived. For instance, the human SH-SY5Y cell line, was derived by subcloning from the parental metastatic bone tumor biopsy cell line SK-N-SH (Biedler et al., 1973). Therefore, it is prudent to use primary cultures because they are not tumor-derived and hence are more likely to exhibit the properties of neuronal cells *in vivo*. Furthermore, CGNs are small and the most numerous unmyelinated neurons, therefore we used primary cultures of rat CGNs as a cellular model and exposed them to diseased CSF to comprehend the pathophysiological mechanisms implicated in MS and prior to that validating the expression stability of commonly used housekeeping genes for their use in future gene expression experiments.

We selected some frequently used housekeeping genes from literature to determine their expression stability in our experimental setting. MS is a major cause of non-traumatic neurological disability deemed to affect more than 2 million people worldwide (Blight, 2011). It manifests as a chronic inflammation in central nervous system (CNS) that leads to demyelination and neurodegeneration. The disease typically manifests at 20–40 years of age when people are in their full employment and sometimes develops into an aggressive stage that alters the lives of patients and their families. Unfortunately the current treatments are only effective in preventing relapses and slowing down progression but not completely ceasing it. Although the pathogenesis of MS is not well understood, accumulating evidence suggests a complex interplay of both genetic and environmental factors (Al-Bader and Al-Sarraf, 2005; Compston and Coles, 2008; Oksenberg et al., 2008). A plethora of gene expression studies have been undertaken in peripheral mononuclear white blood cells (Der et al., 1998; Ramanathan et al., 2001; Wandinger et al., 2001; Bomprezzi et al., 2003; Koike et al., 2003; Sturzebecher et al., 2003; Hong et al., 2004; Iglesias et al., 2004; Satoh et al., 2006), in MS brain tissues (Becker et al., 1997; Whitney et al., 1999; Chabas et al., 2001; Whitney et al., 2001; Lock et al., 2002; Mycko et al., 2003; Tajouri et al., 2003; Lindberg et al., 2004; Mycko et al., 2004) and in CSF (Brynedal et al., 2010). Proteomic approaches have also been used to identify differentially expressed proteins in the CSF of MS patients (Dumont et al., 2004; Hammack et al., 2004; Noben et al., 2006). However, the proteomics analysis of CSF obtained from MS patient is relatively challenging. Since proteins are highly abundant, diversified, and soluble, only some protein subgroups may be detected and others important proteins may fail to be identified by proteomics approach. Thus, it would be prudent to use proteomic analysis along with other approaches such as gene expression profiling using microarray. Another similar but totally distinct neurological disease known as NMO shares many pathological similarities with MS and therefore it was previously considered as its variant. For this reason clinicians often used to encounter difficulty in distinguishing MS from NMO and hence similar treatment was provided to both the category of patients. However, recent research shows that there are some NMO specific IgG antibodies present in the sera of NMO patients, which differentiate both the diseases (Lennon et al., 2004).

In MS, axonal damage is widely accepted as the major cause of persistent functional disability, although its origin is unknown. During the relapsing-remitting disease course the patient's brain itself is capable of repairing the damage, remyelinating the axon and recovering the neurological function. CSF is in contact with brain parenchyma (Rossi et al., 2012, 2014) and a site of deposition of cellular damaged products, which can influence

the cellular physiology of brain cells. It is a promising biofluid in the search for biomarkers and disease associated proteins in MS, both with respect to inflammatory and neurodegenerative processes. Exposure of CGNs with CSF from diseased states can allow us to understand the pathophysiology of MS but prior to that evaluation of housekeeping genes to accurately normalize target genes is a crucial step. Selected housekeeping genes were quantified using real time PCR to accurately normalize target genes in our experimental setting. The expression stability of reference genes was further assessed by *GeNorm* and *NormFinder* algorithms. *GeNorm* program defines the gene stability as the average pairwise variation of a particular gene with all other control genes and ranks the genes according to their average expression stability denoted by *M* (Vandesompele et al., 2002). The gene with minimum *M* value is considered to be highly stable whereas the gene with highest *M* value is least stable and can be excluded. An alternative program, *NormFinder*, ranks the candidate reference genes based on the combined estimates of both intra- and intergroup variations (Andersen et al., 2004).

Materials and Methods

All procedures were approved by the Committee of Animal Care of Prince Felipe Research Center (CIPF), Valencia, in accordance with the regulations of the European Union and Spanish legislation. Informed consent was obtained from all the patients and controls for this study and authorized by the Ethical Committee of the Institute.

Patient Cohort

Patient Population

A total of 59 patients were recruited and CSF samples were obtained from the Department of Neurology, Hospital La Fe and Hospital Clinico, University of Valencia. Out of 59 patients, 21 had inflammatory MS (11 IgM+/+ and 10 IgM +/−), 8 had medullary subtype, 11 had PPMS, 9 had NMO, and 10 were non-inflammatory neurological controls (NIND patients). In CSF, apart from factors related to MS or NMO, there are factors from other diseases that produce their action. This must be considered as "*background noise*" as average population. Mixing of total CSF samples in all clinical forms may potentiate the factors related to MS. Therefore, we mixed CSF samples in all clinical forms.

Multiple sclerosis patients were defined and grouped in different clinical courses, according to the current criteria (Lublin and Reingold, 1996) and diagnosed according to McDonald criteria. They all met the following characteristics: oligoclonal IgG bands (OCGB) present, not in a phase of relapse, and have spent more than a month after the last dose of steroids. Wingerchuk criteria were used to diagnose patients with NMO disease (Wingerchuk et al., 2006). Patients suffered relapses of optic neuritis and myelitis, and two of the three criteria, normal MRI or that did not accomplish the Patty criteria for MRI diagnosis of MS. **Table 1** illustrates the clinical characteristics of the patients.

Patient Characteristics

Inflammatory MS (RRMS and SPMS forms)

MS is categorized into: (1) Relapsing remitting MS (RRMS) that later develops into secondary progressive stage (SPMS); and (2) primary progressive MS (PPMS). Over 95% of patients with MS show oligoclonal bands (OCBs) of IgG in CSF (G+) (Kostulas et al., 1987) and 40% show IgM OCBs in CSF (M+) related to a more aggressive course of disease (Sharief and Thompson, 1991). In our project we also classified and named inflammatory MS into *"IgM+/−"* and *"IgM+/+ subtype"* (see below) on the basis of aggressivity and prognosis that is more complete than just RRMS or PPMS. In addition we have studied separately a set of patients with MS but with a predominant affectation of the spinal cord, because these patients have some peculiarities, and we wanted to explore if they have some differences in light of our experiments. The most aggressive cases termed as *"medullary"* have more spinal injuries.

IgM+/− clinical form of MS

Patients named as *"IgM+/− subtype"* had IgG antibodies (+) but no IgM (−) oligoclonal antibodies detected in the CSF of brain.

IgM+/+ clinical form of MS

Patients named as *"IgM+/+ subtype"* had both IgG antibodies (+) and IgM (+) oligoclonal antibodies detected in the CSF of brain.

Medullary clinical form of MS

All these patients were positive for OCGBs and negative for oligoclonal IgM bands (OCMBs) in CSF of spinal region. The patients accomplished the Swanton criteria for dissemination in time.

Primary progressive MS

These patients are characterized by progressive decline in neurological disability.

Neuromyelitis optica patients

Individuals diagnosed with NMO met at least two of the following three features. (1) Long extensive transverse myelitis (>3 vestibule bodies); (2) Antibodies against aquaporin-4; (3) Normal brain at the first event.

Controls [Non-Inflammatory Neurological Diseases (NIND)]

Individuals who were suspected to have MS but were not diagnosed with MS were classified as controls.

Cerebrospinal Fluid Samples of Patients

Cerebrospinal fluid samples were obtained by lumbar puncture at the time of diagnosis. Samples were centrifuged for 10 min at 700 × *g* and aliquots were frozen at −80°C until use. No patient had received treatment with immunosuppressive drugs, immunomodulators or corticosteroids for at least 1 month prior to the extraction of CSF.

Cerebrospinal Fluid Studies

All the studies were performed by immunologists who were blind to the clinical and MRI data.

TABLE 1 | Clinical characteristics of the patients studied.

Case #	Sex	Working clinical form	Clinical form	Age (years)	Evolution time	Actual EDSS
1	Female	RRMS (+/–)	RRMS	23	5	1.50
2	Female	RRMS (+/–)	SPMS	21	18	4.00
3	Female	RRMS (+/–)	RRMS	36	4	1.50
4	Male	RRMS (+/–)	RRMS	22	6	1.50
5	Female	RRMS (+/–)	RRMS	21	3	3.00
6	Female	RRMS (+/–)	RRMS	30	22	4.00
7	Female	RRMS (+/–)	RRMS	29	10	1.50
8	Female	RRMS (+/–)	RRMS	29	7	1.50
9	Female	RRMS (+/–)	RRMS	28	10	5.50
10	Female	RRMS (+/–)	RRMS	28	4	1.00
11	Female	RRMS (+/+)	RRMS	37	7	3.50
12	Male	RRMS (+/+)	RRMS	32	4	1.00
13	Female	RRMS (+/+)	RRMS	44	5	2.00
14	Female	RRMS (+/+)	RRMS	26	5	2.00
15	Female	RRMS (+/+)	RRMS	14	18	3.50
16	Male	RRMS (+/+)	RRMS	25	11	2.00
17	Female	RRMS (+/+)	SPMS	21	25	8.50
18	Female	RRMS (+/+)	RRMS	17	16	2.00
19	Female	RRMS (+/+)	SPMS	23	18	6.50
20	Female	RRMS (+/+)	SPMS	22	5	4.00
21	Female	RRMS (+/+)	RRMS	29	5	2.50
22	Male	MedMS	SPMS	39	10	4.50
23	Female	MedMS	SPMS	25	6	7.00
24	Female	MedMS	SPMS	25	14	8.00
25	Male	MedMS	SPMS	34	9	6.00
26	Male	MedMS	SPMS	34	6	6.50
27	Female	MedMS	RRMS	23	5	4.00
28	Female	MedMS	SPMS	40	10	7.50
29	Female	MedMS	SPMS	23	28	6.50
30	Female	PPMS	PPMS	54	12	7.00
31	Male	PPMS	PPMS	40	23	6.00
32	Female	PPMS	PPMS	52	14	5.50
33	Female	PPMS	PPMS	38	11	5.50
34	Male	PPMS	PPMS	31	24	6.00
35	Female	PPMS	PPMS	47	14	5.50
36	Male	PPMS	PPMS	49	11	6.00
37	Female	PPMS	PPMS	26	13	6.50
38	Female	PPMS	PPMS	34	6	5.00
39	Female	PPMS	PPMS	39	8	8.50
40	Male	PPMS	PPMS	18	15	8.00
41	Female	NMO	NMO	39	5	9.00
42	Female	NMO	NMO	50	4	7.00
43	Male	NMO	NMO	15	17	4.00
44	Male	NMO	NMO	42	5	3.50
45	Female	NMO	NMO	22	5	2.50
46	Female	NMO	NMO	27	5	2.00
47	Male	NMO	NMO	9	14	1.00
48	Female	NMO	NMO	8	32	4.00
49	Male	NMO	NMO	19	20	8.50
50	Male	CONTROL	CONTROL	23	NA	NA
51	Female	CONTROL	CONTROL	77	NA	NA
52	Female	CONTROL	CONTROL	33	NA	NA
53	Female	CONTROL	CONTROL	32	NA	NA
54	Male	CONTROL	CONTROL	59	NA	NA

(Continued)

TABLE 1 | Continued

Case #	Sex	Working clinical form	Clinical form	Age (years)	Evolution time	Actual EDSS
55	Female	CONTROL	CONTROL	36	NA	NA
56	Female	CONTROL	CONTROL	57	NA	NA
57	Male	CONTROL	CONTROL	37	NA	NA
58	Female	CONTROL	CONTROL	21	NA	NA
59	Male	CONTROL	CONTROL	13	NA	NA

EDSS, Kurtzke expanded disability status scale (method of quantifying disability in MS); MedMS, medullary MS; RRMS, relapsing-remitting multiple sclerosis; PPMS, primary progressive multiple sclerosis; NMO, neuromyelitis optica; +/−, presense of IgG but no IgM antibodies in the CSF; +/+, presense of both IgG and IgM antibodies in the CSF; NA, not applicable.

Oligoclonal band studies

Paired CSF and serum samples were analyzed to detect OCBs (OCGB and OCMB) by isoelectric focusing (IEF) and immunodetection. We used a commercial kit to determine OCGB (Helena BioScience IgG-IEF Kit) and the technique described by Villar et al. (2001) to detect OCMB. Serum samples were diluted in saline before the IEF in order to reach the same concentration range as that of CSF samples. All samples were incubated with 50 mmol/L dithiothreitol at pH 9.5 to reduce IgM. Focusing was performed on a Multiphor II Electrophoresis System (GE Healthcare) at pH 5–8. Proteins were then transferred to a PVDF membrane and analyzed by Western blot. Finally, immunodetection was performed by biotin-conjugate-goat anti-human IgM and streptavidin-alkaline phosphatase (Sigma–Aldrich).

Serum studies

Anti-AQP4 antibody in NMO has a high specificity so as to contribute to early diagnosis and optimized treatment of Devic disease. Serum sample diluted 1:10 in PBS-Tween was used to detect the presence of NMO specific IgG antibodies. Indirect immunofluorescence (IFI) was performed to diagnose NMO (**Figure 1B**). Antibodies against aquaporin 4 were detected using a cell line, which was molecular biologically modified to produce large quantities of aquaporin 4. In this method (EuroImmun IIFT) recombinantly transfected cells act as an antigen substrate to be incubated with diluted serum samples for half an hour.

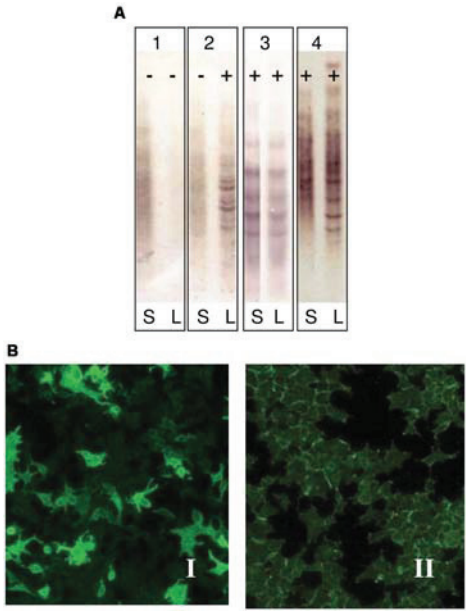

FIGURE 1 | (A) Immunodetection of oligoclonal bands (OCBs) in serum (S) and cerebrospinal fluid (CSF) (L). Pattern 1: No OCBs seen (negative, polyclonal): No OCBs in CSF or serum. No intrathecal Ig synthesis; Pattern 2: OCBs in CSF only (positive): OCBs present in CSF only. Intrathecal IgG synthesis as seen in MS; Pattern 3: Identical OCBs in both (mirror images): Bands in serum mirror those in CSF. This suggests systemic Ig synthesis; Pattern 4: Identical OCBs in both with extra bands in CSF: Identical bands in both serum and CSF with extra bands in CSF. Image demonstrates both intrathecal and systemic Ig synthesis. This is identical as it is seen in MS. **(B)** Indirect immunofluorescence in cells transfected by aquaporin 4 (EUROIMMUN Aquaporin-4 IIFT). Panel I: Anti-AQP4 antibodies observed in the serum of NMO patients (positive sample); Panel II: Absence of anti-AQP4 antibodies in serum sample (negative sample).

Animals

Wistar rats (Harlan Iberica) with weight between 200 and 250 g were used. All animals were raised under controlled conditions with cycles of light/dark (12/12 h), temperature of 23°C and humidity of 60%. Access to water and food (standard rodent feed supplied by Harlan, Teklad 2014 Global 14% Protein Rodent Maintenance Diet) was provided. To obtain offspring, pregnant females were separated and kept in isolated cages during gestation. The maintenance of the animals was performed in the animal facilities unit of Prince Felipe Research Center, Valencia, Spain.

Primary Culture of Cerebellar Granule Neurons

All operations were performed under sterile conditions in vertical laminar flow chamber (Telstar AV-100 and Bio-II-A). The cells were kept in an incubator at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2 (CO2 incubator Thermo Form, model 371). Primary cultures of CGNs were obtained according to previously described modified protocol (Minana et al., 1998). Forebrains were collected from 8 days old Wistar rats, mechanically dissociated and cerebellum was dissected.

Isolated cerebella were stripped of meninges, minced by mild trituration with a Pasteur pipette and treated with 3 mg/ml dispase (grade II) for 30 min at 37°C in a 5% CO_2 humidified atmosphere. After half an hour, dispase was inactivated with 1mM EDTA. Granule cells were then resuspended in basal Eagleś medium (BME, Gibco, ref. 41010) with 40 μg/ml of DNaseI. The cell suspension was filtered through a mesh with a pore size of 90 μm and centrifuged at 1500 rpm for 5 min and thereafter, cell suspension was washed three times with BME. Finally, the cells were resuspended in complete BME medium with Earleś salts containing 10% heat inactivated FBS (fetal bovine serum, Gibco), 2 mM glutamine, 0.1 mg/ml gentamycin and 25 mM KCl. The neuronal cells were counted and plated onto poly-L-lysine coated 6-well (35-mm) culture dishes (Fisher) at a density of 3×10^5 cells/well and incubated at 37°C in a 5% CO_2/95% humidity atmosphere. After 20 min at 37°C, the medium was removed and fresh complete medium was added. Since the purpose of our study was to obtain pure cultures of CGNs, it was necessary to add a chemical that can prevent the growth of non-neuronal cells. Twenty micro liter of cytosine arabinoside (1 mM) was added to each culture plate after 18–24 h to inhibit replication of non-neuronal cells. The cells were kept in an incubator at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2 (CO_2 incubator Thermo Form, model 371). Cells were fed every 3–4 days in culture with 5.6 mM glucose.

Cerebellar granule neurons were stained with Texas Red and FITC dyes. The nuclei of neurofilaments were stained with DAPI. **Figure 2A** shows pure cultures of granule neurons isolated from cerebellum with stained neurofilaments.

Confocal Microscopy

The living cells were always kept at 37°C and 5% CO_2. Cells were analyzed on a Leica TCS SP2 confocal microscope AOBS (Leica Microsystems) inverted laser scanning confocal microscope using a 63× Plan-Apochromat-Lambda Blue 1.4 N.A. oil objective lens. All confocal images were obtained under identical scan settings. Images of 1,024° × 1,024 pixels, 8-bits were collected for each preparation. Best focus was based on highest pixel intensity. Imaging conditions were identical for all the images, and no images were saturated. Metamorph 7.0 (Molecular Devices, Downingtown, PA, USA) was used for image analysis on the images collected.

Selection of Housekeeping Genes

Candidate housekeeping genes were selected from those most commonly used in literature including β-actin (*ActB*), hypoxanthine guanine phosphoribosyl-transferase (*Hprt*), ribosomal protein L19 (*Rpl19*), lactate dehydrogenaseA (*Ldha*), transferrin receptor (*Tfrc*), microglobulin beta-2 (*B2m*), and glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*). The function and references of the genes are listed in **Table 2**. The primers for the selected reference genes from 5′- to 3′- end were as follows: *Actb* forward ATTGAACACGGCATTGTCAC, reverse ACCCTCATAGATGG GCACAG; *Hprt* forward CCTCTCGAAGTGTTGGATACAG, reverse TCAAATCCCTGAAGTGCTCAT; *Rpl19* forward ACCT

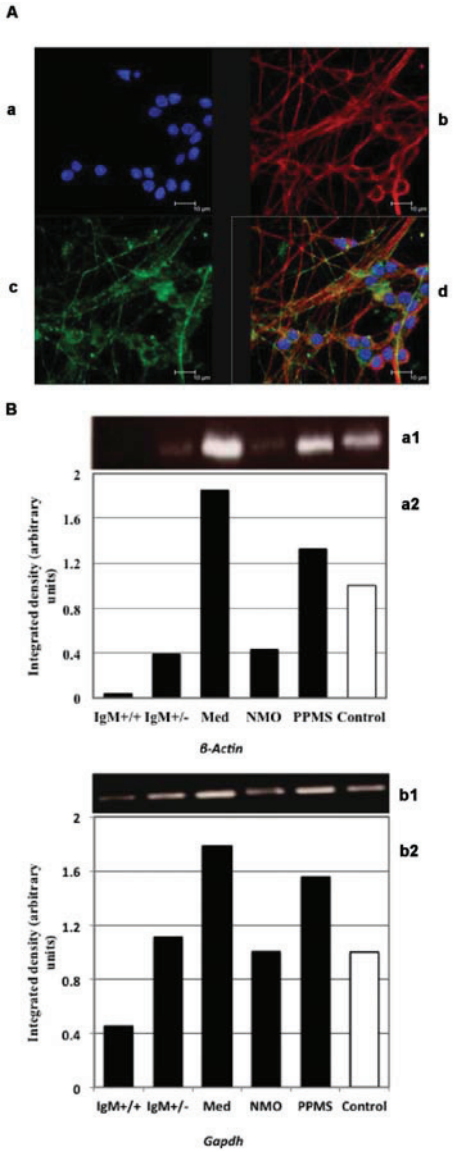

FIGURE 2 | (A) Immunofluorescence of primary cultures of CGNs. (a) DAPI stained nuclei. Neurofilaments stained with (b) Texas red and (c) FITC dyes. (d) Merged images. **(B)** Analysis of β-*actin* (a) and *Gapdh* (b) expression by PCR: (1) Electrophoretic bands; (2) Integrated density obtained by Image J software quantification. The values correspond with the fold change *vs*. control values of the MS clinical forms, NMO, and control patients.

TABLE 2 | Panel of seven candidate housekeeping genes selected for expression analysis.

Gene symbol	Gene name	mRNA accession number	Function	Reference
ActB	β-Actin	NM_031144	Cytoskeletal structural Protein	Stürzenbaum and Kille, 2001
Hprt	Hypoxanthine guanine phosphoribosyl transferase	NM_012583	Metabolic salvage of purines	Everaert et al., 2011
Rpl19	Ribosomal protein L19	NM_031103	Unclear	Zhou et al., 2010
Ldha	Lactate dehydrogenase A	NM_017025	NADH dependent enzyme that catalyzes reduction of pyruvate to lactate	–
Tfrc	Transferrin Receptor	NM_022712	Iron delivery from transferrin to cells	Gorzelniak et al., 2001
B2m	Microglobulin-b-2	NM_012512	Major histocompatibility complex class I	Yurube et al., 2011
Gapdh	Glyceraldehyde-3-phosphate-dehydrogenase	NM_017008	NAD+ dependent enzyme that catalyzes conversion of glyceraldehyde-3-phosphate to 1,3-bis phosphoglycerate	Fort et al., 1985; Harrison et al., 2000; Medhurst et al., 2000; Gorzelniak et al., 2001

GGATGCGAAGGATGAG, reverse CCATGAGAATCCGCTTG TTT; *Ldha* forward AGGAGCAGTGGAAGGATGTG, reverse AGGATACATGGGACGCTGAG; *Tfrc* forward GTTGTTGAG GCAGACCTTCA, reverse ATGACTGAGATGGCGGAAAC; *B2m* forward GTCGTGCTTGCCATTCAGA, reverse ATTTGA GGTGGGTGGAACTG; *Gapdh* forward GGAAACCCATCA CCATCTTC, reverse GTGGTTCACACCCATCACAA.

Treatment, Total RNA Isolation and cDNA Synthesis

Neuronal cell cultures were incubated with 10% v/v CSF from MS (IgM+/−, IgM+/+, medullary, PPMS) patients, NMO patients and controls for 24 h. This step was performed to identify transcriptional changes in future transcriptomic experiments. Total RNA was isolated from cell cultures exposed to the CSF of different experimental conditions (IgM+/−, IgM+/+, medullary, NMO, PPMS, Control) using Quick RNA MicroPrep Kit (Zymo Research Corp.). The RNA concentration was determined spectrophotometrically at 260 nm using the Nanodrop 1000 spectrophotometer (V3.7 software) and RNA purity was checked by means of the absorbance ratio at 260/280 nm. Isolated RNA was stored at −80° and later reverse transcribed to cDNA. The cDNA was synthesized and stored at −20°C. Primers for selected genes were designed using Primer 3 software. PCR was performed in a thermocycler (BioRad) with cycling conditions (94° for 30 s, 40 cycles at 59° for 30 s and 72° for 30 s). Each 25 μl reaction contained 12.5 μl Master Mix (Applied Biosystems), 1 μl gene-specific forward and reverse primers (0.5 μM), 1 μl undiluted cDNA and 10.5 μl DEPC (nuclease free) treated water. Negative controls with no template contained nuclease-free water instead.

Agarose Gel Electrophoresis and Real-time Polymerase Chain Reaction of Selected Housekeeping Genes

The electrophoresis was performed in 1.5% agarose gels, and they were run at 50 V, stained with ethidium bromide, photographed and evaluated with ImageJ software. A DNA ladder control (100 bp, Invitrogen) was also used in the electrophoresis to evaluate DNA fragment size. Real time PCR was performed in a 96-well plate (Roche) incubated in thermocycler (LC480, Roche) with cycling conditions (94° for 15 s, 45 cycles at 60° for 30 s and 72° for 30 s). Each 10ml reaction contained 5 ml SYBR Green Master Mix (Applied Biosystems), 1 μl gene-specific forward and reverse primers (0.5 μM), 1 μl undiluted cDNA and 3 ml DEPC (nuclease free) treated water. Negative controls with no template contained nuclease-free water instead. All samples were run in duplicate and average values were calculated. Data was analyzed using 7300 Sequence Detection Software (SDS) Version 1.3 (Software Roche). Following qRT-PCR, a dissociation curve was run to check the PCR product specificity.

Determination of Reference Gene Expression Stability

To determine the stability of these genes on the basis of their Cp values, we employed comparative ΔCT method. Data are plotted as fold change values which were calculated by $2^{\Delta\,(\mathrm{Ctexp}\ -\mathrm{Ctcontrol})}$. Cp value is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. Invariable genes were later assessed by publicly available software tools named *GeNorm* and *NormFinder*.

Results

Demographic and Clinical Profiles of MS, NMO and NIND Groups

Patients were classified according to detection of OCBs (**Figure 1A**) and of aquaporin antibodies (**Figure 1B**). Baseline characteristics of the study population are described in **Table 3**. Prevalence of MS was found more in women (75%) than in men. Mean age of MS patients was 30.7 ± 9.7 whereas 25.6 ± 15 for NMO patients. According to the clinical classification, the general characteristics of MS patients are described in **Table 4**. There were significant differences observed between the age at beginning

TABLE 3 | General characteristics of series studied.

	Controls ($n = 10$)	MS patients ($n = 40$)	NMO patients ($n = 9$)	p
% Females (n)	60.0 (6)	75.0 (30)	55.6 (5)	0.40 (χ^2)
Age (mean, SD)	40.3 (19.5)	30.7 (9.7)	25.6 (15.0)	0.04 (ANOVA test)
EDSS	n.a.	4.5 (2.3)	4.6 (2.8)	0.94 (t-test)
Evolution time	n.a.	11.1 (6.6)	11.8 (9.7)	0.79 (t-test)

of PPMS and the other two MS forms ($p < 0.003$); between the EDSS of RRMS and the two other MS forms (<0.001); the evolution time between PPMS and RRMS ($p = 0.043$) after Bonferroni correction. **Table 5** shows the characteristics of MS patients according to new proposal and working classification. After Bonferroni correction, significance was due to differences between the age at beginning and the EDSS between medullary MS and PPMS with the inflammatory MS.

We found significant differences in the age at beginning between PPMS and the other two MS forms (RRMS and SPMS) after Bonferroni correction ($p < 0.003$) (**Table 6**). People with PPMS are usually older at the time of diagnosis with an average age of 40. Furthermore, different subtypes of MS help predict disease severity and response to treatment hence their categorization is important. In our study, we found significant differences between the "*Expanded Disability Status Scale*" (EDSS) of RRMS and the two other MS forms (SPMS and PPMS) ($p < 0.001$) (**Table 5**). Although nerve injury always occurs, the pattern is specific for each individual with MS. Disease severity and disability increases from relapsing-remitting to secondary progressive course and in PPMS subtype, symptoms continually worsen from the time of diagnosis rather than having well-defined attacks and recovery. PPMS usually results in disability earlier than relapsing-remitting MS. Significant differences were found in the evolution time from the first to the second episode between RRMS and PPMS ($p = 0.043$). In patients experiencing a progressive course, evolution time was similar in secondary progressive cases and in cases that were progressive from onset (13.5 versus 13.8) (**Table 5**).

According to the new proposal and working classification, inflammatory MS subtypes shared similar age at disease onset (mean = 26.7 versus 26.3 years; $p = 0.005$). Significant differences were found between the age at disease onset in medullary MS and PPMS with the inflammatory MS ($p < 0.005$). The degree of disability as measured by EDSS was similar in medullary MS and PPMS (6.2 versus 6.3) whereas significant differences were found between disability extent in medullary MS and PPMS with the inflammatory MS ($p < 0.001$). IgM+/− represents the less aggressive inflammatory subtype with OCGB in CSF with poor prognosis whereas IgM+/+ signifies a more aggressive category with OCGB and OCMB in CSF with worse prognosis. On the contrary, medullary MS represents the most aggressive subtype of MS with increased neurological disability and dysfunction as compared to inflammatory subtypes. Disability in patients experiencing PPMS worsens over time with no relapses and remission.

Identification of Stably Expressed Housekeeping Genes

PCR of *Gapdh* and β-*actin*

We first quantified *ActB* and *Gapdh* genes using conventional PCR in treated neuronal samples and ran agarose gel electrophoresis. We found that both β-*Actin* and *Gapdh* genes, which are presumed to express at constant levels showed varying band intensity in CGNs when treated with the CSF from MS and NMO patients (**Figure 2B**). From this data we conclude that both *ActB* and *Gapdh* genes are not suitable to normalize gene transcripts in our experimental conditions.

Quantitative PCR of Housekeeping Genes in our Experimental Conditions

Quantitative real time PCR was performed for a group of frequently used reference genes. *GeNorm* and *NormFinder* algorithms were used to assess the most stably expressed genes. Our data suggests *Tfrc, B2m* as the most stable genes followed by *Rpl19* using *GeNorm* software (Average expression stability value denoted by M: 1.09 for *Tfrc* and *B2m;* M: 1.19 for *Rpl19*). Similarly, *Tfrc* showed most stable expression as assessed by *NormFinder* algorithm followed by *Ldha* and *Rpl19* (M: 0.54 for *Tfrc*; M: 0.58 for *Ldha* and 0.97 for *Rpl19*) (**Table 6**). On the other hand, β-*Actin* and *Gapdh* showed highest fluctuation in our experimental conditions with 2.9 and 4.2 as the average expression stability value by *GeNorm*. Therefore their use is strictly discouraged while normalizing gene expression data in studies related to the current one.

Table 6 illustrates candidate housekeeping genes ranked in CGNs treated with CSF from MS/NMO patients according to their expression stability by *GeNorm* and *NormFinder* methods. The C_t values of all the experimental conditions obtained from qPCR experiment were normalized to control. Then we plotted the fold change values for each reference gene tested in distinct disease courses of MS and NMO patients (**Figure 3**). Fold change was calculated by $2^{\Delta(\text{Ctexp} - \text{Ctcontrol})}$.

ActB

We found that the expression of *ActB* gene dropped to 0.2 folds in neurons treated with IgM+/− MS patients and increased again to 1.4 folds in IgM+/+ treated neurons, as compared to control. In neurons treated with medullary CSF the gene expression dropped to 0.04 folds and 0.2 folds in PPMS and increased to 1.78 folds in NMO patients compared to control. Although the variation in the expression level of this gene in all the different experimental conditions is not large as seen by qPCR data, we employed *GeNorm* software to compare the expression stability of all the reference genes with each other and identify the best reference gene out of a group of commonly used reference genes to avoid getting biased results. The software *GeNorm* ranked *ActB* gene as second last unstable gene as compared to the expression levels of other selected reference genes (M value: 2.92 using *GeNorm*). We conclude that this gene varies in our experimental conditions with respect to other selected reference genes. Hence it should not be used to normalize gene expression data in our experimental conditions.

TABLE 4 | Characteristics of MS patients according to the clinical classification.

	RRMS ($n = 18$)	SPMS ($n = 11$)	PPMS ($n = 11$)	*p*
% Females (n)	83.3 (15)	72.7 (8)	63.6 (7)	0.48 (χ^2)
Age (mean, *SD*)	27.3 (7.2)	27.9 (7.3)	38.9 (11.2)	0.003 (ANOVA test)
EDSS	2.4 (1.2)	6.2 (1.5)	6.3 (1.1)	<0.001 (ANOVA test)
Evolution time	8.1 (5.4)	13.5 (7.8)	13.8 (5.7)	0.043 (ANOVA test)

After Bonferroni correction, significance were due to differences between the age at beginning between PPMS and the other two MS forms; between the EDSS of RRMS and the two other MS forms; the evolution time between PPMS and RRMS.

TABLE 5 | Characteristics of MS patients according to new proposal and working classification.

	Inflammatory MS ($n = 21$)		Medullar MS ($n = 8$)	PPMS ($n = 11$)	*p*
	G+/M− ($n = 10$)	G+/M+ ($n = 11$)			
% Females (*n*)	90 (9)	81.8 (9)	62.5 (5)	63.6 (7)	0.40 (χ^2)
Age (mean, *SD*)	26.7 (4.8)	26.3 (8.7)	31.4 (7.0)	38.9 (11.2)	0.005
EDSS	2.5 (1.5)	3.4 (2.2)	6.2 (1.4)	6.3 (1.1)	0.000
Evolution time	8.9 (6.3)	10.8 (5.9)	8.5 (3.1)	13.8 (5.7)	0.154

After Bonferroni correction, significance was due to differences between the age at beginning and the EDSS between Medullar MS and PPMS with the inflammatory MS.

TABLE 6 | Candidate housekeeping genes ranked in cerebellar granule neurons treated with CSF of MS/NMO patients according to their expression stability by *Genorm* and *Normfinder* methods.

Genorm			*Normfinder*		
Ranking order	**Gene name**	**Average *M* value**	**Ranking order**	**Gene name**	**Stability value**
1	***Tfrc***	**1.092**	**1**	***Tfrc***	**0.546**
1	***B2m***	**1.092**	**2**	***Ldha***	**0.589**
2	***Rpl19***	**1.198**	**3**	***Rpl19***	**0.972**
3	*Ldha*	1.253	4	*B2m*	1.102
4	*Hprt*	1.318	5	*Hprt*	1.379
5	*ActB*	2.929	6	*ActB*	6.099
6	*Gapdh*	4.201	7	*Gapdh*	6.953

M, average expression stability measure of seven reference genes with Tfrc the most stable gene and Gapdh the least stable. Lower M value of average expression stability indicates more stable expression while the highest M value indicates variable expression. Bold indicates most stably expressed genes.

Hprt

The data indicates that *Hprt* gene was 0.23 folds downregulated in neurons treated with IgM+/− CSF of MS patients as compared to control. The expression was up regulated 1.7 times in IgM+/+ treated neurons and again down regulated by 0.1 folds, 0.27 folds and 0.4 folds in neurons treated with the CSF of medullary, PPMS and NMO patients as compared to control. According to *GeNorm* program, *Hprt* was ranked third last unstable reference genes with respect to other reference genes (Average expression stability value: 1.3).

Rpl19

Rpl19 gene expression was down regulated by 0.2 and 0.5 folds in IgM+/− and IgM+/+ treated neurons as compared to control. There was only 0.1 folds decrease in *Rpl19* gene expression when neurons were treated with medullary, PPMS and NMO patients as compared to control. Hence, there is a negligible variation in all the experimental conditions as indicated by qPCR data. In agreement with this data *GeNorm* identifies this gene as the third most stable gene (M value: 1.19).

Ldha

There was 0.5 folds downregulation of *Ldha* gene in neurons treated with the CSF of IgM+/− patients with respect to control. The expression level increased up to fourfolds in neurons treated with IgM+/+ treated neurons. In medullary, there was a 0.17 folds decrease in gene expression and we found 0.28 folds and 0.54 folds decrease gene expression in PPMS and NMO patients. The data signifies that the expression of this gene is not constant in all the experimental conditions. The average expression stability (M) value of this gene was 1.25 and was ranked as the fourth stable gene according to *GeNorm* software.

Tfrc

Tfrc gene was up regulated by 1.2 folds in neurons treated with IgM+/− treated neurons as compared to control. In IgM+/+ treated neurons the expression level almost remained the same as compared to control. In medullary patients the expression was reduced by 0.2 folds and in PPMS treated neurons the level was increased by only 1.1 folds which was almost similar

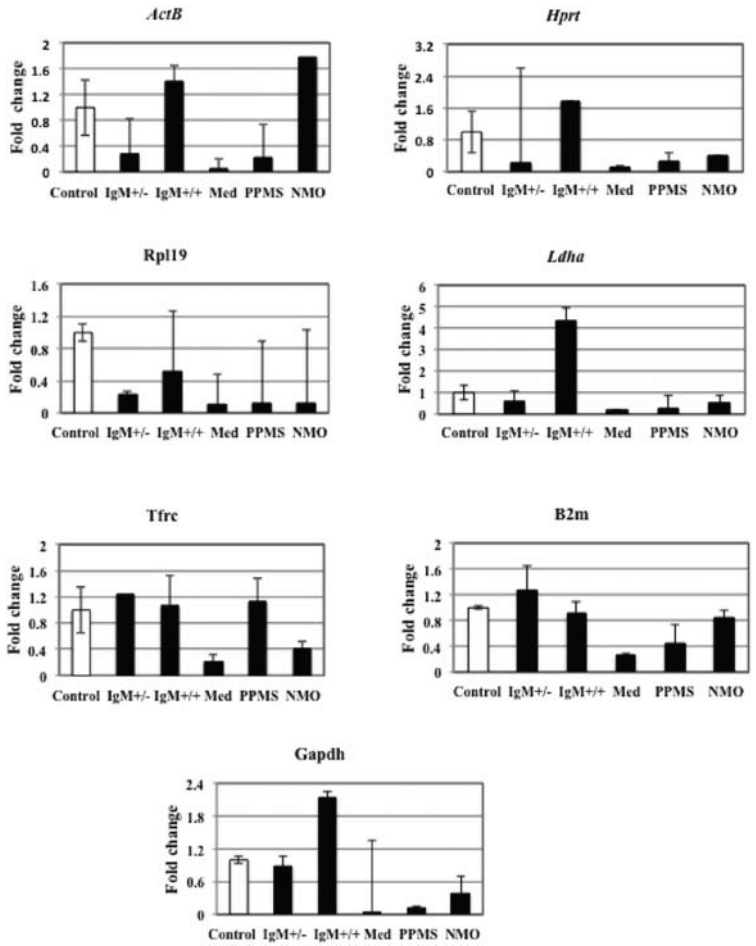

FIGURE 3 | Fold change for each reference gene tested in distinct disease courses of multiple sclerosis (MS). IgM+/+ and IgM+/−: Inflammatory forms of relapsing remitting MS; Med: medullary form; NMO: neuromyelitis optica; Control: other non-inflammatory neurological diseases (NIND).

as compared to control. There was a downregulation of this gene by 0.4 folds in neurons treated with the CSF of MS patients. Overall, there was a negligible variation in the gene expression in different experimental conditions. According to the *GeNorm* algorithm the average expression stability value was 1.092 and it was ranked the best reference gene with respect to others.

B2m

The data indicates that there was 1.2 folds up regulation of *B2m* gene in IgM+/− treated neurons as compared to control. The expression was down regulated by 0.9 folds in IgM+/+ treated neurons which was not a large variation as compared to control. It dropped to 0.2 folds in medullary treated neurons and 0.4 folds in PPMS treated neurons. The expression decreased by 0.8 folds in NMO treated neurons as compared to control. According to the *GeNorm* algorithm the average expression stability value was similar to *Tfrc* average expression stability (M: 1.092) and it was also ranked the best reference gene with respect to others. We conclude that both *Tfrc* and *B2m* with similar average expression stability values should be used to normalize gene expression data in our experimental conditions.

Gapdh

The expression level of *Gapdh* gene was 0.89 folds lower in IgM+/− treated neurons as compared to control. The expression level increased by twofolds in IgM+/+ treated neuons as compared to control. In neurons treated with the CSF of medullary MS patients the gene downregulated by 0.02 folds and by 0.1 fold in neurons treated with the CSF of PPMS patients. Similarly the expression level declined by 0.38 folds in NMO treated patients. According to qPCR data that there is a huge fluctuation in *Gapdh* gene expression in our experimental conditions. Normally, *Gapdh* is used as a housekeeping gene but we find that it is not a housekeeping gene in our experimental conditions. *GeNorm* ranked this gene as the least stable gene with 4.2 as the average expression stability value.

Discussion

Quantitative RT-PCR has recently become the most widely accepted method of quantification for its sensitive, accurate and reliable determination of gene expression levels in cells and tissues. To avoid sample-to-sample variation, normalization of gene transcripts is required. The conventional way to perform normalization is to select a housekeeping gene whose expression is believed to remain stable in all cell types/tissues, during cellular development and under various experimental conditions then relate the expression of gene of interest to that of a housekeeping gene. For many years it has been assumed that the genes such as β-*Actin* and *Gapdh* express constitutively in all cells and tissues. β-*Actin* (*ActB*) is a cytoskeletal protein that maintains the structure and integrity of cells. GADPH, on the other hand, is a key glycolytic enzyme involved mainly in the production of energy. Since both *ActB* and *Gapdh* are involved in maintaining the basic metabolic functions of a cell, they are presumed to express at stable levels. Therefore, they are employed as common internal controls in most of the laboratories. However, several lines of evidence show that their rate of transcription is affected by a variety of factors such as epidermal growth factor, transforming growth factor-β and platelet-derived growth factor while constitutively expressed (Elder et al., 1984; Leof et al., 1986; Keski-Oja et al., 1988). Therefore, their expression may not necessarily be constant in all conditions. Furthermore, GADPH is implicated in non-metabolic processes independent of its metabolic function, such as transcription activation, vesicle transport from endoplasmic reticulum to Golgi apparatus and polymerization of tubulin into microtubules (Kumagai and Sakai, 1983; Durrieu et al., 1987; Muronetz et al., 1994; Zheng et al., 2003; Tisdale and Artalejo, 2007). Previous literature reveals that neuronal apoptosis is associated with suppressed glycolytic activity of GADPH (Burke, 1983; Dastoor and Dreyer, 2001; Makhina et al., 2009). It has been observed that GADPH interacts with other proteins which results in reduced glycolytic activity (Hara et al., 2005). This process may lead to neuroaxonal damage in neurodegenerative diseases such as Huntington's, Parkinson's, and Alzheimer's disease (Vécsei and Pál, 1993; Mazzola and Sirover, 2003; Senatorov et al., 2003; Li et al., 2004; Tsuchiya et al., 2005; Kolln et al., 2010). The realization that these reference genes may fluctuate in different experiments has led to their pre-validation for their expression stability.

This is the first study to the best of our knowledge that reports the most stable HK genes in CGNs treated with CSF from MS/NMO patients. Seven commonly used housekeeping genes were chosen from the available literature. Expression levels of HK genes in different MS clinical forms were quantified by qRT-PCR. Our results reveal that *Gapdh* expression levels changed in all forms (RRMS, PPMS, NMO) as compared to controls. This gene was not among the best reference genes therefore it is strongly advised not to employ it as a control in studies related to current one. Moreover, β-*Actin*, that is often used as a loading control also showed unstable expression in all conditions, though to a lesser extent than *Gapdh*. Transferrin receptor (*Tfrc*) gene was up regulated by 1.2 folds in neurons treated with IgM+/− treated neurons as compared to control. In IgM+/+ treated neurons the expression level almost remained the same as compared to control. In medullary patients the expression was reduced by 0.2 folds and in PPMS treated neurons the level was increased by only 1.1 folds which was almost similar as compared to control. There was a downregulation of this gene by 0.4 folds in neurons treated with the CSF of MS patients. Overall, we see from the data that there was a negligible variation in the *Tfrc* gene expression in different experimental conditions. *Tfrc* is required for iron delivery from transferrin to cells. Microglobulin beta-2 (*B2m*), a component of MHC class I molecule, showed higher expression in IgM+/− treated neurons as compared to control. The expression was down regulated by 0.9 folds in IgM+/+ treated neurons which was not a large variation as compared to control. It dropped to 0.2 folds in medullary treated neurons and 0.4 folds in PPMS treated neurons. The expression decreased by 0.8 folds in NMO treated neurons as compared to control. According to the *GeNorm* algorithm the average expression stability value was similar to *Tfrc* average expression stability (M: 1.092) and it was also ranked the best reference gene with respect to others. We conclude that both *Tfrc* and *B2m* with similar average expression stability values should be used to normalize gene expression data in our experimental conditions. Hypoxanthine guanine phosphoribosyl-transferase (*Hprt*) gene showed fluctuated expression level in different experimental conditions. It plays an important role in purine salvage pathway. According to *GeNorm* program, *Hprt* was ranked third last unstable reference genes with respect to other reference genes (Average expression stability value: 1.3). Ribosomal protein L19 (*Rpl19*) showed negligible down regulation in all the different experimental conditions. *GeNorm* identifies this gene as the third most stable gene (*M* value: 1.19). On the contrary, *Ldha* gene was upregulated in IgM+/+ but down regulated in IgM+/− and medullar clinical form of RRMS. Its expression further lowered in PPMS and NMO. The data signifies that the expression of this gene was not constant in all the experimental conditions and not suitable for normalization of gene transcripts in studies related to the current one.

Overall, *geNorm* and *NormFinder* algorithms identified *Tfrc* and *B2m* the best housekeeping genes and *Gapdh* and *ActB* the most unsuitable genes in our experimental model of MS and

therefore the current study demonstrates the necessity for pre-validation of HK genes for any experimental system. Since both the algorithms are based on different mathematical approaches, the order of genes was not exactly similar. However, both *geNorm* and *NormFinder* rank the traditional reference genes GAPDH and β-actin as most unstable genes. Therefore, we strongly advise to check the expression stability of these genes before using them for normalization purposes.

We conclude from data provided in this study that transferrin receptor (*Tfrc*) and microglobulin beta-2 (*B2m*) as the most stably expressed housekeeping genes in CGNs treated with CSF of MS patients. On the other hand, *Gapdh* and β-*actin* showed highly fluctuated expression indicating their unsuitability for such studies. This study demonstrates the usefulness of pre-validating the expression stability of housekeeping genes for normalization of target gene transcripts in gene expression studies. Our data suggest that it is required to determine the suitability of any common HK genes to be used for normalization in "*Omic*" studies, and even such pre-selection should be a routine step for any experimental system in a laboratory.

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