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Sirtuin 3 expression in the context of Relapsing Remitting Multiple Sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a debilitating disease that attacks the myelin sheath surrounding neurons in the central nervous system resulting in focal demyelinated lesions. This process is immune-mediated in nature and is thought to arise following inflammatory and metabolic alterations leading to loss of the myelin sheath. Following this, axons in the afflicted areas may recover and remyelinate or undergo axonal loss leading to eventual neurodegeneration. Current knowledge of the mechanisms involved in lesion formation and neuronal outcomes is limited. A relatively recently identified family of proteins, the sirtuins, have been found to be strongly implicated in inflammation and aging throughout the body. While some work has identified alterations in sirtuins 1 and 2 within animal models and MS samples, no such investigations have examined the related sirtuin 3 (SiRT3) protein. In our current study, we examined SiRT3 expression in MS lesions from post-mortem tissue as well as mRNA levels within CD14+ cells isolated from MS patients and controls. We found reduced SiRT3 expression within MS lesions and trends towards reduced SiRT3 mRNA levels in females as well as MS patients. Overall, our work supports the hypothesis that SiRT3 plays a role in MS, however, further studies are needed to identify the CNS distribution of SiRT3 in MS patients, how SiRT3 alterations impact CD14+ cells in MS, and whether SiRT3 may play a role in the sex differences observed in MS.

Significance: It is clear that both inflammatory and metabolic processes can play a role in the pathogenesis of MS, however, this requires further investigation. Sirtuin 3 is an NAD+ dependant deacetylase that plays a role in both in metabolism and inflammation and has been implicated in a variety of disease states. Herein, we present the first evidence demonstrating that sirtuin 3 may be involved or at least altered in the context of MS pathology.

INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated disorder which attacks the central nervous system (CNS) and presents with hallmark focal demyelinated plaques. The incidence prevalence of MS continues to increase and worldwide 2.8 million people suffer from the disease.[1] MS is a highly heterogeneous disease and is believed to arise through an interplay between genetic vulnerabilities and environmental factors, however, no definite etiology has yet been confirmed.[2] Most patients are diagnosed between the ages of 15 and 40 and the prevalence is approximately 3 times greater in females compared to males.[3] Early symptoms of the disease manifest as temporary neurological phenomena, such as the loss of sensation, tremors, difficulty speaking, or paralysis.[2] Most patients have slowly worsening attacks which lead to progressive and irreversible neurological and cognitive symptoms over time in a disease category known as Relapsing Remitting Multiple Sclerosis (RRMS).[2,4] While the mechanistic underpinnings that give rise to MS are not currently known, the symptoms are the result of demyelinating lesions and subsequent neuronal loss.[2] These lesions are partially the result of the infiltration of immune cells into the CNS and can be examined histologically post-mortem.[5]

To date, no treatment has reliably altered the long-term outcomes of MS, however, numerous disease-modifying therapies (DMTs) have been identified with the ability to treat symptoms, reduce relapses and slow the course of the disease.[6–8] DMTs can reduce the frequency of relapses and have been demonstrated to slow disease progression in RRMS. Due to the heterogeneous nature of MS and toxicity associated with many MS treatments each patient must be managed at an individual level.[7,9]

Biologically speaking, MS is characterized by focal lesions within the CNS classically occurring in the optic nerve, cerebellum, brainstem, subpial spinal cord, and periventricular white matter.[5] During the progression of a lesion, a large influx of inflammatory macrophages and T-cells results in a hypercellular demyelinated region. During and following myelin loss, axons stripped of their conductive sheath can either atrophy or can remyelinate from surviving oligodendrocytes and/or

newly recruited oligodendrocyte progenitor cells.[5] Over time the lesion sites become hypocellular and inactive sites can be readily identified through a paucity of Luxol fast blood staining.[10]

Microglia are the resident immune cell of the CNS and part of the innate immune system.[11] In a healthy state, microglia possess a ramified morphology and extend long projections that actively survey the microenvironment for markers of cell injury, death or infection (i.e. danger signals).[12,13] Upon contact with these signals, the projections retract and the microglia migrate to the site and begin to produce cytokines and phagocytize debris, damaged cells or pathogens.[14] Concurrently, microglia are able to modulate astrocytes to alter blood brain barrier permeability and recruit circulating peripheral immune cells.[15]

Emerging evidence has implicated microglia as having a significant biological role in the function and dysfunction of neurons. Microglia engage in bilateral communication with neurons through a variety of molecules and receptors including the CX3CL, C1q, BDNF, IL-1B, and TNF-α.[16] Interestingly, in MS specifically, 48 of the 81 genes implicated in MS are highly expressed in the microglia.[17] Furthermore, recent advances in transcriptional profiling have identified differences in microglial protein expression in healthy appearing CNS tissue of MS patients to healthy controls.[18] Additional studies have confirmed these results and found alterations between microglia found in normal appearing grey and white matter of MS patients.[19] One family of proteins that has been found to modulate microglial activation are the sirtuins.[20]

The sirtuin (SiRT) family of proteins are nicotinamide adenine dinucleotide (NAD) linked deacetylases and are highly conserved across mammalian species.[21] Sirtuin activity is linked to intracellular NAD concentration, thus the regulation of sirtuin function is partially mediated by dietary intake and fasting. Sirtuin family members are set apart by cellular localization and downstream targets. Sirtuins 3,4,5 are found mainly localized to the mitochondria, while sirtuins 1,6,7 are in the nucleus and SiRT2 is found in the cytoplasm. Mutations within these genes have been linked to a variety of illnesses, including macular degeneration[22], obesity[23], and cancer.[24–26] Despite this, there is substantial evidence to suggest that some rare alleles have beneficial effects and indeed upregulated expression of sirtuins is protective in several disorders, including cardiovascular disease and Parkinson's disease.[27,28] Thus far, sirtuins 1,2, and 3 have been well characterized. Despite their different sub-cellular locations these three proteins have highly similar protein targets including immunoregulatory proteins FOXO3a and the p65 subunit of the NF-kB complex.[29]

Given the role of inflammatory microglia and T-cells in MS, the sirtuin family of proteins has been extensively examined in the past decade.[30] Most research has focused on sirtuin 1 and sirtuin 2, as well as several genome wide association studies reporting risk alleles in SiRTs 4 and 5.[30,31] Upregulation of SiRT1 through genetic mouse models and *in vitro* activators protects axonal density, reduces neuronal death and protects from gross symptoms in the experimental autoimmune encephalomyelitis (EAE) model of MS.[20,32,33] Experiments examining human MS patients have found that SiRT1 levels are decreased in glatiramer acetate (a commonly prescribed DMT) non-responders and these levels drop in the plasma of MS patients during relapses.[34]

The story with SiRT2 is largely similar although less well-characterized. SiRT3 has been found to be downregulated in a host of inflammatory models involving CNS tissue over the past decade.[35–37] With regards to MS specifically, one preliminary paper by Rice *et al.* reported reduced SiRT3 immunohistochemical labelling in the brains of MS patients, however, they did not examine plaques specifically, identify potential cell types of interest, or present statistical data to back up their assertation.[38] While SiRT3 acts on many of the same targets as SiRT1 and SiRT2, its physiologic location inside the mitochondria means that it plays a more specific role in mitochondrial dysfunction and internal oxidative stress than other sirtuins.[39,40]

Further research into SiRT3 could possibly help explain the loss of some neurons relative to others in MS and may be a potential therapeutic target with fewer off-target effects than existing treatments. In macrophages, increased SiRT3 levels leads to reduced ROS generation while overexpression acts to increase antioxidant production through the FOXO3a pathway both in tissue culture and rodent models.[41] SiRT3 has also been implicated in the fractalkine signalling pathway, whereby increased SiRT3 levels enhances CX3CR1 expression and microglial motility while reducing inflammatory status.[42–44] In this current investigation, we sought to determine whether SiRT3 expression differed within MS lesions and whether myeloid-derived cells within peripheral blood (CD14+) reflected similar changes.

METHODS

Samples

All experiments involving human participation were approved by the Newfoundland Health Research Ethics Board. MS patients were recruited through the Health Research Innovation Team in Multiple Sclerosis (HITMS) at Memorial University of Newfoundland, St. John's, NL, Canada. Sample selection was conducted based on a pilot study (data not shown) raising the question of sex effects. All male RRMS samples present in the HITMS study were selected with the following criteria: 1. Diagnosis of RRMS 2. Male sex and 3. Not on active treatment at time of collection. Female samples closest in age to the male samples were selected on the same three criteria. Control samples closest in age to each RRMS sample donated from healthy male and female volunteers were selected. Venous blood was previously drawn from relapsing–remitting MS (RRMS) patients and healthy controls with informed consent. PBMCs were isolated following ficoll-density gradient centrifugation, and CD14+ monocytes were subsequently isolated to ~95% to 98% purity using anti-CD14 magnetic beads (Miltenyi). To examine post-mortem MS tissue, CNS sections from subcortical white matter lesions and control regions in MS patients who died of complications of MS were obtained from autopsies conducted by Eastern Health between 1999 and 2019. Paraffin-embedded CNS tissue from these samples was serially sectioned and mounted on consecutive slides. Samples included in the study were selected on the basis of having known inactive MS lesions and normal adjacent tissue in the same paraffin block.

Antigen Retrieval

Slides were rinsed in three changes of xylene substitute for 3 minutes each and then sequentially in 100% EtOH, 100% EtOH, 95% EtOH, 70% EtOH and distilled water for 3 minutes each to remove paraffin and rehydrate the tissue. Slides were then submerged within a Coplin jar inside of a 1L beaker of sodium citrate buffer (10mM Sodium citrate, 0.05% Tween 20, pH 6.0) and microwaved for 20 minutes before cooling to room temperature in 10mM phosphate buffered solution (PBS).

Immunohistochemistry

Following antigen retrieval, sections were rinsed twice in PBS-T (10mM PBS, 0.05% Tween 20) for five minutes each and placed in blocking solution (10% normal goat serum, 1% horse serum in PBS) for 30 minutes. Slides were then incubated in blocking solution with 1:100 SiRT3 antibody (Sigma #54072). Slides were washed through three x five-minute PBS changes and incubated for one hour in blocking solution with 1:200 anti-rabbit biotinylated IgG (Vector Labs BA-1000) and washed thrice more in PBS. HRP was attached to the biotin using the Vectastain Elite ABC kit (PK-6102) following the included instructions for one hour. Slides were washed through three five-minute PBS changes and then incubated with DAB peroxidase substrate (Vector Labs SK-4100) for five minutes and rinsed three more five-minute PBS washes. Slides were then dipped in tap water for ten seconds, coated in hematoxylin QS (Vector Labs H-3404) for thirty seconds and rinsed in running tap water for ten seconds. Slides were allowed to dry for 24 hours and then dehydrated and cover slipped with Permount (Fisher Scientific SP15100).

Imaging

Slides were imaged using Cytation5 plate reader at 4X to define lesion areas and 10X to acquire images for analyses. A total of 4 areas within each lesion and immediately adjacent (4-600ums from the border) were imaged along with serial sections stained with Luxol Fast Blue and H&E for anatomical comparison. A no primary control was completed (data not shown) and no non-specific labelling was detected.

RNA Isolation and qPCR

Cells were lysed in QiaZOL reagent (Qiagen 79306) and stored at –80°C. Total RNA was isolated by RNeasy column extraction with a DNase treatment step (Qiagen, 74004). RNA was quantified using a Nanodrop. For gene expression assays, RNA (200 ng) was reverse transcribed using M-MLV reverse transcriptase (Invitrogen, 28025013). SiRT3 expression assays were performed using SiRT3 TaqMan probes (Thermofisher Scientific, 4331182) and normalized to the endogenous control gene GAPDH.

Analyses

Images were analysed using FIJI to extract determine the number of nuclei, SiRT3 positive cells and relative staining intensity of the non-nuclei areas of the tissue. The average between the four regions was taken and used for analysis. RNA expression fold changes were calculated using the ΔΔCt method using GAPDH as an endogenous control. Graphpad Prism 6 software was used to conduct paired t-tests for immunohistochemical results while a Student's t-test was used to determine overall SiRT3 RNA changes and a two-way ANOVA used to examine the interplay of sex and disease status.

RESULTS

Using post-mortem CNS tissue, we examined SiRT3 protein expression within MS lesions and adjacent CNS tissue (Figure 1) and observed an overall mean integrated density (Figure 2A) increase indicating reduced SiRT3 expression in lesioned tissue compared to control regions ($p=0.0256$). There was no significant alteration in the average number of nuclei per field (Figure 2B, $p>0.10$) nor in the ratio of SiRT3+ nuclei to SiRT3- nuclei (Figure 2C, $p>0.10$).

Utilizing CD14+ cells isolated from the whole blood of MS patients and healthy controls, we assessed mRNA expression levels of SiRT3. No statistically significant differences were observed (Figure 3A, $p>0.10$), however, when stratifying the data based on sex, both females and MS patients trended towards reduced SiRT3 mRNA expression (Figure 3B, $p=0.0875$ and $p=0.1066$ respectively) while there was no interaction ($p>0.10$). Collected demographics are shown in Table 1. No effects of age or disease duration were observed (data not shown).

DISCUSSION

Over the past decade, research has rapidly defined a role for the sirtuin family proteins in a variety of diseases, including cancer, heart disease, Parkinson's disease, and MS. Sirtuins 1 and 2 have both been linked to MS. Sirtuin 1 is increased in acute and chronic lesion sites and decreased in PBMCs in relapse phase[34,45] while some sirtuin 2 isoforms are decreased in MS lesions matching data from rodent EAE model comparators.[46] Despite these links, to date there has been very little literature published regarding a potential role for SiRT3 in MS. SiRT3 shares many pathways both up and down stream with other sirtuin family proteins, however, its location within the mitochondria, dysfunction of which is implicated in MS pathogenicity may contribute to metabolic and inflammatory disease pathways apart from other sirtuins.[47,48] We set out to determine whether SiRT3 may be linked to MS pathology through the exploration of cells and tissues from MS patients and healthy controls. A previous report from Rice *et al.* in 2012 asserted that SiRT3 expression was reduced in non-lesioned gray matter of MS patients compared to controls, however, no statistical analyses was presented, and no further papers have explored SiRT3.[38]

In this study, we compared SiRT3 protein expression levels in inactive MS lesions to adjacent parenchyma. Our findings in Figures 1 and 2 support the hypothesis that SiRT3 may be altered in the CNS of MS patients, specifically we found a reduction in SiRT3 within the lesion. In the brain regions examined, we found that SiRT3 was expressed in nearly every cell (Figure 2B), however, no gross expression changes between lesion and non-lesion locations appeared evident. Variability between lesions, both within and between individuals has been previously established and obtaining a larger sample set may have enabled an analysis of SiRT3 expression to be conducted based on lesion classification.[10] Based on evidence from other neurodegenerative diseases, we hypothesized that SiRT3 expression levels would be reduced during acute inflammatory processes within active lesions. Furthermore, it was anticipated that SIRT3 levels would return to baseline (or become elevated) in inactive and post-demyelinating lesions given the overall reduction of pro-inflammatory cell signalling.

Determining whether SiRT3 levels are altered in MS pathology may provide further insight into the pathology of the disease and help inform treatment responses in individual patients. The ability to predict treatment response using a biomarker readily assessed in whole blood would simplify treatment initiation and reduce the time between diagnoses and effective treatment. An important consideration in determining whether SiRT3 levels are altered is the means to determine this *in vivo* using a non-invasive approach (i.e. optimal predictive biomarker). Given the involvement of microglia (a macrophage) in MS lesions we opted to investigate levels of SiRT3 specifically in CD14+ (macrophage) cells. To this end, we extracted CD14+ cells from the whole blood of healthy controls and MS patients. Samples were matched for age and sex; all MS samples analyzed were derived from DMT-naïve individuals. No overall impact of MS status on SiRT3 mRNA levels were observed (Figure 3A), however, when stratifying the data based on sex and disease, we noted a bi-modal distribution in male patients whereby SiRT3 expression levels appeared to be either greater than the mean or less than it while female samples tended to cluster around the mean. Overall, female samples and MS samples both trended to have reduced SiRT3 expression, although no interaction was noted (Figure 3B). Previous studies examining sex differences in SiRT3 expression have been performed. For example, SiRT3 expression in cardiac myocytes decreased with age in females, but not males.[49] This is possibly due to alterations in estradiol mediated SiRT3 increases, however, few papers have investigated this.[50,51] In addition, animal studies have found that SiRT3 expression is decreased in male mice and contributes to greater ischemic kidney damage and poor metabolic responses to high fat diet.[51,52]

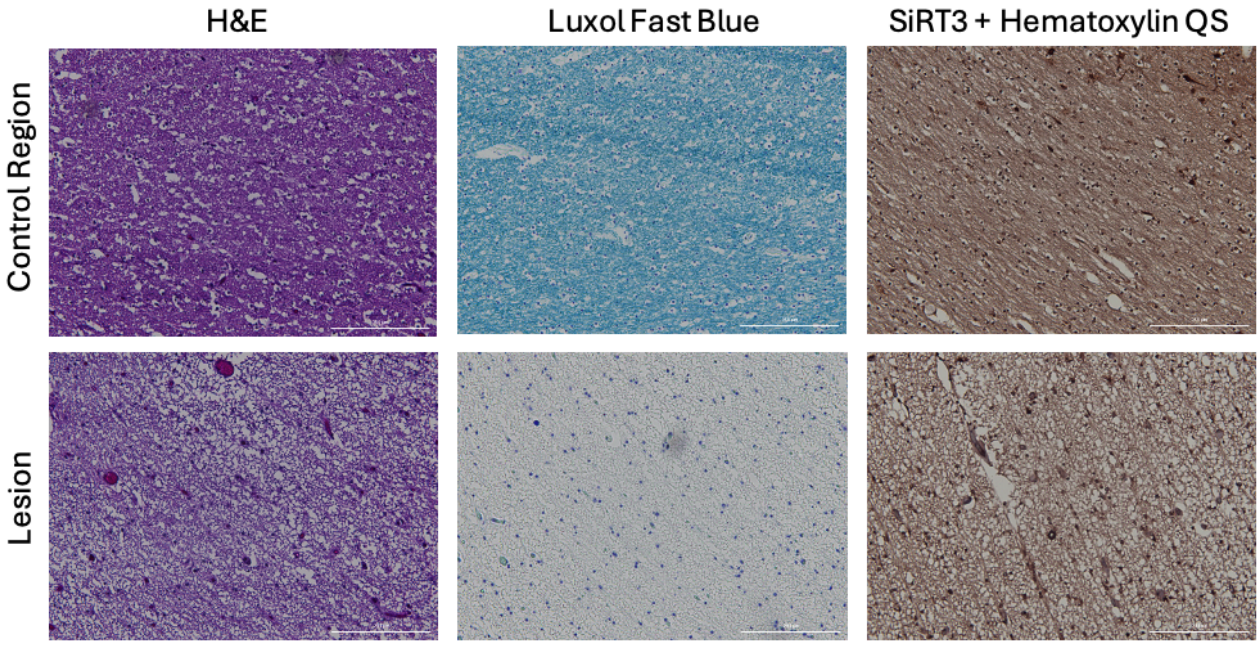

Figure 1: Representative images of human brain tissue (n=3) immunohistochemically stained for Sirt3 in order to identify expression patterns within and adjacent to demyelinated lesions. All lesions and control regions were located in the subcortical white matter of specimens and demonstrated reduced Luxol fast blue and H&E staining. Control sites were randomly selected adjacent to each lesion.

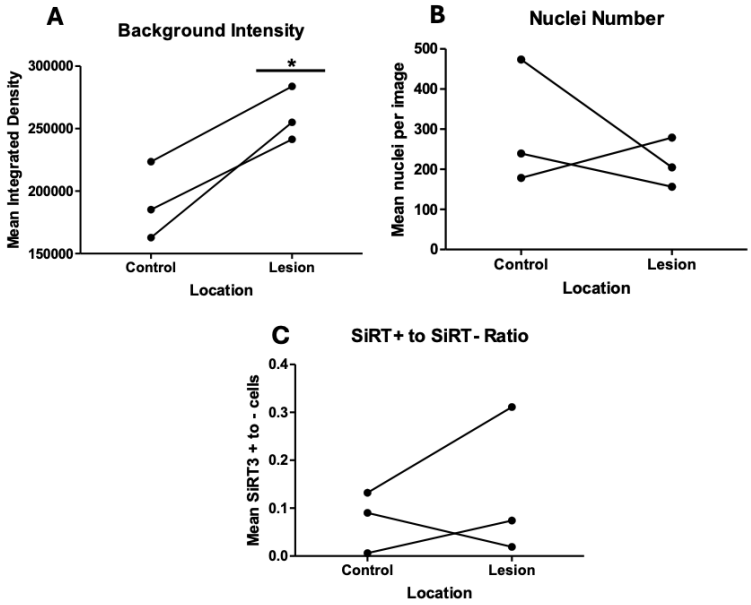

Figure 2: Staining in **Figure 1** was assessed by examining the integrated density of non-nuclei associated staining **(A),** through looking at total nuclei counts **(B)**, and examining the ratio of SiRT3+ cells to SiRT3- cells **(C)**. No statistically significant findings were identified in **B** or **C** using a paired t-test, however, there was an overall decrease (p=0.0256) in **A**, non-nuclei associated SiRT3 staining based on increased mean integrated density.

Table 1: CD14+ Cell Participant Demographics

Group	Average Age	Average Disease Duration
Male RRMS n=8	51.8 +/- 2.2	13.3 +/- 3.3
Female RRMS n=5	50.2 +/- 2.4	17.0 +/- 4.0
Male Control n=8	51.6 +/- 2.9	0 +/- 0
Female Control n=7	50.8 +/- 1.6	0 +/- 0

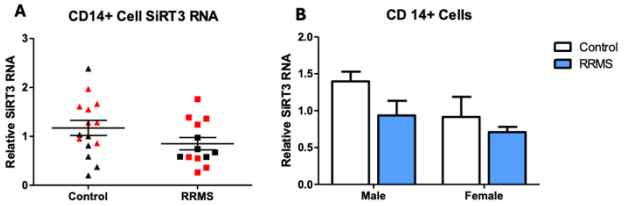

Figure 3: CD14+ cells isolated from peripheral blood of treatment negative MS patients and healthy controls was probed for SiRT3 RNA using Taqman primers, with means and standard error plotted. Male samples are represented in red. A) In age and sex matched samples RRMS samples appeared to have lower SiRT3 expression (p=0.12). B**)** Breaking down the population in B we found trends toward lower SiRT3 in females (p=0.08), RRMS patients (p=0.1066) but no interaction (p=0.520).

Importantly, MS is a highly heterogenous disease and a large proportion of our samples were obtained from participants who were DMT-naive and older than the typical age of first presentation. Due to these factors, it is likely that our sample represents individuals with less aggressive forms of RRMS and thus our data may not be fully reflective of the MS population. The average age of study participants could have also influenced our findings given that MS is typically diagnosed in younger individuals; the average age in our study was 51, which may also complicate identification of sex differences as some females may have been undergoing or post menopause. Further work using samples collected at first presentation with later blood draws during remission and treatment in a larger number of individuals would enable a greater level of analysis. A data set such as this could inform the timeline of any SiRT3 changes and could potentially identify whether SiRT3 levels predict treatment response or disease course. Our findings suggest that SiRT3 may be a protein of interest for further MS research and may contribute in some way to the sex differences observed in the clinical population.

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