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Chapter

Enkephalin Therapy Improves Relapsing-Remitting Multiple Sclerosis

Chirag L. Patel, Ian S. Zagon, Gary A. Thomas and Patricia J. McLaughlin

Abstract

Multiple sclerosis (MS) is accompanied by decreases in serum endogenous enkephalin/endorphins and alterations in inflammatory cytokines. This retrospective analysis of serum levels was conducted in 53 patients with established relapsing-remitting MS treated with the disease-modifying therapies (DMT) glatiramer acetate, dimethyl fumarate or with the biotherapeutic low dose naltrexone (LDN) to elevate enkephalins, an off-label alternative. Opioid growth factor (OGF), an inhibitory endogenous opioid involved in modulating cellular replication, was measured and correlated to serum β-endorphin, IL-17A and TNFα. Results revealed that MS leads to a significant reduction in OGF levels in subjects on DMTs, but patients on LDN had OGF levels comparable to non-MS controls. Individuals on DMTs had significantly elevated TNFα levels, while IL-17A levels were significantly elevated only in patients taking dimethyl fumarate. A direct correlation was established between OGF and IL17A indicating a potential interaction between the OGF-OGFr axis and pro-inflammatory T-helper cells providing insight into the disease etiology.

Keywords: relapsing-remitting multiple sclerosis, serum cytokines, opioid growth factor, low-dose naltrexone, biomarkers

1. Introduction

Multiple sclerosis (MS) is a demyelinating disorder with an underlying neuroinflammatory disease process affecting approximately 2.5 million people worldwide [1]. Traditionally, the disease has a greater prevalence in locations which are geographically north of the equator. In a study from 2015, North America and Europe had an average prevalence of greater than 100/100,000 individuals, whereas East Asia and Sub-Saharan Africa had rates of 2/100,000 individuals [2].

1.1 Environmental and genetic risk factors

To better understand this disparity, an umbrella systemic review looked at the possible environmental risk factors involved in the development of multiple sclerosis [3]. An analysis of 44 potential risk factors was filtered down to 3 which were found to be profoundly significant: anti-Epstein-Barr Virus Nuclear Antigen IgG seropositivity, infectious mononucleosis, and smoking [3]. The hypothesis

behind anti-EBVNA IgG suggests that late adolescence exposure to EBV leads to infectious mononucleosis with significantly elevated IgG titers when compared to individuals who were exposed at a younger age [4, 5]. These titers in turn correlate to an increased risk of developing multiple sclerosis. One study done on this, "high-hygiene" population found that individuals of the same age who were not infected with EBV had a 10-fold lower risk of developing multiple sclerosis when compared to their EBV infected counterparts [4]. The pathology linking EBV titers to the initiation of multiple sclerosis are not yet clear, however it may increase the risk for an autoimmune type response as was seen with Systemic Lupus Erythematous and EBV [4, 6].

Several studies have shown a direct correlation between cigarette smoking and incidence of multiple sclerosis [4, 7–10]. There is some variability in the literature between gender and age groups. One study suggests cigarette smoking at a younger age (<26.4 years) is associated with a 50% increased risk that was alleviated in individuals who were older [8]. A Canadian study comparing gender and smoking history and found that 71.5% males diagnosed with multiple sclerosis had previously smoked compared to 63.6% of females [10]. Smoking has previously been defined in several pathologies including cancer, asthma, atherosclerosis and heart disease, but within multiple sclerosis, the mechanism is still not understood.

Genetic risk factors have come to the forefront of current research as some have been linked with modulation of the immune response. Initial studies linked loci of the Major Histocompatibility Complex (MHC) and Human Leukocyte Antigen (HLA) as contributing risk in the development and progression of multiple sclerosis [4, 11–14]. In particular, HLA-*DRB1 and DQB1* gene loci were thought to play a role in developing the inherent autoimmunity associated with the disease [11, 14–16].

1.2 Diagnosis of multiple sclerosis

In 2001, the McDonald criteria were created to streamline the diagnosis of multiple sclerosis even with its heterogeneous clinical presentation. The initial criteria introduced the utility of magnetic resonance imaging (MRI) and integration of multiple clinical symptoms while removing "clinically definite" and "possible multiple sclerosis" as alternatives [17]. Since 2001, 3 additional revisions have been made to the initial McDonald criteria: 2005, 2010 and 2017 [18–21]. Diagnosis of multiple sclerosis is now approached based on dissemination of time (temporal) and space. Dissemination in space is defined by either clinical presentation or MRI. Clinically, an individual must have symptoms which are distinct to different anatomical locations of the central nervous system. Usually, individuals present with optic neuritis or ocular symptoms and later acquire gait disturbances or peripheral weakness [18, 22]. On MRI, dissemination in space requires T2 evident lesions located in at least two distinct zones such as periventricular, infratentorial, juxtacortical or within the spinal cord [18, 22]. Dissemination in time requires the presence of a gadolinium-enhancing lesion on MRI, indicating an acute or active lesion, along with a non-enhancing lesion [18, 22]. The presence of a new lesion alone can meet the criteria if it is performed on a follow up scan. Essentially, dissemination in time seeks to distinguish multiple sclerosis symptomatology both typical and atypical from other neurological disorders which may share certain characteristics. As of McDonald 2010, CSF analysis is not required in order to make a definitive diagnosis [19–22]. Analysis of CSF typically presents with mildly elevated white blood cell count, protein, and IgG oligoclonal bands which are not typically seen in serum analysis [22]. IgG oligoclonal bands can be found in 90% of multiple sclerosis patients, but it may have a greater role in distinguishing individuals with clinically isolated syndrome (CIS) [20]. Some studies demonstrated that CSF

oligoclonal bands increase the specificity of MR imaging in adults with CIS and pediatrics with radiologically isolated syndrome (RIS) [23, 24]. Beyond the diagnosis of multiple sclerosis, CSF analysis may help distinguish other immune mediated neurological and non-neurological pathologies.

1.3 Clinical presentation of multiple sclerosis

The disease course of multiple sclerosis is usually defined into four clinical subtypes: clinically isolated syndrome (CIS), relapsing-remitting multiple sclerosis, secondary progressive multiple sclerosis, and primary progressive multiple sclerosis. A majority of patients have a disease course defined by stages of relapses and remission which may later translate into the secondary progressive form [25]. Clinically, relapses are defined as distinct episodes of neurological dysfunction which can present as a wide array of symptoms including sensory defects of the limbs, visual loss, motor defects, gait disturbances, vertigo, heat sensitivity (Uhthoff phenomenon), Lhermitte sign and fatigue [22, 26]. However, multiple sclerosis can present atypically in younger individuals, making it difficult to diagnosis properly. The initial episodes are followed by periods of remission where the patient will fully or partially regain normal function and be deemed neurologically stable [27]. In the long term, as some individuals transition from the relapsing-remitting form to secondary progressive, relapses no longer occur, yet patients will experience worsening neurological function [27]. After 10 years of disease, 50% of individuals with relapsing-remitting multiple sclerosis will convert to secondary-progressive, whereas after 25 years of the disorder, more than 90% of the individuals have secondary progressive multiple sclerosis [14].

Prior to establishing criteria for dissemination in time and space, individuals may present with clinically isolated syndrome (CIS) or radiologically isolated syndrome (RIS). Clinically isolated syndrome is defined as a clinical episode resembling a multiple sclerosis attack, after which there is full or partial recovery of neurological function [14, 20, 27]. Studies have shown that individuals with CIS may have an increased rate of conversion to multiple sclerosis especially with the presence of CSF oligoclonal bands and gadolinium enhancing lesions on MRI [14, 18, 20]. Incidental findings of cerebral and spinal cord plaques without a clinical phenotype are defined as radiologically isolated syndrome (RIS). In a 5-year study of 451 patients with RIS, 34% of individuals developed a clinical event for which 9.6% were defined as primary progressive multiple sclerosis [28]. Interestingly, this study was able to strongly correlate the presence of cervical or thoracic spinal cord lesions with the first clinical event [28]. With this increase risk of progression to multiple sclerosis, the utility of disease-modifying therapies and longitudinal monitoring of CIS and RIS has become a priority for clinicians.

1.4 Inflammation in multiple sclerosis

The direct cause of multiple sclerosis still eludes the scientific community; however, several hypotheses have emerged which have been utilized to develop disease-modifying therapies (DMTs) and alter the course of disease. Traditionally, multiple sclerosis has been considered a demyelinating disease of the white matter tracts leading to peripheral symptomatology with an underlying autoimmune cause. Recently, demyelination located in the cerebral cortex and deep gray matter has emerged as a marker of progressing neurological disability [13, 29, 30]. Damage seen to the CNS with infiltration of immune cells suggests the role of peripheral immune response leading to damage of the blood brain barrier prior to established demyelination. Peripheral inflammation in this case may be a result of a foreign

pathogen (virus), autoimmune activation, or a combination of multiple events. Malpass [31] characterized the term "outside-in" based on evidence of cortical inflammation originating in the subarachnoid space in multiple sclerosis patients which transitioned into the white matter [32]. This further strengthens the argument for modulation of the peripheral inflammatory response which is facilitating early multiple sclerosis disease through activation of CD4 T lymphocytes. Once within the CNS, regional activation of microglia, astrocytes and macrophages occurs through expression of cytokines and chemokines from infiltration of T lymphocytes [13, 29, 30, 33]. Activation of specific subsets of CD4 lymphocytes dictates which cytokines are released. Pro-inflammatory cytokines are expressed after direct activation of Th1 and Th17 cells, whereas anti-inflammatory cytokines are a product of Th2 and T-regulatory cells [34–36]. Histopathology of the CNS white matter plaques has shown an abundance of macrophages, CD8, CD4 T lymphocytes, and B lymphocytes [13, 29, 30, 35]. Over time, the prolonged inflammation and infiltration of T and B lymphocytes leads to a disruption of the axonal-glial interaction which results in an increase in gray matter atrophy and axonal loss with marked demyelination [13, 29]. During the remission phase following each flare, there is some evidence to suggest a possible role for regulatory T cells with the induction of Foxp3 [37]. It is possible that during remission, a baseline level of inflammation is present; however, the expression of specific phenotypes of T lymphocytes shifts toward anti-inflammatory/regulatory rather than pro-inflammatory mechanisms. Based on these observations, vitamin D has emerged as a potential homeopathic regulator of immune cell function [2, 3, 38].

1.5 Current disease-modifying therapies

Currently, there is no definitive treatment for multiple sclerosis. Most approved therapies are focused on controlling peripheral inflammation and preventing migration across the blood brain barrier thereby reducing the incidence of acute flares [13, 30, 39, 40]. A majority of these therapies are administered orally, as an injection or through an infusion. With both invasive and non-invasive methods, side effects include increases in liver enzymes, injection site reactions, nausea, diarrhea, and most importantly, progressive multifocal leukoencephalopathy (PML) [39]. The primary therapies prescribed at our institution are Copaxone®, Gilenya®, and Tecfidera®. Copaxone®, glatiramer acetate is an immunomodulator that is FDA approved to reduce the frequency of relapses. Gilenya®, fingolimod, is also an immunomodulator that targets reduction in the number of relapses more than progression of disease. Tecfidera®, dimethyl fumarate, is a combination of fumaric acid esters that was originally approved for oral treatment of psoriasis. Because of the inconsistent or incomplete outcomes from treatment, as well as the evolving nature of the disorder, other biotherapies have been used as adjuvants.

1.6 Enkephalins as therapeutic treatment

One alternative biotherapeutic is the use of low doses of naltrexone (LDN), an opioid receptor antagonist. LDN is often used as an adjuvant to disease-modifying therapy to target fatigue associated with either the disorder or the medication. LDN has a strong profile of safety and tolerability [41–44]. Pilot studies utilizing LDN demonstrated that MS patients had an improvement in peripheral spasticity and mental health composite scores without inducing any side effects [42, 45]. In a retrospective analysis which compared LDN patients with LDN plus Copaxone® patients, there was no significant difference between the groups with respect to MRI, complete blood count, liver enzymes and the 25-foot walk test [44].

A number of published studies, clinical trials and anecdotal stories have supported the use of LDN as a beneficial therapy for multiple sclerosis [41–48]. The mechanism of action for this general antagonist is to block the interaction of Opioid Growth Factor (OGF) (chemically termed [Met⁵]-enkephalin) from interacting at the nuclear-associated receptor OGFr. In addition to being a neurotransmitter, OGF is an inhibitory growth factor that suppresses proliferation of cells, including T and B cells associated with autoimmune disorders. Naltrexone was initially developed to treat opioid use disorder and alcoholism at a dosing of 50 mg where it acts as an opioid receptor antagonist for mu, delta, and kappa opiate receptors. At a dosage less than 5 mg, LDN acts as a biotherapeutic and modulates the activity of endogenous enkephalins and endorphins [49, 50]. In the multiple sclerosis patient population, endogenous [Met⁵]-enkephalin (i.e., OGF) is reduced when compared to non-multiple sclerosis patients [51]. It is hypothesized that the reduction in serum levels of this inhibitory growth factor are unable to control the increase in T cell proliferation that occurs with immune-related flares. These T cells are the source of other pro-inflammatory cytokines that exacerbate the symptomatology of multiple sclerosis. The decreased serum levels of OGF appear to be compensated by low dosages of naltrexone (LDN).

1.6.1 Laboratory studies on LDN

Animal studies have been used to study both the mechanisms of LDN, as well as to establish the role of LDN as a biotherapy in mice with experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. In these studies, mice were immunized with antigens against myelin proteins—either myelin oligodendrocyte glycoprotein 35–55 or proteolipid peptides 139–151. The key component in the mechanistic pathway is the duration of opioid receptor blockade. This work is detailed in animal studies [51–57]. LDN produces an intermittent blockade of OGFr preventing OGF from binding and thereby increasing cellular replication, similar to what is seen with high doses of Naltrexone (HDN) through a prolonged blockade of OGFr [49]. Conversely, after the blockade has subsided, within 4–6 h, there is over proliferation of endogenous OGF and OGFr and a resulting exaggerated expression of p16 and p21 leading to promotion of cellular senescence [49].

A newly discovered pathway involves the antagonism of Toll-like receptor 4 (TLR4) by LDN as means to reduce neuroinflammation or persistent pain [58, 59]. Specifically, activation of Toll-like receptor 4 (TLR4) initiates the release of inflammatory cytokines: interleukin 1 (IL-1), tumor necrosis factor α (TNFα), nitric oxide (NO), and interferon β (IFNβ) [48, 60]. Currently, the relationship of the Toll-like receptor pathway to pro-inflammatory cytokine activation is not completely understood.

1.6.2 Clinical studies on LDN

Several studies have been conducted to evaluate multiple sclerosis patients who are prescribed LDN. The use of enkephalins, particularly OGF, has been demonstrated to be safe and tolerable in Phase I and Phase II studies of pancreatic cancer [61, 62]. LDN is widely used for treatment of other autoimmune disorders including Crohn's disease [63] and fibromyalgia [64].

1.6.3 Clinical studies on LDN and multiple sclerosis

Clinical trials using LDN for multiple sclerosis were conducted more than a decade ago, and most likely because it is widely used with no side-effects,

government agencies are reluctant to support new trials. Three clinical trials have suggested that LDN increased the quality of life of patients with relapse-remitting or secondary progressive multiple sclerosis, without serious adverse effects [42, 43, 45]. Two retrospective studies examining charts of patients prescribed LDN alone, as well as in combination with the disease-modifying therapy Copaxone®, revealed no exacerbation of the disease or any substantial side effect for patients in either cohort [44]. In this study, the average length of disease was 14 years, with an average of 3 years on LDN alone. Clinical laboratory data revealed that patients on LDN alone had no significant differences in their blood chemistry, nutrition or liver data from patients on disease-modifying therapies.

However, these studies did not measure serum enkephalins, endorphins, or cytokines in an effort to gain more information on the mechanism of action for this biotherapeutic. A small study obtained stored serum from relapsing-remitting multiple sclerosis subjects and reported that enkephalin (i.e., OGF) levels were depressed relative to controls [51]. In the few individuals on LDN alone, serum OGF levels were elevated 2-fold in comparison to multiple sclerosis subjects on Copaxone® alone, suggesting that LDN may be effective at restoring serum enkephalin. Given this information, studies on both the mouse model of EAE and multiple sclerosis have been pursued to evaluate select cytokines that may be dysregulated in multiple sclerosis and possibly modulated by LDN (and enkephalin levels) and restored to normal levels.

2. Clinical study: enkephalin regulation of IL-17 in multiple sclerosis

A retrospective clinical study was designed to examine serum levels of cytokines and endogenous peptides, including [Met^5]-enkephalin.

2.1 Methods

Patients were identified through the Institute of Personalized Medicine at the Pennsylvania State University College of Medicine and had a clinically definitive diagnosis of relapsing–remitting multiple sclerosis [17, 18, 21, 36]. Selected individuals were between 18 and 70 years of age, of which 17 were males and 36 were females. Cohorts were established based on the disease-modifying therapy each of these patients was receiving. The five groups included multiple sclerosis patients receiving dimethyl fumarate, glatiramer acetate, low-dose naltrexone, or no disease-modifying therapy and a control group of non-multiple sclerosis patients. The 13 non-multiple sclerosis patients were recruited from the Neuroscience Institute and were age and gender matched to the study population. All patients were de-identified to the study team.

Blood was collected and stored in non-heparinized ethylenediaminetetraacetic acid (EDTA) tubes to prevent formation of a clot [65, 66]. Whole blood was centrifuged at 4°C for 10 min at 2500 rpm [65, 66]. Serum was aliquoted and stored long term at −80°C in 200 μL vials to prevent repeated freeze and thaw cycles.

Serum was analyzed using commercially produced sandwich or competitive enzyme labeled immunosorbent assay (ELISA). Opioid growth factor (MBS990622), β-endorphin (MBS770600), IL17A (MBS00565) and IL17 (MBS772095) ELISA kits were manufactured by MyBioSource (San Diego, CA); whereas the TNFα ELISA kit (EK0525) was purchased from BosterBio (Pleasanton, CA). Assays were completed following the manufacturer's recommendations; all samples and standards were run in duplicate and averaged for the data analyses. Chemiluminescence was measured using a BioTek microplate spectrophotometer and Gen5 software at 450 nm.

To ensure redundancy and reproducibility, duplicate samples were run on multiple ELISA kits from the same manufacturer.

Data analysis was performed using GraphPad Prism 8.0 software. Parametric data were analyzed using one-way analysis of variance (ANOVA) with post-hoc comparisons made using Newman-Keuls. Correlations were determined using Pearson's Correlation Coefficient (R). Significance was determined with a p value less than 0.05.

2.2 Results

A cohort of 53 patients was analyzed in this study, 40 of whom were individuals with an established diagnosis of relapsing-remitting (RR) multiple sclerosis and 13 who did not have multiple sclerosis. Patients with multiple sclerosis who received no disease-modifying therapy were designated as controls along with the 13 individuals with no diagnosis of multiple sclerosis. Within the control population, 17 were females and 7 males, whereas the study population had 19 females and 10 males. The age distribution between the cohorts of patients on treatment ranged between 25 and 69 years of age while the control cohort ranged in age from 25 to 78 years. Length of disease in the multiple sclerosis patient group was similar between the no-drug, dimethyl fumarate and LDN group which had mean lengths of 14.3, 10.4 and 16.5 years, respectively.

Serum OGF levels differed substantially between multiple sclerosis patients who declined therapy and control subjects considered non-multiple sclerosis (**Figure 1A**). OGF levels were more than three-fold higher in non-multiple sclerosis cohort relative to the no-drug cohort (93 ± 26 pg/ml). Because there is no diagnosis provided for the no-drug volunteers, it is difficult to more fully assess these data. Mean serum levels for RR-multiple sclerosis subjects prescribed disease-modifying therapy were comparable to serum values for the no-drug cohort, and significantly less than serum values for the non-multiple sclerosis cohort (**Figure 1B**). RR-multiple sclerosis patients receiving dimethyl fumarate had serum OGF values of 125 ± 22 pg/ml whereas those individuals receiving glatiramer acetate had serum levels of 136 ± 30 pg/ml. RR-multiple sclerosis subjects receiving LDN had mean OGF serum levels of 217 ± 29 mg/ml; these values did not differ from the non-multiple sclerosis cohort. Correlations between serum OGF levels and age or length of disease for cohorts of no drug, DMTs, or LDN are presented in **Figure 2A** and **B**, respectively. The association between age and serum OGF had an overall significance, with dimethyl fumarate subjects revealing a corresponding decrease in OGF serum values with increasing age (p = 0.03). Regarding the length of disease, RR-multiple sclerosis subjects taking LDN had comparable OGF serum values irrespective of having disease for 2 months or 33 years (**Figure 2B**). RR-multiple sclerosis subjects not taking any drugs (no-drug cohort) also displayed a biphasic response, suggesting that length of disease does not impact OGF serum. Analyses of OGF serum values by gender indicated that females had a pattern similar to that in **Figure 1B**. A majority of the males in this study received dimethyl fumarate and had serum OGF levels that ranged from 25 to 200 pg/ml; the small number of male subjects in the other cohorts prevented data analyses.

Serum β-endorphin levels were also measured in three cohorts of RR-multiple sclerosis subjects and non-multiple sclerosis subjects. Levels of serum β-endorphin ranged between ~1 ng/ml (no-drug and dimethyl fumarate cohorts) to the mean of 2.3 ng/ml (non-multiple sclerosis cohorts); individual samples ranged as high as 4 ng/ml in the non-multiple sclerosis cohort. Despite the variation, significant two-fold increases were recorded for non-multiple sclerosis and glatiramer acetate cohorts relative to no drug and dimethyl fumarate RR-multiple sclerosis
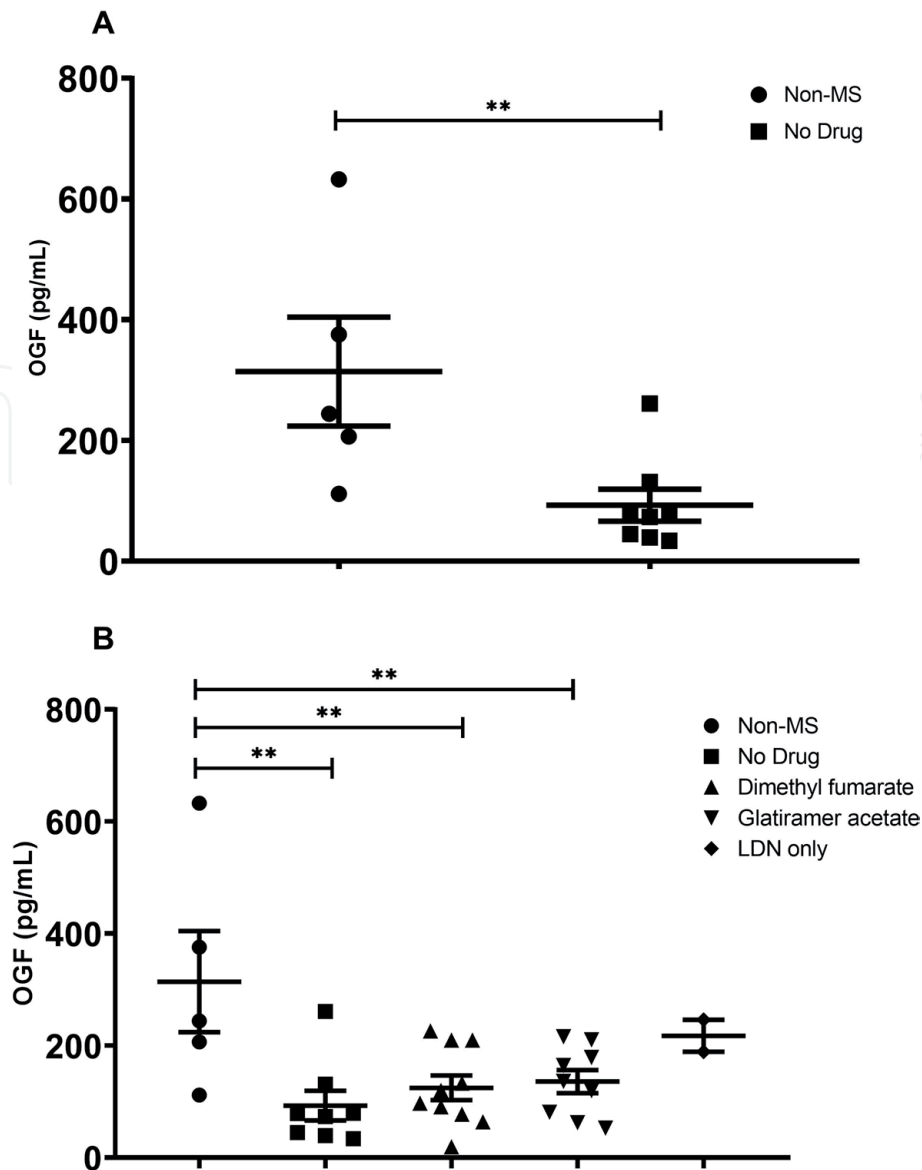
Figure 1.
Scatterplots of serum OGF levels (pg/ml) in (A) control subjects who were either non-multiple sclerosis subjects or (B) RR-MS patients who were not receiving any therapy (no drug) or RR-multiple sclerosis patients receiving dimethyl fumarate, glatiramer acetate, or low dose naltrexone (LDN). One-way ANOVA or t-tests were used to show the differences between groups. Significantly different from non-multiple sclerosis values at p < 0.01 (**).

individuals. Associations between age and β-endorphin revealed that endorphin levels were relatively stable within the population of RR-multiple sclerosis subjects on DMT.

With regard to serum levels of inflammatory cytokines, expression levels of two cytokines in serum are presented in **Figure 3**. Serum levels of IL-17 (A) and TNFα (B) were measured in RR-multiple sclerosis subjects on DMTs, LDN, or no-drug, as well as non-multiple sclerosis controls. Mean IL-17A (not shown) concentrations ranged from 27 ± 3 pg/ml (dimethyl fumarate) to 39 ± 10 pg/ml (glatiramer acetate group). Only one sample of blood from RR-multiple sclerosis patients on LDN was assayed; serum IL-17A value was 46 pg/ml. No differences in serum levels were noted between any therapeutic treatment and controls. IL-17 levels ranged from the mean 0.12 ng/ml recorded for non-multiple sclerosis cohort to 0.92 ± 0.35 ng/ml for the dimethyl fumarate group (**Figure 3A**); levels of the IL-17 cytokine recorded for these subjects differed significantly from glatiramer acetate and no-drug cohorts. RR-multiple sclerosis patients had a mean IL-17 cytokine level of near 0, whereas some individuals using dimethyl fumarate had more than 3 pg/ml IL-17 levels.
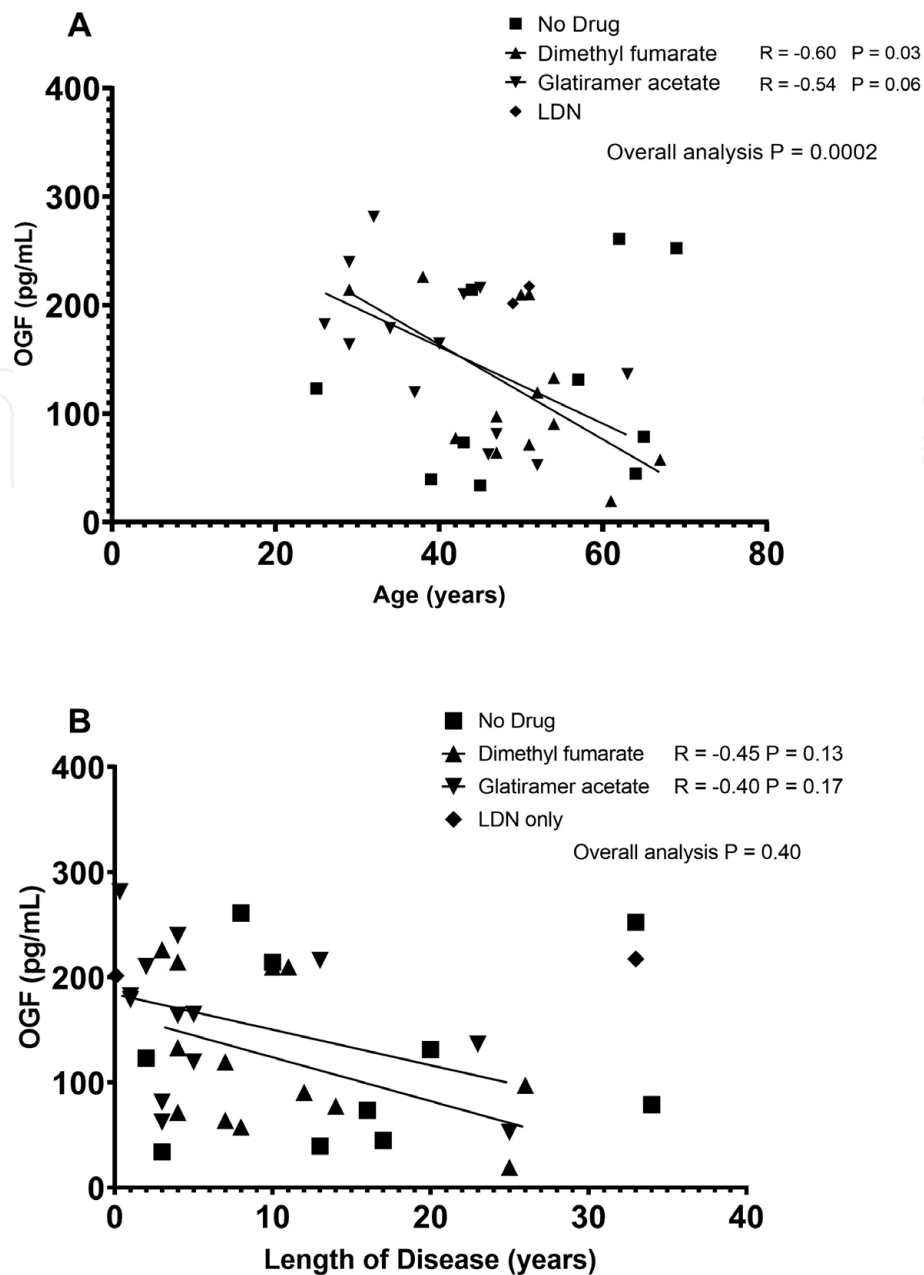
Figure 2.
Expression levels of serum OGF (pg/ml) measured by ELISA as a function of the age (years) of subject (A) or the length of disease (years) (B). Correlations were determined by Pearson's Correlation Coefficient (R) tests. P values less than 0.05 were considered significant.

Non-multiple sclerosis and RR-multiple sclerosis patients not taking any DMT had comparable TNFα values (16 ± 10 and 22 ± 3 pg/ml, respectively) (**Figure 3B**). RR-multiple sclerosis cohorts on DMTs had TNFα serum values that were at least 6-fold higher and differed significantly from the non-multiple sclerosis levels. Correlation analyses indicated that OGF (**Figure 4A**) and IL-17A (**Figure 4B**) were positively associated (R = 0.82), whereas TNFα values did not correlate (R = 0.20) with OGF serum values.

2.3 Discussion and summary

Cohorts of RR-multiple sclerosis patients on disease-modifying therapy, as well as a group of individuals diagnosed with RR-multiple sclerosis and taking LDN only were compared with control volunteers. in an assessment of peripheral serum levels of two endogenous neuropeptides—OGF (chemically termed methionine enkephalin) and β-endorphin, as well as 3 cytokines that are known to be involved
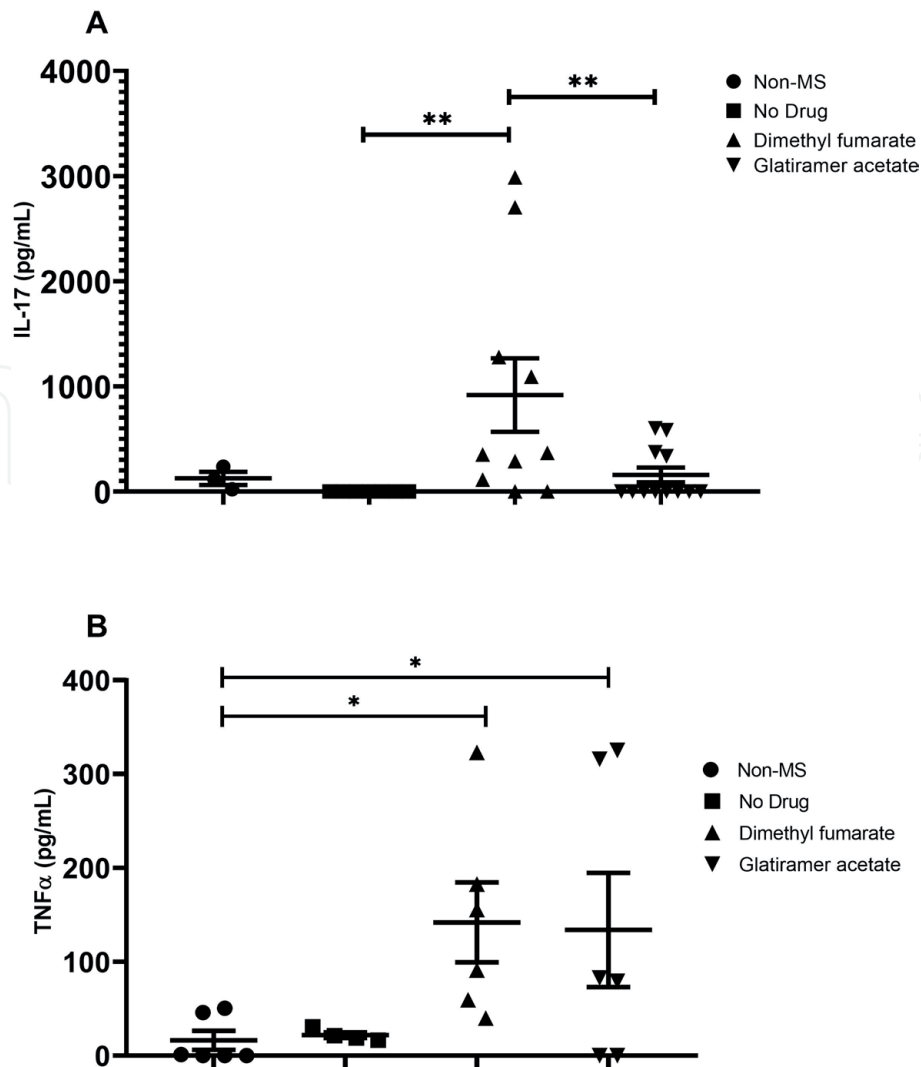
Figure 3.
Scatterplots of serum cytokines IL-17 (A) and TNFα (B) measured by ELISA tests (pg/ml) in RR-multiple sclerosis patients receiving DMTs or no drug; controls include volunteer non-multiple sclerosis subjects. Data was analyzed using one-way ANOVA with subject comparisons made by Newman-Keuls tests. Significantly different at p < 0.05 (*) and p < 0.01 (**).

in RR-multiple sclerosis, specifically IL-17, IL-17A, and TNFα [67–71]. Inclusion criteria restricted our analyses to RR-MS subjects to those receiving glatiramer acetate or dimethyl fumarate. A few RR-multiple sclerosis subjects in this study were only prescribed LDN as an off-label product.

The restricted access to blood samples and corresponding patient data in this retrospective study limited the findings, as did inclusion of RR-multiple sclerosis patients on no drug. No rationale was reported in the REDCap database to explain why the RR-multiple sclerosis patients had denied therapy. It is conjured that some multiple sclerosis patients who have stabilized over a long period of time request to be removed from therapy.

The focus of this study was to determine the relationship between serum expression levels of endogenous neuropeptides, specifically OGF, and a select group of pro-inflammatory cytokines. OGF levels in the serum of RR-multiple sclerosis subjects declined with age and were not associated with the length of disease. In general, the OGF levels of RR-multiple sclerosis patients using DMTs, or those on no therapy, were substantially lower than RR-multiple sclerosis subjects on LDN. This relationship between OGF and RR-multiple sclerosis has been reported previously [51], suggesting that the biotherapeutic LDN treatment is producing enkephalins. Clinical studies have reported that LDN treatment resulted in perceived increased
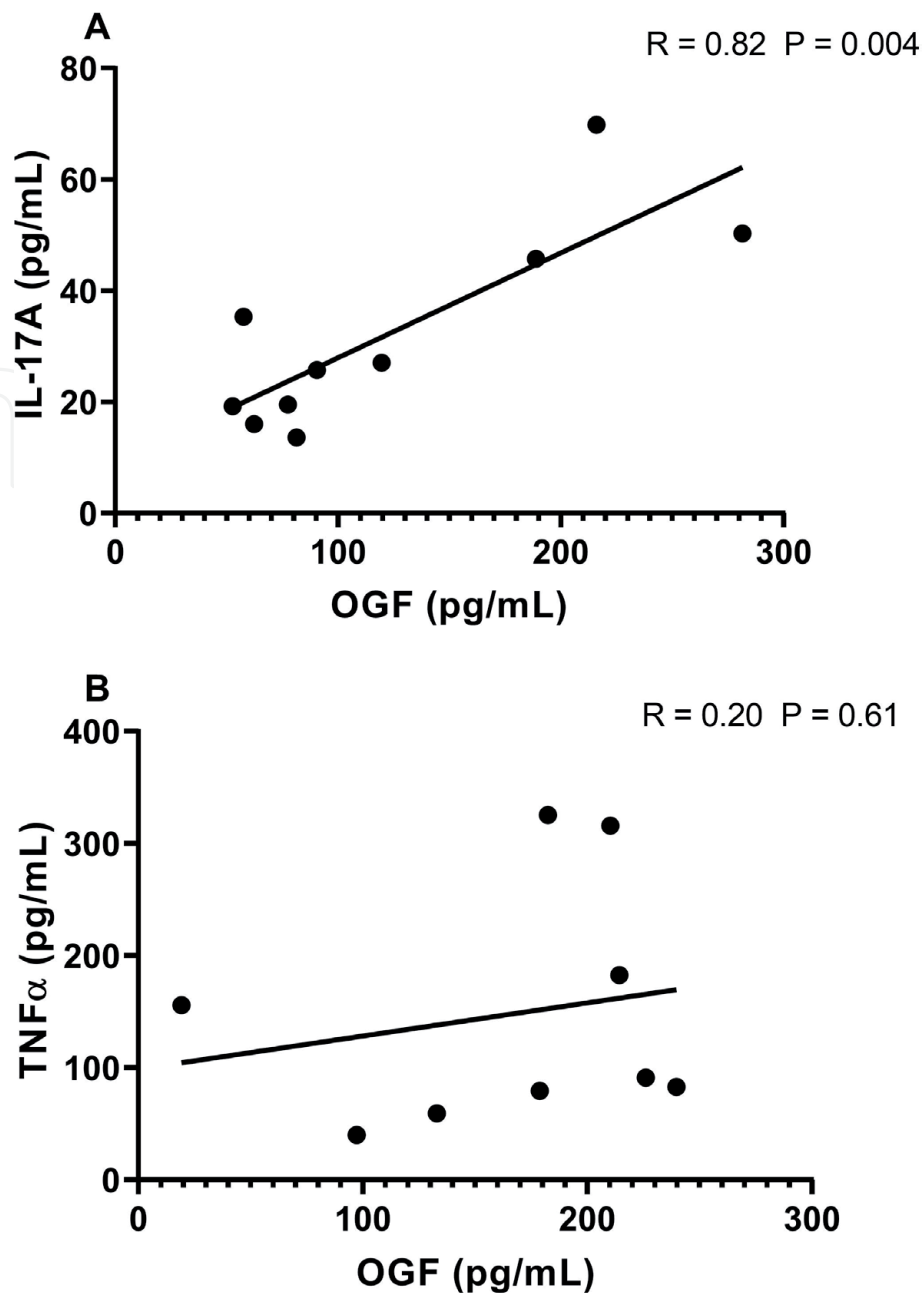
Figure 4.
Associations of cytokine expression between IL-17A (pg/ml) and OGF (pg/ml) (A) or TNFα (pg/ml) and OGF (B) for RR-multiple sclerosis subjects receiving a DMT. Data were analyzed by Pearson's Correlation Coefficient (R) tests. P values less than 0.05 were considered significant.

quality of life [42, 43] and stabilization of disease symptomatology [44]. A second neuropeptide, β-endorphin, is known as the "feel-good" hormone and has been reported to increase during exercise and pregnancy [72] and to increase during multiple sclerosis remission [73]. In this study, β-endorphin levels were comparable in all subjects suggesting that these RR-multiple sclerosis patients had stabilized endorphins. Based on the scatter gram plots of individual expression levels of endorphin, some of the individuals in the glatiramer acetate group may have been in remission. Moreover, whereas OGF levels fluctuate with RR-multiple sclerosis and different therapies, β-endorphin does not appear to be altered by DMTs in RR-multiple sclerosis.

The proinflammatory cytokine IL-17 may be a prime indicator of RR-multiple sclerosis progression [69, 71]. The IL-17 family of cytokines is produced by CD4⁺ T-helper cells known as Th17 cells. At least 7 polymorphisms have been identified, with IL-17A and IL-17F most frequently associated with neutrophils activation in

autoimmunity [67, 69]. IL-17A has been identified in the CNS following migration of Th17 cells in response to a pro-inflammatory event [74]. Clinical studies have reported that IL-17A may be responsible for breakdown of the blood-brain barrier facilitating the entrance of other inflammatory cytokines into the CNS [71]. IL-17A has been shown in preclinical studies to accelerate glial activation leading to neuroinflammation and neurodegeneration [68]. Thus, it would be expected that IL-17A levels may be lowest in RR-multiple sclerosis individuals who are in remission or most responsive to therapy [67]. Moreover, recent preclinical studies have reported that both IL-17A and IL-17F increase pro-inflammatory cytokine IL-6 secretion [70].

In the present study, TNFα, a proinflammatory marker has been shown to increase in RR-multiple sclerosis [75, 76] and to be consistently elevated regardless of the status of disease type (i.e., clinically isolated syndrome, primary progressive, or relapsing-remitting), with serum levels 40–50% higher in multiple sclerosis subjects relative to controls. Preclinical studies have reported an association between TNFα and impaired memory in mice with EAE [77] and implicated astrocyte signaling as the downstream target of overexpression of the cytokine. Despite studies whereby TNFα knockdown mice had more severe EAE [69] and therapeutic administration of the cytokine protected against the disease [74], this cytokine is reported to be a major contributor to cognitive deficits related to late stages of multiple sclerosis. In the present study, age, gender, number of relapses, or length of disease had no effect of TNFα levels, but in general, the expression levels of this cytokine were elevated in RR-multiple sclerosis subjects relative to cohorts of RR-multiple sclerosis patients presumably in remission or non-multiple sclerosis.

LDN is an off-label therapeutic used in substantially lower dosages than prescribed for drug overdose or alcohol use (3 vs. 50 mg). Its use is increasing worldwide [78, 79] and is consistently reported to be well-tolerated over extended periods of time [46]. Small clinical trials have reported few, if any, side effects [42, 43]. In this study, OGF serum levels appear to be correlated with IL-17A. Alternatively, whereas β-endorphin has been associated with the propensity to avoid alcohol or opioids, it was not related to RR-multiple sclerosis. The serum levels of cytokines IL-17A and TNFα did not appear to be discriminating biomarkers at least with the current population of RR-multiple sclerosis subjects. Perhaps with additional study, and possibly other DMTs, these cytokines will be able to discern progression of disease.

3. Conclusions

In summary, the preclinical and clinical data illustrate that enkephalin levels (i.e., OGF) are decreased in animals with EAE and humans with relapsing remitting multiple sclerosis. LDN as a biotherapy is associated with elevating enkephalins and from all aspects, appears to reduce symptomatology of multiple sclerosis. LDN therapy to upregulate the body's own production of enkephalins has been shown by a number of clinical trials to be a safe adjuvant, or primary, treatment for RR-multiple sclerosis. LDN continues to be associated with stabilizing multiple sclerosis and does not appear to interfere with other disease-modifying therapies. Whether enkephalin levels directly or indirectly alter the therapeutic pathways is unclear at this time. Moreover, it is difficult to determine at this point whether specific disease-modifying therapies are more advantageous for manipulating enkephalin levels. However, it is evident that restored serum enkephalin expression is associated with reduced inflammatory cytokines and better patient outcome. Moving forward, studies will be conducted to determine the mechanistic role of

OGF in modulating both pro and anti-inflammatory cytokines. Collectively the data from published studies as well as the new data presented in this report demonstrate that the biotherapeutic LDN and resulting enkephalin (specifically OGF) levels play a role in disease progression of multiple sclerosis. By integrating animal model work and patient serum analysis, future studies will try and understand the role of OGF during initiation of disease and definitive diagnosis of disease. The efficacy of LDN alone needs to be evaluated in prospective, randomized, controlled studies, but unfortunately this design is not forthcoming as most physicians will prescribe the off-label drug based on its safety record.

Author declaration

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