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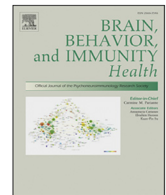
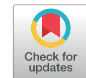


# Depression and anxiety in patients with multiple sclerosis treated with interferon-beta or fingolimod: Role of indoleamine 2,3-dioxygenase and pro-inflammatory cytokines



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ABSTRACT

Depression/anxiety (D/A) occurs in up to 50% of multiple sclerosis (MS) patients. Proinflammatory cytokines induce classical symptoms of depression. Activation of the inflammatory response also triggers production of indoleamine 2,3-dioxygenase (IDO), which catabolizes tryptophan, the amino acid precursor of serotonin and melatonin. It has been suggested that IDO is the link between the immune and serotonergic systems.

This study aimed to quantify the levels of IDO and pro-inflammatory and anti-inflammatory cytokines in patients with MS and depression, according to treatment with interferon-beta (IFN-β) or fingolimod. The study inclusion criteria were age 18–60 years and a clinical and radiological diagnosis of MS. One hundred and thirty-two patients diagnosed by McDonald's criteria and followed up at Brasília District Hospital, Brazil, with relapsing-remitting MS were identified as potential study participants. Thirty-five of these patients were identified to be receiving treatment with fingolimod or IFN-β and to have a diagnosis of D/A. IDO and pro-inflammatory and anti-inflammatory cytokine levels were compared between these 35 patients and 18 healthy controls. The level of IL-10 (an anti-inflammatory cytokine) was lower in both the fingolimod-treated ($P < 0.001$) and IFN-β-treated ($P < 0.01$) patient groups than in the control group. IFN-β-treated patients showed increased IDO expression and decreased inflammatory cytokine levels. In contrast, fingolimod-treated patients showed significantly decreased expression of IDO and significantly increased levels of proinflammatory cytokines produced by innate immune cells, including tumor necrosis factor-alpha and interleukin-6.

The agents used to treat MS maintain symptoms of D/A in patients with MS via different mechanisms.

## 1. Introduction

An estimated 2.5 million persons worldwide have multiple sclerosis (MS), and there is evidence suggesting that the disease may be increasing in prevalence (Ascherio and Munger, 2016; Benito-León, 2011). MS manifests as inflammation and degeneration of white and gray matter in the central nervous system (CNS) and is caused by a combination of genetic and environmental factors. The inflammatory phase of MS follows a pattern of relapse and remission (relapse-remitting MS [RRMS]). The relapses in turn are characterized by inflammatory exacerbations. The triggers for relapse are not well understood. The current treatments for MS target molecules in the inflammatory phase (Thompson et al., 2018; Brownlee et al., 2017; Buzzard et al., 2017). Therefore, the inflammatory phase of MS remains a focus of neuroimmunology research.

Behavioral, affective, and cognitive abnormalities are common in patients with MS (Paparrigopoulos et al., 2010). Rates of depression are higher in patients with MS (especially in younger patients) than in the general population (Marrie et al., 2017; Simpson et al., 2016). Approximately one third of patients with MS and major depression do not receive adequate treatment (World Health Organization -WHO, 2018).

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Moreover, although mood disorders are common in these patients, they are underdiagnosed, causing considerable morbidity and further deterioration of quality of life.

The biological basis for depression/anxiety (D/A) includes signaling through neurotransmitters and the hypothalamic-pituitary-adrenal axis, with a more recent focus on inflammation. Interactions between these pathways lead to elevated proinflammatory cytokine levels and D/A (Hestad et al., 2017). These pathways may be altered in patients with MS who have been treated with cytokines or other biological response modifiers (Hestad et al., 2017).

In the early 1990s, the relationship between the immune system and depression was attributed to an imbalance between proinflammatory and anti-inflammatory cytokines. Higher levels of proinflammatory cytokines (interleukin [IL]-1(Smith, 1991), tumor necrosis factor alpha [TNF-α], and IL-6, interferon-gamma [IFN-γ]) were found to be associated with development and/or worsening of depression (Kwidzinski and Bechmann, 2007; Maes et al., 1997).

The participation of tryptophan (Trp) as a precursor to serotonin was of fundamental importance in understanding the pathogenesis of depression. Trp is an essential amino acid formed by an indole ring and is obtained from the diet. Serotonin is synthesized from 1% of the Trp available in the body. Approximately 99% of Trp is metabolized in the liver by the action of tryptophan 2,3 dioxigenase (TDO) under normal conditions (Guillemin et al., 2001; Eynard et al., 1993). In the case of inflammation, infection or oxidative stress, the enzyme indoleamine 2,3 dioxigenase (IDO), also responsible for the degradation of Trp, can be synthesized in extra hepatic tissues such as lung, placenta, kidney, spleen, lymph nodes and brain (Braidy and Grant, 2017; Yuwiler et al., 1977).

The Kynureine pathway is the main route for Trp metabolism, and contributes to several fundamental biological processes. Trp is continuously oxidized by TDO in liver cells. In other types of cells, IDO is catalyzed under certain pathophysiological conditions, which consequently increases the formation of Kynureine metabolites (Lim et al., 2017). The relationship between cytokines and tryptophan metabolites are present in normal individuals, without acute or chronic inflammation (Deac et al., 2016).

The most important production site for serotonin is the population of enterochromaffin cells in the intestinal mucosa, which expresses TPH1. Thereafter, serotonin is transported and distributed through platelets, but does not cross the blood-brain barrier (BBB). Other precursor indole amines, such as tryptophan and 5-HTP, access the brain and target neurons, establishing local biosynthesis in the serotonin CNS guaranteed by neuronal TPH (TPH2). Thus, the transport of tryptophan via BHE becomes a crucial step in the process of the synthesis of serotonin in the CNS (Strasser et al., 2017).

IDO catabolizes tryptophan and its intermediate metabolites, which can modulate neurotransmitters, including serotonin and melatonin, and inflammatory pathways in the peripheral nervous system and CNS (Mancuso et al., 2015). This may explain in part the risk of depression in patients with diseases such as hepatitis C or MS who are treated with these cytokines (Choi et al., 2017).

IDO is produced by various cells, including macrophages, dendritic cells, and fibroblasts (Carlin et al., 1989; Myint et al., 2009). Studies in murine and human microglia show that IFNs stimulate production of IDO (Guillemin et al., 2001; Jürgens et al., 2009). This pathway plays a role in the pathogenesis of neuroinflammatory and neurodegenerative disorders (Mancuso et al., 2015).

Recent studies suggest that mood changes in patients with MS are induced by intrathecal inflammation and that proinflammatory cytokines may affect mood. IL-2 is a major cytokine involved in this effect (Kallaur et al., 2016; Mancuso et al., 2015). A catalytic analysis study found a difference in IDO metabolite levels between periods of relapse and stability (Mancuso et al., 2015) and confirmed the important role of IDO in the natural history of MS. Therefore, IDO is believed to be the link between the immune and serotonergic systems (Alberati-Giani et al., 1996; Rossi et al., 2017).

The Fingolimod (Gilenya®, Novartis Pharma AG) is the first oral drug that has increased the therapeutic arsenal of MS in recent years. The mechanism of action binds to sphingosine 1- phosphate receptors (S1PRs) on lymphocytes leading to retention of circulating lymphocytes in the lymph nodes. This reversible reduction in the number of peripheral blood lymphocytes is postulated to be mechanistically important in MS, decreasing the recirculation of autoreactive lymphocytes and preventing their infiltration into the central nervous system (Brinkmann et al., 2010; Kira et al., 2014).

The aim of this study was to determine the relationship between IDO and proinflammatory cytokine activity in patients with MS and D/A according to whether they are treated with fingolimod or IFN-β.

## 2. Materials and methods

### 2.1. Participants

One hundred and thirty-two of 150 patients with RRMS diagnosed by McDonald's criteria and followed up at Brasília District Hospital, Brazil, between October 2016 and October 2017 were identified as potential study participants. The study inclusion criteria were age 18–60 years and a clinical and radiological diagnosis of MS. Patients treated with antipsychotic or anxiolytic agents, those diagnosed with an infectious or neoplastic disease, those with a psychiatric illness other than D/A, and women who were pregnant were excluded.

Thirty-five patients who met the eligibility criteria were recruited and evaluated by the same neurologist on the MS care team. All patients were receiving fingolimod or IFN-β and underwent collection of blood samples and other data, magnetic resonance imaging (MRI) of the brain, and neuropsychological testing at the same time and stage of the disease. Eighteen healthy subjects were recruited as a control group from the technical team and researchers in the neuroimmunology laboratory and other laboratories in the Institute of Biology, University of Campinas.

The study protocol was approved by the Ethics Committee of State Secretary for Health of the Federal District, Brazil (CAAE: 22477313.9.0000.5553/Opinion: 660.753) and performed in accordance with the national and international research regulations (REF Resolution no. 466/2012 and Document of the Americas). The study is registered with the National Commission of Ethics in Research through the Brazil platform. Written informed consent was obtained from all study participants after providing a detailed explanation of the purpose and nature of the research.

### 2.2. Clinical assessment

All patients had clinical and neuraxial MRI evidence of RRMS according to the 2010 revised McDonald criteria (Polman et al., 2011) None showed signs of relapse at the time of evaluation or collection of serum samples. Use of immunosuppressant therapy and disease activity (annual rate of relapse, disease duration, patient age at onset of disease) and severity (progression according to the Expanded Disability Status Scale score) (Kurtzke, 1983) were comparable between the patients receiving fingolimod and those receiving IFN-β. Clinical and demographic data were collected during follow-up visits.

All the patients with MS were evaluated using the Hospital Anxiety and Depression Scale (HADS, for anxiety and depression) (Zigmond and Snaith, 1983) (Pais-Ribeiro et al., 2007) and Beck Depression Inventory (BDI, for depression) (Beck et al., 1961) (Gomes-Oliveira et al., 2012) under the supervision of a neuropsychologist.

### 2.3. Laboratory assessments

Samples were obtained from the patient and control groups at the time of recruitment and during follow-up in the outpatient clinic. Approximately 40 mL of blood were obtained by peripheral venous puncture in sterile heparinized tubes. The blood samples were

centrifuged at 1200 rpm for 10 min, after which the plasma was discarded and the leukocyte concentrate was washed in Hank's balanced salt solution (HBSS; Sigma-Aldrich, St Louis, MO, USA) supplemented with sodium bicarbonate. After homogenization, the cells were carefully placed in Histopaque-1077 Ficoll solution (Sigma-Aldrich) and centrifuged at 1400 rpm for 30 min to isolate the peripheral blood mononuclear cells (PBMC). The mononuclear cells between the HBSS and Ficoll layers were then collected, washed in HBSS, and suspended in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich) with 2-mercaptoethanol (Thermo-Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (Cultilab, Campinas, Brazil), 1% L-glutamine (Life Technologies, Carlsbad, CA, USA), and 1% Pen Strep (10,000 IU/mL penicillin and 10,000 μg/mL streptomycin; Life Technologies). Cell viability was assessed by the Trypan blue exclusion method using an optical microscope (Strober, 2001).

Sterile 96-well polystyrene cell culture plates were coated with 2 μg/mL of anti-human CD3 antibody (BD Pharmingen, San Diego, CA, USA) for in vitro stimulation of T lymphocytes in each well over 24 h at 4 °C. After incubation, the solution was gently aspirated; $2 \times 10^5$ PBMC were added into each well in the same medium in which the cells were suspended and supplemented with 2 μg/mL of anti-human CD28 antibody (BD Pharmingen). The culture plate was maintained for 48 h in a $CO_2$ incubator at 37 °C with injection of 5.1% $CO_2$. The material was then removed and centrifuged in conical tubes at 1400 rpm for 10 min. The supernatant was separated and stored at -80 °C for quantification of cytokine levels at a later date. The stimulated PBMC were suspended in lysis buffer (RNeasy Micro Kit, Qiagen, Hilden, Germany) for real-time polymerase chain reaction testing.

The stimulated mononuclear cells from the cell culture were lysed for total mRNA extraction according to the manufacturer's instructions (RNeasy Micro Kit). The extracted mRNA was then converted to complementary DNA using a conversion kit (Applied Biosystems, Waltham, MA, USA). The complementary DNA was then suspended with TaqMan primers for IDO 1, IFN-γ, transforming growth factor-beta (TGF-β; Thermo Fisher Scientific), and ROX Reference Dye (Sigma-Aldrich) in Master Mix (LuminoCT; Sigma-Aldrich). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The assay was performed using a real-time PCR system (9700 Plus; Applied Biosystems).

IL-17A, IFN-γ, TNF-α, IL-10, IL-6, IL-4, and IL-2 levels were quantified in the supernatant of the mononuclear (lymphocyte and monocyte) cultures. The concentration of each cytokine was analyzed by flow cytometry using a Cytometric Bead Array kit for human Th1/Th2/Th17 cytokines (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. The samples were diluted in wash buffer when necessary. Acquisition was performed on a dual-laser BD FACSCalibur™ flow cytometer (BD Biosciences) and the data were analyzed using FCAP Array software (BD Biosciences).

All the above-mentioned substances were aliquoted by molecular biologists, who were blinded to study group allocation, at the Laboratory of Neuroimmunology, Institute of Biology, University of Campinas.

### 2.4. Statistical analysis

The data are presented as the mean and standard deviation and were tested for normality using the Shapiro-Wilk test. Gene expression and cytokine levels were compared between two groups using the Mann-Whitney test and between three groups using the Kruskal-Wallis test. All statistical analyses were performed using GraphPad Prism v.6 software (GraphPad Software Inc., La Jolla, CA, USA). A $P$-value <0.05 was considered statistically significant.

## 3. Results

The characteristics of the study participants are summarized in Table 1. All patients with RRMS had a diagnosis or depression, anxiety, or both (D/A) according to their HADS and BDI scores. The patients were divided in two groups according to whether they were receiving fingolimod or IFN-β as their disease-modifying drugs. The assay results were compared between the two patient groups and with those in the control group.

**Table 1**
Characteristics of patients and healthy subjects.

| | RRMS-Fingo | RRMS–IFN–β | Healthy Control |
|---|---|---|---|
| Number of subjects | 20 | 15 | 18 |
| Gender (F: M) | 14 : 6 | 13 : 2 | 10 : 8 |
| Age in years (range) | 34 (18–51) | 44 (28–60) | 30 (21–52) |
| EDSS (range) | 2.4 (1–5) | 2.4 (0–3.5) | not apply |

RRMS: Relapsing Remitting Multiple Sclerosis; Fingo: Fingolimod; IFN-β: Interferon-beta; F: female; M: male; EDSS: Expanded Disability State Scale.

Mononuclear cells from the control group and the patient groups were cultured in vitro with anti-CD3 and anti-CD28 to stimulate T lymphocytes. After 48 h, these cells and the secreted molecules were separated for evaluation. The cytokines in the supernatant were quantified by flow cytometry using a Cytometric Bead Array assay[25]. Concentrations of pro-inflammatory and anti-inflammatory cytokines associated with innate and adaptive immunity are shown in pg/mL in Fig. 1.

The IL-17A levels in both patient groups were similar to the level in the control group (Fig. 1A). However, the IFN-γ, IL-4, and IL-2 levels were lower in both patient groups than in the control group (Fig. 1B–D).

Levels of TNF-α and IL-6 (both innate immunity cytokines) were higher in the fingolimod-treated patient group than in the control group (both $P < 0.01$) but were comparable between the IFN-β-treated patient group and the control group (Fig. 1E and F).

The level of IL-10 (an anti-inflammatory cytokine) was lower in both the fingolimod-treated ($P < 0.001$) and IFN-β-treated ($P < 0.01$) patient groups than in the control group (Fig. 1G).

The results for IDO, TGF-β, and IFN-γ gene expression determined in the cultured cells by real-time polymerase chain reaction are shown in Fig. 2. IDO expression was significantly higher in the IFN-β-treated patient group than in the control group ($P < 0.001$) but was similar between the fingolimod-treated patient group and the control group ($P > 0.05$; Fig. 2A).

Expression of IFN-ɣ mRNA was similar between the fingolimod-treated and IFN-β-treated patient groups but was lower in both patient groups than in the control group (Fig. 2B). TGF-β expression was comparable between the fingolimod-treated patient group and the control group ($P > 0.05$) but was significantly lower in the IFN-β-treated patient group than in the control group ($P < 0.001$; Fig. 2C).

IDO transcription and synthesis, which is regulated mainly by dendritic cells, is complex and depends on external factors, including cytokines and other mediators. In the context of Th1 immunity, the IFN-ɣ/STAT1 axis is essential for IDO transcription. Other molecules implicated in modulation of IDO include TGF-β, TNF-α, and IL-6 [11].

IDO mRNA expression was elevated and secreted inflammatory cytokine levels were diminished in the IFN-β-treated patient group. Conversely, IDO mRNA expression was lower and the levels of proinflammatory cytokines produced by innate immune system cells, such as TNF-α and IL-6, were elevated in the fingolimod-treated patient group.

## 4. Discussion

The findings of this study indicate that the inflammatory mechanism responsible for maintenance of D/A in patients with MS depends on the treatment received. Patients with MS and D/A who were treated with IFN-β showed a significant increase in IDO expression whereas those treated with fingolimod showed a significant increase in expression of proinflammatory cytokines, such as TNF-α and IL-6.

Recombinant forms of IFN-β are widely used as first-line treatment in patients with relapsing MS. The mechanism of action of IFN-β is complex

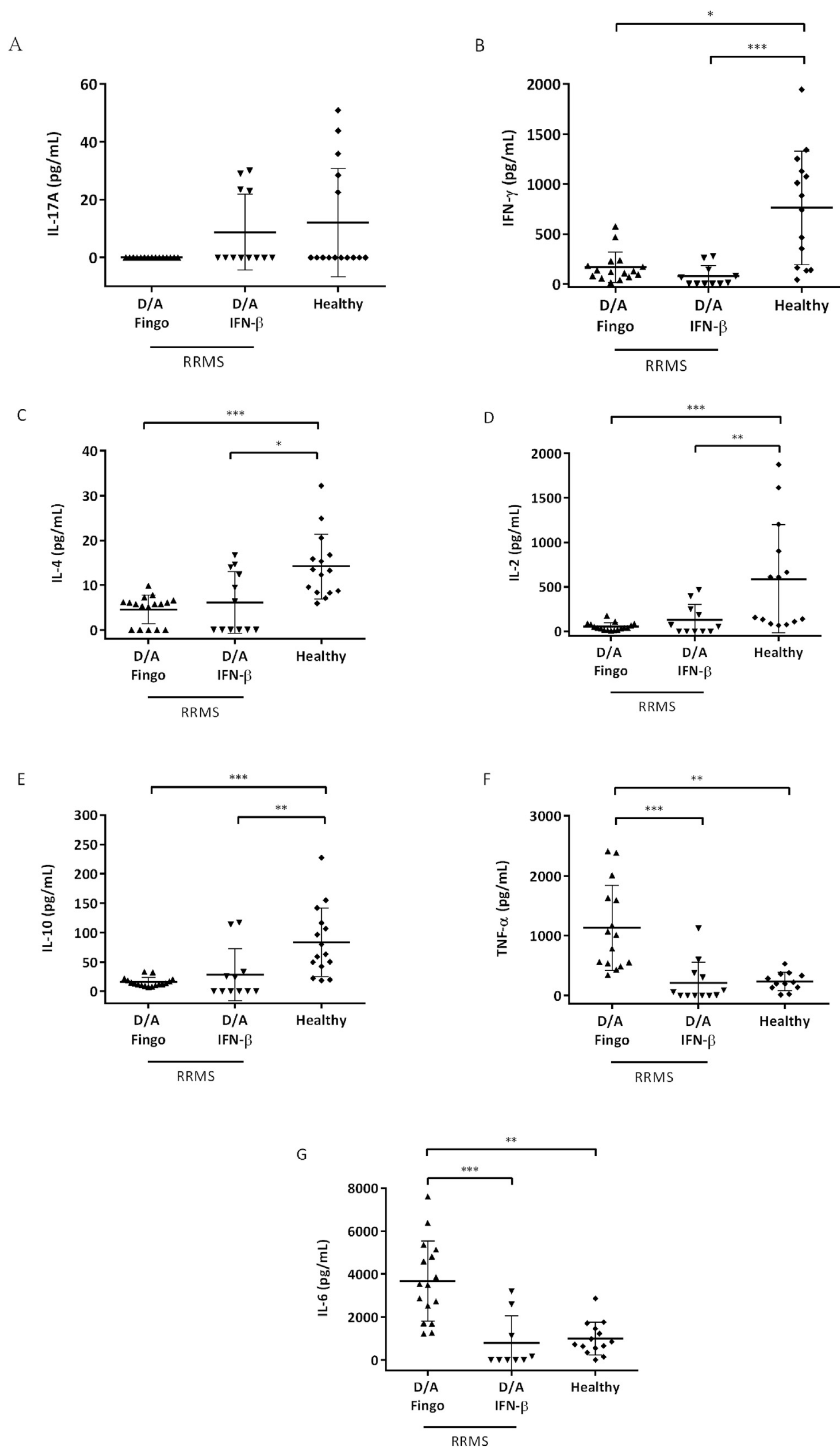
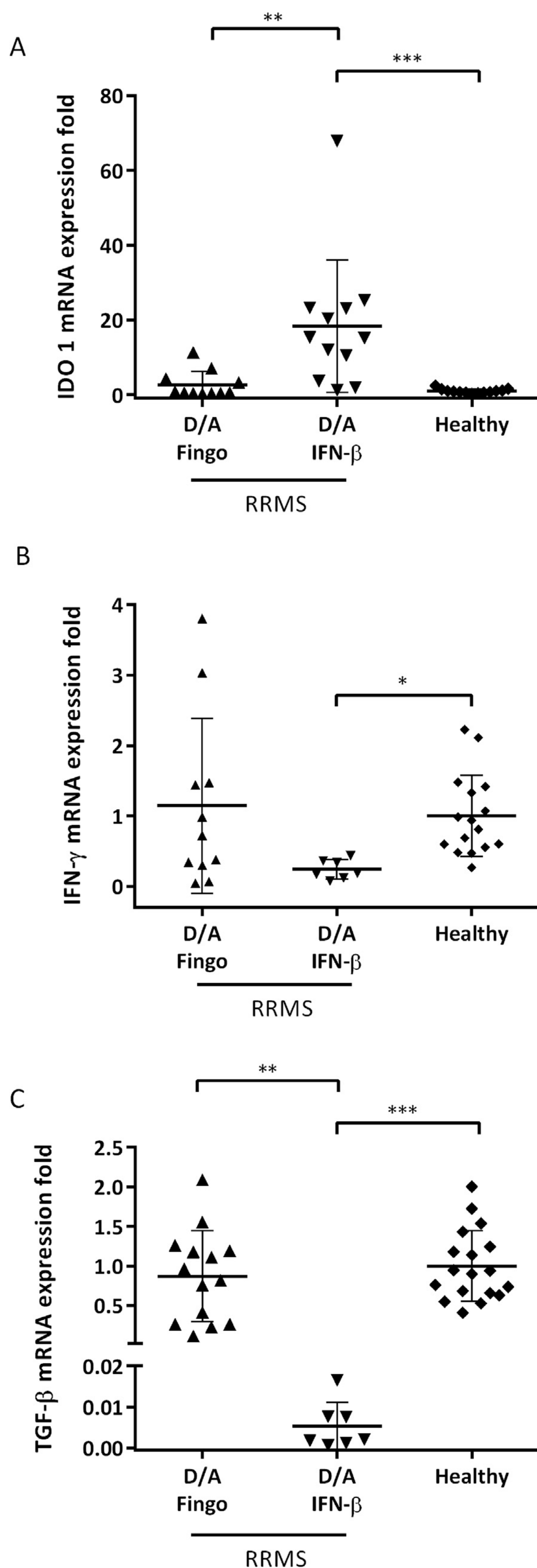



**Fig. 1. Comparison of cytokine levels in the supernatant of cultured PBMC in patients with RRMS with those in healthy controls.** We quantified the cytokines with the Cytometric Bead Array method. Levels of (A) IL-17A, (B) IFN-γ, (C) IL-4, (D) IL-2, (E) IL-10, (F) TNF-α, and (G) IL-6. RRMS patients were under treatment with Fingolimod (Fingo) or IFN-β. The data are represented by mean and standard deviation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. RRMS, Relapsing-Remitting Multiple Sclerosis; D/A, depression/anxiety; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.




*(caption on next column)*

**Fig. 2.** Comparison of IDO, IFN-γ and TGF-β mRNA expression levels in cultured PBMC stimulated in vitro in patients who have RRMS with those in healthy controls. mRNA Expression of (A) IDO, (B) IFN-γ, and (C) TGF-β. We used qPCR to measure expression levels. GAPDH was the housekeeping control gene. We normalized the data by the mean expression in the healthy control. RRMS patients were under treatment with Fingolimod (Fingo) or IFN-β. The data are represented by mean and standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. RRMS, Relapsing-Remitting Multiple Sclerosis; D/A, depression/anxiety; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; qPCR, quantitative polymerase chain reaction; TGF- β, transforming growth factor beta.

and includes multiple levels of cellular function but appears to involve downregulation of expression of proinflammatory cytokines. Our finding of a significant decrease in production of IFN-γ and IL-2 with normal IL-17 levels (all cytokines involved in the pathogenesis of MS) are consistent with previous observations from the Arreola and collaborators who discussed one of the catabolic pathways of the Kynurenine-Tryptophan system that uses the enzyme IDO to generate metabolites from tryptophan. This pathway is positively regulated when immune cells become activated and begin to secrete IFN-α, IFN-β and IFN-γ, TNF-α, TGF-β, IL-1β and IL-2, which consumes tryptophan significantly and limits its availability for the production of 5-HT. L-quinurenine, 3-hydroxy-L-quinurenine and 3-hydroxyanthranilic acid can negatively modulate immune responses (Arreola et al., 2015).

We detected increased expression of IDO in addition to beneficial effects of IFN-β. The increased expression of IDO in patients treated with IFN-β is in agreement with numerous studies in the literature. Higher levels of IDO accelerate degradation of tryptophan, inducing an imbalance in the kynurenine-tryptophan pathway and promoting an increase in levels of metabolites such as kinuric acid, quinolinic acid, picolinic acid, and nicotinamide adenine dinucleotide. Physiologically, this imbalance may lead to neurodegeneration, mood changes, behavioral and sleep disorders, and energy depletion (Arreola et al., 2015; Lim et al., 2017). Our results are consistent with the finding of a higher risk of depression in IFN-treated patients. Previous studies have shown that IFN-β induces the kynurenine pathway and synthesis of quinolinic acid by human macrophages, which are associated with neurodegeneration (Guillemin et al., 2001; Pokryszko-Dragan et al., 2012). Moreover, IFN-β increases IDO activity and modifies the KYN/tryptophan ratio in patients with MS (Filiano et al., 2016; Mohr et al., 2001). Production of IDO is associated with IFN-γ levels. In this study, patients treated with IFN-β were found to have significantly reduced expression of IFN-γ mRNA, which is associated with a significant decrease in IFN-γ production. The levels of proinflammatory cytokines required to activate IDO and degrade tryptophan in kynurenine are not known. However, the hypothesis remains that in chronic inflammatory diseases such as MS, even normal levels of cytokines can disrupt autoregulation of the inflammatory response, reducing production of serotonin, which can lead to a depressive disorder.

Fingolimod is the first oral agent approved by the US Food and Drug Administration for patients with RRMS (Brinkmann et al., 2010). Fingolimod is a sphingosine-1-phosphate (S1P) receptor antagonist, which acts by binding to S1P receptors on lymphocytes, resulting in maintenance of these cells in lymph nodes. Therefore, the autoreactive lymphocytes are not able to leave the peripheral circulation or cross the blood-brain barrier (Brinkmann et al., 2010). Consistent with this mechanism, we found a significant decrease in production of the proinflammatory cytokines released by Th1 lymphocytes (IFN-γ, IL-2) and Th17 (IL17) and Th2 cytokines (IL-4, IL-10). Expression of IDO is reduced in patients with MS treated with fingolimod in contrast with those patients treated with IFN-β. Although myeloid cells express S1P receptors, our experiments demonstrated diminished T lymphocyte-derived cytokines and increased monocyte-derived cytokines, suggesting that immature myeloid cells may remain in the peripheral blood of fingolimod-treated patients with MS who develop D/A. Cytokines

produced by cells involved in the innate immune response, such as TNF-α and IL-6, were significantly increased in relation to those in healthy individuals without MS. Recent studies in depression suggest that cytokines such as IL-6 are risk factors for depression (Khandaker et al., 2020). Proinflammatory cytokines have been presumed to affect serotonin synthesis and reuptake in the CNS (Pokryszko-Dragan et al., 2012). Anti-inflammatory cytokines regulate the intensity and duration of sickness behavior, probably by inhibiting production of proinflammatory cytokines and attenuating their signaling (Guillemin et al., 2001; Pokryszko-Dragan et al., 2012). Our data demonstrated a significant reduction in expression of IL-10 in fingolimod-treated patients with D/A, although the TGF-β levels were comparable with those in healthy individuals. These findings suggest that normal production of TGF-β was insufficient to reduce the increasing levels of TNF-α and IL-6.

Noteworthy is the earlier finding that proinflammatory cytokines such as IL-1 and TNF-α may induce excessive production of S1P (Alvarez et al., 2007; Brinkmann et al., 2010), leading to activation of S1P receptors. The balance between S1P levels in the tissues and body fluids is crucial to the maintenance of immune cells trafficking (Matloubian et al., 2004). It is possible that proinflammatory cytokines produced by patients with MS who develop D/A may interfere with the action of fingolimod. Therefore, these patients should be monitored carefully for worsening of MS in response to a potential increase in production of S1P and consequent migration of autoreactive cells to the CNS.

Among the limitations of this research, we list that our results are conclusive of a cross-sectional study, and a cohort study should be carried out later to confirm it. This type of study could provide data on the group of MS patients before starting to use of disease-modifying drugs. Furthemore, we did not investigate sleep disorders and all aspects of the influence of physical exercise on depression and anxiety such as the measurement of serotonin, melatonin and other metabolites of the kynurenine pathway that were not considered for laboratory evaluation and can be important confounding factors (Dubuisson et al., 2017; Maes et al., 2016).

## 5. Conclusions

Overall, our data demonstrate that certain agents used to treat MS have mechanisms of action that can promote a depressive state in these patients. An understanding of these pathways is crucial when following up patients with MS, considering that excessive production of proinflammatory cytokines may lead to episodes of depression.

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## Declarations of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2020.100162.

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