Multiple Sclerosis and Related Disorders 44 (2020) 102243

Contents lists available at ScienceDirect

Multiple Sclerosis and Related Disorders

journal homepage: www.elsevier.com/locate/msard

Original article

Whole transcriptome analysis of multiple Sclerosis patients reveals active inflammatory profile in relapsing patients and downregulation of neurological repair pathways in secondary progressive cases

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ARTICLE INFO

Keywords:
Multiple Sclerosis
Transcriptome
gene expression
RRMS
SPMS

ABSTRACT

Background: Multiple sclerosis (MS) is an inflammatory autoimmune neurologic disease that causes progressive destruction of myelin sheath and axons. Affecting more than 2 million people worldwide, MS may presents distinct clinical courses. However, information regarding key gene expression and genic pathways related to each clinical form is still limited.
Objective: To assess the whole transcriptome of blood leukocytes from patients with remittent-recurrent (RRMS) and secondary-progressive (SPMS) forms to explore the gene expression profile of each form.
Methods: Total RNA was obtained and sequenced in Illumina HiSeq platform. Reads were aligned to human genome (GRCh38/hg38), BAM files were mapped and differential expression was obtained with DeSeq2. Up or downregulated pathways were obtained through Ingenuity IPA. Pro-inflammatory cytokines levels were also assessed.
Results: The transcriptome was generated for nine patients (6 SPMS and 3 RRMS) and 5 healthy controls. A total of 731 and 435 differentially expressed genes were identified in SPMS and RRMS, respectively. *RERE, IRS2, SIPA1L1, TANC2* and *PLAGL1* were upregulated in both forms, whereas *PAD2* and *PAD4* were upregulated in RRMS and downregulated in SPMS. Inflammatory and neuronal repair pathways were upregulated in RRMS, which was also observed in cytokine analysis. Conversely, SPMS patients presented IL-8, IL-1, Neurothrophin and Neuregulin pathways down regulated.
Conclusions: Overall, the transcriptome of RRMS and SPMS clearly indicated distinct inflammatory profiles, where RRMS presented marked pro-inflammatory profile but SPMS did not. SPMS individuals also presented a decrease on expression of neuronal repair pathways.

1. Background

Multiple Sclerosis (MS) is the most prevalent neurological autoimmune disease, causing significant clinical impairment of patients. Clinical courses of MS such as relapsing remitting (RR) and progressive forms have been suggested to be distinct entities (Zeydan and Kantarci, 2018). The transition from relapsing-remitting to progressive forms is not totally understood, but it is likely to be associated with previous lesion load, age, gender and low vitamin-D levels (Zeydan and Kantarci, 2018; Scalfari et al., 2018; Ascherio et al., 2014).

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https://doi.org/10.1016/j.msard.2020.102243
Received 20 January 2020; Received in revised form 21 April 2020; Accepted 25 May 2020

Pathologically the RRMS form presents an important inflammatory component, whereas neurodegenerative and noninflammatory profiles prevail in the progressive form (Lublin et al., 2014). Previous transcriptome studies in MS patients evaluated the expression of miRNAs and mRNAs using cDNA microarrays as well as real-time quantitative PCR and Next Generation Sequencing (NGS) approaches in order to understand the transcriptional effects of MS treatment (Angerer et al., 2018; Freiesleben et al., 2016; Guo et al., 2014; Smets et al., 2018; Gurevich et al., 2015; Moreno-Torres et al., 2018). Many studies dedicated to understand the role of distinct therapies in the transcriptome of MS patients. Regardless the drugs studied most of them reported altered level of expression of pro-inflammatory genes and pathways such as IL-2, IL-6 and others (Gurevich et al., 2015; Moreno-Torres et al., 2018; Mousavi Nasl-khameneh et al., 2018; Azoulay et al., 2009).

However, there have been limited information of overall gene expression of different forms of MS. Certainly, this may allow us to evaluate what genes and metabolic pathways are abnormally regulated in such conditions. Comprehensive and comparative analysis of transcriptomic alterations in RRMS and secondary progressive forms has not yet been undertaken.

Here we assessed the transcriptome of blood leukocytes obtained from MS patients presenting relapsing remitting (RRMS) and secondary progressive (SPMS) forms to deeply explore and compare gene expression profiles, highlighting the main disease-associated canonical pathways.

2. Methods

2.1. Study population

MS patients were diagnosed according to McDonald and Polman criteria (Angerer et al., 2018; Freiesleben et al., 2016; Polman et al., 2011; McDonald et al., 2001) and were enrolled in the study according to their clinical status. Healthy individuals with no family history of MS were also included as controls. Serum and total blood were collected from: i) Relapsing Remitting Group (RRMS) that included relapsing remitting MS patients under confirmed relapsing condition at the time of sampling and Expanded Disability Status Scale (EDSS) $<=4.5$. Samples were collected before corticosteroids treatment; ii) Secondary Progressive Group (SPMS), composed of patients diagnosed >10 years; EDSS $>= 6$; high neuronal lesion load with at least 1 lesion in every site of the Central Nervous System (CNS); iii) non-MS Control Group (CG), composed of age and gender matched healthy individuals with no familial history of MS. This study was approved by the ethical comittees from *Santa Casa de Misericórdia de São Paulo and Faculdade de Medicina da Universidade de São Paulo* (protocol # 620.741 e 896.341). All participants were informed and signed the consent form.

2.2. RNA extraction and purification

Peripheral blood mononuclear cells (PBMCs) were obtained after incubating 1mL of total blood with EL buffer (Qiagen) and centrifugation. RNA was extracted (Trizol-chloroform method) and 40U of RNAse inhibitor Ambion (California, USA) was added to avoid RNA degradation. The RNA was treated with DNAse as recommended (DNAse-Free Turbo – Ambion) and the absence of genomic DNA was confirmed by Real-Time PCR with primers to endogenous gene with multiple copies (HERV-W) without reverse transcriptase. RNA amount and quality were assessed after the procedure, through Bioanalyzer electrophoresis with Agilent RNA 6000 pico kit (Santa Clara, USA).

2.3. RNASeq

RNA samples were sequenced on Illumina HiSeq 2500 platform using pair-ended method. Briefly, library construction was performed with 100ng of total RNA using *TruSeq Stranded Total RNA sample kit* with *Ribo Zero Globin* (Illumina) or *GLOBINclear-Human kit* (Ambion). Around 10nM of the cDNA were used to construction and dispersion of clusters through the flowcell, which was performed in the cbot with HiSeq PE Cluster v4 cbot kit (Illumina). The sequencing was performed with HiSeq SBS v4 kit (Illumina) in two separate runs.

2.4. Bioinformatics pipeline

FastaQ files were assessed through fastQC software and aligned to human genome (GRCh38/hg38) using STAR Aligner (Dobin et al., 2013) with high stringency parameters, such as single hit reads and only one mismatch allowed per read. Low quality reads and short reads were discarded and BAM files were quantified with Salmon program (Patro et al., 2017). DESeq2 (Love et al., 2004) was used to identify differentially expressed transcripts ($p<0.05$), which were exported to a .csv file and uploaded in Ingenuity IPA (Qiagen) to perform pathway analysis.

2.5. Cytokine quantification in serum samples

A panel with selected pro inflammatory cytokines and chemokynes including IL-6, IL-1β, TNF-α, IFN-γ IP/CXCL10, MCP-1 and soluble CD14 (sCD14) was tested in an expanded cohort to validate the differential expression transcripts as suggested by the NGS transcriptome data. To quantify the cytokines we used a designed model of Luminex xMAP platform and for sCD14 the Quantikine ELISA for sCD14 (RnD Systems).

Table 1
Clinical and demographic information of individuals included in the study.

Groups	MS Groups RR (3)	SP (6)	Controls CG(5)
Gender	2 women 1 men	4 women 2 men	4 women 1 men
Age (median)	25-54 (45)	36-60 (48,5)	29-57 (40)
Duration of the relapse*	1-6 days (5 days)	NA	NA
EDSS (median)	1-4.5 (2)	6-8 (6.5)	NA
Duration of disease	4-20 years (4.5)	10-20 years (15)	NA
Current treatment	1 wo treatment 1 Avonex 1 βInterferon	6 Natalizumab◆	NA

*Only applicable for RRG, ◆Less than 6 infusions

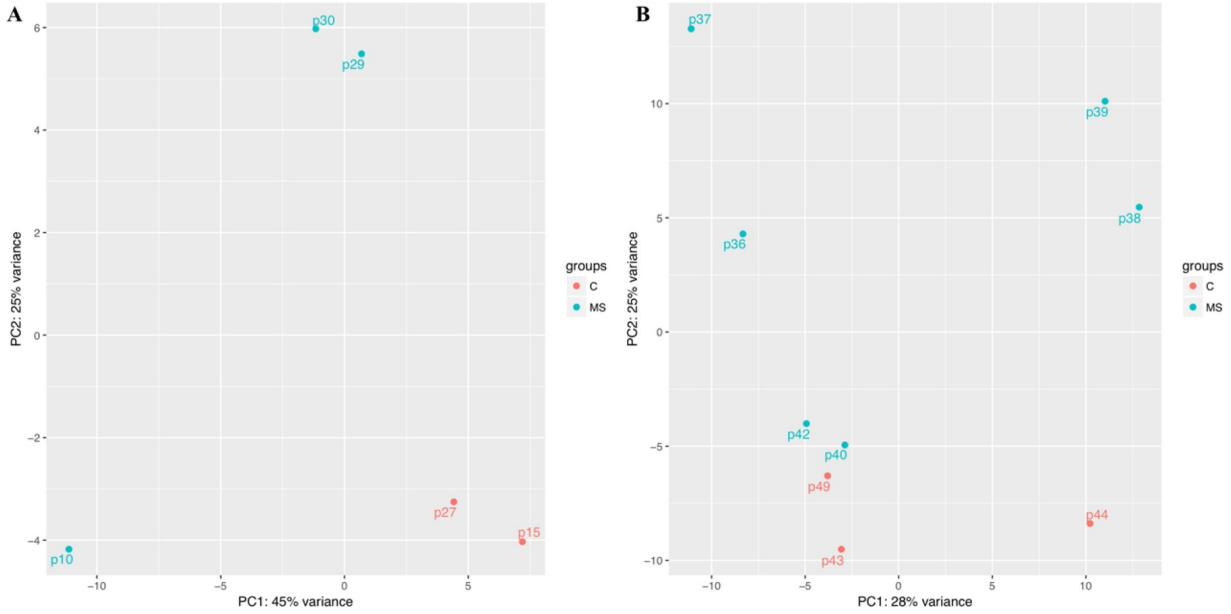

Figure 1. PCA analysis of normalized reads. A) PCA for RRMS (blue) versus healthy controls (red); B) PCA for SPMS (blue) versus healthy controls (red).

3. Results

3.1. Patients

The global transcriptome was assessed for 14 samples including 9 MS patients (3 RRMS and 6 SPMS) and five age and gender matched healthy controls (Table 1). Relevant clinical data is given in (Table 1).

3.2. Differential expression and pathway analysis

Above 21 million paired-end mapped reads were obtained per sample leading to a transcriptome coverage varying from 30 to 46x.

Since we performed the RNA sequencing in two separate runs, with distinct library kits we performed separate Principal Component Analyzes (PCA) comparing RRMS x controls and SSG x controls to avoid batch effects. In both PCA, MS cases grouped apart from controls (Fig. 1).

Differential expression analysis on DESeq2 revealed 731 genes differentially expressed in SPMS compared to controls and 485 genes differentially expressed in RRMS ($P < 0.05$). (S5 and S6)

We also identified 44 genes that were differently expressed in both RRMS and SPMS, of those *RERE, IRS2, SIPA1L1, TANC2* and *PLAGL1* were upregulated in both groups, whereas *RPS27* and *RPS29* were downregulated in both groups. Interesting the gene *PAD4* was upregulated in RRMS but downregulated in SPMS. Heatmaps showing the expression levels of each of these genes per individual are given in (Figures S1 and S2). The differentially expressed genes for each group were used to perform core analysis and comparisons on Ingenuity IPA (Qiagen), when clinically relevant pathways were assessed for both MS groups compared to controls. Core analysis for RRMS versus controls revealed 12 canonical pathways affected with biological support to the disease (Table 2) and in SPMS 15 affected canonical pathways (Table 3) were identified. The pathways are also represented on Figures S3 and

Table 2
Canonical pathways revealed from RNA-Seq analysis of RRG compared to controls.

Affected Pathway	Biological function	*Z score
IL-12	Pro-inflammatory response	0.06
Th1	Intracellular immune response	1.32
IL-7	Pro-inflammatory response	1.342
PEDF(Pigment epithelium derived factor)	Neuronal survival factor promotion	1.633
ILK(Integrin linked kinase	Integrin connections to cytoskeleton	1.897
IL-2	Lymphocyte growth and homeostasis	2
Integrin signaling	Cell adhesion molecules interaction with blood brain barrier	2.121
Paxilin	Cell mobility, adhesion to extracelullar matrix	2.45
IL-6	Pro-inflammatory response	2.646
Dendritic cell maturation	Adaptative and innate immune response enhancer	2.646
NFkβ	Adaptative and innate immune response cytokines	2.828
Lymphocyte leakage	Diapedesis promotion	3.05

* Z score values are related to more active pathways in cases as compared to controls (positive scores). No down-regulated pathways were seen for RRG as compared to controls.

Table 3
Canonical pathways revealed from RNA-Seq analysis of SSG compared to controls.

Affected Pathway	Biologic function	Z Score*
NGF (Neuronal Growth Factor)	Cell growth and proliferation of neuronal cells	-3.31
IL-8	Pro inflammatory response	-3.3
IL-1	Pro inflammatory response	-3.3
B cell receptor	B cell activation	-3.1
IL-6	Pro inflammatory response	-3.05
P13k (AKT)	Cell growth and proliferation	-2.7
VEGF (Vascular endothelial growth factor)	Cell growth and development of endothelial tissue	-2.6
iNOS	Cell signaling	-2.6
Neurotrophin	Neuronal cell growth	-2.6
B Lymphocyte activation factor	B cell activation	-2.23
Toll Like Receptor	Humoral and cell responses	-2.12
Neuregulin	Maintenance and growth of neuronal cells	-1.4
EIF4	Cell growth, proliferation and development	-1.13
mTOR	Cell growth, proliferation and development	-0.33
EIF2 (Eukaryotic initiation factor)	Apoptosis, cell damage	2.6

* Z score values are related to more active pathways in cases as compared to controls (positive scores), whereas down-regulated pathways are given by negative-scores.

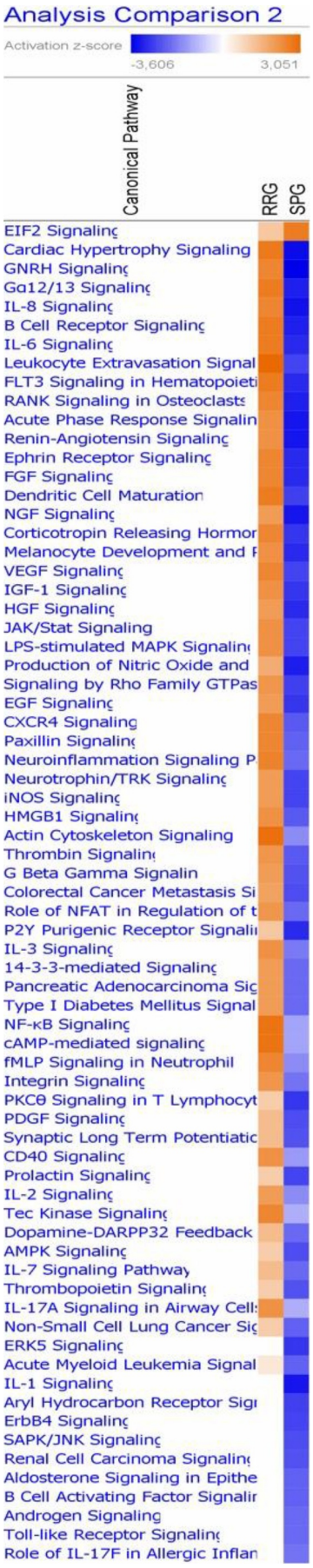

Figure 2. Comparison analysis of up and down regulated pathways for RG and SPMS.

S4. Comparisons of the altered pathways were performed (Fig. 2) and the pathways with stronger biological associations to MS are given in Tables 2 and 3.

Since analyses were always performed against the control group, we sought to analyze whether the genes responsible for the observed alterations of the pathways in both groups were the same. As illustrated in (Figure 3), the genes contributing of IL-6 pathway alterations in RRMS and SPMS were distinct, suggesting that these pathways are up or downregulated by distinct mechanisms.

3.3. Cytokine analysis

The quantification of cytokines (IL-6, IL-1β, TNF-α, IFN-γ, IP/CXCL10, MCP-1) and sCD14 were done after the RNA sequencing with the aim of validating some of the differentially expressed genes/pathways. To this, a larger number of individuals was evaluated (16 RRMS, 7 SPMS and 26 healthy controls). This analysis confirmed the abnormal expression of four pro-inflammatory cytokines observed in RRMS transcriptome analysis (IL-6, IL-1β, TNF-α, IFN-γ, $p < 0.01$). The only chemokine upregulated in SPMS group was MCP-1(Fig. 4).

4. Discussion

RRMS and SPMS are distinct entities (Zeydan and Kantarci, 2018); whereas RRMS displays an intense inflammatory condition, with acute clinical signs and periods of remission (Lublin et al., 2014), SPMS have a more degenerative profile (Lublin et al., 2014). The transcriptome analysis of patient-derived PBMCs revealed several upregulated pathways in RRMS related to inflammatory response. Oppositely, secondary progressive patients presented upregulated pathways linked, fundamentally, to a non-inflammatory profile but with transcripts that suggest an exhaustion of the neuronal repair, as evidenced in Table 3.

RRMS patients had activated canonical pathways that include integrin signaling and diapedesis promotion, indicating a typical immuno-inflammatory state with pro-inflammatory cytokines, vascular permeability and neuronal repair also activated. In fact, alterations in some of these pathways were already reported in MS, including IFNγ, IL-12, IL-2, and IL-6 (Balashov et al., 1997; Balashov et al., 1998; Sun et al., 2015; Malekzadeh et al., 2015; Wylezinski and Hawiger, 2016). Some integrins are current targets for MS treatment, in an attempt to avoid the migration of T-lymphocytes through the blood-brain barrier, such as Natalizumab (Polman et al., 2006). Nearly all studies focused on MS transcriptome analysis have compared groups receiving different drugs or before and after treatment (Gurevich et al., 2015; Moreno-Torres et al., 2018; Mousavi Nasl-khameneh et al., 2018; Azoulay et al., 2009). Interestingly, despite the drugs evaluated, the results revealed pro-inflammatory genes and/or pathways that are found to be altered in MS prior the treatment consistent with data obtained here, as well IL-2, IL-6, IL-12, Th1 (Moreno-Torres et al., 2018).

In addition to the upregulated pro-inflammatory cytokines, pathways involved in the migration of lymphocytes were also activated in RRMS. Among them, Paxillin, a focal adhesion adaptor protein that interact with integrins and potentially contribute for immune response in different sites (Liu et al., 1999; Kummer et al., 2010). Paxillin can also interact with proteins involved in the modification of cytoskeleton organization (Zaidel-Bar et al., 2003). Therefore, it is possible that when upregulated, Paxilin may increase lymphocyte adhesion and migration.

Intersection analysis of the differently expressed genes revealed that 5 genes were upregulated and 2 were down regulated in both groups of MS patients. Among these genes we highlight the function of *RERE*, formerly known as Athrophin, that play a role on the development of the cerebellum, specially Purkinje cells (Kim and Scott, 2014). In fact, lesions in the cerebellum are frequent disorder in MS patients (Smets et al., 2018; Kalron et al., 2018), and over expression of this

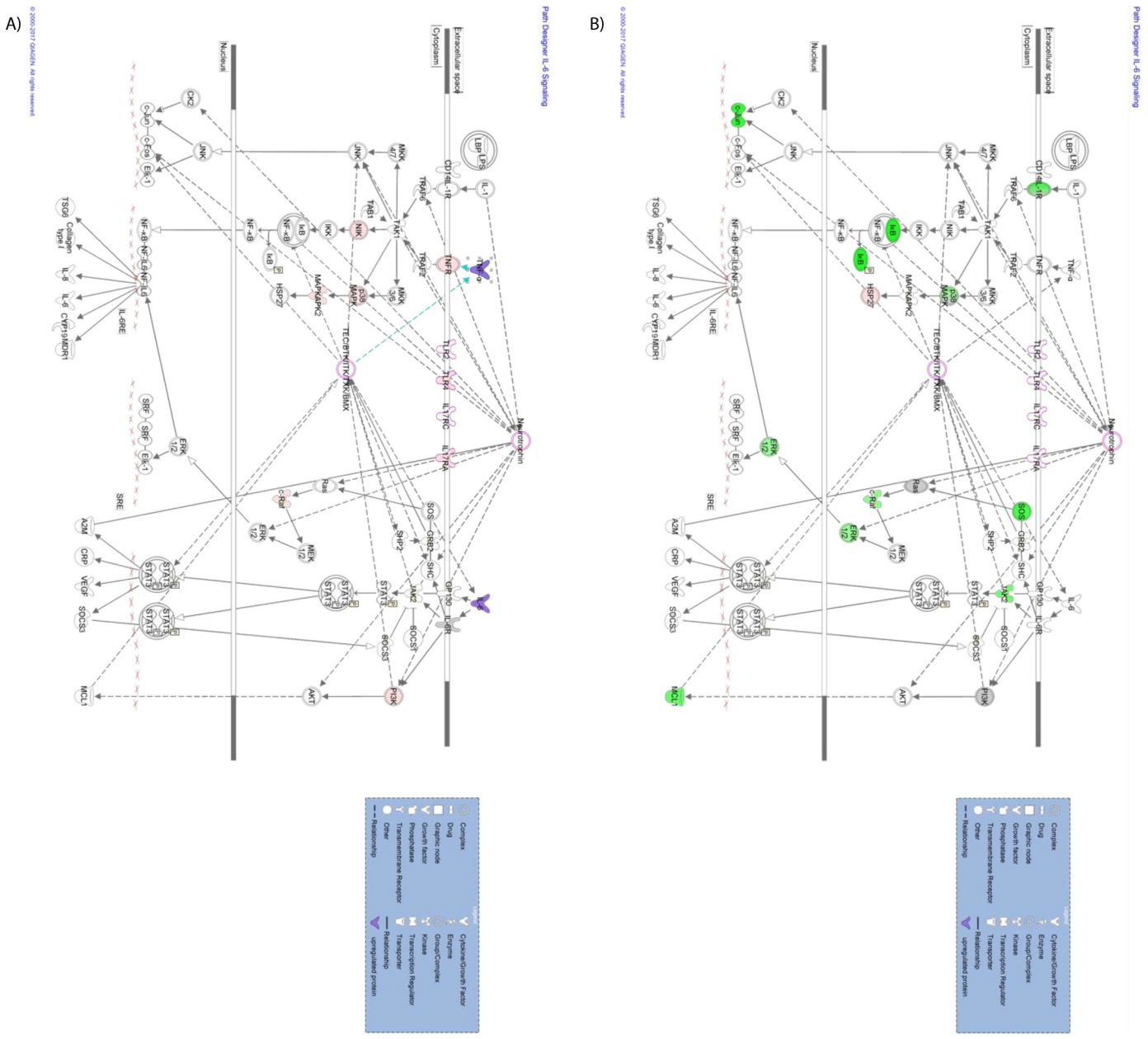

Figure 3. IL-6 pathway genic network. A) IL-6 pathway related genes in RRMS. B) IL-6 pathway related genes in SPMS. Genes in red/pink are upregulated, genes in green are downregulated, and genes in blank were not differentially expressed in MS patients. Genes in purple represent upregulated protein observed in cytokine quantification.

gene seems to be an alternative route for neuronal repair in the cerebellum. *TANC2*, other gene found to be up regulated in MS patients seems to play a role in synapsis signaling and plasticity and differentiation of neurons (Gasparini et al., 2017). Therefore, these findings suggest a signature of the transcriptional profile of some genes in Multiple sclerosis regardless the clinical conditions of the patients

The transcriptome data is in agreement with previous findings that described several differentially expressed genes and affected pathways in MS patients based on distinct approaches, such as microarray. For example, some of the upregulated genes found in the present study in RRMS patients as *HBA-1* and *2, TNFRS, FC6R3, ANPEP, IL6R, DYSF, ENTPD, MX1* were already reported as overexpressed in MS (Angerer et al., 2018; Guo et al., 2014,; Herrmann et al., 2016; Koch et al., 2018). Nevertheless, our transcriptome data revealed several other differentially expressed genes that have not been previously reported, regardless the same inflammatory outcome in MS patients observed previously (Guo et al., 2014; Hendrickx et al., 2017).

Peptidylargininedeiminase family (PAD) is involved in physiological processes as immune responses, and cell signaling. PAD2 and PAD4 in particular are expressed in the brain and the peripheral blood cells and it has been demonstrated that their upregulation may contribute to citrullination of myelin basic protein (MBP) in MS patients (Calabrese et al., 2012; Wood et al., 2008). Here we also confirmed that PAD4 is upregulated in RRMS but surprisingly, PAD2 and PAD4 were downregulated in SPMS group. PAD2 have been repeatedly reported to have a detrimental effect in MS by preventing MBP to form compact sheaths, letting myelin to remains immature (Beniac et al., 2000; Musse et al., 2008). Interestingly, however, the role of PAD2 in MS appears to be more complex and some controversial. For instance, the progression of EAE was not impaired in PAD2 knockout mice (Raijmakers et al., 2006). In another study, mice lacking PAD2 display impaired motor function and also a decrease in the number of

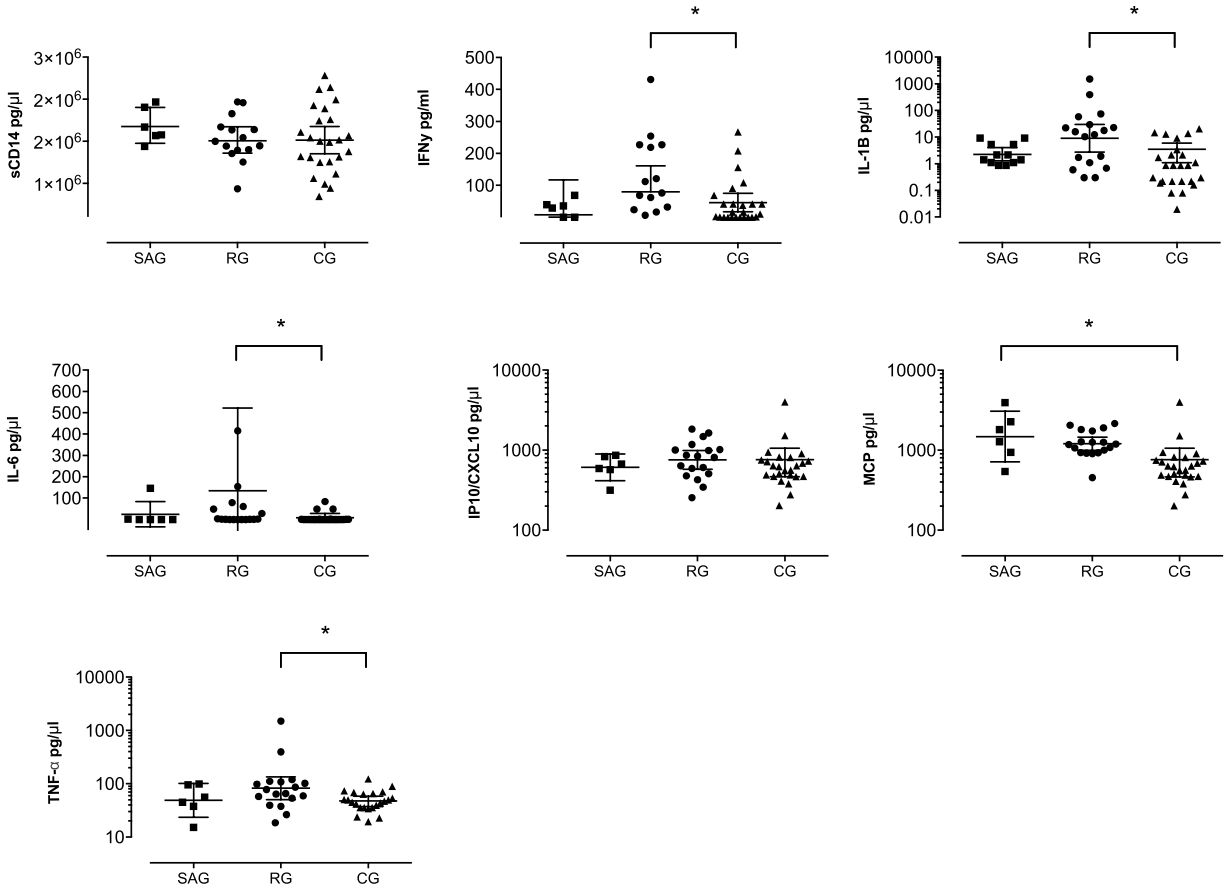

Figure 4. Analysis of cytokine levels in MS patients and controls. * = $p < 0.01$

myelinated axons (Falcão et al., 2019). The authors also showed that PAD2-mediated citrullination may act as an epigenetic modulator and is required for oligodendrocytes differentiation and myelination. Therefore, the downregulation of this gene as well PAD4 in secondary progressive group may be linked to the degenerative profile and re-myelination failure. The role of PAD enzymes in other proteins citrullination and regulation needs further investigations.

Patients with Diabetes Mellitus (DM) present increased probability to develop MS compared to non-DM individuals (Dorman et al., 2003; Hou et al., 2017; Bechtold et al., 2014). Also, the risk for DM is increased in MS patients, as compared to non-MS (Dobson and Giovannoni, 2013; Marrosu et al., 2002), revealing an striking interrelation between both conditions. In fact, both diseases share the inflammatory profile and previous transcriptomic analysis revealed differentially expressed genes related to inflammatory response in DM (Evangelista et al., 2014). Accordingly, pathways such as cytokine signaling and other genes were commonly differentially expressed in this study and DM, as *CCR1, CD46, TNF, IL6* and *RXRA*, suggesting that both diseases share particular pathways involved in autoimmunity.

Regarding SPMS, a non-inflammatory profile was evident, with decreased expression of pro-inflammatory pathways as IL-1, IL-6, IL-8 and B-cell response genes. Inflammation may be present in all stages of MS, including SPMS; however, the degree of inflammation appears to reflect disease-activity, which is more intense in acute MS and RRMS as compared to more advanced stages of the disease (Frischer et al., 2009). The SP group studied here presented EDSS >6 and no recent relapses, agreeing to the decline of the inflammatory process previously observed at this stage. It is important to highlight that the Neuregulin pathway, which promotes oligodendrogenesis and remyelinization (Kataria et al., 2018) was also downregulated. Similarly, low levels of this protein and also mutations in the correspondent gene were related to the progressive forms of MS (Kataria et al., 2018; Bahadori et al., 2015). Additionally, other pathways related to neuronal growth and neuronal regeneration were also previously found to be downregulated or non-functional in MS, including neurotrophin and nerve grow factor (Minnone et al., 2017; Acosta et al., 2015) as well as the galanin gene. Neurotrophin is class of proteins of the family of grow factors, and among several functions, they play a role in synaptic physiology (Shinoda et al., 2019) and in myelin repair (Zhu et al., 2014). Mutations in Brain Derived Neutrophin (BNDF) gene are related to the progression of MS (Nociti et al., 2018).

Nerve growth factor (NGF) regulates the synthesis of several neurological receptors, as neurotransmitters and neuropeptides. Its effects in local immune response have also been explored and several immune cells as monocytes, macrophages, dendritic, T and B cells have receptors to NGF and respond to this protein altering the proliferation, maturation and cytokine release among others (Minnone et al., 2017; Thorpe et al., 1987). Studies on inflammatory and autoimmune diseases have revealed an increase in NGF at the sites of inflammation and its activity appears to be bidirectional, as NGF participates in neuronal repair during inflammation process in Experimental Autoimmune Encephalomyelitis (EAE), decreasing the inflammation and contributing to the neurological recovery (Acosta et al., 2015). Additional experiments in EAE clearly demonstrated that increasing the NGF levels reduces disease severity by diminishing tissue inflammation and by preventing the full development of EAE lesions and immune cell infiltrates (Villoslada et al., 2000; Arredondo et al., 2001).

Therefore, the NGF downregulation detected in our RNA-Seq data may probably be related to the inability to control the neurological recovery, contributing to the maintenance of a chronic inflammation status and neurodegeneration observed in these individuals.

Other studies focused on transcriptome analysis of distinct

degenerative neuropsychiatric conditions, such as Alzheimer's disease, demonstrated several differentially expressed genes (Berchtold et al., 2014; Sekar et al., 2015), including *ARHGEF11* and *CD93* that were also altered in RRMS group. However, none of the affected pathways of SPMS and RRMS were observed in the previous studies, suggesting that the degeneration process in both diseases is probably an outcome from distinct pathways. The observation of genes involved these pathways may reveal hallmark signatures of genetic profiles associated to these diseases.

Together with the reduced sample size, using total PBMC rather than brain lesion samples might be a limitation of this study. However, most transcriptome studies using RNA-Seq and other methods (Angerer et al., 2018; Guo et al., 2014; Herrmann et al., 2016; Koch et al., 2018; Hendrickx et al., 2017) consider the whole PBMCs as a representative cellular group since these cells usually reflect patterns of expression that operate in the disease, even when the studied samples are not representative of the diseased tissue.

Another limitation includes distinct treatment approaches in the subjects of the groups. All patients from SP group were receiving Natalizumab (NTZ) for less than 6 months, and of the three RR individuals, one was receiving Avonex, other received B-Interferon, and the third was drug naïve. Avonex is also a class of B-interferon and both may produce similar effects on the patients' transcriptome. In fact, the results of all three individuals were similar (see Figure S1) and distinct from the controls. We can't exclude, however, the possibility that part of the transcriptome alterations observed in SPMS are derived from the treatment since NTZ is capable of changing the patients inflammatory mechanism and of altering the number of circulating immune cells (Börnsen et al., 2012) as well as the expression of some pro- and anti-inflammatory proteins (Ramos-Cejudo et al., 2011). Nevertheless, the immunological effects of NTZ are mostly observed in long-term treated patients (Jilek et al., 2010; Arru et al., 2014). Short effects were also observed (24h after the first infusion) but they apparently support a pro-inflammatory phenotype, with increased IL-2 and IL-17 (Benkert et al., 2012). The very opposite scenario we observed here, with decreasing pro-inflammatory biomarkers in SPMS. Therefore our observations are unlikely to be NTZ-derived artifacts.

In summary, data described here agree with the two different scenarios for MS according to its clinical course. The pathways altered correspond to the main characteristics observed in each stage. Critically, the downregulation of NGF found in SP group was not totally unexpected. Others have observed a decrease in this protein in SP individuals in comparison to RRMS and CIS, where inflammation is more prominent (Villoslada and Genain, 2004). Comparing particular activated and inactivated pathways in the transcriptome of both groups suggest that the ability to keep the balance between inflammation and repair is crucial to control the progression of the disease. In this sense, the release of NGF during the inflammation may play a key role in the repair of inflammation-induced damages.

Credit author statement

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Declaration of Competing Interest

The authors declare no conflict of interest

Acknowledgements

ED-N is a research fellow from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. This work was funded by Fundação de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP) projects # 2015/05958-3 and # 2013/24223-9.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2020.102243.

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