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PBMCs protein expression profile in relapsing IFN-treated multiple sclerosis: A pilot study on relation to clinical findings and brain atrophy

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ABSTRACT

This cross-sectional study investigated with two-dimensional gel electrophoresis coupled to MALDI-TOF and MRI the relationship between PBMCs protein expression profile and whole-brain atrophy in 16 unselected RR-MS IFN-treated patients compared with 6 RR IFN-untreated and 12 matched healthy control subjects. Grey/white matter fraction, T1/T2 lesion load and clinical variables were considered too.
Twenty six proteins showed significant differential expression among RR IFN-treated patients and control samples. Four of these (IN35, GANAB, PP1B, SEPT2) resulted correlated with clinical and MRI findings in RR IFN-treated MS patients.
Future clinical applications remain to be validated by other techniques and confirmed by a larger study.

1. Introduction

Relapsing–remitting multiple sclerosis (RR-MS) is a chronic demyelinating disease of the central nervous system (CNS) with autoimmune and degenerative components.

The main pathogenetic hypothesis arises from the activation of peripheral autoreactive T-cells that adhere to the blood brain barrier and transmigrate into the brain parenchyma. In this phase, many soluble inflammatory mediators released by immune cells or even resident cells can induce neuronal and axonal loss (Sospedra and Martin, 2005), the major determinants of persistent neurological disability and disease progression (Filippi and Rocca, 2005; Trapp et al., 1999; Neumann, 2003). Despite many evidence suggest the pivotal role of this inflammatory environment in the development of neurodegeneration in MS (Giuliani and Yong, 2003; Noseworthy et al., 2000), the interactions between inflammation, demyelination and neurodegeneration are still under investigation (Wolfang, 2007).

In the last decade, several MR-based parameters provided surrogate markers in monitoring disease progression. Moreover, the brain atrophy has been proposed to monitor MS evolution and represents an additional exploratory measure of outcome in recent clinical trials (Miller et al., 2002). On the contrary, no blood-derived species of clinical relevance is available at present. Lastly, the so called disease modifying therapies like interferon-beta (IFN-β) acting on the peripheral T-cells, reduce the disease activity and the clinical progression, with a MRI-detectable effect in preventing lesion burden and cerebral atrophy development in RR-MS (Hardmeier et al., 2005; Richert et al., 2006), by suggesting a critical role of peripheral blood mononuclear cells (PBMCs) immune response and modulation in developing CNS inflammation and atrophy.

In this study, MRI was applied in combination with two-dimensional gel electrophoresis (2-DE) separation of PBMC proteins to identify clinically relevant correlations between PBMCs protein expression profile and clinical and MRI parameters in a class of RR IFN-β treated patients. Furthermore, proteomic modifications depending on therapy were addressed by a comparison with a diseased untreated group.

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2. Materials and methods

2.1. Study population

Sixteen (nine male, seven female) consecutive unselected RR-MS, IFN-treated subjects have been enrolled in a case control cross-sectional proteomic and MRI study at the MS unit of "F. Ferrari" Casarano Hospital Neurology Division (LE), Italy. All MS patients were previously diagnosed according to the standard criteria (McDonald et al., 2001) and imaged in a steroid- and exacerbation-free period of at least three months. Patients were included in the study according to the following criteria: (i) RR phase of the disease; (ii) age between 23 and 65 years; (iii) administration of IFN-β-1a-based therapy from one year at least; and (iv) availability to express written informed consent. Patients treated with immunosuppressive drugs were excluded from this study. Patients with autoimmune diseases (LES, rheumatoid arthritis etc.) or other comorbidity conditions were excluded. All the patients received, when appropriate, symptomatic therapy not interfering with immune conditions.

Patients were classified as RR according to standard disease course criteria (Lublin and Reingold, 1996) and treated with interferon β-1a at 30 μg I.M. weekly according to international guidelines (Jacobs et al., 1996). Disability was scored on the Expanded Disability Status scale (EDSS) (Kurtzke, 1983) within 48 h from MRI acquisition. Twelve (seven male, five female) age and sex matched healthy control subjects have been imaged and ten of these underwent the blood sampling. Moreover, we considered a sex/age matched IFN-untreated MS group constituted by six patients enrolled and classified according to the same standard criteria. These patients expressed the same disease duration than IFN-treated group. Approval was provided by the local hospital and central ethic committee.

2.2. MRI protocol

MR imaging was performed on a Philips Gyroscan 1.5 T apparatus. Two-dimensional 28 axial slices of the brain were obtained using interleaved slices (3 mm), a 230 mm field of view, by using T2-weighted turbo spin-echo (TSE) (repetition time [TR]/echo time [TE]/MAT: 6158/110/304×512), PD-weighted TSE (TR/TE/MAT: 1697/30/352×512) and unenhanced T1-weighted turbo fluid attenuation inversion recovery (FLAIR) (TR/TE/inversion time [TI]/MAT: 2165/22/860/304×512) sequences. Subjects were positioned inside the magnet according to the European Community guidelines (Miller et al., 1991). All the axial slices were acquired and analysed from the base of the cerebellum to the vertex, with a caudal cut-off point defined as the most cranial cord slice not-containing cerebellum.

2.3. MRI image analysis

Image analysis was performed on a Windows computer-assisted system according to the highly reproducible semiautomatic local thresholding technique for lesions segmentation previously published (Grimaud et al., 1996; Rovaris et al., 1997). In particular, the lesions were first outlined on each PD-weighted axial slice and the latter was used as a reference to increase confidence in lesion detection on T2-weighted axial slices.

T1 lesions were delineated with semiautomatic local thresholding technique according to a sequence that expresses a low signal (short TR, short TE), with an intensity level ranging between those of the cerebrospinal fluid (CSF) and the grey matter and corresponding to a high level intensity signal region on the T2-weighted sequence (high TR and high TE).

T1-2 lesions were delineated as regions of interest (ROI) and the corresponding volume expressed in cubic millilitres was obtained for each sequence by multiplying the total area of ROIs by the slice thickness. The number of T1 and T2 lesions was also calculated.

To assess the reproducibility of the measurements, five determinations were performed on the same images set for all patients, quantifying the intra-observer T1LL and T2LL coefficient of variation (COV = 100% × SD / mean) between the repeated measurements. The mean test–retest interval was 17.3 days (SD = 23.3).

Table 2
Radiological features of MS and control subjects.

	CC			RRt			
	Range	Mean	SD	Range	Mean	SD	*p*
BPF	0.90–0.75	0.84	0.05	0.85–0.73	0.79	0.02	0.03
GMF	0.43–0.60	0.54	0.07	0.54–0.48	0.50	0.05	0.01
WMF	0.22–0.42	0.29	0.06	0.33–0.21	0.26	0.04	0.01
T1LL				0.03–20.53	5.03	5.93	
T2LL				0.46–40.18	9.82	11.90	
N°T1				1.00–45.00	20.57	11.22	
N°T2				7.00–65.00	33.05	15.89	

BPF: brain parenchymal fraction.
GMF: grey matter.
WMF: white matter fraction.

2.4. Selective and whole-brain atrophy

The brain atrophy was calculated on T1-weighted conventional spin-echo sequences. The brain parenchymal fraction (BPF), representing the ratio of brain parenchymal volume to the total intracranial volume (TIV) within the brain surface contour, was used to measure global brain atrophy (Rudick et al., 1999). Selective brain atrophy was calculated from the grey (GMF) and white matter fraction (WMF) normalized against TIV using the formulas: normalized GMF = GMF / TIV; normalized WMF = WMF / TIV. Evaluation of total and partial brain volumes were performed using the well validated SIENAX tool (Smith et al., 2002).

2.5. PBMC isolation and lysis

Heparinized blood (15 ml) was collected by venipuncture 24–32 h after the IFN-β injection and immediately processed. To minimize the platelets contamination, blood was centrifugated at 150 ×*g* for 5 min and the platelet-enriched supernatant was removed. Blood was then diluted with PBS (Phosphate Buffered Saline) solution and layered on a Ficoll density gradient (GE Healthcare). PBMCs were isolated by centrifugation (400 ×*g* for 40 min) and processed within 1 h. After the isolation of the lymphocyte layer, cells were washed twice in PBS and lysed in 0.5 ml of sample buffer (7 M Urea, 2 M Thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and protease inhibitor cocktail). The sample was sonicated 3 times × 5 s on ice avoiding the formation of foaming (low amplitude). Protein concentration was determined by the Bradford assay (Bio-Rad Protein assay).

Table 1
Clinical and demographic features of MS treated (RRt), untreated (RRu) and healthy control (HC) subjects.

	RRt (*n* = 16)			HC (*n* = 12)			RRu (*n* = 6)			*p* value
	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	
Disease duration	2.0–14.0	7.43	4.5				2.9–13.9	7.83	3.9	$p = 0.15$
Age at scanning	23.0–65.0	42.6	11.1	22.2–64.3	41.8	10.6	20.9–66.0	42.9	12.2	$p^{1,2,3} \geq 0.22$
EDSS	0.0–5.0	2.0	1.7							
Sex ratio (♀/♂)	56%/44%			58%/42%			57%/43			

$p^{1,2,3}$: *p* value of comparison between HC/RRt, RRu/RRt, RRu/HC groups respectively.