Analysis of serum protein biomarkers in clinical forms of Multiple Sclerosis

Brichette-Mieg I.[1,4], Aliaga-Gaspar P.[1,4], Rodríguez-Bada J.[1], Alonso-Torres A.[1,2,3], Urbaneja-Romero P.[1,2,3], Reyes-Garrido V.[1,2,3], Ortega-Pinazo J.[1], Hurtado-Guerrero I.[1,3], Oliver-Martos B.[1,2,3,4], Leyva L.[1,2,3,4]

1. *Neuroimmunology and Neuroinflammation Group. UGC Neurociencias, Instituto de Investigación Biomédica de Málaga-IBIMA. Hospital Regional Universitario de Málaga, Spain.* 2. *Red Andaluza de Investigación Clínica y Traslacional en Neurología (Neuro-Reca) Andalucía Spain.* 3. *Red Española de Esclerosis Múltiple (REEM), Spain*. 4. *Universidad de Málaga, Spain.*

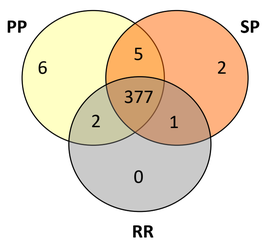
Introduction: Multiple sclerosis (MS) is an autoimmune disease that presents a significant inter- and intraindividual heterogeneity, which puts it among the most difficult disease to diagnose. Thus, there is still an unmet need to develop and validate new biomarkers in the field of MS (1). An important mechanism to regulate the activity of the immune system is the generation of soluble receptors by alternative splicing or proteolysis (2). The combination of membrane proteins and their soluble isoforms is a main source for the search of non-invasive biomarkers in MS. For that purpose, an exploratory phase with -omic techniques for the identification of potential biomarkers, focused on cytokine and chemokine receptors, as well as on the proteins involved in their signaling pathways was carried out.

Objective: The aim of this study was to identify soluble proteins associated with the different clinical forms of MS, to validate this –omic results by enzyme-linked immunosorbent assay (ELISA), and to establish whether a differential expression can provide insight into some of the mechanisms underlying the pathogenesis of the different clinical forms.

Material and Methods: **Quantitative proteomic analysis** was carried out in serum samples from 18 untreated patients with MS (7 RRMS, 7 SPMS and 4 PPMS) and seven healthy subjects (HC), matched in aggregate by age and gender. Total protein fractions were analyzed using nano-liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS / MS) on a MALDI-TOF / TOF UltrafleXtreme mass spectrometer. Data analysis was performed using 4 search engines (Mascot, OMSSA, X! TANDEM and Myrimatch) and a target database constructed from sequences in the proteome using "Homo sapiens at Uniprot Knowledge base" as a reference. Before analyzing the samples, they were purified to remove the majority proteins (Top 10), quantified and normalized to the same protein concentration. For data analysis the proteins were normalized in the "Total Peptide Amount" mode and the ratios were calculated using the Protein Abundance Based approach. Hypothesis testing was carried out using an ANOVA based on the abundance of each of the proteins.

Validation of the results was carried out in an independent cohort of 60 untreated MS patients (20 of each clinical form) and 20 HC by commercial ELISAs (Antibodies-online and Vitro S.A.). The abundance of each protein among the different clinical forms was initially compared by means of Kruskal-Wallis test (KW). Then, differences between two matrices were assessed with the matrix pairwise comparison and adjusted for multiple testing with Bonferroni correction (P_{adj}).

Results of the Exploratory Phase

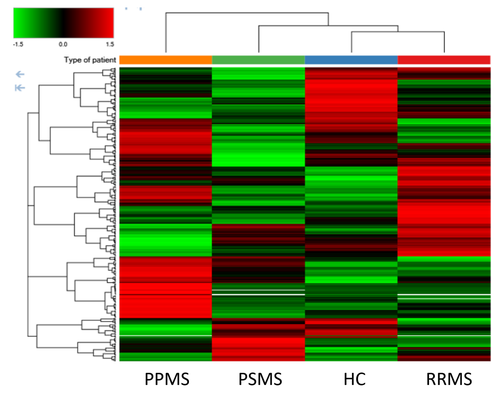


In the proteomic analysis of the serum of the exploratory cohort, 1432 proteins were identified, of which 393 met the following criteria:

1) They were "Master" proteins within their protein group;
2) The percentage of false positives was <1%;
3) More than 2 unique peptides were detected in the protein.

There were 377 proteins detected in the three clinical forms, 6 exclusive proteins in PPMS patients, 2 exclusive proteins in SPMS patients and none in RRMS patients.

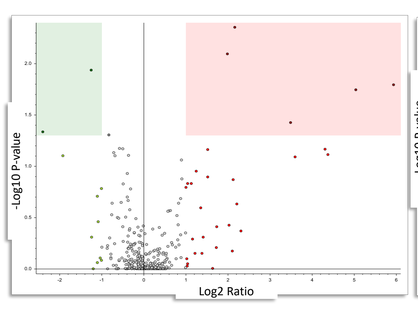
Every MS clinical form showed a unique profile, perfectly distinguishable from controls and from the other MS clinical forms.



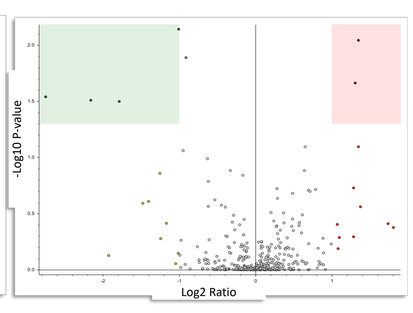
Proteins deregulated in each clinical form

Proteins having a fold change < 0.5 or > 2 *(Log_2 ratio < -1 or > 1)* and found to be statistically different [p < 0.05 *(–Log_{10} p value > 1.3)*] between the groups were considered deregulated.

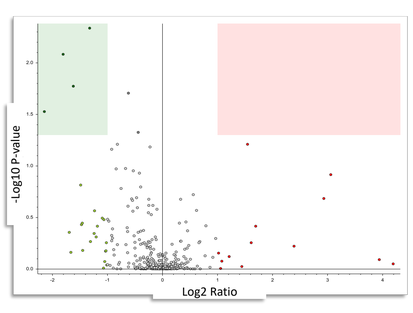
PPMS versus HC



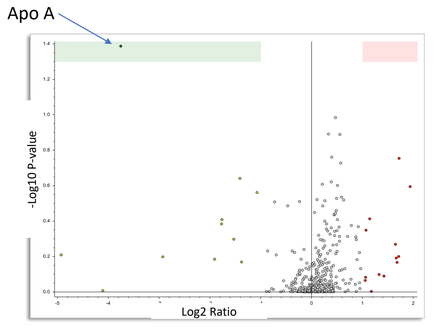
RRMS versus HC



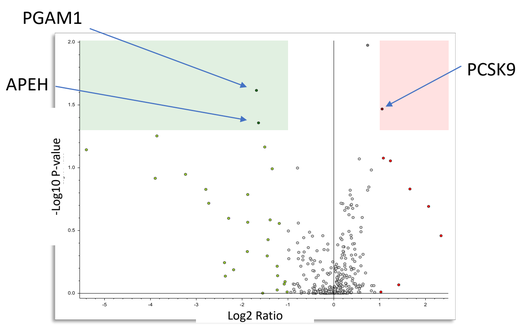
SPMS versus HC



RRMS versus SPMS



RRMS versus PPMS



Proteins found to differ significantly between each of the clinical forms and HC are listed in the following tables:

PPMS versus HC	AR
Protein S100-A6 (**S100A6**)	61.32
Neutrophil elastase (**ELANE**)	32.89
Peroxiredoxin-6 (**PRDX6**)	11.28
Phosphoglycerate mutase 1 (**PGAM1**)	4.49
ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2 (**BST1**)	3.97
Complement factor H-related protein 5 (**CFHR5**)	0.42
Low affinity immunoglobulin gamma Fc region receptor III-A (**FCGR3A**)	0.19

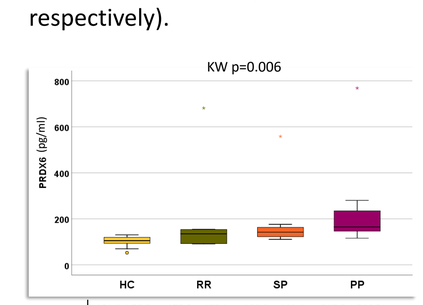
RRMS versus HC	AR
Hepatocyte growth factor-like protein (**MST1**)	2.55
Complement factor H-related protein 2 (**CFHR2**)	2.47
Isoform 2 of Alpha-1B-glycoprotein (**A1BG**)	0.50
Immunoglobulin kappa constant (**IGKC**)	0.29
Low affinity immunoglobulin gamma Fc region receptor III-A (**FCGR3A**)	0.22
Immunoglobulin kappa light chain (**IGKL**)	0.15

SPMS versus HC	AR
Low affinity immunoglobulin gamma Fc region receptor III-A (**FCGR3A**)	0.33
Immunoglobulin kappa light chain (**IGKL**)	0.23
Immunoglobulin kappa constant (**IGKC**)	0.29
Alpha-actinin-1 (**ACTN1**)	0.40

- Apolipoprotein A (**APO A**) was less abundant in RRMS than in SPMS patients (AR= 0.12).
- Those proteins decreased in RRMS compared to PPMS were Phosphoglycerate mutase 1 (**PGAM1**) and Acylaminoacid releasing enzyme (**APEH**) (AR of 0.312 and 0.322, respectively), whereas Proprotein convertase subtisilin/kexin type 9 (**PCSK9**) was more abundant in RRMS patients (AR = 2.077).

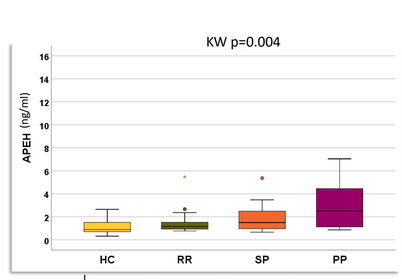
Results of the Validation Phase

Although, many of the results of the proteomic analysis were not validated by commercial ELISAs in an independent cohort, our analysis confirmed the abundance of **PRDX6** in PPMS patients versus HC (P_{adj}=0.003). Even if in the proteomic analysis **APEH** abundance did not reach an AR of 2 in PPMS relative to controls, assessment in a higher sample size during the validation phase showed that this protein was more abundant in PPMS patients than in controls (P_{adj}=0.004). The higher ratio of **APEH** in PPMS compared to RRMS was also confirmed (p=0.027), but statistical significance was lost after Bonferroni correction. On the other side, **CFHR2** was significantly less abundant in PPMS patients than in HC (P_{adj}=0.025).
Regarding RRMS patients, **BST1** showed a higher abundance in this clinical form than in PPMS patients and HC (P_{adj}=0.040 and 0.030, respectively). **MST1** analysis showed opposite results to the proteomic phase, as a lower AR was found in RRMS patients than in HC and PPMS patients (P_{adj}=0.014 and 0.027, respectively). Finally, **PCSK9** was also less abundant in RRMS patients than in PPMS, SPMS patients, and HC (P_{adj}=0.024, 0.010, 0.012, respectively).



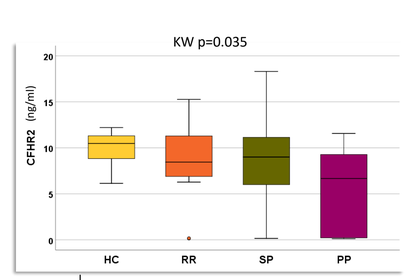
	HC	RR	SP	PP
Mean ± SD	101.6 ± 25.9	179.2 ± 178.4	186.9 ± 141.1	247.0 ± 216.3
Median	104.8	134.9	141.8	164.7

	P-value	P adj
SP vs. HC	**0.011**	0.065
PP vs. HC	**0.001**	**0.003**



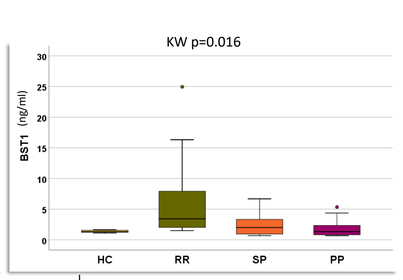
	HC	RR	SP	PP
Mean ± SD	1.16 ± 0.65	1.53 ± 1.09	1.88 ± 1.23	4.65 ± 7.59
Median	0.89	1.17	1.50	2.51

	P-value	P adj
SP vs. HC	**0.045**	0.270
PP vs. HC	**0.001**	**0.004**
PP vs. RR	**0.027**	0.159



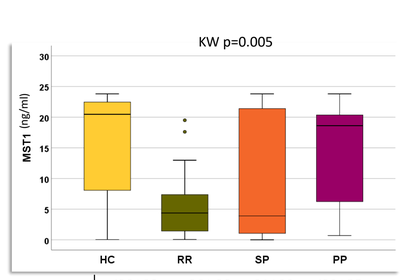
	HC	RR	SP	PP
Mean ± SD	9.9 ± 1.8	8.3 ± 4.5	8.7 ± 5.1	4.9 ± 4.6
Median	10.5	8.4	8.7	6.7

	P-value	P adj
PP vs. HC	**0.004**	**0.025**



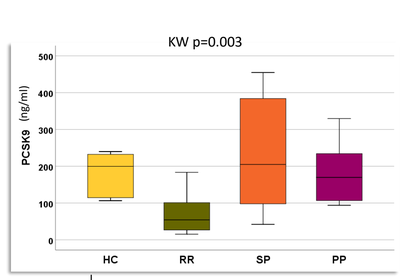
	HC	RR	SP	PP
Mean ± SD	1.37 ± 0.19	6.75 ± 7.80	6.18 ± 11.93	1.97 ± 1.62
Median	1.35	3.42	2.00	1.33

	P-value	P adj
RR vs. HC	**0.005**	**0.030**
RR vs. PP	**0.007**	**0.040**



	HC	RR	SP	PP
Mean ± SD	15.8 ± 9.2	5.6 ± 5.5	9.1 ± 9.9	15.3 ± 7.7
Median	20.5	4.4	3.9	18.8

	P-value	P adj
RR vs. PP	**0.004**	**0.027**
RR vs. HC	**0.002**	**0.014**



	HC	RR	SP	PP
Mean ± SD	183.1 ± 55.9	68.9 ± 52.7	231.7 ± 162.6	186.4 ± 84.5
Median	199.7	54.2	204.8	169.8

	P-value	P adj
RR vs. PP	**0.004**	**0.024**
RR vs. SP	**0.002**	**0.010**
RR vs. HC	**0.002**	**0.012**

Conclusions:

- The proteins we found to differ between PPMS and HC are involved in "*neutrophil degranulation*", "*response to oxidative stress*", and "*regulation of complement activation*". Those that differ between RRMS patients and both the HC and the PPMS patients are involved in "*regulation of cell-matrix adhesion*", "*regulation of macrophage activation*", "*regulation of receptor recycling and internalization*" and "*lipoprotein metabolism*".
- These results support the use of proteomic techniques as a high throughput method for the identification and discovery of blood-based biomarkers, useful in the diagnosis and prognosis of MS.
- The combination of PRDX6, APEH, CFHR2, BST1, MST1, and PCSK9 abundance in serum could facilitate the ongoing challenge of diagnosing MS and distinguishing between PPMS and RRMS clinical forms.

References:
1. Ziemssen T, Akgün K, Brück W. Molecular biomarkers in multiple sclerosis. J Neuroinflammation 2019;16(1):272.
2. Hurtado-Guerrero I, et al. Antiviral, Immunomodulatory and Antiproliferative Activities of Recombinant Soluble IFNAR2 without IFN-ß Mediation. J Clin Med 2020;9(4):959.

Funding*:* This work was supported by PI18/00551 (ISCIII, Spanish Ministry of Science, Innovation and Universities) and co-founded by AEI/FEDER, UE, PI: Leyva L & Oliver-Martos B, and by CPC-21/132 EME-REEM to Leyva L.