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Identification of peripheral biomarkers for immune-modulatory treatment efficacy in Multiple Sclerosis patients

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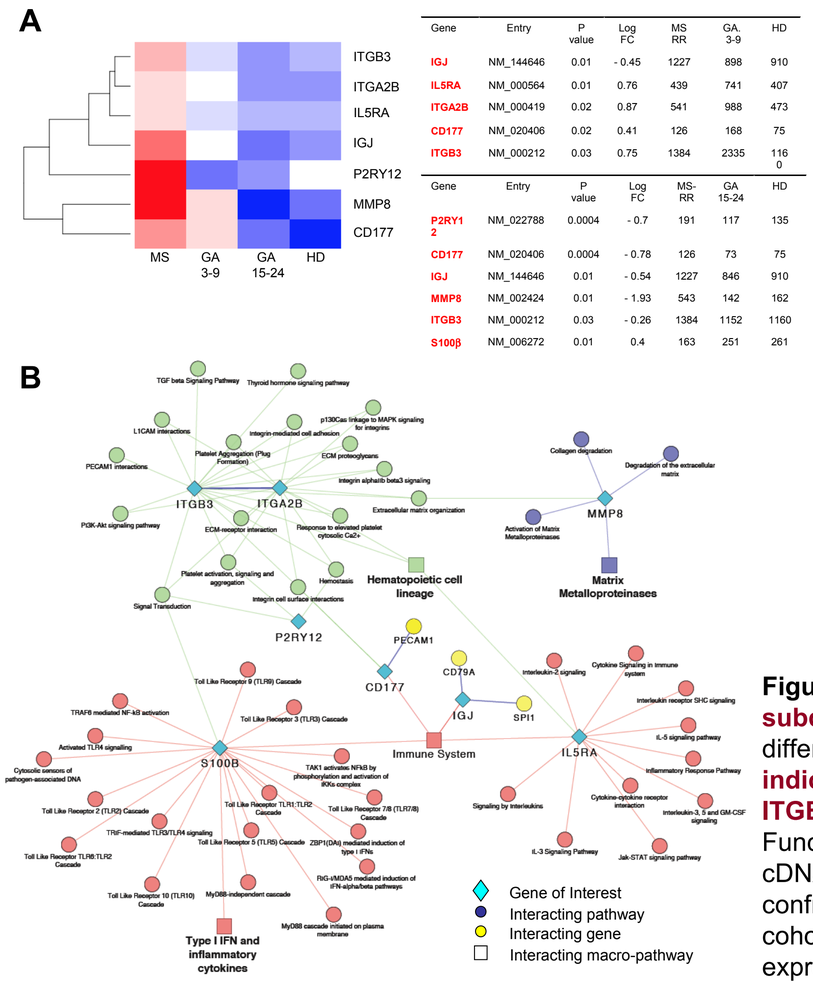
Introduction & Aims: Multiple sclerosis is a very heterogeneous neuroimmunologic disorder, which still lacks complete pathogenic characterization. The vast majority of patients is affected by a relapsing-remitting form of MS, for which different immune-modulating therapies exist. Nonetheless, so far no single biomarker correlates with clinical activity or, even more importantly, with treatment response. The aim of the study is to analyse the molecular and protein phenotype of immune competent peripheral blood cells to identify novel biomarkers for treatment responsiveness. In the present study, we enrolled relapsing-remitting MS patients (RR-MS) for microarray analysis followed by a longitudinal study, to detect a molecular signature of immune-modulatory treatment at a peripheral level. We first performed a real-life one-shot whole-blood microarray study on Glatiramer Acetate (GA) early (3-9 months of treatment) and late (15-24 months of treatment) responding patients, to identify a specific set of differentially expressed genes. Secondly, the selected biomarkers were validated in peripheral blood leukocytes, via qPCR and cytofluorimetry, in a longitudinal follow-up study of RR-MS patients treated with GA, Interferon-beta (IFNβ), Fingolimod, and a cohort of RR-MS patients non responding to first-line drugs.

Microarray and validation study



Gene	Entry	P value	Log FC	MS RR	GA. 3-9	HD
IGJ	NM_144646	0.01	- 0.45	1227	898	910
IL5RA	NM_000564	0.01	0.76	439	741	407
ITGA2B	NM_000419	0.02	0.87	541	988	473
CD177	NM_020406	0.02	0.41	126	168	75
ITGB3	NM_000212	0.03	0.75	1384	2335	1160

Gene	Entry	P value	Log FC	MS-RR	GA 15-24	HD
P2RY12	NM_022788	0.0004	- 0.7	191	117	135
CD177	NM_020406	0.0004	- 0.78	126	73	75
IGJ	NM_144646	0.01	- 0.54	1227	846	910
MMP8	NM_002424	0.01	- 1.93	543	142	162
ITGB3	NM_000212	0.03	- 0.26	1384	1152	1160
S100β	NM_006272	0.01	0.4	163	251	261

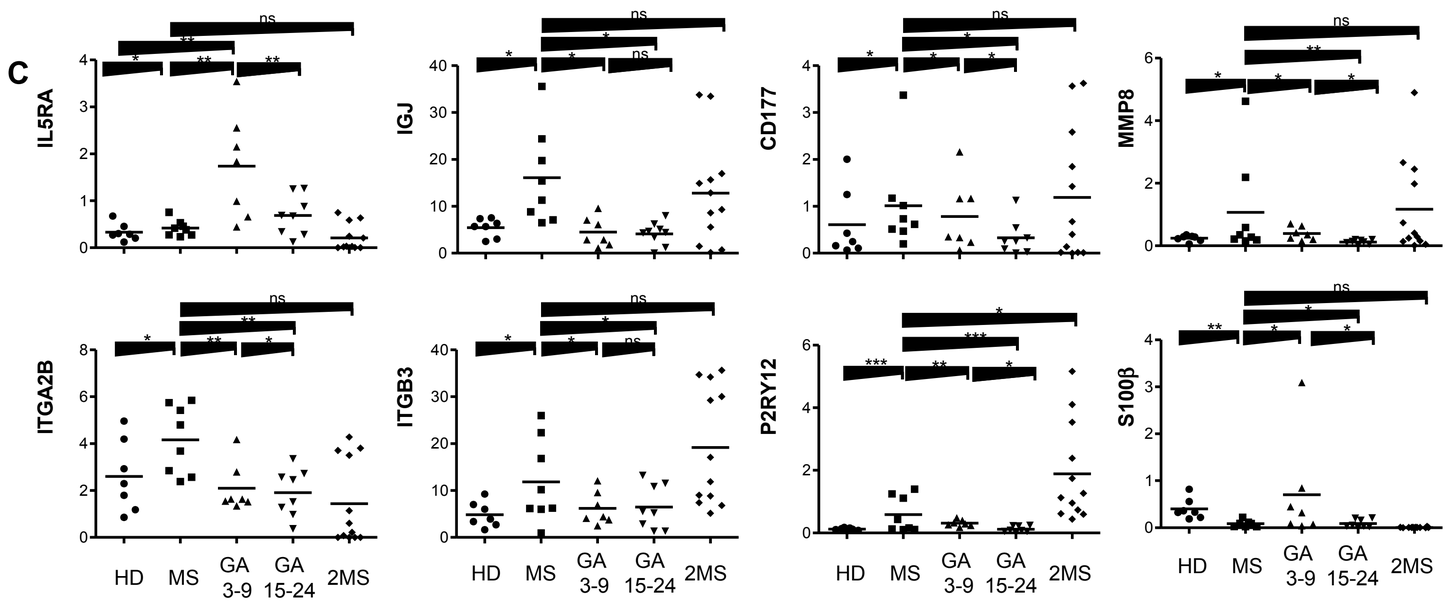
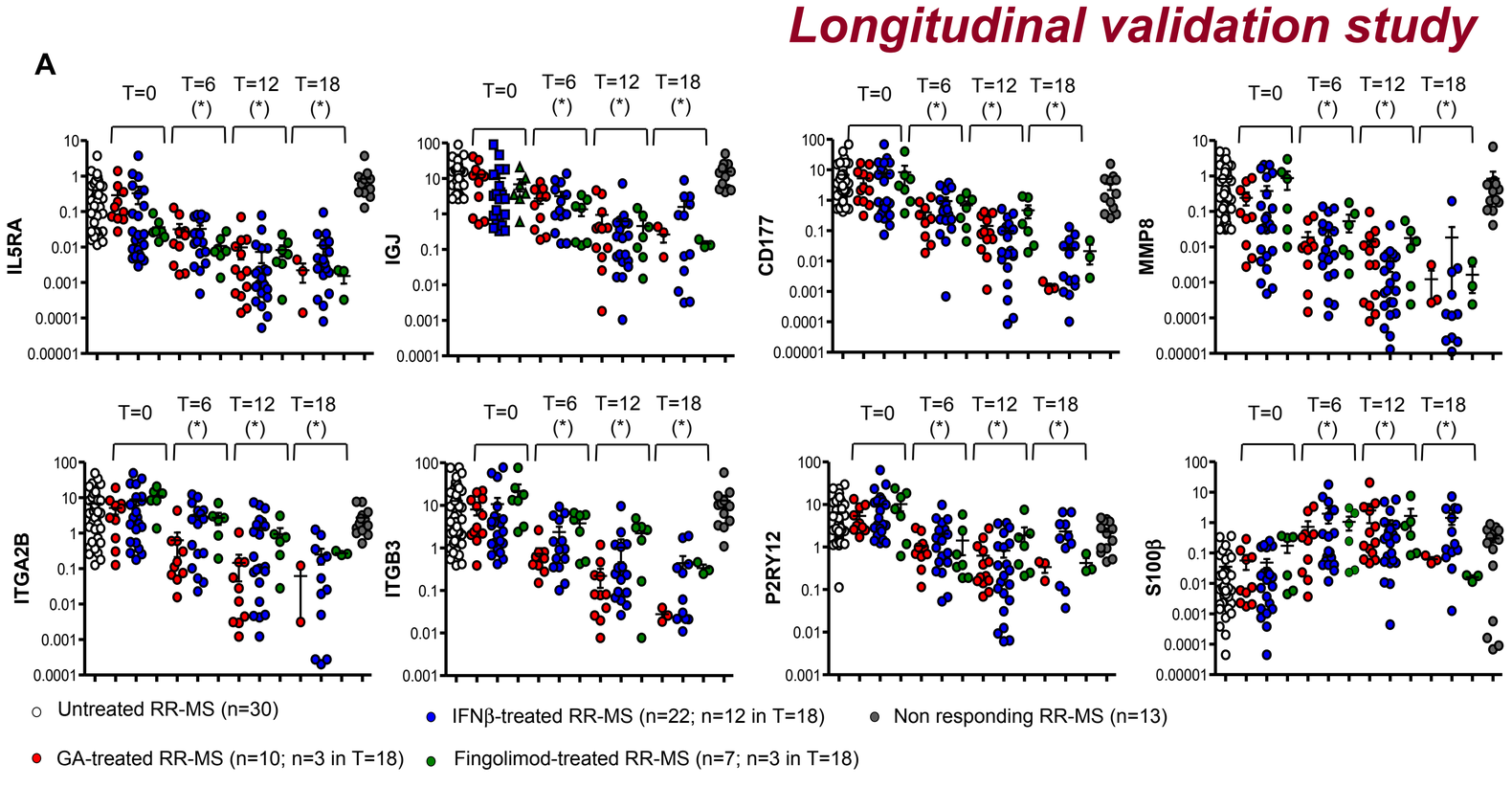


Figure 1. Microarray analysis of a cohort of 52 healthy donors (HD) and 32 RR-MS patients (MS), 20 of which treated with GA, subdivided in two groups according to treatment duration (3-9 months, n=9; 15-24 months, n=11). Microarray analysis resulted in 212 differentially expressed genes following short GA treatment, and 336 following long GA treatment**. We selected 8 genes as treatment efficacy indicators,** on the basis of Log fold expression, and biological relevance in immune-modulation of the central nervous system**: ITGA2B, ITGB3, IL5RA, IGJ, P2RY12, MMP8, CD177 and S100β** (**A**). The selected genes showed tight interconnections, as represented by the Functional Annotation Network (**B**), thus forming a specific **signature** of GA **immune-modulatory treatment**. qPCR analysis on whole-blood cDNA validated the microarray data (**C**), regarding the gene expression of **IL5Ra, IGJ, CD177, MMP8 ITGA2B, ITGB3, P2RY12 and S100β**, confronting HD (n=7), RR-MS (MS; n=8), RR-MS GA treated for 3-9 months (GA3-9; n=7) or 15-24 months (GA15-24; n=8), and a second cohort of newly-enrolled RR-MS patients (2MS; n=12). Gene expression values were normalized to the housekeeping gene GAPDH and expressed as $2^{-Dct}\times100$. Statistics: Kruskal-Wallis test with Dunn's multiple comparison. * =P<0.05, ** =P<0.01, *** =P<0.001.

Longitudinal validation study



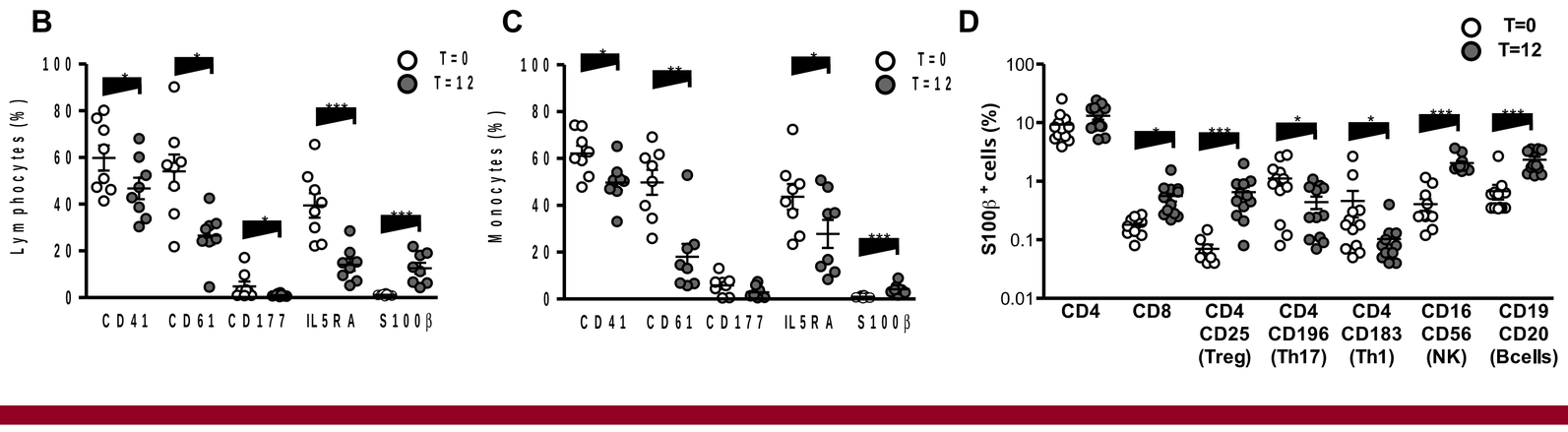


Figure 2. The immune-modulatory signature highlighted in the microarray study was confirmed through a longitudinal validation study, on 82 RR-MS patients. (A) qPCR analysis of PBL gene expression in RR-MS patients under GA (n=10), IFNβ (n=22) and Fingolimod (n=7) treatment. Gene expression for **IL5Ra, IGJ, CD177, MMP8, ITGA2B, ITGB3, P2RY12 and S100β** was analysed in patients PBL sampled before therapy initiation (T=0) and at T=6, T=12 and T=18 months (T=18: n=3 for GA and Fingolimod; n=12 for IFNβ), and compared to PBL of untreated RR-MS patients (n=30) and RR-MS patients non responding to GA, IFNβ or Fingolimod therapies (n=13). Values in (**A**) are normalized to GAPDH housekeeping gene, expressed as $2^{-Dct}\times100$ and represented in logarithmic scale. Kruskal-Wallis test with Dunn's multiple comparison. * =P< 0.05 for the following comparisons: T=0vs.T=6, T=0vs.T=12, T=0vs.T=18 for each treatment. No significant difference was observed between Untreated RR-MS and each T=0 samples, nor between Untreated RR-MS and Non responding RR-MS samples. (**B-D**) Protein levels of **CD41 (ITGA2B), CD61 (ITGB3), CD177, IL5RA and S100β** were detected via cytofluorimetric analysis in circulating lymphocytes (**B**) and monocytes (**C**) of RR-MS patients before immune-modulating treatment initiation (T=0) and after 12-month treatment (T=12). The analysis comprised 11 RR-MS patients under GA (n=4), IFN-β (n=4) and Fingolimod (n=3) therapy. Results showed a decrease in percentages of positive cells for CD41, CD61, CD177 and IL5RA gated on total lymphocytes (**B**) and monocytes (**C**), while highlighting an increase in both S100β+ lymphocytes (**B**) and S100β+ monocytes (**C**), at T=12. Among the S100β+ lymphocytes, detailed sub-population analysis (**D**) showed an increase in S100β+ Treg cells (CD4+ CD25+), S100β+ CD8 cells, S100β+ NK cells (CD16+CD56+) and S100β+ Bcells (CD19+CD20+), accompanied by a decrease in S100β+ effector Tcells, both Th17 (CD4+CD196+) and Th1 (CD4+CD183+). Statistics: Mann-Whitney test: * =P<0.05, ** =P<0.01, *** =P<0.001.

Altogether the results validated both at molecular and at protein level the signature of effective immune-modulatory treatment, characterized by down-regulation of IL5RA, MMP8, CD177, IGJ, ITGA2B, ITGB3, P2RY12 and up-regulation of S100β in PBL of RR-MS patients treated with GA, IFNβ and Fingolimod.

Results & Conclusions: The microarray study identified a GA-modulated signature of gene expression, comprising both typical leukocyte-related genes (CD177, IGJ), integrins (CD41, CD61), markers of inflammation (IL5RA), extracellular matrix proteases (MMP8), and atypical leukocyte-related genes, such as purinergic metabotropic receptors (P2RY12) and calcium binding proteins (S100□). The computational analysis revealed strong inter-connections between the selected genes or their related pathways. The eight selected biomarkers (CD177, IGJ, CD41, CD61, IL5RA, MMP8, P2RY12, S100□) were then confirmed both at molecular and at protein level in a follow-up study in which samples were obtained every six months of treatment with first-line (GA, Interferon-beta) and second-line (Fingolimod) drugs. Thus, a specific immune-modulatory signature of eight biomarkers was identified, highlighting a common immune-modulatory mechanism of action in three different MS drugs and monitoring their effective treatment in responsive patients.