Human Molecular Genetics, 2011, Vol. 20, No. 18 **3606–3619**
doi:10.1093/hmg/ddr281
Advance Access published on June 15, 2011

Global map of physical interactions among differentially expressed genes in multiple sclerosis relapses and remissions

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Received April 12, 2011; Revised and Accepted June 10, 2011

Multiple sclerosis (MS) is a central nervous system autoimmune inflammatory T-cell-mediated disease with a relapsing–remitting course in the majority of patients. In this study, we performed a high-resolution systems biology analysis of gene expression and physical interactions in MS relapse and remission. To this end, we integrated 164 large-scale measurements of gene expression in peripheral blood mononuclear cells of MS patients in relapse or remission and healthy subjects, with large-scale information about the physical interactions between these genes obtained from public databases. These data were analyzed with a variety of computational methods. We find that there is a clear and significant global network-level signal that is related to the changes in gene expression of MS patients in comparison to healthy subjects. However, despite the clear differences in the clinical symptoms of MS patients in relapse versus remission, the network level signal is weaker when comparing patients in these two stages of the disease. This result suggests that most of the genes have relatively similar expression levels in the two stages of the disease. In accordance with previous studies, we found that the pathways related to regulation of cell death, chemotaxis and inflammatory response are differentially expressed in the disease in comparison to healthy subjects, while pathways related to cell adhesion, cell migration and cell–cell signaling are activated in relapse in comparison to remission. However, the current study includes a detailed report of the exact set of genes involved in these pathways and the interactions between them. For example, we found that the genes *TP53* and *IL1* are 'network-hub' that interacts with many of the differentially expressed genes in MS patients versus healthy subjects, and the epidermal growth factor receptor is a 'network-hub' in the case of MS patients with relapse versus remission. The statistical approaches employed in this study enabled us to report new sets of genes that according to their gene expression and physical interactions are predicted to be differentially expressed in MS versus healthy subjects, and in MS patients in relapse versus remission. Some of these genes may be useful biomarkers for diagnosing MS and predicting relapses in MS patients.

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune inflammatory T-cell-mediated disease, believed to result from a misdirected immune attack against central nervous system (CNS) myelin antigens. The damage of myelin in MS leads to neurological dysfunction (1–3). MS attacks mainly young adults (usually between ages 20 and 40), and is predominant in women. In northern Europe and the USA, the disease has prevalence of around 100 per 100 000 (2). Thus, it has a significant socioeconomic impact.

The trigger of the autoimmune process in MS is unknown. It is believed that MS occurs as a result of some combination of genetic, environmental and infectious factors (4), and possibly other factors such as vascular problems (5). For example, studies of identical twins have demonstrated a concordance

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of 30% to develop MS (3), suggesting that the genetic background has a relatively limited but significant role in triggering MS.

Symptoms of MS are unpredictable and include, among others, motor weakness, loss of balance and muscle coordination, slurred speech, cognitive decline, sensory impairment and bladder dysfunction.

In 85% of the patients, the disease has a relapsing–remitting course, which is characterized by the onset or deterioration of the neurological symptoms (relapses), which are followed by partial or complete recovery (remissions). Relapses are the consequence of complex immunological and neurodegenerative processes. They result in the development of new acute inflammatory lesions or the activation of old lesions within the CNS, and are associated with myelin and axonal loss (1,2,6). Accordingly, relapses are the visible clinical expression of the complicated immunopathological mechanisms that occur in the CNS.

As was demonstrated in numerous previous studies, transcriptional profiling of peripheral blood mononuclear cell (PBMC) is a useful tool for identifying gene expression signatures that are related to MS and other autoimmune diseases (7–16, reviewed in 17). A possible explanation for the advantageousness of PBMC in this context is the fact that autoreactive immune cells initiate the autoimmune inflammatory process against the corresponding target organs (18–24).

The aforementioned studies concerning gene expression in MS were based solely on measurements of mRNA levels. The first stage of gene expression regulation is transcription (i.e. changes in mRNA levels). However, several steps in the gene expression process may be regulated, including transcription, post-transcriptional modifications, such as RNA splicing, translation and post-translational modifications of a protein (25). Thus, studying changes in gene expression should give a very limited and coarse depiction of the regulatory changes that occur in the disease. Indeed, it was shown that in human and other organisms, the gene mRNA levels explain only ~30% of their protein abundance variance (26,27). In addition, mRNA measurements usually suffer from various elements of noise and bias (28).

The goal of the current study is to improve the understanding of the regulatory changes in PBMC in MS, by incorporating in addition to gene expression, prior knowledge about protein–protein interactions (PPIs) in human. By integrating this additional data, we were able to identify genes that undergo regulatory changes in MS, which are not necessarily at the transcription level. It has been shown that such an approach is useful for identifying markers related to metastasis (29) and yields better results compared with analyses based solely on gene expression; the current paper is the first time that such an approach was employed for the analysis of an autoimmune disease. In autoimmune diseases, as in cancer metastasis, many of the regulatory changes are posttranscriptional; thus, such an approach should be beneficial in both cases.

We identified proteins that have many PPIs with differentially expressed genes (in terms of mRNA levels) in MS compared with healthy subjects, and in MS relapse when compared with MS remission. Such genes have not previously been reported in large-scale studies of mRNA levels in MS.

With high probability, such genes undergo transcriptional and/or post-transcriptional regulation. Thus, our approach enables uncovering suspected transcriptional and post-transcriptional regulatory changes in the disease.

Indeed, as demonstrated in the following sections, we supply a more accurate and comprehensive analysis of the genes and pathways involved in MS than previously reported.

RESULTS

We analyzed a DNA-microarray gene expression dataset that included a total of 164 subjects. To detect genes that are differentially expressed (in terms of mRNA levels) in the disease, we employed a statistical method which takes into account their demographical parameters and batch effects (Materials and Methods). Next, to better understand the molecular mechanisms related to MS, the data of the differently expressed genes were integrated with large-scale measurements of PPI and protein–DNA interactions (PDIs, Materials and Methods, Fig. 1).

At all stages, we analyzed two datasets. Each dataset was related to a comparison of two groups of patients or healthy subjects:

(i) The first dataset included patients with MS (relapse or remission, 123 patients) that were compared with healthy subjects (41 subjects). This dataset was utilized to understand regulatory processes that appear in both relapse and remission stages of MS.
(ii) The second dataset included MS patients with relapse (34 patients) that were compared with MS patients with remission (75 patients with active disease, EDSS >0, Materials and Methods). This dataset was used to better understand the regulatory processes that are specific to each stage of the disease.

We named the first dataset MS/healthy and the second dataset Rel/Rem. The properties of these datasets (e.g. age, gender, disease duration of the analyzed patients) appear in Table 1 (see also Supplementary Material, Table S1).

Differentially expressed genes in MS and their projection on the PPI and PDI networks: a global (network-level) view

Gene expression data were normalized, statistically over/under-expressed genes were found in each dataset based on the analysis of variance [ANOVA (30)] and the false discovery rate [FDR; (31) see the Materials and Methods section for more technical details regarding the normalizations]. We found that 1268 and 938 genes were significant according to ANOVA (P-value ≤ 0.05) in the MS/healthy and the Rel/Rem databases, respectively.

In both datasets, no gene passed the FDR criterion based on the ANOVA P-values (Table 2 for the list of genes with the most significant ANOVA P-value; see also Supplementary Material, Tables S2 and S3 for the entire list of P-values) demonstrating the essentiality of integrating additional information sources in order to augment the differentially expressed genes signature.

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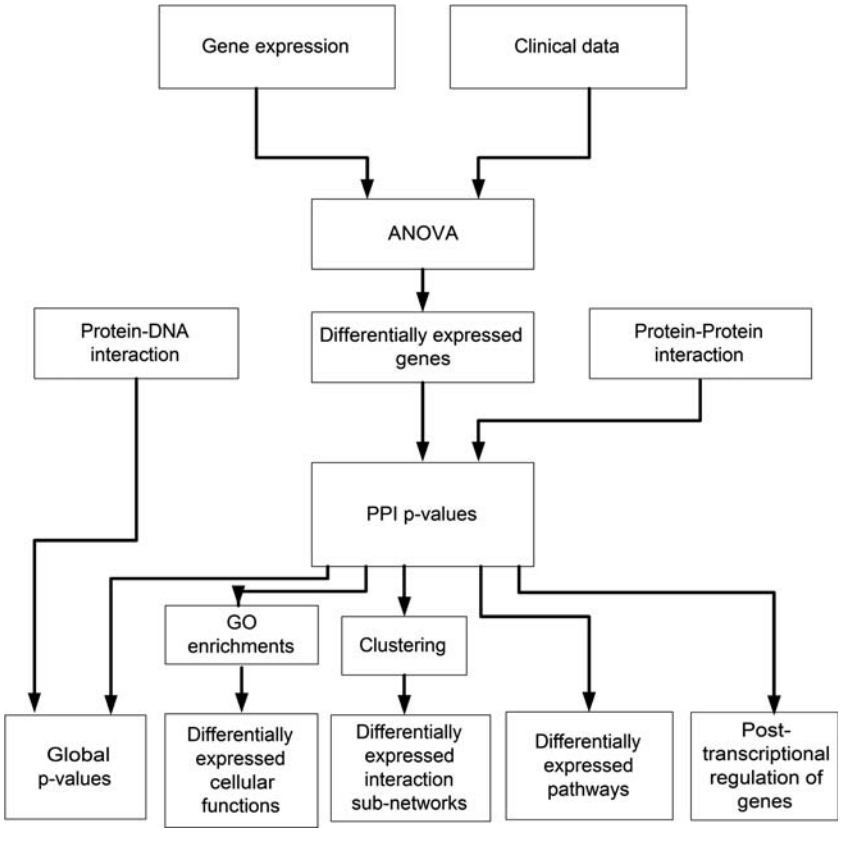

Figure 1. Flow diagram of the analysis performed in this study.

Table 1. Clinical and demographical characteristics of the patients/subjects in the analyzed datasets

Group	Age	Disease duration	Annual relapse rate	EDSS	F/M
(A)					
All ($n = 164$)	35.5 ± 10.31	N/A	N/A	N/A	99/65
MS patients ($n = 123$)	35.6 ± 10.8	6.16 ± 6.15	1.58 ± 1.66	2.25 ± 1.45	78/45
Healthy subjects ($n = 41$)	35.1 ± 8.7	N/A	N/A	N/A	21/20
(B)					
All ($n = 109$)	36.6 ± 10.47	6.6 ± 6.31	1.46 ± 1.59	2.54 ± 1.28	72/37
Patients in relapse ($n = 34$)	34.0 ± 8.4	6.14 ± 4.13	1.0 ± 0.65	3.29 ± 1.2	25/9
Patients in remission ($n = 75$)	37.7 ± 11.14	6.8 ± 7.1	1.7 ± 1.8	2.21 ± 1.18	47/28

(A) The entire dataset (MS/healthy—MS patients versus healthy subjects). (B) The subset of patients in relapse and in remission (Rel/Rem dataset). See also Supplementary Material, Table S1.

At the next stage, we computed for each gene a *P*-value that was based on the number and expression levels of genes that have protein interaction with it (but not including mRNA levels of the gene itself). We named this *P*-value PPI *P*-value. Genes that have a relatively high number of PPIs with differentially expressed genes have a higher probability to undergo regulatory changes, possibly post-transcriptional, themselves and thus have more significant PPI *P*-values.

We found that 398 and 257 genes were PPI significant (*P*-value <0.05) in the MS/healthy and Rel/Rem databases, respectively (see Supplementary Material, Tables S2 and S3 for the entire list of *P*-values).

Figure 2 includes a global view of the projection of the differentially expressed genes on the PPI network. In the remainder of this section, we report several global *P*-values that are related to the expression levels of the entire set of genes and the physical interactions between them. The *P*-values were based on comparisons to random networks with similar properties to the original ones (Materials and Methods).

The first global *P*-value tests if differentially expressed genes tend to be close to each other in the PPI network, as expected for the real biological signal. We found that this global *P*-value was significant in the case of the MS/healthy dataset (*P*-value $= 0.05$) but was not significant in the case

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Table 2. Genes with the most significant *P*-values (ANOVA and PPI) for the MS/healthy dataset (A) and for the Rel/Rem dataset (B)

ANOVA *P*-values				Protein interaction *P*-values			
Gene	Fold change	*P*-value	Description	Gene	Fold change	*P*-value	Description
(A)							
GNAI1	−1	9.80E − 06	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	JUN	1	1.52E − 14	jun oncogene
PDE3A	1	5.93E − 05	Phosphodiesterase 3A, cGMP-inhibited	SMAD3	−1	1.43E − 11	SMAD family member 3
PANK4	−1	8.99E − 05	Pantothenate kinase 4	RB1	1	1.26E − 10	Retinoblastoma 1
STK38	−1	9.99E − 05	Serine/threonine kinase 38	JUNB	1	6.48E − 10	jun B proto-oncogene
PARP4	−1	0.0001179	Poly (ADP-ribose) polymerase family, member 4	FOS	1	1.13E − 09	FBJ murine osteosarcoma viral oncogene homolog
PDCD10	−1	0.0001555	Programmed cell death 10	JUND	1	1.12E − 08	jun D proto-oncogene
MMP9	1	0.0001914	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	TP53	−1	7.71E − 08	Tumor protein p53
OR2W1	−1	0.0002247	Olfactory receptor, family 2, subfamily W, member 1	TRAF1	−1	1.75E − 07	TNF receptor-associated factor 1
PLEKHF1	−1	0.0002551	Pleckstrin homology domain containing, family F (with FYVE domain) member 1	BRCA1	−1	1.15E − 06	Breast cancer 1, early onset
SOCS1	1	0.0003106	Suppressor of cytokine signaling 1	TBP	−1	1.21E − 06	TATA box binding protein
(B)							
GNG4	1	7.12E − 06	Guanine nucleotide binding protein (G protein), gamma 4	PTPRD	1	2.21E − 10	Protein tyrosine phosphatase, receptor type, D
C6orf103	1	2.53E − 05	Chromosome 6 open reading frame 103	PDGFRB	0	6.55E − 09	Platelet-derived growth factor receptor, beta polypeptide
GPR116	−1	2.66E − 05	G protein-coupled receptor 116	STAT5A	0	6.55E − 09	Signal transducer and activator of transcription 5A
KIAA1654	1	2.68E − 05	KIAA1654 protein	PTPRF	1	1.67E − 08	Protein tyrosine phosphatase, receptor type, F
CCL14–CCL15	1	4.00E − 05	Chemokine (C-C motif) ligand 14/chemokine (C-C motif) ligand 15	PPFIA1	0	1.67E − 08	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 1
ST7L	1	4.26E − 05	Suppression of tumorigenicity 7 like	PTPRS	1	4.40E − 08	Protein tyrosine phosphatase, receptor type, S
ACCN3	1	0.000108689	Amiloride-sensitive cation channel 3	STAT5B	−1	4.68E − 08	Signal transducer and activator of transcription 5B
ALOX15	1	0.000117418	Arachidonate 15-lipoxygenase	PTPRA	−1	1.65E − 07	Protein tyrosine phosphatase, receptor type, A
CARD14	1	0.000120208	Caspase recruitment domain family, member 14	PPFIA2	1	1.65E − 07	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 2
LGR4	1	0.000132248	Leucine-rich repeat-containing G protein-coupled receptor 4	SCNN1B	0	1.65E − 07	Sodium channel, non-voltage-gated 1, beta

All the PPI *P*-values pass the FDR.

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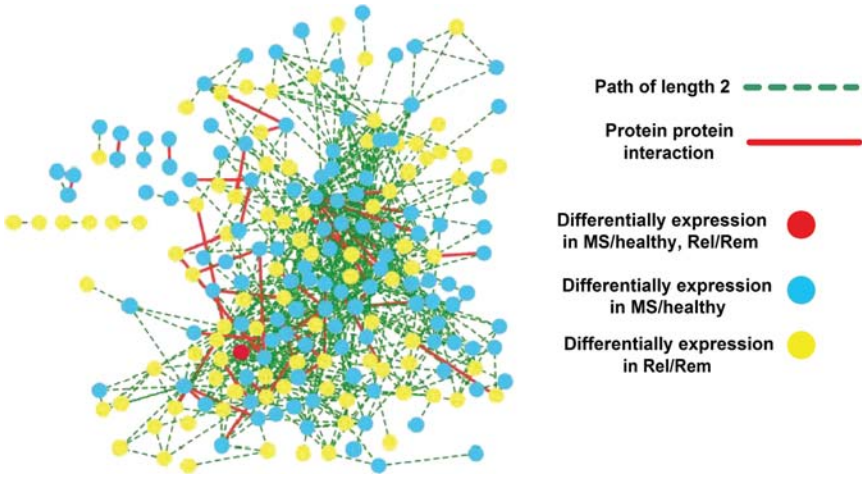

Figure 2. Global view on genes that is differentially expressed in MS and the PPIs between them. The graph includes edges of two types: continuous edges correspond to PPI; dashed edges corresponding to paths of length two (via a gene that is not differentially expressed). The graph also includes three types of nodes: differentially expressed in MS/healthy and Rel/Rem (red), differentially expressed in MS/healthy (blue), differentially expressed in Rel/Rem (yellow). Differentially expressed genes in MS tend to be close to each other in the PPI network (*P*-value = 0.05).

of the Rel/Rem dataset (*P*-value = 0.2). The result suggests that the two stages of the disease are relatively similar in terms of the gene expression signature; thus, in general, the differentially expressed genes in the Rel/Rem database do not seem to be clustered in the protein interaction network.

In the second global *P*-value, we checked if the number of genes with significant PPI *P*-values is higher than in random networks with similar properties (Materials and Methods). This number was significantly higher than expected in randomized networks for the MS/healthy dataset (275 in the real data versus 88 in the randomized data; *P*-value 0.01) but non-significant in the Rel/Rem dataset (Materials and Methods).

The third global *P*-value was related to the number of significantly expressed genes (according to the ANOVA *P*-value) that also have PPI with other significantly expressed genes (see exact details in the Materials and Methods section). We found that in both datasets, this number was higher than expected from random permutations with similar properties (69 genes in the real dataset versus a mean of 34 in the randomized gene networks for the MS/healthy dataset, *P*-value < 0.01; and 40 genes in the real dataset versus a mean of 22 in the random networks, *P*-value < 0.05 for the Rel/Rem dataset; Materials and Methods). Thus, we deduce that *P*-values based on more refined measures do detect significant global changes also in the Rel/Rem dataset. In the following sections we report additional, more specific and highly significant *P*-values related to functional groups of genes and single genes, which are differentially expressed in the Rel/Rem dataset.

PDI analysis of 111 transcription factors (TFs) and their targets (a total of 5787 interactions, Materials and Methods) revealed that 8 and 16 TFs changed their expression according to the ANOVA *P*-value in the Rel/Rem dataset and the MS/healthy dataset, respectively. We found 31 and 119 pairs of TFs and their targets that are both ANOVA significant in the Rel/Rem dataset and in the MS/healthy dataset, respectively. The number of such pairs was significantly higher compared with randomized networks (Materials and Methods) in the case of the MS/healthy dataset (mean number of genes in the real data: 119 versus mean number of genes in the randomized data: 53; *P*-value = 0.03) but not in the Rel/Rem dataset. The resultant lists of genes that are differentially expressed and have differentially expressed TFs appear in Supplementary Material, Tables S2 and S3.

Enriched gene ontology groups for genes with significant ANOVA or PPI *P*-values

To gain a picture of the cellular processes that are specific to MS and the cellular processes that are differentially expressed in relapse versus remission, we performed a gene ontology (GO) enrichment analysis of the genes with significant ANOVA *P*-values and PPI *P*-value (Materials and Methods). Some cellular functions with the highest enrichment *P*-values, which pass FDR, are depicted in Table 3 (all the enriched cellular functions can be found in Supplementary Material, Tables S4–S7).

In the MS/healthy dataset, the list of enriched cellular processes that were based on the genes with ANOVA significant *P*-values includes the following functional groups: positive regulation of cell death, chemotaxis, inflammatory response (all *P*-values < 0.0014; Table 3). These cellular processes are known to be key mechanisms in MS.

Almost all the cellular functions that were enriched according to genes with ANOVA significant *P*-values were also enriched based on genes with PPI significant *P*-values. However, the enrichment results according to the PPI significant *P*-values included additional relevant cellular functions that were not discovered by the ANOVA-based enrichment. For example, the cellular functions positive regulation of NF-kappaB TF activity, immune system development and regulation of caspase activity (all *P*-values < 0.0021; Table 3) were uncovered only by the PPI-based enrichment.

In the case of the Rel/Rem dataset, the following cellular functions were enriched based on the genes with ANOVA

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Table 3. Some of the cellular processes that were significantly enriched in (A) MS/healthy dataset and (B) Rel/Rem dataset

	Gene expression *P*-value	PP-net *P*-value
(A)		
Term		
Positive regulation of cell death	4.16E − 06	1.03E − 17
Chemotaxis	9.9E − 06	–
Regulation of transcription from RNA polymerase II promoter	0.00076	3.41E − 55
Inflammatory response	0.0014	1.73E − 06
Positive regulation of NF-κB transcription factor activity	–	0.00094
Immune system development	–	7.22E − 08
B cell differentiation	–	0.00217
Regulation of caspase activity	–	0.000367
(B)		
Cellular process		
Cell adhesion	0.00012	4.99E − 07
Cell migration	0.000034	8.15E − 10
Cell–cell signaling	2.2E − 06	1.77E − 07
Regulation of cell motion	0.0007	1.48E − 10
Positive regulation of cell proliferation	–	5.95E − 08
Synaptic transmission	–	4.61E − 06
T cell receptor signaling pathway	–	0.0000395
T cell activation	–	0.000349
Regulation of T cell proliferation	–	0.0007
T cell differentiation	–	0.0009
Positive regulation of natural killer cell-mediated cytotoxicity	–	0.00245
Regulation of apoptosis	–	1.26E − 06

Full tables appear in the Supplementary material based on the ANOVA *P*-values and based on the PP network *P*-values (Materials and Methods).

significant *P*-values: cell adhesion, cell migration, cell–cell signaling and regulation of cell motion (all *P*-values $<$ 0.0007; Table 3).

A similar analysis based on genes with significant PPI *P*-values again revealed additional relevant cellular functions: positive regulation of cell proliferation, T cell receptor signaling pathway, T cell activation, regulation of T cell proliferation, T cell differentiation and positive regulation of natural killer cell-mediated cytotoxicity and regulation of apoptosis (all *P*-values $<$ 0.00245; Table 3).

These results demonstrate that many of the regulatory changes in MS cannot be detected based solely on gene mRNA levels; however, incorporating PPIs can further the discovery of additional relevant regulatory changes in MS.

Genes with significant PPI *P*-values

The genes with the highest PPI *P*-values appear in Table 4 (see the entire list in Supplementary Material, Tables S2 and S3). In this case, many of the *P*-values passed the FDR threshold (16 genes for the Rel/Rem dataset and 11 for the MS/healthy dataset). Several of the genes that passed the FDR test are known to be key-apoptotic genes such as *TP53* and *TRAF1* that mediate the anti-apoptotic signals from tumor necrosis factor (TNF) receptors, and the anti-apoptotic gene *JUND*. Other genes that passed the FDR are related to growth and proliferation (e.g. *FOS* and *BRCA1*) and transcription (e.g. *TBP*, *SMAD3*, *JUN*).

The list of genes that passed the FDR test according to the PPI *P*-value in the Rel/Rem dataset included many genes that related to phosphorylation (e.g. *PTPRD, PTPRF, PPFIA1, PTPRS, PTPRA, PPFIA2*).

Interestingly, when we focused on genes with known single nucleotide polymorphisms (SNPs) that are associated with MS (32) (*HLA-DRB1, IL2RA, CD58, IL7R, PLP1, MAG, MOG*), we discovered that many of them have significant *P*-values (ANOVA or PPI) in at least one of the analyzed databases (Table 4); specifically, we found enrichment in the number of genes with SNPs associated with MS among the PPI significant genes in the Rel/Rem dataset (hyper-geometric *P*-value = 0.001).

One interesting gene is *IL7R* which is a receptor for interleukin 7 (IL7). IL7 has been shown to play a critical role in the V(D)J recombination during lymphocyte development (33). This protein also controls the accessibility of the TCR gamma locus by STAT5 and histone acetylation (34). Knockout studies in mice have suggested that blocking apoptosis is an essential function of this protein during differentiation and activation of T lymphocytes (35). We found that *IL7R*, which does not have a significant ANOVA *P*-value, has a significant PPI *P*-value in the Rel/Rem dataset (*P*-value = 0.00003), but not in the MS/healthy dataset. Thus, this result may suggest that protein levels of *IL7R* tend to change between relapse and remission periods of MS, even though no change is observed at the mRNA level of this gene.

Sub-networks of genes with both ANOVA and PPI significant *P*-values

Next we analyzed the specific set of genes that have both ANOVA and PPI significant *P*-values. We also considered the PPIs between these genes (Materials and Methods). The resultant protein interaction networks corresponding to these genes appear in Figure 3 (MS/healthy) and Figure 4 (Rel/Rem; see also Supplementary Material, Tables S8 and S9). As can be seen in Figure 3, the MS/healthy network includes genes related to transcription, regulation of proliferation and apoptosis, inflammatory response, response to cytokine stimulus and T cell activation (all *P*-values $<$ 0.004). The Rel/Rem network (Fig. 4), on the other hand, includes genes related to protein amino acid dephosphorylation, and cell adhesion (all *P*-values $<$ 0.003).

Clusters of genes with both ANOVA and PPI significant *P*-values

We clustered the sub-networks mentioned in the previous subsection to find modules (clusters) of genes with relatively more PPIs between them ((36); Materials and Methods). The resultant networks and the clusters appear in Figure 3 (for the MS/healthy dataset) and Figure 4 (for the Rel/Rem dataset). We verified that the modularity of the resultant clustering is higher than in random networks with similar properties (*P*-value $<$ 0.01; see details in the Materials and Methods section), suggesting that these clusters indeed have biological significance.

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Table 4. ANOVA *P*-values, PPI *P*-values and the *P*-value ranks for genes with SNPs related to MS (32)

Symbol	Entrez	*P*-value PPnet	Rank PPnet	*P*-value ANOVA	Rank ANOVA
HLA-DRB1	3123 100133661	Rel/Rem: pval = 1	Rel/Rem: 1884	Rel/Rem: pval = 0.3134	Rel/Rem: 4556
	3127	MS/cont: pval = 1	MS/cont: 2135	MS/cont: pval = 0.0491	MS/cont: 1244
IL2RA	3559	Rel/Rem: 0.0382	Rel/Rem: 208/13523	Rel/Rem: Pval = 0.8932	Rel/Rem: 12130
		MS/cont: 1	MS/cont: 2277/13523	MS/cont: pval = 0.9509	MS/cont: 12951
CD58	965	Rel/Rem: 1	Rel/Rem: 943/13523	Rel/Rem: pval = 0.9266	Rel/Rem: 12588
		MS/cont: 1	MS/cont: 1244/13523	MS/cont: pval = 9.3791E−004	MS/cont: 42
IL7R	3575	Rel/Rem: 0.0000335	Rel/Rem: 24	Rel/Rem: pval = 0.6162	Rel/Rem: 8498
		MS/cont: 1	MS/cont: 2290	MS/cont: 0.8654	MS/cont: 11984
PLP1	5354	Rel/Rem: 1	Rel/Rem: 2844	Rel/Rem: pval = 0.062	Rel/Rem: 1126
		MS/cont: 1	MS/cont: 3050	MS/cont: pval = 0.3548	MS/cont: 5735
MAG	4099	Rel/Rem: 0.0646257	Rel/Rem: 280	Rel/Rem: pval = 0.927	Rel/Rem: 12597
		MS/cont: 1	MS/cont: 2564	MS/cont: pval = 0.9969	MS/cont: 13488
MOG	4340	Rel/Rem: 1	Rel/Rem: 2431	Rel/Rem: pval = 0.06997	Rel/Rem: 1240
		MS/cont: 1	MS/cont: 2666	MS/cont: pval = 0.2466	MS/cont: 4312

The MS/healthy network includes many apoptotic genes and modules that will be discussed in details in the following section. One striking module includes the cytokine *IL1B* and its receptor, which are over-expressed in MS. *IL1* is known to have an important role in activation of lymphocyte proliferation. Specifically, it induces TNF-alpha release by endothelial cell, proliferation of CD4+ cells, IL-2 production, co-stimulates CD8+/IL-1R+ cells, induces proliferation of mature B cells and immunoglobulin secretion, induces IL-6 and granulocyte colony-stimulating factor secretion and stimulates expression of fibroblast growth factor and epidermal growth factor (EGF). In addition, it was found that MS patients tend to have polymorphisms in this gene and its receptor (37–39).

The Rel/Rem network includes modules related to phosphorylation, the growth factor receptors (EGFR) and cell adhesion.

The epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, is a hub in one of the modules. EGFR (and some of the proteins interacting with it) is over-expressed (in terms of mRNA levels) in relapse relative to remission. EGFR signaling is initiated by ligand binding to the extracellular domain of EGFR, and following activation, phosphorylation of cytoplasmic substrates occurs, and a signaling cascade is launched. As a result, many cellular responses are derived. Specifically, there are changes in gene expression, cytoskeletal rearrangement, anti-apoptosis, increased cell proliferation and adhesion. The EGFR cluster is enriched with genes related to adhesion (*P*-value = 0.03; Fig. 4); thus, the genes that appear in the EGFR cluster suggest that in our context (relapse versus remission in MS), EGFR mainly plays an important role in adhesion.

Pathways analysis of genes with significant *P*-values (ANOVA and PPI)

In order to better understand the molecular mechanisms that are involved in MS/healthy signatures, we combined the genes with significant ANOVA *P*-values with those with significant PPI *P*-values, and analyzed them with Ingenuity software (http://www.ingenuity.com). We considered the canonical pathways of Ingenuity and found that the most striking biological pathways that were enriched with these genes were related to IL1–IL8-induced inflammation ($P = 4.7 \times 10^{-19}$; Fig. 5A) and suppression of Fas ligand (FASL) and P53-dependent apoptosis ($P = 1.1 \times 10^{-12}$; Fig. 5B). IL-1 is produced by activated macrophages, B cells and fibroblasts: it is a pivotal pro-inflammatory cytokine that is centrally involved in local and systemic responses in the immune system, which lead to typical effects of inflammation. Its deregulation, prolonged synthesis and release in chronic inflammation contribute to diseases such as MS. There are two forms of IL-1 encoded by distinct genes, *IL-1α* and *IL-1β*, both of them were found to be over-expressed in the MS/healthy signature. The IL-1 receptor has two subunits, IL-1R1 and IL-1RAP, that upon ligand binding form a complex; in accordance with the previous results, the genes encoding *IL-1* receptor were also up-regulated.

Binding of IL-1 to its cell-surface receptor activates G-proteins, which in turn stimulate adenylate cyclase activity, which leads to an increase in the intracellular level of cAMP. Cyclic AMP activates PKA which then activates the pro-inflammatory NF-κB pathway as demonstrated by the significant *P*-value of the two subunits of *NFKB1*. Activated NF-κB, JNK1 and p38MAPK are translocated to the nucleus where, either directly or via the transcriptional regulators c-Jun and c-Fos (both found to be over-expressed), they induce genes that regulate inflammation.

Another mechanism of NF-κB, c-Jun and c-Fos activation involves the recruitment of IRAK and TRAF6 (Fig. 5A).

Interleukin 8 (IL-8) is a member of the C-X-C family of chemokines, which plays a central role in inflammation. Activation by IL-8 can trigger inflammation in cells like neutrophils, which leads to chemotaxis, granule release and increased cell adhesion. Interestingly, over-expressed IL-8 similarly to IL1 can also induce nuclear TF-kappa B (NF-κB) through a TRAF6-dependent pathway, and activate cyclooxigenase (PTGS2) and prostaglandin type of inflammation, which we found in the MS/healthy signature. This last mechanism is also supported by the significant over-expression of *IL1A* and *Ap1* genes. In our context, the most imperative result of IL8 activation is the up-regulation of matrix metalloproteinase (MMP2 and MMP9), which is involved in the blood brain barrier damage mechanism in MS. The activation of

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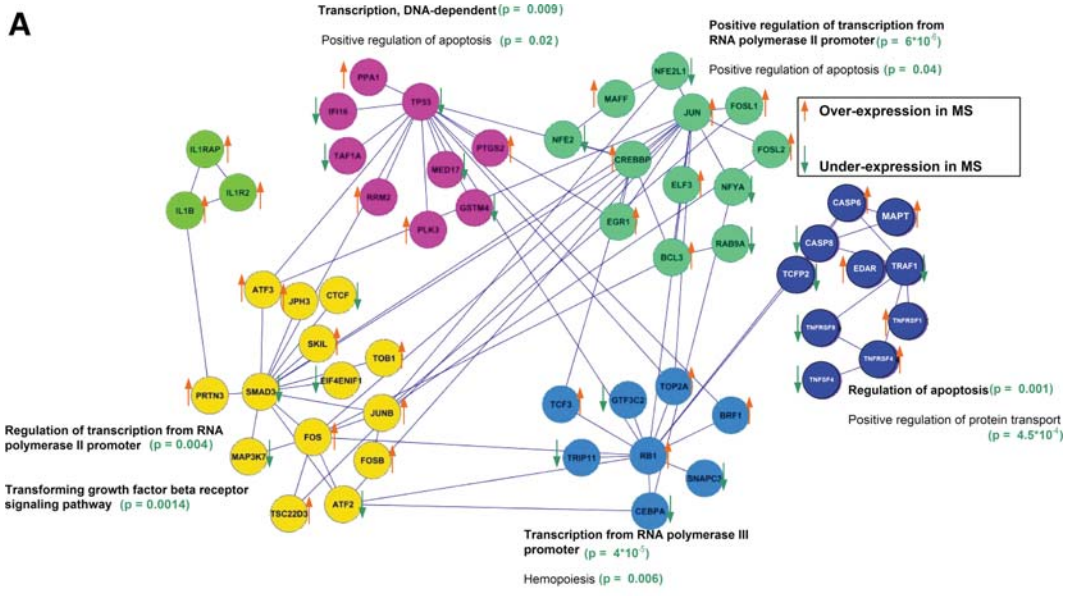

B

Term	P-value
Transcription, DNA -dependent	6.74E -10
Regulation of cell proliferation	4.11E -07
Positive regulation of apoptosis	4.09E -06
Response to abiotic stimulus	2.91E -04
Release of cytochrome c from mitochondria	0.004234
Leukocytedifferentiation	3.18E -05
T cell activation	2.95E -04
Macromolecular complex subunit organization	3.89E -04
Inflammatory response	2.30E -06
Cytokine-cytokine receptor interaction	4.85E -06
Transmembrane receptor protein serine/threonine kinase signaling pathway	0.001338
Response to cytokine stimulus	1.71E -06
Positive regulation of developmental process	0.000297
Regulation of protein transport	1.85E -04
Regulation of cytokine production	0.001523
Protein import into nucleus, translocation	5.20E -04

Figure 3. Differentially expressed interactions in the MS/healthy dataset. The graph includes only genes that were differentially expressed and also have significant PPI *P*-values. The graph was clustered [Materials and Methods (36); each cluster has a different color], and for each cluster we performed a functional enrichment analysis (Materials and Methods). (**B**) The functional enrichments for the entire graph.

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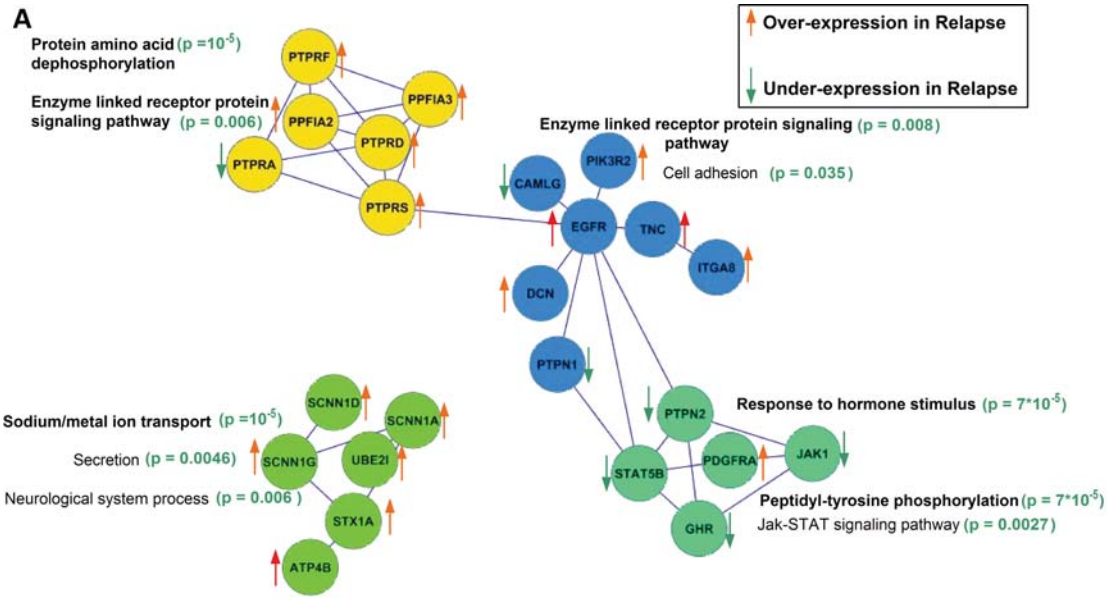

B

Term	P-value
Enzyme linked receptor protein signaling pathway	$8.44*10^{-11}$
Protein amino acid dephosphorylation	$3.29*10^{-5}$
Cell adhesion	0.002986
Response to endogenous stimulus	$8.5*10^{-4}$
Regulation of neurological system process	$6.44*10^{-5}$
Regulation of synaptic transmission	$5.53*10^{-4}$

Figure 4. Differentially expressed interactions in the Rel/Rem dataset. (**A**) The graph includes only genes that were differentially expressed with significant PPI *P*-values. The graph was clustered [Materials and Methods (36); each cluster has a different color], and for each cluster we performed a functional enrichment (Materials and Methods). (**B**) The functional enrichments for the entire graph.

inflammatory cellular response in the MS/healthy signature was also demonstrated by the over-expression of the CXCR4 signaling mechanism. As can be seen in Figure 5A, the *CXCR4* receptor and its specific *CXCL12* chemokine were over-expressed. This over-expression should lead to an increased phosphorylation of multiple focal adhesion components.

The down-regulation of apoptotic mechanisms in MS has been demonstrated in previous studies (40). Here, by combining gene expression and PPIs, we were able to reveal more precisely the mechanisms of apoptosis deficiency in the MS/healthy signature (Fig. 5B). We found that FASL, which mediates apoptosis, and its effector CASP8 proteolitic protein, was suppressed in MS. The downstream apoptotic cascade includes the genes *Daxx*, *JNK*, *BcL-XL*, *CIAP*, *CASP3* and *CAD*, which had significant PPI *P*-values. Another apoptotic pathway which appears in the MS/healthy signature is initiated by the binding of TNF family ligands to their receptors inefficiently due to under-expression of TRAF1/2 adaptor protein, which facilitate binding to pro-*caspase 8* and pro-caspase (Fig. 5B). In addition, we found clues which suggest that the *p53* signaling pathway is also suppressed in MS: first, as we previously mentioned, we found that *p53* is under-expressed; secondly, we found that *p53* masters regulatory proteins such as MDM2, WTN1, p300 and PCAF, which have highly significant PPI *P*-values; thirdly, we found that *cJUN*, which is known as an antagonist of *p53*, is over-expressed in MS. Downstream negative regulators of cell cycle, like retinoblastoma 1 (RB), were also over-expressed. Thus, the suppression of immune cell's apoptotic mechanisms could lead to expansion of autoimmune cell clones and maintenance of autoimmunity.

The Rel/Rem signature (Fig. 5C) characterized by activation of lymphocyte migration mechanisms ($P < 5.7 \times 10^{-4}$) includes a large group of significantly over-expressed cytokines and chemokines (according to the ANOVA test). Specifically, the set of over-expressed genes in the Rel/Rem dataset included the protein CCL14, a cytokine which induces alterations in intracellular calcium concentrations, enzymes released in monocytes and the chemokine CCBP2. These proteins physically interact with each other and are critical for the recruitment of effector immune cells to the inflammation site (Fig. 5C; 41,42).

The activation of chemotaxis of lymphocytes and monocytes is demonstrated by the over-expression of members of

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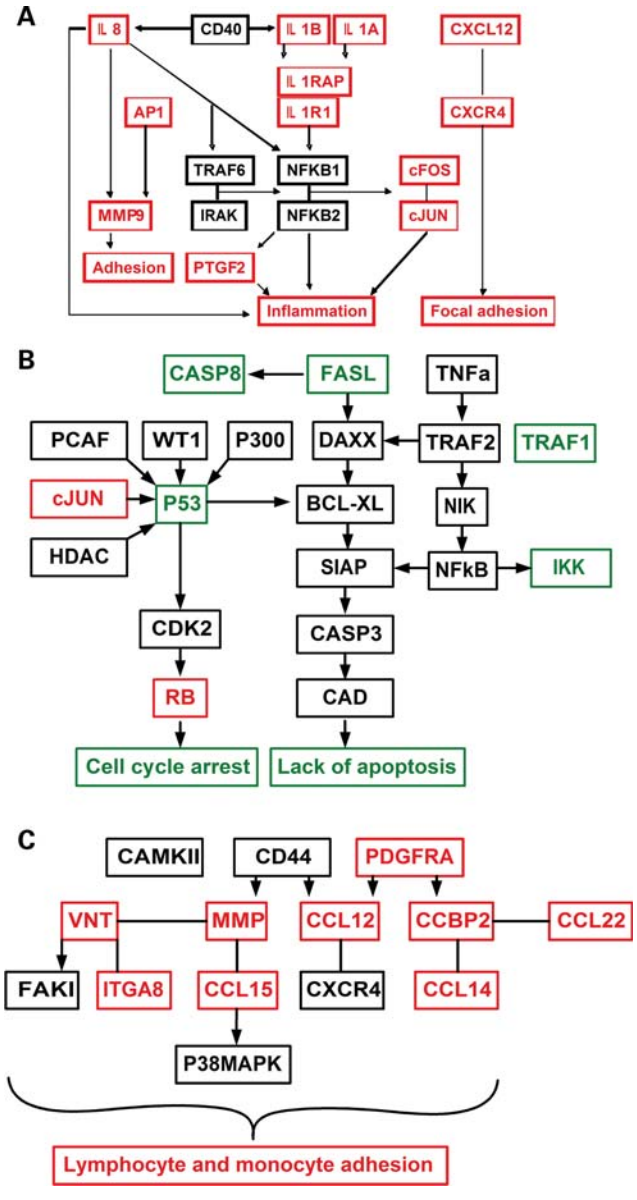

Figure 5. Pathway analysis by Ingenuity based on the significant (PPI and ANOVA) genes in MS/healthy (**A**, **B**) and Rel/Rem (**C**). Two signaling pathways found to be enriched by Ingenuity in the MS/healthy database: the inflammation pathway (A) and the apoptosis pathway (B); one pathway was significant in the Rel/Rem database: the lymphocyte and monocyte adhesion pathway (C). Red/green nodes denote ANOVA significant over/under-expression, respectively; black nodes denote genes with significant PPI *P*-values (but with non-significant ANOVA *P*-value).

the MIPI family such as CCL15, which induces changes in intracellular calcium concentrations, and acts via the CCR1 receptor (Fig. 5C; 41,42).

An important chemokine which is over-expressed in Rel/Rem is *CCL22* (Fig. 5C). It plays a role in trafficking dendritic cells and natural killer cells to the inflammatory sites of monocytes, and chronically activated T lymphocytes. It also displays a mild activity in primary activated T lymphocytes, though has no chemoattractant activity for neutrophils, eosinophils and resting T lymphocytes (41,42).

Another piece of evidence that supports lymphocyte activation in Rel/Rem is the over-expression of *CCL12* (Fig. 5C), which is known to play a role in the pathogenesis of diseases that are characterized by monocytic infiltrates, such as psoriasis, rheumatoid arthritis and MS (43–45).

In addition, we found that the *VNT* molecule, which is found in serum and tissues, and promotes cell adhesion and spreading, is over-expressed in acute relapse (Fig. 5C). We also uncovered that acute MS relapse is characterized by stimulation of pro-inflammatory molecules such as MMP1 and ALOX15 (Fig. 5C; Supplementary Material, Table S3).

To complete the picture of lymphocyte activation and monocyte trafficking in inflammatory sites, we report a few additional relevant proteins with significant PPI *P*-values (Fig. 5C). This set of proteins includes (Fig. 5C, Supplementary Material, Table S3) IL2R and *CXCR4*—a receptor for CCL12, and the focal adhesion kinase (P38MAPK). In addition, it incorporates CD44 (Fig. 5C), a cell-surface glycoprotein that is involved in cell adhesion and migration, as well as interaction with other ligands, such as osteopontin, collagens and MMPs. CD44 also binds to chemokines in the intracellular matrix. Interestingly, the receptor of TGFB [PDGFRA (46)], which is an upstream regulator that increases the expression of CCL12 and CCBP2, was over-expressed in the Rel/Rem database as well (Fig. 5C).

DISCUSSION

In this study, we reported the first systems biology study of gene expression in MS patients under remission and relapse, encompassing 164 large-scale measurements of gene expression in PBMC, including clinical and demographical characteristics of the patients, batch effects and physical interactions between proteins. Using this approach, we were able to report the regulatory changes that occur in the disease with a much higher resolution than before (8,12). The results that are reported in this study can be classified into three major levels of resolution: global, pathways and gene groups and single genes.

At the global level, when considering the expression levels of all the genes and the physical interactions between them, we show that there is a strong global signal related to the disease in the MS/healthy dataset. Even though there are clear differences between the two disease stages (relapse and remission), and despite the fact that there are specific genes and pathways that are differentially expressed between the two stages of the disease, we discerned a considerably weaker global signal in the Rel/Rem dataset. Thus, roughly speaking, most of the MS gene expression signature remains relatively constant in relapses and remissions.

At the pathway and gene modules level, the current study includes a detailed report of modules and pathways that are involved in the disease (MS/healthy dataset) and its two stages (Rel/Rem dataset), including the exact set of relevant proteins, and the interaction mechanisms among them (see Figs 3–5 that includes only genes with significant PPI and/or ANOVA *P*-values). Previous papers (8,12) included only a high-level report of the pathways that appear to be related to the disease (e.g. T-cell activation, apoptosis and

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inflammation). Thus, the pathways outlined in this paper improve our understanding of the disease phenotypes at the signaling pathways level. For example, we found that EGFR is a 'hub' which interacts with many ANOVA significant genes in the Rel/Rem database, possibly to promote improved adhesion. Thus, it is plausible that the gene expression of the EGFR sub-network is a good candidate for relapse prediction.

At the single gene level, we employ novel statistical analyses that overcome some of the biases related to gene expression analysis, and the fact that many of the regulatory mechanisms are not transcriptional. Thus, we detail a robust list of potential biomarkers of the disease in PBMC (Tables 2 and 4 and Supplementary Material, Tables S1–S3, S8 and S9). To the best of our knowledge, many of these genes are novel in this context, and have not been reported in previous studies (8,12). For example, to the best of our knowledge, the genes *SMAD3, RB1, FOS, TP53, BRCA1* and *TBP* with significant *P*-values in the MS/healthy dataset (see Table 2) and the genes *PTPRD, PDGFRB, STAT5A, PTPRF, PPFIA1, PTPRS, PPFIA2* and *SCNN1B* with significant *P*-values in the Rel/Rem dataset (Table 2) are novel potential biomarkers in the MS context.

Finally, since gene expression in PBMC has been utilized in the study of other autoimmune diseases (7,15,16), the approach employed in this study can be useful in the case of these diseases as well.

MATERIAL AND METHODS

Population study

The clinical and demographical characteristics of each of the analyzed dataset (MS/healthy and Rel/Rem) appear in Table 1.

In the Rel/Rem dataset, we did not include patients whose Expanded Disability Status Scale (EDSS) (47) was zero.

All the MS patients were diagnosed with definite MS according to the McDonald criteria (48). MS relapse was defined as the onset of new objective neurological symptoms/signs or the aggravation of existing neurological disability, not accompanied by metabolic changes, fever or other signs of infection, and lasting for a period of at least 48 h accompanied by objective change of at least 0.5 in the EDSS score. Confirmed relapses and EDSS scores were recorded consecutively.

RNA isolation and microarray expression profiling in MS

PBMCs were separated on Ficol-Hypaque gradient, and total RNA was purified, labeled, hybridized to a Genechip array (HU133A-2) and scanned (Hewlett Packard, GeneArray-TM scanner G2500A) according to the manufacturer's protocol (Affymetrix Inc., Santa Clara, CA, USA).

The normalization and analysis of the gene expression datasets

We used the Sheba MS center recorded computerized clinical follow up and blood gene expression measurement dataset. This dataset includes information regarding clinical variables such as: age, gender and EDSS at the time of blood sampling.

The following steps of the data analysis were performed by MATLAB:

(i) Expression values were computed from raw CEL files by applying the Robust Multi-Chip Average (RMA) background correction algorithm. We averaged the expression levels of all the probes of each gene, and each of the datasets underwent quantile normalization.
(ii) In the next step, in each of the datasets (MS/healthy and Rel/Rem), we computed significantly over/under-expressed genes based on ANOVA (30), considering batch effects such as the scan date of each chip, and the age and gender of each patient.

The drug regimen of each patient

Supplementary Material, Table S1 includes the drug regimen of each patient during the month of blood withdrawal (last column). We found that differing treatment is not a variable that has a significant effect on the gene expression pattern: the Spearman ranked correlation between the ANOVA *P*-values of all the genes when considering only the untreated patients versus the ANOVA *P*-values when considering all the patients is close to perfect ($r = 0.943$), i.e. genes that are relatively more significant based on the untreated group are also more significant based on the entire group of patients (roughly speaking the ranking of the genes' *P*-values does not change).

However, when we considered all the patients there we uncovered 16% more significant genes (the statistical power increases). Thus, we decided to employ all the patients.

The PPI network of human

The human PPI network was gathered from public databases (49,50) and from recently published papers (51,52). The final reconstructed network includes 7915 proteins and 28972 PPIs (a subset of 6850 proteins and 25931 PPIs appears in the analyzed chips).

PDI network of human

This network was downloaded from the TRED database (53). It includes 112 TFs, 2964 proteins that are regulated by these TFs and a total of 7298 PDIs. When considering only the genes that appear on the analyzed Genechip array, there are 111 TFs, 6069 proteins that are regulated by the TFs and a total of 5787 PDIs.

Gene-specific *P*-values based on the PPI network

For each protein in the PPI network, we computed a hypergeometric *P*-value that was based on the number of ANOVA significant proteins that interact with it, the total number of ANOVA significant and the topology of the PPI network. Roughly speaking, a protein that interacts with a larger number of interacting genes that are ANOVA significant proteins will have a more significant *P*-value. We named this *P*-value PPI (PPI network *P*-value) and calculated it as follows:

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Let n_i denote the number of proteins interacting with gene i, let m_i be the number of protein interactions with gene i that have significant ANOVA P-values, let N be the total number of proteins in the network and let M be the total number of proteins with significant ANOVA P-values in the network. At the first stage, we computed for each gene an initial P-value; let *PPI0* denote this P-value. The initial P-value of gene i is:

$$\sum_{j=m_i}^{n_i} \frac{\binom{M}{j} * \binom{N-M}{n_i-j}}{\binom{N}{n_i}}$$

To get a more refined P-value, we repeated iteratively (till convergence) on the procedure described above in the following way:

In iteration k compute for each gene a *PPIk* P-value that is based on the *PPIk-1* P-values computed in the previous iteration using the formula above, with the only change being we replace ANOVA significant genes with genes that are *PPIk-1* significant (i.e. M is the total number of proteins with significant *PPIk-1* P-values and m_i is the number of protein interactions with gene i that have significant *PPIk-1* P-value).

In this paper, we considered three sets of genes:

(i) Genes that satisfy two conditions: (1) significant ANOVA P-value and (2) significant *PPI*-network P-value. Such genes appear in Figures 3 and 4.
(ii) Genes that satisfy only condition (2). Such genes appear in the left sides of Tables 2 and 3.
(iii) Genes that satisfy only condition (1). Such genes appear the right sides of Tables 2 and 3.

A global *P*-value related to the total number of PDIs

In this subsection, we describe a global P-value which is related to the number of genes with ANOVA significant P-values, such that each of them is regulated by at least one ANOVA significant TF.

The aforementioned global P-value related to the number of genes was computed as follows:

Repeat 100 times:

(1) Randomly choose a subset of genes of size M.
(2) Assume that the subset of genes that was chosen in 1 includes the ANOVA significant genes, and compute the number of genes that are ANOVA significant and are regulated by at least one ANOVA significant TF.
(3) Compute the empirical probability (frequency) that the number of genes with ANOVA significant P-values that are regulated by at least one TF which is ANOVA significant, obtained in a random network, is larger (or equal) than the number of such genes in the original network.

A global *P*-value for the number of genes with significant PPI-network *P*-values

A global P-value which is related to the number of genes with significant PPI-network P-values was computed as follows:

Repeat 100 times:

(1) Randomly choose a subset of genes of size M.
(2) Assume that the subset of genes that was chosen in 1 includes the ANOVA significant genes, and compute for all genes the PPI P-values mentioned above.
(3) Compute the empirical probability (frequency) that the number of genes with significant PPI P-values obtained in the random network is larger (or equal) than the number of genes with significant PPI P-values obtained in the original network.

A global *P*-value for the distance between ANOVA significant genes in the PPI network

The aim of the global P-value described in this sub-section was to demonstrate that genes with ANOVA significant P-values tend to be close to other genes with ANOVA significant P-values in the PPI network. Therefore the observed changes in the gene expression are not random.

This global P-value was computed as follows:

(1) Find for each gene that is ANOVA significant, the distance to the closest gene in the PPI network that is also ANOVA significant. Compute the mean distance.

Repeat 100 times:

(2) Randomly select a subset of M genes and assign them to the nodes of the PPI network such that the degree distribution of these nodes will be identical to the degree distribution of ANOVA significant nodes in the original graph.
(3) Find the mean distance between each of the M random ANOVA significant nodes and its closest neighbor, and compute the mean distance.
(4) Compute the empirical probability (frequency) that the random network has a smaller (or equal) mean distance than the mean distance in the original one.

Clustering the differentially expressed networks

The clustering of differentially expressed networks was performed by the Newman algorithm (36) for finding communities in biological networks. The algorithm was implemented in *MATLAB*. This algorithm detects subsets (clusters) of proteins that include relatively many protein interactions between them (compared with other parts of the network). By definition, a network is modular if it can be divided to modules/sub-networks with a large number of PPIs between proteins within the same module, and less PPIs between proteins that reside in different modules.

We analyzed the PPI network consisting *only* of genes with both significant PPI and ANOVA P-values, with the aforementioned Newman algorithm

We used the modularity score that is described in the work of Girvan and Newman (36). To evaluate the significance of

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the modularity score of the resultant clustering, we compare it to the modularity score of randomized inputs as follows:

Let n denote the number of nodes (proteins) in the original network. Let *u1,v1*, *u2*and *v2* denote four proteins (nodes) in the network and let (*u1,v1*) denote PPI between proteins *u1* and *v1*.

Each random network was generated in the following way:

(1) Start with the original PPI sub-network.
(2) Repeat $10\times n$ times on the following steps:
    (a) Choose a random pair of edges (*u1,v1*) and (*u2,v2*) in the network.
    (b) Replace them with a new pair of edges (*u1,v2*) and (*u2,v1*).

GO enrichment

GO enrichment analysis of genes with significant ANOVA *P*-values and/or of genes with significant PPI *P*-values ($P < 0.05$ in both cases) was performed by David (54) (http://david.abcc.ncifcrf.gov/). In all cases, we considered GO groups with enrichment *P*-values that passed the FDR. To this end, we utilized the FDRs that were reported by David, and we reported only cases with FDR of <5%.

FDR test for the *P*-values of single genes

In this case, we considered the nonparametric approach of reference (31) with a threshold of $q = 0.05$.

Fold change

To estimate the fold change in one condition versus the second (e.g. relapse versus remission) while taking into account the batch effects and additional variables, we performed a multivariate linear regression (55) in which the Rel/Rem (or MS/control) is the dependent variable and all the other (Table 1) previously mentioned variables (batch effects, clinical and demographical variables) are the independent variables. The scan date was represented by a set of dummy binary variables, and other variables were either continuous or binary. The sign of the coefficient related to the expression levels determined the fold change in one condition versus the other. For example, in the case of the Rel/Rem database, we set the dependent variable to be '1' in the case of relapse (and '0' otherwise). Thus, a positive coefficient of the expression levels variable corresponds to increased expression level of the gene in relapse in comparison to remission.

Similar results were obtained when we used partial correlations (Spearman or Pearson) between the Rel/Rem (or MS/control) variable and the expression levels, given all the other variables.

ACKNOWLEDGEMENTS

We thank Ms Hadas Zur for helpful comments.

Conflict of Interest statement. None declared.

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