Gene expression modulation induced by fingolimod in relapsing remitting multiple sclerosis

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INTRODUCTION

Fingolimod (FTY) is an oral treatment approved for relapsing-remitting (RR) multiple sclerosis (MS)[1]. Fingolimod is a modulator of sphingosine-1phosphate receptor (S1PR).The interaction between receptor and drug cause the internalization and degradation of receptor and lead to the sequestration of lymphocyte into the lymph nodes[2].

Very few gene expression experiments have been performed at whole genome level to assess the transcriptional changes induced by the treatment.

In this study we want investigate the molecular mechanisms underlying FTY action, by looking at transcriptional alterations induced by the treatment in whole blood.

MATERIAL AND METHODS

Patients enrolment:

We enrolled in the study adult RRMS patients, who started FTY at San Raffaele Hospital MS center. Patients were sampled before treatment start and after 6-months of therapy. In order to avoid confounding effects related to previous therapies, we excluded from the study subjects with history of relapses or corticosteroid treatment in the month before FTY, patients treated with interferon, BG-12, teriflunomide or immunosuppressants in the 3 months before and patients treated with Natalizumab in the year before FTY start.

Gene Expression:

RNA from whole blood was extracted from PAXgene blood tube using PAXgene Blood miRNA Kit (Qiagen) according to the manufacturer's protocol. RNA quality control was performed by means of concentration estimation using the NanoDrop® ND-1000 Spectrophotometer. We also performed an agarose gel to evaluate the integrity of RNA and to verify that there was no contamination with genomic DNA.

The genome expression profiling was assessed using the Illumina® HumanHT-12v4.0 Expression BeadChips. Samples preparation was performed according to the manufacturer's protocol. Beadchips were imaged using the Illumina iScan and the fluorescent hybridization signals were assessed using Illumina GenomeStudio software.

Differentially expressed genes (DEGs) were identified using Limma[3] software, including white blood cell and lymphocyte counts as covariates. As additional tool, we used the CellMix[4] R package to deconvolute the expression data according to cell types.

Enrichment Analysis:

The lists of differential expressed genes (DEGs) were analysed in term of pathway enrichment according to Gene Ontology.

RESULTS

Patients:

We analysed whole-blood gene expression data for 24 patients treated with FTY. Table 1 reports the demographic and clinical characteristics.

Table 1: Demographic and clinical characteristics of enrolled patients.

Characteristics	Value
Female:Male	18:6
Age at treatment start, years (range)	39.9 (28.5 – 64.3)
Median EDSS at treatment start, (range)	2.0 (1.0-4.5)
Disease duration, years	10±7

Differential expression analysis:

Given the particular mechanism of action of the drug, the lymphocyte count at six-months follow up is significantly lower compared to baseline. For this reason, we had to introduce into the model as covariate the logarithm of white blood cells count and lymphocyte count. We found 478 differentially expressed genes (DEGs) at a 15% FDR (Experimental DEGs). Table 2 reports the top 10 DEGs.

We also used the CellMix package to deconvolute the expression data according to cell types, based on a sample signature tool that is able to estimate the fraction of different cells type starting from whole blood. The proportion of significantly changed cell types was used as covariate in the analysis of gene expression. For this analysis, the best obtainable FDR is ~35%; we obtained 135 up-regulated and 181 down-regulated genes. Table 3 and Table 4 report respectively the top 10 up-regulated and the top 10 down-regulated genes, with their nominal p-value.

When comparing the DEGs found with the two approaches (experimental *vs.* deconvoluted data) we observed a significant overlap for the down-regulated genes, but no overlap was found for the up-regulated genes.

Figure 1: Cells count at baseline and at six-months follow up

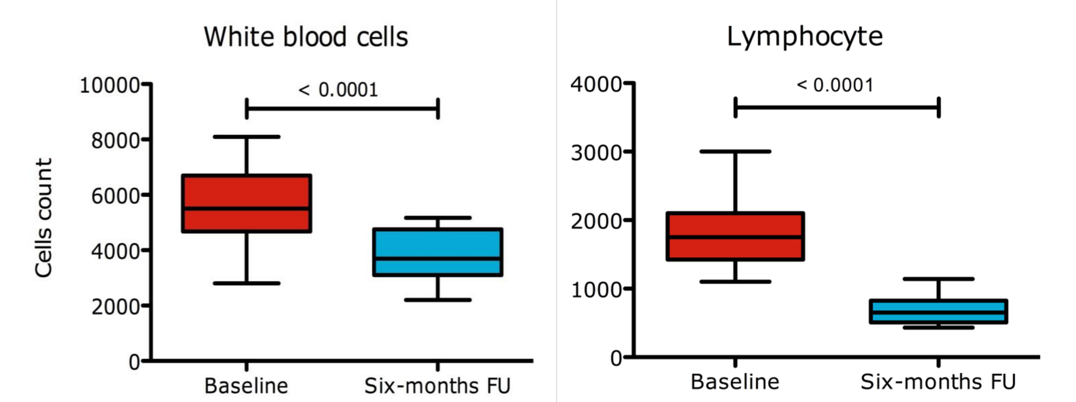


Table 2: Top10 DEGs – Experimental data

Probe ID	Gene symbol	Log Fold Change	Adjusted p-value
ILMN_1763487	CTLA4	-2.399	0.0002583
ILMN_1745112	FAM102A	-2.53	0.0002583
ILMN_2383058	SIRPG	-2.651	0.0004115
ILMN_1676470	TCF7	-2.354	0.0008345
ILMN_1715131	CCR7	-4.778	0.0008345
ILMN_2264011	GRAP	-1.788	0.0008345
ILMN_1726928	TCEA3	-2.266	0.00102
ILMN_2213136	LEF1	-3.714	0.001734
ILMN_2401779	FAM102A	-2.51	0.001734
ILMN_1770768	SLAMF1	-2.234	0.003257

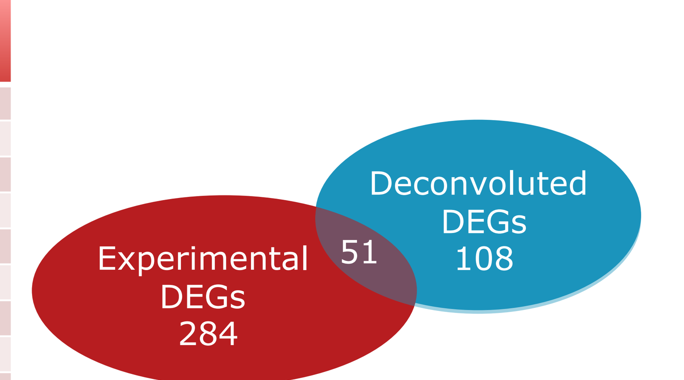
Table 3: Top 10 Up regulated genes – Deconvoluted data

Probe ID	Gene symbol	Log Fold Change	p-value
ILMN_1845475		1.494	0.0001423
ILMN_1674098	SORBS2	1.185	0.0001974
ILMN_3303979	LOC729177	1.901	0.0002081
ILMN_1736300	SEMA5B	1.154	0.0002403
ILMN_1668460	PRTN3	1.831	0.000307
ILMN_1705035	FBXL7	1.376	0.0003395
ILMN_1917315		1.37	0.0004259
ILMN_1720373	SLC7A5	1.821	0.0004352
ILMN_1740784	LOC390829	1.038	0.0006834
ILMN_3238797	FAM72A	1.086	0.0008861

Table 4: Top 10 Down regulated genes – Deconvoluted data

Probe ID	Gene symbol	Log Fold Change	p-value
ILMN_1726928	TCEA3	-2.069	1,31E-02
ILMN_1725417	NELL2	-2.348	2,12E-02
ILMN_1752502	HKDC1	-1.709	5,09E-02
ILMN_2198878	INPP4B	-1.481	6,39E-02
ILMN_2116827	RGPD1	-1.94	9,32E-02
ILMN_1898453		-1.613	0.0001603
ILMN_1728132	LDHB	-1.425	0.000185
ILMN_1763487	CTLA4	-1.754	0.0001922
ILMN_1755862	PFAS	-1.408	0.0001965
ILMN_1793870	IL1RAP	-1.497	0.0001996

Figure 2: Overlap of experimental and deconvoluted down-regulated genes



Enrichment Analysis:

For the enrichment analysis, both experimental and deconvoluted DEGs list were analysed in terms of GeneOntology enrichment.

In Table 5, 6, 7 and 8 the top significant categories for the "Biological Process" ontology are reported.

Table 5: Top Biological processes enriched in Up-regulated genes – Experimental data

Term	Odds Ratio	p-value	Expected count	Count	Size
Oxalate transport	68.84	0.0006171	0.0378	2	10
Sulfate transmembrane transport	68.84	0.0006171	0.0378	2	10
Inorganic ion transmembrane transport	4.774	0.0006545	1.966	8	520
Sulfate transport	55.06	0.0009007	0.04536	2	12
Cellular response to epinephrine stimulus	55.06	0.0009007	0.04536	2	12

Table 6: Top Biological processes enriched in Down-regulated genes – Experimental data

Term	Odds Ratio	p-value	Expected count	Count	Size
Lymphocyte activation	4.21	1.98E-10	9.326	33	543
Leukocyte activation	3.581	7.90E-09	10.79	33	628
Adaptive immune response	4.785	1.63E-08	5.307	22	309
Regulation of immune system process	2.738	1.74E-08	22.02	50	1282
Regulation of lymphocyte activation	4.571	1.77E-08	5.805	23	338
Cell activation	3.075	4.85E-08	14.07	37	819

Table 7: Top Biological processes enriched in Up-regulated genes – Deconvoluted data

Term	Odds Ratio	p-value	Expected count	Count	Size
Porphyrin-containing compound biosynthetic process	33.07	1.43E-05	0.1519	4	24
Tetrapyrrole biosynthetic process	28.75	2.33E-05	0.1708	4	27
Porphyrin-containing compound metabolic process	22.79	5.28E-05	0.2088	4	33
Cofactor biosynthetic process	7.899	0.0001991	0.8479	6	134
Regulation of mitochondrial electron transport, NADH to ubiquinone	161.2	0.0002352	0.02531	2	4

Table 8: Top Biological processes enriched in Down-regulated genes – Deconvoluted genes

Term	Odds Ratio	p-value	Expected count	Count	Size
Lymph node development	50.69	2.54E-07	0.1486	5	16
Lymphocyte differentiation	4.893	4.25E-05	2.516	11	271
Leukocyte differentiation	3.951	7.77E-05	3.686	13	397
Biological regulation	2.528	8.82E-05	78.22	96	8424
Hematopoietic or lymphoid organ development	3.185	0.0001021	6.054	17	652
Lymphocyte activation	3.342	0.0001447	5.042	15	543

CONCLUSIONS

FTY treatment appears to induce a down-regulation of genes involved in lymphocyte activation and immune response.

These data could be driven by FTY mechanism of action, although we took into account the drug effect on lymphocyte count at the analytical level, by including the lymphocyte count as covariate and also by applying deconvolution tools.

On the contrary, there is less overlap between the list of pathways up-regulated by FTY according to experimental and deconvoluted data, pointing to more complex mechanism which need to be confirmed in additional cohort of patients.

A replication study is ongoing on 24 additional patients; in these samples we will perform additional experiments on sorted cells, in order to confirm data obtained in whole blood and to better understand the effect of FTY at cellular level.

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DISCLOSURES

F. Esposito received honoraria from TEVA and Merck. L. Moiola received honoraria for speaking at meetings or for attending to advisory board from Sanofi-Genzyme, Biogen-Idec, Novartis and TEVA. B. Colombo received travel grant from Biogen-Idec, Merck, Bayer, Genzyme. V. Martinelli has received honoraria for consulting and speaking activities from Biogen-Idec, Merck, Bayer, TEVA, Novartis and Genzyme. G. Comi has received compensation for consulting services with the following companies: Novartis, Teva, Sanofi, Genzyme, Merck, Biogen, Excemed, Roche, Almirall, Chugai, Receptos, Forward Pharma and compensation for speaking activities from Novartis, Teva, Sanofi, Genzyme, Merk, Biogen, Excemed, Roche. F. Martinelli Boneschi has received compensation for activities with Teva Neuroscienze as speaker and/or advisor. E. Mascia, P. Provero, L. Ferre', F. Clarelli, G. Sferruzza, C. Guaschino and M. Radaelli nothing to disclose.

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