Transcriptional modulation induced in T and B lymphocytes by fingolimod treatment in Relapsing Remitting Multiple Sclerosis patients

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Introduction and Aim

Fingolimod (FTY-Gilenya®) is a second-line oral drug approved for Relapsing Remitting Multiple Sclerosis (RRMS), known to reduce peripheral lymphocytes counts by preventing their egress outside lymph node. Our study was aimed at detecting transcriptional changes induced after 6 months of follow-up by FTY treatment in immune cell subtypes, B cells and T cells, to elucidate its mechanism of action at molecular and pathway levels.

Patients and Methods

Patients

We selected a cohort of patients affected by RRMS, diagnosed according to McDonald Criteria, followed at San Raffaele MS center and treated with FTY.

Exclusion criteria were:

- Patients treated with IFN-beta, BG-12, teriflunomide or immunosuppressant in the 3 months before FTY start
- Patients treated with Natalizumab in the 9 months before FTY start
- Patients with history of relapses or corticosteroid treatment in the month before FTY start.

Twenty-four RRMS were finally included in the study

Clinical and demographic features (n = 24)	
Female : Male ratio	2
Age at disease onset, years (± sd)	27.9 ± 8.4
Age at treatment start, years (± sd)	36.0 ± 9.0
Disease duration, years (± sd)	7.6 ± 7.5
Median EDSS at treatment start, (range)	2.5 (1.0 – 5.0)
Annualize relapse rate before treatment (± sd)	1.2 ± 1.8

Table 1 - Clinical and demographic characteristics of the patients enrolled

Transcriptomic assay

Whole blood samples were obtained for each patient on the day of FTY start and after 6 months of treatment. Peripheral blood mononuclear cells were isolated through density gradient centrifugation. T cells (CD 3+) and B cells (CD 20+) were isolated by means of MACS human MicroBeads system with positive selection procedure. RNA was isolated both from B and T lymphocytes, libraries were obtained using Illumina TruSeq Stranded mRNA sample Prep Kit and sequenced on Illumina NextSeq500 platform.

Statistical analysis:

1. Differential expression analysis

Transcript levels after 6 months of therapy were contrasted with baseline according to a paired design, evaluating differential expression with DESeq2 [1] on Trascript Per Million (TPM) counts. Genes with fold-change (FC) > 2 (<0.5) and adjusted P-value < 0.05 were declared differentially expressed genes (DEGs).

2. Pathway analysis

Pathways enriched of DEGs were detected by means of hypergeometric test, adopting KEGG database as pathway repository. Pathways were also tested for subpaths activation state using MinePath tool [2], which exploits the internal pathway topology of KEGG graphical maps. Subpaths are gene sequences linked with Boolean operators that take into account functional relationship among genes.

We conducted a self-contained gene-set analysis on KEGG Sphingolipid Signaling Pathway, since this pathway could be of prior interest for FTY mechanism of action, to evaluate overall involvement of the pathway in the transcriptional induction. ROAST Rotation tests were employed to this end [3].

3. Network analysis

Cell-type specific protein-protein interactions were obtained from GIANT database, focusing on interactions with a posterior probability > 0.7. We extracted from these networks the largest connected components and performed module detection of DEGs enriched sub-networks.

We used Network Analyzer and Centiscape plugin to perform a centrality-based analysis to find, according to different metrics, genes with higher network centrality.

Genes	6 months			Baseline		
	S_1	S_2	S_3	S_4	S_5	S_6
A	1	0	1	0	1	1
B	1	1	1	1	1	1
D	1	1	1	1	1	1
C	0	0	0	1	1	1

Sub-paths	Boolean operations	6 months			Baseline		
		S_1	S_2	S_3	S_4	S_5	S_6
A → B	A & B	1	0	1	0	1	1
B → D	B & D	1	1	1	1	1	1
D –| C	D ⊗ C	1	1	1	0	0	0
A → B → D	((A & B)) & (B & D)	1	0	1	0	1	1
B → D –| C	(B & D) & (D ⊗ C)	1	1	1	0	0	0
A → B → D –| C	((A & B) & (B & D)) & (D ⊗ C)	1	0	1	0	0	0

Fig. 1 - SubPaths generation from gene activation state using Boolen operators

Results

DEGs analysis

	T Cells			B Cells		
	Gene	FC	Padj	Gene	FC	Padj
UP-REGULATED GENES	*CX3CR1*	5.96	6.38E-26	*FCGR3B*	6.26	1.12E-29
	LILRB1	5.09	1.17E-30	*CX3CR1*	6.03	6.15E-35
	FASLG	4.8	2.63E-50	*CXCR2*	5.40	2.99E-24
	FCGR3B	4.66	1.14E-23	*FCGR3A*	4.90	4.75E-37
	CMKLR1	4.66	1.65E-23	*TLR8*	4.76	1.21E-23
	KLRD1	4.65	1E-41	*CMKLR1*	4.45	4.16E-17
	ASCL2	4.59	2.79E-36	*SH2D1B*	4.38	4.22E-20
	FCGR3A	4.58	5.95E-32	*CLEC12A*	4.24	4.26E-23
	PLEK	4.44	9.77E-39	*PRSS23*	4.18	2.12E-23
	PDGFD	4.43	3.79E-38	*P2RY13*	4.03	6.43E-18
DOWN-REGULATED GENES	*EDAR*	0.14	2.79E-34	*ADTRP*	0.20	4.46E-22
	NOG	0.14	2.68E-34	*CCR7*	0.21	3.94E-41
	SCML1	0.17	6.05E-46	*MDS2*	0.22	4.36E-32
	CCR7	0.18	5.49E-31	*SLC22A17*	0.23	9.43E-18
	FOSB	0.19	1.04E-13	*FHIT*	0.25	1.64E-28
	MDS2	0.19	9.26E-34	*TCEA3*	0.25	2.93E-15
	TSHZ2	0.2	1.13E-45	*GAL3ST4*	0.27	9.37E-22
	JUN	0.21	6.64E-18	*CACNA1I*	0.29	5.54E-16
	AK5	0.22	3.47E-44	*CA6*	0.29	1.06E-24
	PLAG1	0.22	6.7E-35	*ANKRD55*	0.29	4.56E-14

Table 2 - Top DEGs in T and B cells. Red highlighted genes related with lymphocytes migration. Green highlighted genes related with immune function

Our study reveals a larger proportion of up-regulated genes as compared to down-regulated ones both for T (n_{up}=313, n_{down}=240) and B cells (n_{up}=400, n_{down}=104). This pattern confirms results emerged from previous studies on T cells in a German cohort treated with FTY [3] [4]. Our study overall replicated significance, direction and effect sizes observed in top DEGs from the previous study.

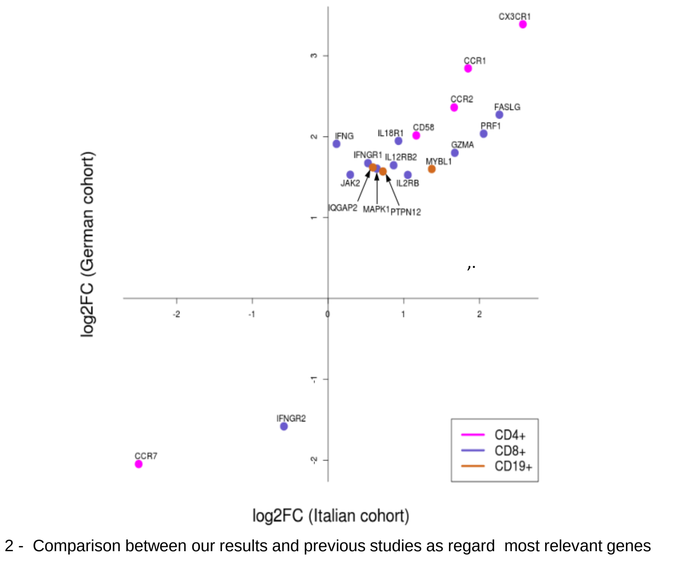

Fig. 2 - Comparison between our results and previous studies as regard most relevant genes

Pathways analysis

	Enrichment analysis			SubPath analysis		
	Pathway	P value	FDR	Pathway	P value	Activated SP 6m/BI
T cells	Cytokine-cytokine receptor interaction	4.29E-14	1.26E-11	Ras signaling pathway	3,01E-06	78/2
	Chemokine signaling pathway	2.17E-06	3.19E-04	Natural killer cell mediated cytotoxicity	2,30E-08	74/3
	Natural killer cell mediated cytotoxicity	9.21E-06	9.03E-04	Fc gamma R-mediated phagocytosis	5,11E-04	70/0
	Osteoclast differentiation	1.95E-05	1.18E-03	MAPK signaling pathway	1,00E-09	62/33
	Hematopoietic cell lineage	2.00E-05	1.18E-03	Rap1 signaling pathway	1,04E-04	58/0
	Cell adhesion molecules (CAMs)	4.84E-05	2.37E-03	Terpenoid backbone biosynthesis	1,59E-01	37/0
	Pathways in cancer	6.91E-05	2.90E-03	Regulation of actin cytoskeleton	2,26E-06	25/11
	Neuroactive ligand-receptor interaction	1.11E-04	4.09E-03	Chemokine signaling pathway	3,70E-03	24/3
	Graft-versus-host disease	1.49E-04	4.50E-03	P53 signaling pathway	2,28E-04	23/0
	Staphylococcus aureus infection	1.53E-04	4.50E-03	Focal adhesion	2,31E-03	23/5
B cells	Cytokine-cytokine receptor interaction	3.42E-10	1.01E-07	Rap1 signaling pathway	6,67E-07	184/0
	Osteoclast differentiation	4.84E-09	7.12E-07	Glycerophospholipid metabolism	1,55E-06	117/1
	Malaria	1.18E-07	1.16E-05	Fc- gamma R-mediated phagocytosis	1,28E-06	102/1
	Neuroactive ligand-receptor interaction	3.88E-07	2.86E-05	Ras signaling pathway	3,61E-06	64/2
	Staphylococcus aureus infection	1.07E-06	6.28E-05	Inositol phosphate metabolism	7,70E-03	58/0
	African trypanosomiasis	3.30E-05	1.62E-03	Platelet activation	5,18E-04	55/1
	Complement and coagulation cascades	3.98E-05	1.67E-03	Pentose phosphate pathway	1,15E-02	52/0
	Transcriptional misregulation in cancer	5.17E-05	1.88E-03	Regulation of actin cytoskeleton	3,33E-05	47/2
	Natural killer cell mediated cytotoxicity	5.76E-05	1.88E-03	MAPK signaling pathway	1,00E-09	45/49
	Hematopoietic cell lineage	2.37E-04	6.97E-03	Natural killer cell mediated cytotoxicity	7,77E-04	35/2

Table 3 – Pathway analysis

Subpaths analysis identified *RAP1A*, *RAC1* and *ZAP70* as nodes of several subpaths significantly activated at 6 months.

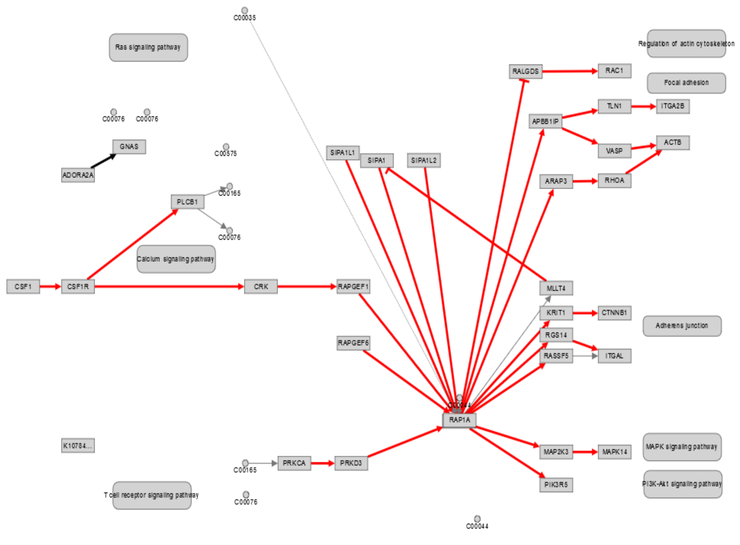

Fig.3 - Rap1a signaling pathway, subpaths activated at 6 months in B cells are highlighted in red

Although Sphingolipid signaling pathway did not stand out among pathways significantly enriched of DEGs or activated subpaths, it turned out to be modulated as of self-contained test ($p < 0.001$).

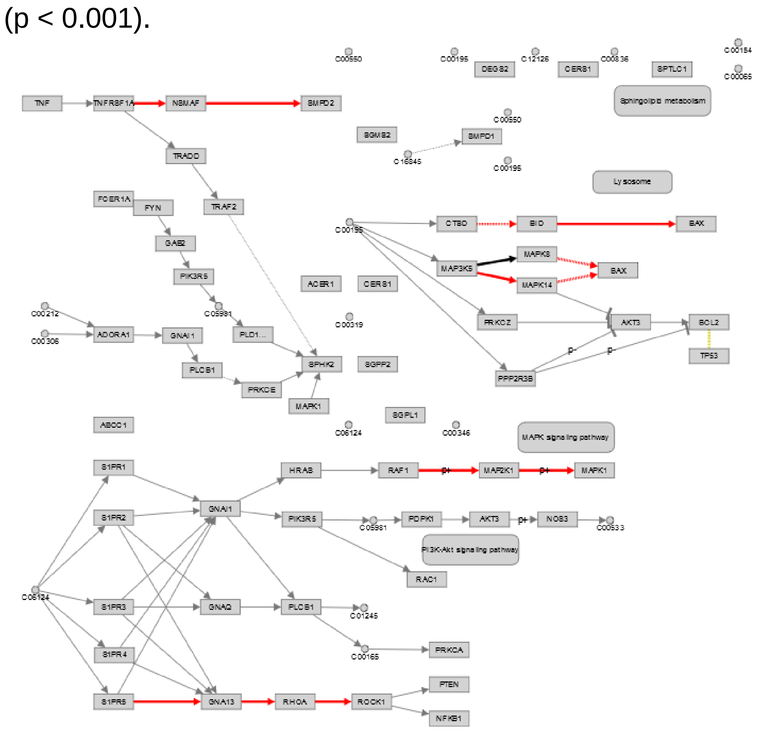

Fig.4 – Sphingolipid signal pathway, subpaths activated at 6 months in T cells are highlighted in red

Network analysis

Network analysis identified, according to different centrality metrics, several genes related with immune function and cell migration.

IFNGR1, *LYN* and *CD45* resulted as central nodes up-regulated in B cells while *IFI30* and *CCL5* resulted as central nodes up-regulated in T cells. Both for T and B cells we observed several hubs related with ribosome function, as *RPS6*, that resulted consistently down-regulated.

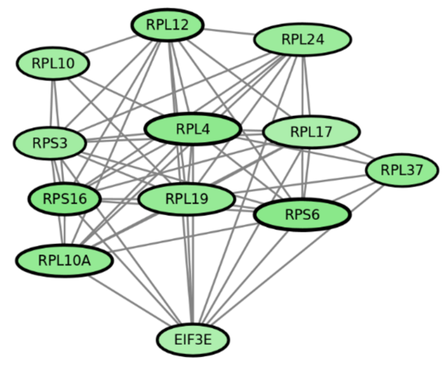

Fig.5 – Network representation of hub genes selected according to topological measures in T cells

Conclusions and Perspectives

Taken together these data suggest an increase in lymphocytes migration propensity and inflammatory profile associated with a lower activation state, as demonstrated by network analysis. This effect could be explained by the already known changes in lymphocytes subpopulations compositions after FTY treatment. As previously shown by several studies, FTY induces an enrichment of TEM and reduction of TCM and naïve T cells [5]. Moreover B cells subpopulations are modulated with an increase in transition and naïve B cells and reduction in memory B cells [6].

Sphingolipid signaling pathway showed an activation of a subpath downstream the *S1PR5* both in T and B cells. This can represent a compensatory reaction to FTY induced S1P1 internalization.

Perspectives:

- Functional studies to confirm the observed results
- Assessment of the changes induced by FTY in specific cell subtypes
- Investigation of a possible association between gene expression modulation at 6 months and response to treatment.

References

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This study was supported by the "Fondazione Italiana Sclerosi Multipla", project 2013/R/13