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Short Communication

Dysregulation of Gene Expressions in Multiple Sclerosis: *TNFSF13B* and Other Candidate Genes

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Abstract

Background: In previous investigations of combined miRNAs/mRNAs expressions in neurodegenerative diseases like Multiple Sclerosis (MS) and Amyotrophic Lateral Sclerosis (ALS) we have targeted some interesting genes and molecular pathways that needed further confirmation. **Methods**: By nanofluidic qPCR analysis, we aimed to verify the expression of genes that resulted differentially expressed in the previous analyses. Data from MS patients — either the pediatric and the adult occurrence of the disease (adMS and pedMS, respectively) — was compared to age-matched healthy groups. As neurological controls we recruited a cohort of ALS subjects, considering published searches of possible genetic similarities between the two diseases. **Results**: The main results confirmed the involvement of most of the investigated genes in pedMS and adMS, like *BACH2* and *MICAL3*. On the other hand, suggestive MS candidate genes like *TNFSF13B* showed an interesting trend possibly influenced by interfering factors, such as concomitant disease-modifying treatments; it is worth noting that *TNFSF13B* was one of the genes upregulated in ALS compared to age-matched adMS patients, together with the transcription factor *TFDP1*. **Conclusions**: Although with caution due to the small sample size, this study confirms the interest in transcriptomic analysis supported by integrated and educated bioinformatics evaluations, to shed further light in complex neurological diseases.

Keywords: Multiple Sclerosis (MS); pediatric MS; Amyotrophic Lateral Sclerosis (ALS); gene expression; nanofluidic qPCR analysis; candidate genes

1. Introduction

Multiple Sclerosis (MS) is a chronic dysimmune disease of the Central Nervous System (CNS) that mainly affects young adults, predominantly females. From a pathogenic point of view, MS is considered a multifactorial disorder, in which predisposing genetic traits interact with environmental factors (e.g., vitamin-D levels, toxics) thus triggering the onset of the disease [1]. At present the whole picture of MS pathogenesis is still missing; several factors, i.e., the wide heterogeneity of clinical and/or neuroradiological phenotypes, might prevent definitive conclusions on this issue, and they may also explain the different response to treatments that has been observed in MS patients. Understanding the interplay of these (and others) key factors will be of invaluable help in the effort of shedding light on these still progressive and irreversible processes, a fundamental step forward the implementation of Personalized Medicine.

A valuable support for the solution of this issue is represented by the availability of innovative extensive approaches in the genetic analysis like massive high-throughput RNA sequencing that uncover the involvement of multiple molecular pathways in each pathogenic process. As examples, transcriptomics studies showed that dysregulated microRNAs (miRNAs, endogenous small RNAs binding the target sites of protein-coding genes, which lead to their degradation or the repression of translation) characterize MS, suggesting that alterations in miRNA regulatory pathways may contribute significantly to the pathogenesis of the disease [2]. On the other hand, it is plausible that these findings may lead to developing "miRNAs therapeutics" in MS as in other Neurodegenerative Diseases (NDDs) [3].

In the last few years, we have addressed the attention towards the identification of peripheral circulating miRNAs able to monitor the course of NDDs like MS and Amyotrophic Lateral Sclerosis (ALS), and some of their most characteristic phenotypic aspects; at the same time, by looking at their target genes and the molecular pathways that they implicated, we have pictured interesting scenarios for both these complex disorders that needed further confirmation [4–6]. To possibly make progress in this direction, here we presented an additional study based on nanofluidic qPCR, in which we aimed to validate some of the previous results, by following the hypothesis that differentially expressed (DE) genes may uncover e.g., Single Nucleotide Polymorphisms (SNPs) or other genetic markers worthy to be investigated for possible association with MS. To this purpose we considered either the pediatric and the adult occurrence of MS compared to age-matched healthy subjects, and to a cohort of ALS patients as neurological controls,

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Table 1. Main demographic and clinical features of the studied population.

	Females/Males	Age at sampling	Age at disease onset
		Mean years (SD)	Mean years (SD)
Pediatric RR-MS (19)	10/9	15.5 (2.7)	12.7 (3.2)
Adult RR-MS (30)	18/12	37 (11.4)	24.7 (9.6)
ALS (30)	15/15	67.1 (10.0)	65.1 (9.9)
Pediatric HC (20)	6/14	8.8 (3.3)	
Adult HC (20)	10/10	45.3 (12.8)	

RR, relapsing remitting; MS, multiple sclerosis; ALS, amyotrophic lateral sclerosis; HC, healthy subjects.

given some genetic similarities between autoimmune diseases like MS, and ALS [7].

2. Materials and Methods

2.1 Study Population

The analysis was performed on 119 subjects divided in 19 pediatric MS (pedMS), 30 adult MS (adMS), 30 ALS, and two age-matched control groups (HC), respectively 20 pediatric (pedHC) and 20 adults (adHC). Clinical and instrumental criteria for the recruited patients have been detailed elsewhere [4–6] and their main features are summarized in Table 1. All the MS were Relapsing-Remitting subjects in the remission phase of their disease.

2.2 Selection Criteria for Genes to be Validated

A total of n. 112 genes were analyzed in the whole study population; they were selected among those DE in previous analysis from the same Group, as it follows:

- n. 81 genes contained candidate SNPs (in intragenic regions) already associated with MS in published papers [8–12];

- n. 56 of these genes were confirmed miRNAs targets in previous PedMS paper from the same Group (mean read count >25, fold change >1.5, adj-$p < 0.05$) [4];

- n. 6 genes with rare variants associated with MS [13];

- n. 21 genes with interesting expression values in previous reports (mean read count >90, fold change >25, adj-$p < 0.0005$) [4];

- n. 4 endogenous controls, selected among those with quite stable expression in our previous study (*SDHA*, *UBE2D2*, *TRAP1*, *PP1B*) [4].

2.3 Sample Processing and Analysis by QuantStudio 12K-Flex

Peripheral blood mononuclear cells (PBMC) extraction was followed by RNA isolation under standardized conditions using commercial RNA isolation kit under the manufacturer protocol [4–6]; RNA quality and quantity were assessed by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and Qubit 2.0 Fluorometer (ThermoFisher Scientific, 168 Third Avenue, Waltham, MA USA), respectively. Extracted RNA samples were stored at –20 °C until use.

Quantification of mRNA expression in the participants was performed by nanofluidic qPCR analysis (QuantStudio 12KFlex RealTime PCR system, ThermoFisher Scientific, 168 Third Avenue, Waltham, MA USA). All samples were prepared by following standardized reverse transcription (RT) and preamplification procedures (ThermoFisher Scientific, 168 Third Avenue, Waltham, MA USA). Data analysis was performed using the software package Expression Suite (Life Technologies, ThermoFisher Scientific, 168 Third Avenue, Waltham, MA USA). Each sample was analyzed in technical replicates and its relative miRNA expression levels was calculated using the $2^{-\Delta\Delta CT}$ method.

2.4 Statistical Analysis

The first step of the analysis was a quality control of the obtained qPCR values. Since all the samples were tested in duplicate, it was possible to analyze both the trend of the expression of each gene and the trend of the entire set of genes obtained in the single technical replicate. All replicates that had more than 30% null values (undetected expressions) or that showed a majority of outlier expressions (outside 1.5 times the interquartile range above the upper quartile and below the lower quartile) were then filtered out.

Then the expression estimates (Mean N-Quant values) were recalculated, and statistical comparisons were made using Student's *t*-test, adjusted with False Discovery Rate. Adjusted p-values ≤ 0.05 were considered statistically significant. The following comparisons were tested: adMS vs adHC, pedMS vs pedHC, adMS vs pedMS, adMS vs ALS, ALS vs adHC. R statistical software (https://www.r-project.org/) was used for *t*-test application and for plot generation.

To identify functionally related genes, we performed a functional analysis (GO-term) by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/). DAVID is a gene functional enrichment program that provides a large series of functional annotation tools and pathway databases (e.g., KEGG, Biocarta, Reactome databases). The statistical significance was determined using the one-tailed Fisher's exact test followed by the Benjamini correction; adjusted p-value < 0.05 was set as the threshold value. Details of methods and softwares used for the analysis have been already published [4]. The Database for Human Transcrip-

Table 2. List of the 22 significant DE genes.

Ensamble Gene ID	GENE	pedMS vs pedHC (adj-p-value)	adMS vs adHC (adj-p-value)	adMS vs ALS (adj-p-value)
ENSG00000216490	*IFI30*	0.002	0.03	0.0006
ENSG00000008130	*NADK*	0.001	0.006	0.003
ENSG00000198176	*TFDP1*	0.007	0.03	0.005
ENSG00000133318	*RTN3*	0.001	0.004	0.01
ENSG00000180644	*PRF1*	0.03	0.04	
ENSG00000105397	*TYK2*	0.01	0.02	
ENSG00000149212	*SESN3*	0.04	0.003	0.01
ENSG00000154122	*ANKH*	0.008	0.006	0.02
ENSG00000061273	*HDAC7*	0.003	0.009	0.001
ENSG00000158470	*B4GALT5*	0.0005	0.005	0.008
ENSG00000204103	*MAFB*	0.0005	0.03	0.02
ENSG00000111275	*ALDH2*	0.001	0.04	0.02
ENSG00000112182	*BACH2*	0.008	0.01	
ENSG00000141034	*GID4*	0.02	0.006	0.006
ENSG00000136826	*KLF4*	0.004	0.02	0.02
ENSG00000196092	*PAX5*	0.05	0.03	
ENSG00000025039	*RRAGD*	0.008	0.02	0.01
ENSG00000243156	*MICAL3*	0.004	0.02	
ENSG00000183621	*ZNF438*	0.008	0.02	0.02
ENSG00000124191	*TOX2*	0.03	0.003	0.008
ENSG00000005471	*ABCB4*	0.04	0.04	0.03
ENSG00000160305	*DIP2A*	0.03	0.05	0.02

The genes are indicated with their correspondent Ensembl Gene IDs and the approved name (first two column on the left); the subsequent columns showed the significant results (adjusted p-values) of the comparisons between pedMS vs pedHC, adMS vs adHC, and adMS vs ALS.

tion Factor Targets (hTFtarget) was also consulted (http://bioinfo.life.hust.edu.cn/hTFtarget#!).

3. Results

Two genes (*POMC* and *NLRP8*) failed the amplification in almost all the samples, so they were excluded from the analysis. **Supplementary Table 1** summarized all the performed comparisons between groups. PedMS and pedHC significantly differed for 63 DE genes, whereas the adult groups (adMS vs adHC) were distinguished by 38 DE genes; 10 DE genes resulted from the comparison between adMS and pedMS, whereas adMS were characterized by 40 genes DE from ALS (**Supplementary Table 1**).

PedMS and adMS shared 22 DE genes from their age-matched control groups (Table 2); among them, *BACH2* and *MICAL3* that have been already reported implicated in MS were both downregulated in pedMS and adMS compared to their age-matched controls (adj-$p < 0.01$ and adj-$p < 0.02$, respectively) (Fig. 1). Seventeen of these genes were also DE in the comparison between adMS and ALS (Table 1).

It is also worth mentioning that, although they did not reach statistical significance in some of the comparisons, by looking at their median/mean expressions most of the genes showed coherent trends between the groups. As an example, *TNFSF13B*, one of the most promising candidate genes in MS as in other autoimmune diseases, resulted significantly upregulated in pedMS vs pedHC (adj-p = 0.001), with a trend to significance between adMS vs adHC (adj-p = 0.06) (Table 1 and Fig. 1).

Indeed, the gene belongs to a set of 17 (*B2M*, *TNFSF13B*, *ODF3B*, *IFIH1*, *PRKRA*, *ZNF438*, *SCO2*, *NADK*, *PRF1*, *TYK2*, *HDAC7*, *ALDH2*, *CD86*, *KLF4*, *RRAGD*, *PRRG4* and *CELSR3*) that were grouped by their expression trend among conditions (coexpression), the lower being observed in pedHC (Fig. 2). The functional analysis revealed that they were involved in immune response and positive regulation of T cell proliferation (GOterms-BP: GO:0006955 and GO:0042129), as well as components of cytosol (GO:0005829-CC) and functionally implicated in protein bindings (GO:0005515-CC). The KEGG-pathway analysis did not show significant results after the correction for multiple tests; however, several interesting molecular pathways seem to be involved, e.g., type-I diabetes mellitus, autoimmune thyroid disease, allograft rejection and intestinal immune network for IgA production (adj-p = 0.079).

On the other hand, the expression trend of *MICAL3* was found concordant with other 9 genes (*IGHV1-69-2*, *TCL1A*, *IGLV3-19*, *KLHL14*, *IGLL5*, *RRM2*, *ADD2*, *TUBB6*, *PAX5*) (Fig. 2) and significantly implicated in the antigen binding (GO:0003823-Molecular Function, adj-p =

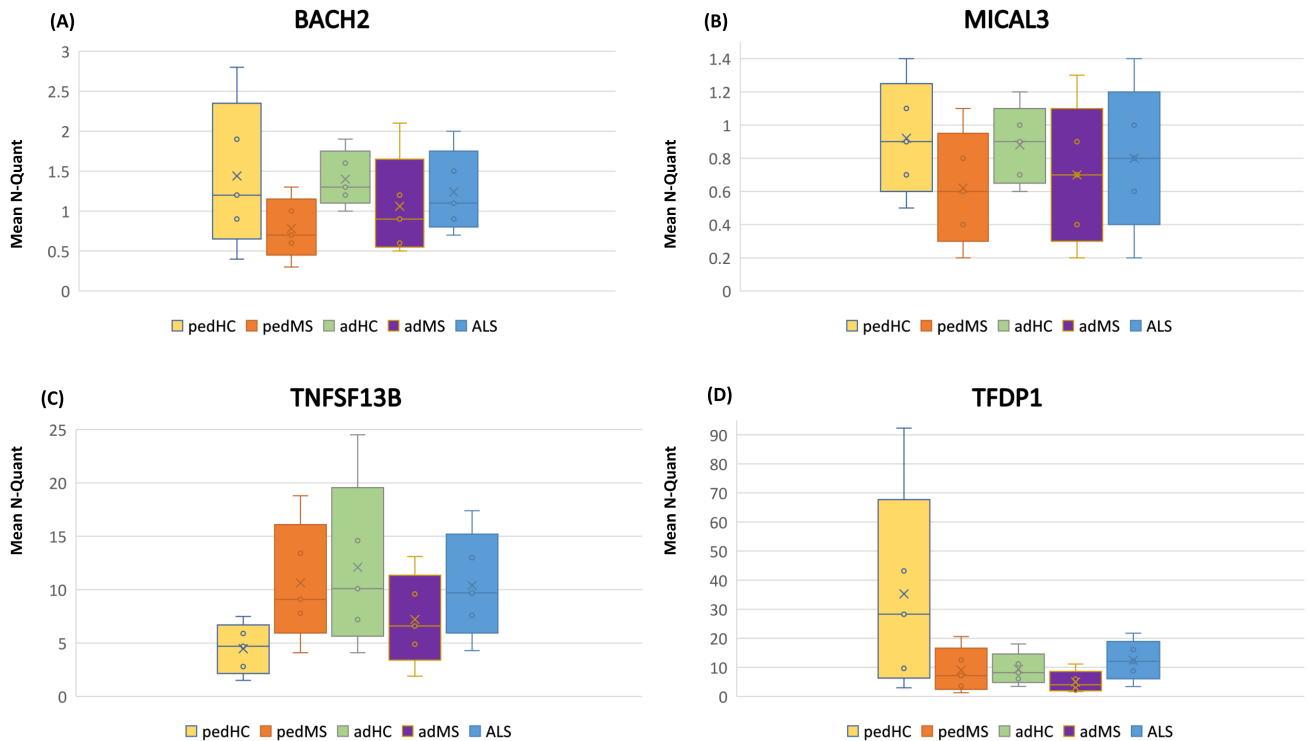

Fig. 1. Examples of DE genes by disease groups (pedMS, pedHC, adMS, adHC, ALS). The horizontal lines in each box represent the median of the gene expression, the box lengths show the range within which the central 50% of values fall, with the box edges at the first and third quartiles; the limit lines represent the range of values. Y-axes indicate the gene expression values, as reported in the methods session. (A) pedHC vs pedMS, adj-p = 0.008. adHC vs adMS, adj-p = 0.01. (B) pedHC vs pedMS, adj-p = 0.004; adHC vs adMS, adj-p = 0.02. (C) pedHC vs pedMS, adj-p = 0.001; adHC vs adMS, adj-p = 0.06 (trend to significance); adMS vs ALS, adj-p = 0.03. (D) pedHC vs pedMS, adj-p = 0.001; adHC vs adMS, adj-p = 0.03; adMS vs ALS, adj-p = 0.005.

0.04) as well as in other immunological activities (Goterms-BP), such as complement activation (GO:0006956), positive regulation of B-cells activation (GO:0050871), B-cell receptor signaling pathway (GO:0050853), and immunoglobulin receptor binding (GO: GO:0034987-MF) (adj-p > 0.05). Of interest, *TUBB6* is a crucial player in the KEGG-84617 pathway already implicated in NDDs like Alzheimer's Disease (AD), Parkinson's and Hungtinton diseases, ALS.

The most numerous set of genes grouped by their coexpression (concordant within the comparisons) was composed by 18 genes (*YBX3*, *UBXN6*, *GNAS*, *CDC34*, *FAM117A*, *SELENBP1*, *OSBP2*, *FAM210B*, *MAF1*, *CARM1*, *ANKH*, *RANBP10*, *IGF2BP2*, *CHPT1*, *CISD2*, *AQP1*, *RAB6B* and *KLC3*) (Fig. 2) implicated in protein binding (GO-terms Molecular Functions) as well as they are components of cytosol, cytoplasm and nucleus (GO-terms Cellular Components: GO:0005829, GO:0005737 and GO:0005634, respectively).

4. Discussion

In this validation study we confirmed most of the DE genes reported by previous analysis [4]. We are aware that some of the results cannot be conclusive due to the size of the study groups; nevertheless, in our view they allow us to build potentially interesting hypotheses on several issues. Furthermore, the age-matched analyses that we performed in MS gave us the opportunity to uncover differences, if confirmed, not only related to the onset of the disease but also to other hypothetical interfering factors (see below). Given the potentially common features between the two diseases [7], we also had the opportunity to use ALS as neurological controls of MS by looking into genes of interest, although this study was not meant to verify the potentially shared genetic bases of MS and ALS.

Twenty-two genes were "in common" between pedMS and adMS by means that they were significantly DE compared with their age-matched HCs, whereas sometimes a trend to significance was found in the adult comparison. As mentioned before, we cannot rule out that this event might be due to a sample bias; on the other hands we may hypothesize that other interfering factors may have occurred.

As an example, the expression data refers to the significance of the *TNFSF13B* gene encoding the cytokine and drug target B-cell activating factor (BAFF). The pathogenic role of BAFF in MS has been witnessed by the higher expression in active lesions and in cerebrospinal fluid (CSF)

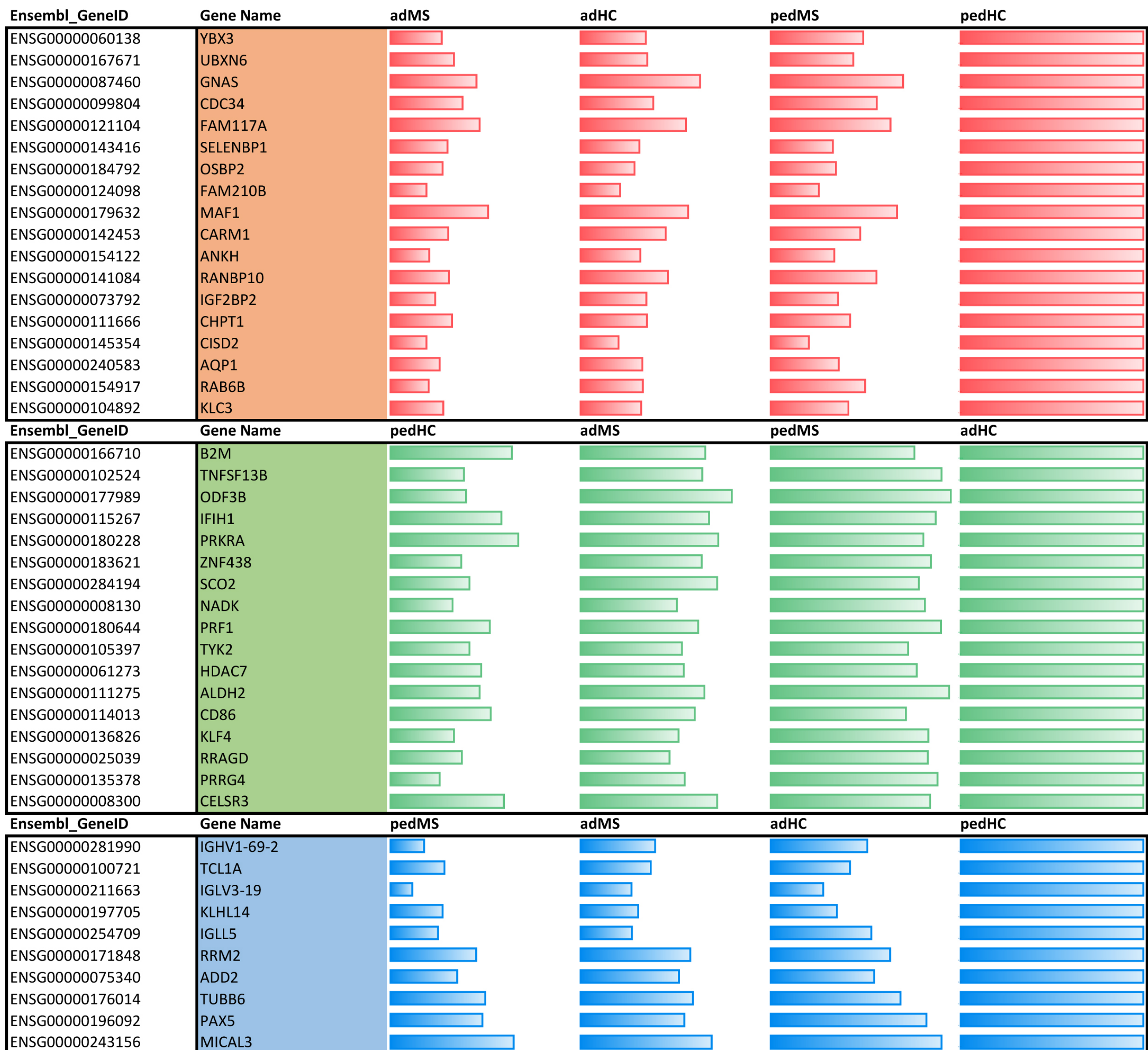

Fig. 2. Coexpression analysis of genes by mean expression in sample conditions. In the first group (n. 18 genes), the means were lower in adMS vs adHC vs pedMS vs pedHC; in the second group (n. 17 genes), the trend was from pedHC to adMS and pedMS to adHC. The third group (n. 10 genes) showed the lower mean values in pedMS vs adMS vs adHC vs pedHC.

during relapses, although other investigations like GWAS failed to demonstrate unquestionable association with MS [14]. To shed light on this issue, a recent study combining genetic, transcriptomic and functional approaches was able to demonstrate a significant role of BAFF in several autoimmune diseases like Systemic Lupus Erythematosus (SLE) and MS in Sardinia, Italy, leading to significant changes of the levels of B-cells and other humoral variables [15]. Changes of BAFF levels were also reported in case of concomitant drugs like IFNbeta-1a and Fingolimod, two of the most used and effective drugs in MS, whose administration was associated with increased protein levels *via* significant changes in the peripheral B-lymphocytes pathways [16,17].

In our study we found that the *TNFSF13B* expression was upregulated between pedMS and pedHC, but quite similar in the adult groups' comparison (with a possible shift in the opposite direction of changes). Of interest, 9/19 pedMS patients were under concomitant disease-modifying treatments (DMTs) at the time of the sampling (7 with IFN-b1a, 1 natalizumab and 1 fingolimod treatments), whereas all the adMS subjects were receiving one of the available DMTs (5 IFNbeta-1a, 14 natalizumab, 3 dimethyl fumarate, 8 fingolimod). On this ground we may hypothesize that the different DMTs may have impacted the gene expression levels, together with the age at the sampling and the disease activity that also represent additional interfering factors [18,19].

Furthermore, in our observation *TNFSF13B* was one of the resulting DE genes between ALS and adMS, its expression being upregulated in ALS. This finding seems to further support the known implication of the immune system dysregulation in the pathogenesis of ALS and other NDDs [20]. Impaired BAFF-R signals led to accelerated disease progression in animal models of ALS [21], which may be a reasonable consequence considering that BAFF is a cytokine member of the Tumor Necrosis Factor superfamily, so its level is functionally related to the survival of several cell lines enclosed neurons. In contrast, a recent study reported the association with *TNFSF13B* mutations and the Fazio-Londe syndrome, an inherited trait with ALS-like features. The Authors hypothesized that this mutation, associated with an increased BAFF level, may be causative of the concomitant immunological dysregulation, expressed by inverted CD4-to-CD8 ratio, polyclonal gammopathy, and elevated alpha-1 levels [22]. Deep focused investigations should be performed to disentangle this issue.

Some potentially interesting tips derive from other DE genes. As an example, in our study the gene *TYMP* encoding for the protein tymidine phosphorylase was found downregulated from pedMS, adMS and ALS to adHC and pedHC, although for some of these comparisons there was only a trend to significance (adMS vs adHC). TYMP was reported as the second key astrocyte-derived permeability factor that, *via* Vascular Endothelial Growth Factor A (VEGFA) interaction, plays an essential role in the blood-brain-barrier (BBB) disruption observed in Experimental Autoimmune Encephalomyelitis lesions, the animal model of MS [23]. Several evidence confirmed that a BBB breakdown also characterized NDDs like ALS [24], thus suggesting that the involvement of this gene should be further investigated in both MS and ALS.

We also found that *MAFB*, one of the 6 genes implicated in both MS and ALS, is a crucial Transcription Factor (TF) for microglia function and homeostasis, as it has been already implicated in several CNS diseases like MS and Alzheimer's Disease [25]. It is intriguing to note that *TFDP1,* another TF that regulates several genes involved in cell cycle progression [26], resulted significantly DE in all the comparisons between the MS sub-groups (Fig. 1). In MS as in other NDDs, transcription factors have been proven to build complex co-regulatory networks with their targeted genes, able to play central roles e.g., in MS autoimmune response [5]. In the present study, *TFDP1* targets *TYMP* as many other DE genes of our analysis (e.g., *BACH2*, *TNFSF13*, *MICAL3*), confirming that it may act modulating the expressions of more complex molecular networks.

5. Conclusions

As mentioned, the study suffers from some limitations mostly due to the small sample size, as well as functional studies should be performed to confirm their impact on the diseases. However, the results were in line with published evidence, to which we believe we added some interesting points of discussion and further hypotheses to test.

Transcriptomic data are influenced by several factors so, to obtain reliable data, stringent enrollment criteria should be followed in future studies, i.e., for the identification of matched controls and the concomitant therapies, as well as the clinical stage of their diseases Confirmed and novel DE genes should be further investigated to possibly uncover e.g., rare genetic variants or non-coding variations possibly associated with MS or any of its phenotypes.

Abbreviations

MS, multiple sclerosis; NDDs, neurodegenerative diseases; ALS, amyotrophic lateral sclerosis; pedMS, pediatric MS; adMS, adult MS; pedHC, pediatric healthy controls; adHC, adult healthy controls; SNP, single nucleotide polymorphism; PBMC, peripheral blood mononuclear cells; DE, differentially expressed; DMT, disease-modifying treatment; CSF, cerebrospinal fluid; GWAS, genome-wide association studies; BBB, blood-brain barrier; EAE, experimental autoimmune encephalomyelitis; AD, Alzheimer's Disease; CNS, central nervous system; TF, transcription factor.

Author Contributions

ML is the senior investigator who designed and supervised the study, contributed to the molecular experiments, supervised the clinical data and wrote the manuscript; she is also the PI of the FISM grant that provided for the financial coverage of the study (see below for details); AC performed the statistical and bioinformatics analysis and contributed to drafting the manuscript; NN performed the molecular experiments and contributed to drafting the manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of Azienda Ospedaliera Policlinico, University of Bari (Prot. 0070059/CE, 18/09/2015). Subjects or their legal tutors (for those under the age of 18 years) signed written informed consent forms (according to the Declaration of Helsinki) at the time of the enrollment.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2201004.

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