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# IL-21 and IL-21-producing T cells are involved in multiple sclerosis severity and progression

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ABSTRACT

Multiple sclerosis is a common neuroinflammatory disease of the central nervous system causing nervous system defects and severe physical disability. IL-21 is a proinflammatory cytokine produced mainly by Th-17 and Tfh cells which its exact role in MS was not yet clearly understood. In the present study we aimed to investigate the possible correlation of IL-21 gene expression, methylation, and its serum levels with MS severity and progression. The results showed that IL-21 mRNA level and serum level were significantly increased in patient group compared with control group ($p = 0.02$ and $p < 0.0001$ respectively). Moreover, we found a strong positive correlation between IL-21 mRNA levels and EDSS scores ($r = 0.637$, $P < 0.0001$), IL-21 mRNA levels and Progression Index ($r = 0.540$, $P < 0.0001$), IL-21 serum levels and EDSS scores ($r = 0.617$, $P < 0.0001$), and IL-21 serum levels and Progression Index ($r = 0.527$, $P < 0.0001$) in MS patients. Additionally, we found that the methylation level of IL-21 promoter region was decreased in patient group compared with the control group ($p < 0.0001$). We also found that methylation level of IL-21 gene promoter is negatively correlated with the IL-21 mRNA level ($r = -0.263$, $p = 0.02$), serum level ($r = -0.249$, $p = 0.03$), EDSS scores ($r = -0.276$, $p = 0.01$) and Progression Index ($r = -0.430$, $p = 0.0001$). Data showed that the increased percentages of IL-21-producing Tfh-like, Th-17 and Th1 cells in patients are positively correlated with MS severity and progression. The results of our study suggest a pro-inflammatory and booster role for IL-21 in the MS pathogenesis and progression.

## 1. Introduction

Multiple sclerosis (MS) is a common, chronic and neuro-inflammatory disease of the central nervous system (CNS), that affects both the white and gray matter causing nervous system defects and severe physical disability [1–3]. MS is more common in females and usually occurs between the ages of 20–40 years [3,4]. MS can be diagnosed by clinical manifestations and radiological evidence of inflammatory lesions in the white and gray matter [5]. The inflammatory lesions are characterized by demyelination, inflammation, axonal loss and gliosis [6]. The disease course is initially relapsing-remitting (RRMS) in about 85% of patients which characterized by episodes of acute neurological dysfunction followed by full or partial recovery. Most RRMS cases develop to a progressive course namely secondary progressive MS (SPMS) after 10–20 years of disease evolution which characterized by continuous neurological deterioration. About 10% of patients have a progressive disease course from onset which called primary progressive MS (PPMS). The least common form (< 5%) of

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disease is progressive relapsing MS (PRMS) which is similar to PPMS but with overlapping relapses [7,8]. Although the exact etiology of MS is unclear, environmental factors such as infections along with individuals genetic background and immune dysregulation have been shown to be involved in MS initiation and development [2,9]. Numerous studies have reported the associations between MS risk and vitamin D levels or sun exposure, Epstein–Barr virus infection, and smoking [9]. There are evidences which demonstrate the role of autoreactive T cells specially Th-17 cells that are activated at peripheral sites through molecular mimicry, bystander activation or the co-expression of T cell receptors (TCRs) with different specificities [10–15]. These cells can traffic to the CNS and are found at high frequency in active MS lesions along with activated B cells and monocytes and can initiate an inflammatory cascade followed by axonal damage, demyelination of the CNS and plaques formation leading to disruption of neuronal signaling [11,16,17].

IL-21 is a pro-inflammatory cytokine produced by activated TCD4 + cells specially Th-17 cells and follicular helper T cells (Tfh) and also by natural killer cells [18–21]. It can affect on different immune cells, including lymphocytes, dendritic cells, macrophages, natural killing cells, and epithelial cells [22–25]. IL-21 can enhance proliferation of lymphoid cells, differentiation of B cells into plasma cells and in synergy with IL-15 can increase cytotoxicity of CD8 + T cells and natural killer cells [18]. Recent studies have demonstrated that B cells, antibodies and IL-21- producing T-follicular helper-like (Tfh-like) cells also play a significant role in MS pathogenesis [26–28]. The therapeutic effect of anti-CD20 antibodies also support the importance of humeral immunity and somehow IL-21 in the pathogenesis of MS as it can induce differentiation of B cells into plasma cells [29,30]. Moreover, in synergy with TGF-β, IL-21 can induce RORγt and IL-17 expression in naive T cells and augments the differentiation of Th17 cell [31–33]. IL-21 receptor (IL-21R) is a heterodimeric receptor formed by a unique IL-21R-α chain and the common γ-chain (CD134), which is expressed on T cells, NK cells, B cells and dendritic cells [18,24,34]. Polymorphisms in IL-21 gene have been reported to be associated with systemic lupus erythematosus (SLE) [35] indicating that IL-21 is a susceptibility locus for autoimmune diseases. Recently, we investigated the possible association of IL-21 gene polymorphisms with the MS clinical profiles and found an association of the IL-21 gene polymorphism with disease progression [36]. Nohra et al. have reported association of IL-21R polymorphisms with EAE and MS [37]. Moreover, experimental studies using IL-21 and IL-21R knock-out mice have shown that IL-21 can potently induce Th-17 differentiation and suppresses Foxp3 expression resulting in protection against experimental autoimmune encephalomyelitis [21]. Conversely, other studies have reported that IL-21 may has a protective role in EAE and is not associated with disease progression [38,39]. Tzartos et al. have studied IL-21/IL-21R gene expression in MS lesions by in situ hybridization and immunohistochemistry and reported increased expression of IL-21 in lymphocytes infiltrated in both acute and chronic lesions demonstrating the possible role of IL-21 in CNS lesions in MS [40]. Based on these conflicting data we aimed to investigate the possible correlation of IL-21 gene expression, methylation, and its serum levels with MS severity and progression.

## 2. Materials and methods

### 2.1. Study subjects

A total of 76 MS patients (28 Males and 48 females) aged between 14–51 years with clinically definite RRMS were recruited to the Department of Neurology, Imam Reza Hospital, Tabriz University of Medical Sciences. Neurological assessments and MRI evaluations were performed by experienced neurologists unaware of patients' clinical characteristics and treatment at Neurosciences Research Center according to the revised 2010 McDonald Diagnostic criteria. All patients underwent examinations including current and past medical histories evaluation, neurological examination, brain MRI and laboratory tests.

All of the patients were in the remission clinical phase, defined as the period of recovery with no relapse episodes within the last 3 months prior to the time of enrollment in the study. Activity of the disease was evaluated using the resonance magnetic imaging (RMI) with gadolinium. The presence of activity was determined when gadolinium-positive lesions were observed. The disability status of subjects was quantified by Kurtzke's Expanded Disability Status Scale (EDSS) and their Progression Index (PI) was defined as the ratio of the EDSS score and disease duration. We also enrolled 40 age- and sex-matched healthy individuals as control group matched for ethnicity that were recruited from the Tabriz Blood Transfusion Center which had no history of chronic infectious diseases, asthma, allergy and autoimmune diseases. Written informed consent was provided by all subjects. Patients which had been treated with immunosuppressive agents and/or anti-inflammatory drugs were excluded from the study.

### 2.2. Preparation of human PBMCs, RNA extraction and cDNA synthesis

PBMCs were isolated using Ficoll density gradient centrifugation (1.077 g $ml^{-1}$, Merck) and were immediately processed after collection. Total RNA extraction from PBMCs was done using TRIzol reagent (Sigma-Aldrich, Missouri, United States) according to the manufacturer's recommendations. cDNA synthesis was carried out using universal cDNA synthesis kit (QIAGEN, Hilden, Germany) by using 2 µl of the extracted RNA.

### 2.3. Quantitative real-time PCR

Real-time PCR was done for testing IL-21 gene expression in patients and control group using 2X SYBR Green Pre-mix (QIAGEN, Hilden, Germany) in a Light Cycler 96 system (Roche Life Science, Penzberg, Germany) and The $2^{-\Delta\Delta CT}$ method was applied to calculate gene expression relative to the housekeeping control. The PCR samples were incubated 10 min-initial step at 95℃ followed by 40 cycles of 10 s on 95 °C, 30 s on 58 °C and 30 s on 72 °C. Table 1 shows the primer sequences used for qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control.

### 2.4. Measurement of IL-21 serum level

Serum samples of patients and control group were collected and freezed at −80 °C until use. Serum IL-21 levels were measured using enzyme-linked immunosorbent assay (ELISA) kit (ebioscience, San Diego, California, USA), with a threshold of detection of 8 pg/ml. All measurements were done in duplicate, and mean values were obtained

### 2.5. Methylation specific quantitative PCR

SYBR Green reagent (Thermo Fisher Scientific) was applied for MS-qPCR. As the negative and positive controls we used the 5-azacytidine treated DNA and SssI enzyme-treated DNA samples respectivelym. Primers for IL-21 are listed in Table 2. We calculated the demethylation rate of IL-21 using a formula defined previously [41] as

**Table 1**
List of IL-21 primer pairs used for qRT-PCR and MS-QPCR.

| Primers | | | Product size | Tm |
|---|---|---|---|---|
| qRT-PCR | F | CTAAAGTCAGCAAATACAGGAAAC | 122 | 56 |
| | R | GAAGGGCATGTTAGTCTGTG | | |
| MS-QPCR | MF | AATAGGTAAGATGTTAGGGG | 195 | 55 |
| | MR | AACAAAAATTAATAAAATCAACGAA | | |
| | UMF | AATAGGTAAGATGTTAGGGG | 195 | 55 |
| | UMR | AACAAAAATTAATAAAATCAACAAA | | |

**Table 2**
The detailed demographic and clinical characteristics of subjects.

| Characteristic | Patient Group | Control Group | P value |
|---|---|---|---|
| Number | 76 | 40 | – |
| Age (years, Mean ± SD) | 33.7 ± 8.3 | 30.1 ± 5.8 | NS |
| Gender (female/male) | 48/28 | 26/14 | NS |
| Age at disease onset (years, Mean ± SD) | 28.7 ± 7.5 | – | – |
| Disease duration (years, Mean ± SD) | 5.1 ± 4.1 | – | – |
| EDSS (Mean ± SD) | 3.6 ± 1.2 | – | – |
| Progression index (PI) (Mean ± SD) | 2.2 ± 0.92 | – | – |

EDSS: Expanded Disability Status Scale; NS: Not Significant.

follows: 100/[1 2(C CG C CG)] 100 + − × t t %, in which the CtTG signifies the cycle threshold (Ct) attained by the unmethylated primers and the CtCG signifies the cycle threshold attained by methylated primers. The primers specific for Methylation and demethylation of IL-21 were designed using the Gene Runner software and MethPrimer website. We applied high resolution melting (HRM) analysis based on a general protocol. As a positive control, a 100% methylated DNA, and as a negative control, unmethylated DNA was used.

### 2.6. Antibodies and flow cytometry

For the detection of immune cells and cytokines, cell suspensions were incubated with the following antibodies: PE-Cy5-conjugated anti-CD4, FITC-conjugated anti-IFN-γ and PE-conjugated anti-IL-17 all from eBioscience (San Diego, CA) and percp/cy5.5-conjugated anti-CD3, allophycocyanin-conjugated anti-CXCR5 and PE-conjugated anti-PD-1 from BioLegend (San Diego, CA, USA). Isotype controls were included to enable correct compensation and confirm antibody specificity. For cell surface staining, cells were incubated with Fc Block (2.4G2; BD Biosciences) for 15 min prior to staining with fluorescently labelled antibodies for 20 min on ice. For intracellular staining, cells were incubated for 4 h at 37 °C with 5% CO2 in the presence of phorbol myristate acetate (PMA), ionomycin and Golgiplug (Monensin; all from Alexis Biochemicals, San Diego, CA). Flow cytometry was performed on a BD FACS Canto II (BD Biosciences). Data were analyzed using Flowjo software (Treestar Inc., Ashland, OR, USA).

### 2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.01 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). All data are presented as mean ± standard deviation (SD). We used students T test to compare the differences of IL-21 gene expression, serum levels and IL-21-producing T cell subpopulations percentage between patient group and healthy control group. The correlation of clinical data with IL-21 gene expression, serum levels, promoter methylation level and IL-21-producing T cells paercentage in patients group were analyzed by Pearson's correlation test. Statistical significance was assumed at the $P < 0.05$.

## 3. Results

### 3.1. Descriptive characteristics

In the present study, 64% of subjects were females and 36% were males. All patients had EDSS ≤ 6 and mean of EDSS was 3.6 ± 1.2. Patients suffered from MS about 5.1 ± 4.1 years. Index of Progression (IP) was 2.2 ± 0.9. The detailed demographic and clinical characteristics of subjects are listed in Table 2.

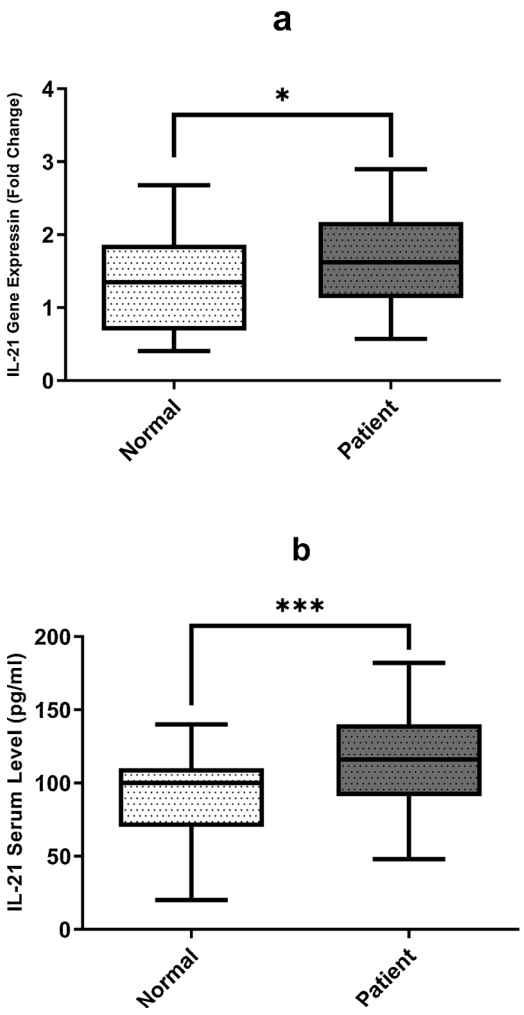


**Fig. 1.** IL-21 Gene Expression and its Serum Levels were increased in MS Patients. a. IL-21 mRNA level was significantly increased in PBMCs of patient group compared with its levels in control group ($p = 0.02$). b. The level of IL-21 was significantly elevated in serum of patients compared with control group ($p < 0.0001$). *$P < 0.05$, **$P < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

### 3.2. IL-21 gene and protein levels were increased in MS patients

Fig. 1a shows the differences of IL-21 mRNA expression levels between patient and control groups. The level of IL-21 mRNA was 1.65 ± 0.63 in patient group while in control group itwas 1.34 ± 0.67. Data showed that IL-21 mRNA level was significantly increased in PBMCs of patient group compared with its levels in control group ($p = 0.02$). Moreover, we investigated plasma level of IL-21 by ELISA. The IL-21 serum level in patient group was 115.7 ± 32.10 pg/ml while in control group it was 90.6 ± 29.22 pg/ml. The results showed that the level of IL-21 was significantly elevated in serum of patients compared with control group ($p < 0.0001$; Fig. 1b).

### 3.3. The IL-21 gene expression and its serum levels are correlated with disease severity and progression

We used Pearson correlation test to evaluate the possible relationship between the IL-21 gene expression and serum levels with disease severity and progression. We found a strong positive correlation between IL-21 mRNA levels and EDSS scores in MS patients ($r = 0.637$, $P < 0.0001$; Fig. 2a). Moreover, IL-21 mRNA levels were positively correlated with Progression Index (PI) of patients ($r = 0.540$, $P < 0.0001$; Fig. 2b). We also found a significantly positive correlation

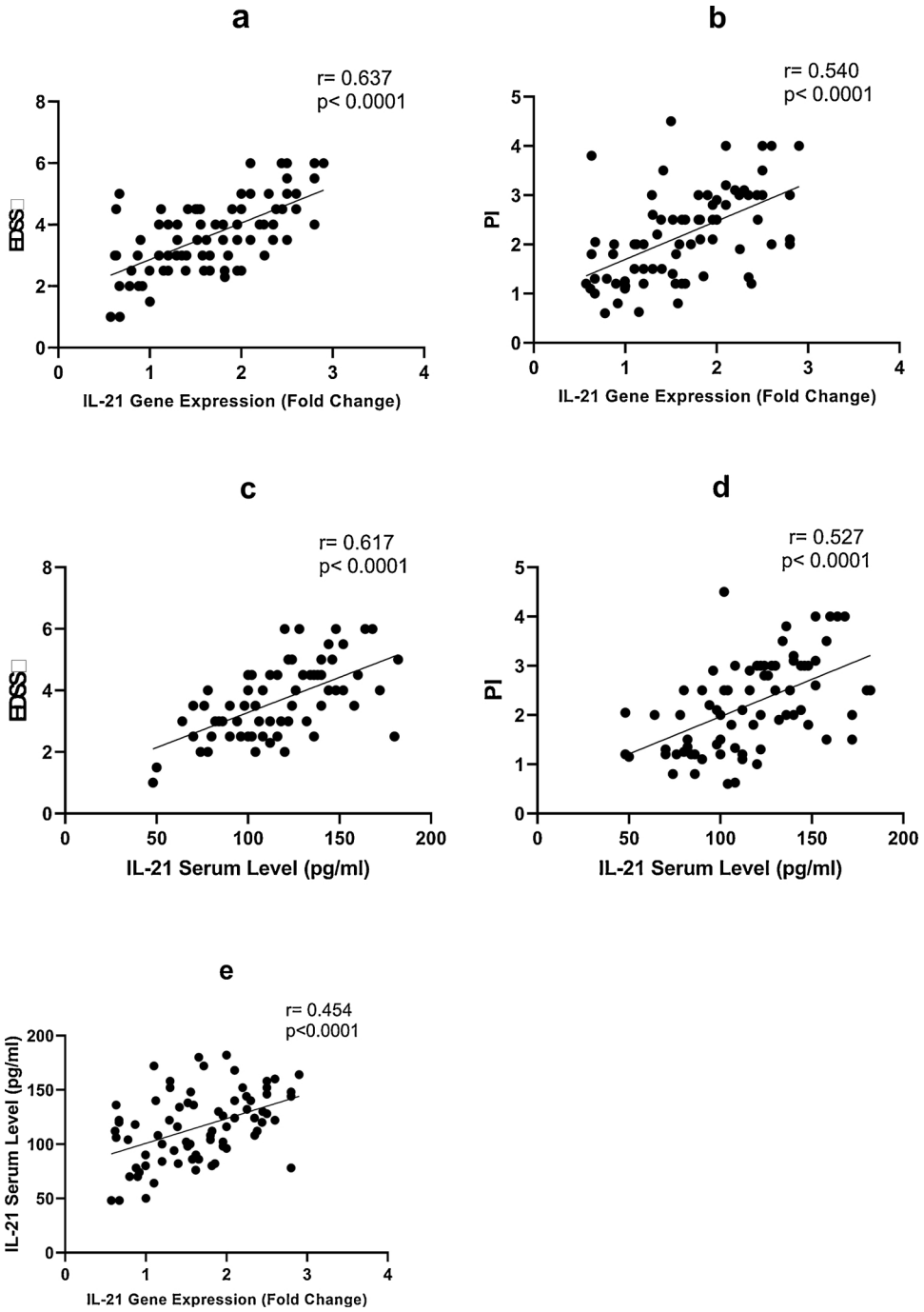


**Fig. 2.** Correlation of IL-21 gene expression and its serum levels with disease severity and progression. a. Strong positive correlation between IL-21 mRNA levels and EDSS scores in MS patients ($r = 0.637$, $P < 0.0001$). b. IL-21 mRNA levels were positively correlated with Progression Index (PI) of patients ($r = 0.540$, $P < 0.0001$). c. A significantly positive correlation between IL-21 serum levels and EDSS scores of patients ($r = 0.617$, $P < 0.0001$). d. Positive correlation of serum levels of IL-21 and PI ($r = 0.527$, $P < 0.0001$). e. positive correlation between IL-21 gene expression and serum level ($r = 0.454$, $P < 0.0001$).

between IL-21 serum levels and EDSS scores of patients ($r = 0.617$, $P < 0.0001$) (Fig. 2c). Moreover, a positive correlation was seen between IL-21 serum levels and PI ($r = 0.527$, $P < 0.0001$ (Fig. 2d). There was also a positive correlation between IL-21 mRNA level and its serum level ($r = 0.454$, $p < 0.0001$).

### 3.4. Reduced IL-21 promoter methylation is correlated with MS severity and progression

We used the melting curve analysis to confirm specificity of the primers for detection of methylated and unmethylated regions on DNA. The methylation-specific qPCR analysis demonstrated a remarkable change in methylation status on promoter region of IL-21 gene. We found that the methylation level (% M) of the promoter region of IL-21 gene was decreased in patient group compared with the control group ($p > 0.0001$). We also found that methylation level of IL-21 gene promoter negatively correlated with the IL-21 mRNA level ($r = -0.263$, $p = 0.02$; Fig. 3b), serum level ($r = -0.249$, $p = 0.03$; Fig. 3c), EDSS scores ($r = -0.276$, $p = 0.01$; Fig. 3d) and PI ($r = -0.430$, $p = 0.0001$; Fig. 3e).

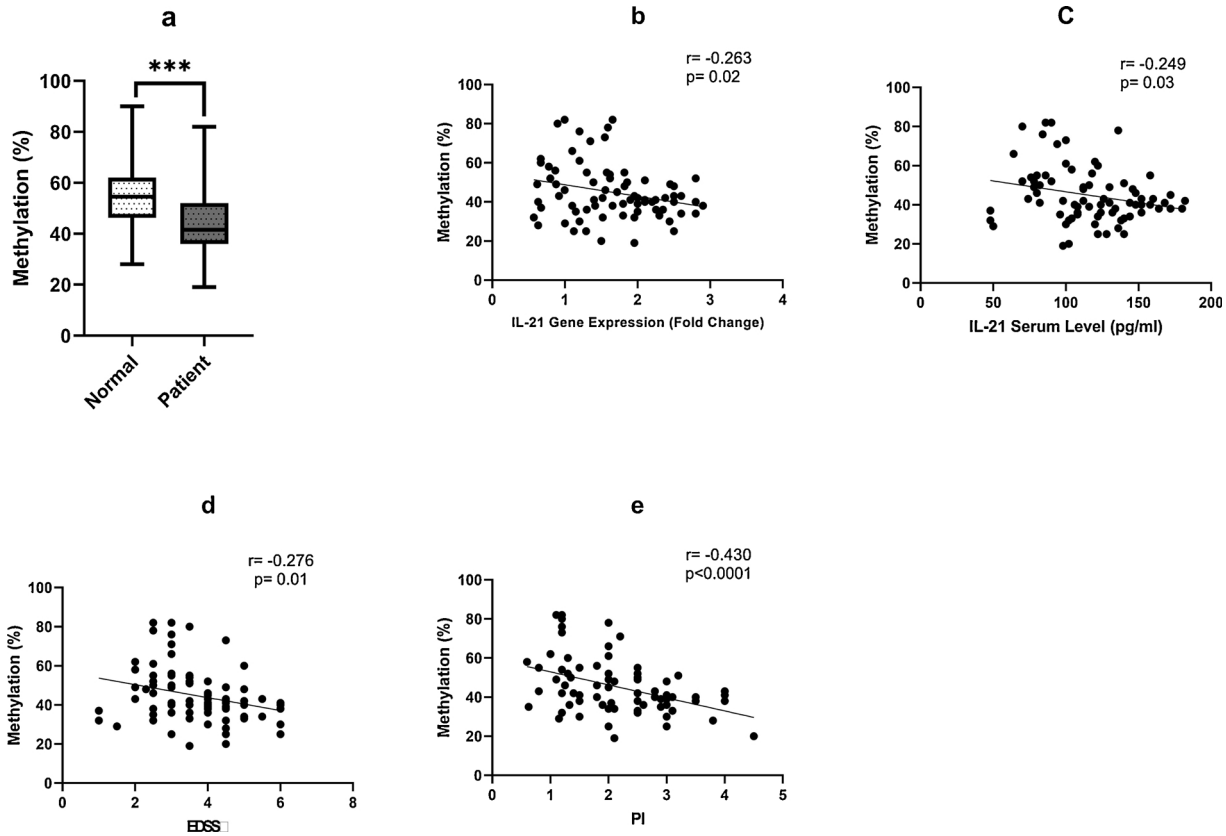


**Fig. 3.** IL-21 promoter methylation in patients and normal group and its correlation with MS severity and progression. a. The methylation level of promoter region of IL-21 gene was decreased in patient group compared with the control group ($p > 0.0001$). b. Methylation level of IL-21 gene promoter was negatively correlated with the IL-21 mRNA level ($r = -0.263$, $p = 0.02$). c. Methylation level of IL-21 gene promoter was negatively correlated with the IL-21 serum level ($r = -0.249$, $p = 0.03$) d. Methylation level of promoter of IL-21 gene was negatively correlated with the EDSS scores ($r = -0.276$, $p = 0.01$) e. Methylation level of promoter of IL-21 gene was negatively correlated with PI ($r = -0.430$, $p = 0.0001$).

### *3.5. IL-21-producing T cell subpopulations were increased in MS patients*

Frequencies of IL-21-producing CD3+ CD4+ CXCR5+ PD1+ Tfh-like cells, Th1 cells and Th17 cells in PBMCs of MS patients and healthy controls were evaluated by flow cytometry. Representative dot plots of these cells were shown in Fig. 4. Percentages of CD4+ IFN-γ+ Th1 cells in MS patients were higher than that in healthy controls (11.08 ± 2.9% vs. 10.05 ± 2.2%). Data showed that Th1 cells percentage was significantly increased in PBMCs of patient group compared with control group ($p = 0.04$; Fig. 4a and d). Moreover, frequencies of CD4+ IL-17+ Th17 cells in MS patients were significantly increased compared with healthy controls (2.37 ± 0.66% vs. 1.88 ± 0.7%; $P = 0.0003$; Fig. 4b and 4e).Moreover data showed statistically significant increase of Tfh-like cells in MS patients compared with healthy controls. (8.26 ± 2.18% vs. 5.95 ± 1.49%; $p < 0.0001$ Fig. 4c and f).

### *3.6. IL-21-producing T cell subpopulations percentage are correlated with IL-21 gene expression, promoter methylation and serum levels in MS patients*

We tested the possible correlation between IL-21-producing Tfh-like, Th-17 and Th1 cells percentage and IL-21 gene expression, promoter methylation and serum levels in patients. We found that Tfh-like, Th-17 and Th1 cells percentage were positively correlated with IL-21 serum level in MS patients ($r = 0.479$, $p < 0.0001$; $r = 0.432$, $p < 0.0001$; and $r = 0.231$, $p = 0.04$ respectively; Fig. 5a–c). Moreover, our data showed that the percentage of Tfh-like, Th-17 and Th1 cells in MS patient also are positively correlated with IL-21 gene expression level ($r = 0.452$, $p < 0.0001$; $r = 0.444$, $p < 0.0001$ and $r = 0.326$, $p = 0.004$ respectively; Fig. 5d–f). There was also a partially negative correlation between IL-21 promoter methylation percentages and the percentage of Tfh-like, Th-17 and Th1 cells in patients ($r = -0.046$, $p = 0.62$; $r = -0.179$, $p = 0.12$; $r = -0.150$, $p = 0.25$ respectively; Fig. 5g–i).

### *3.7. IL-21-producing T cell subpopulations percentages are correlated with MS severity and progression*

We tested the possible correlation between IL-21-producing Tfh-like, Th-17 and Th1 cells percentage and patients clinical data. Data showed that IL-21-producing Tfh-like, Th-17 and Th1 cells percentages were positively correlated with EDSS score of MS patients ($r = 0.271$, $p = 0.02$; $r = 0.295$, $p = 0.009$; and $r = 0.377$, $p = 0.0008$ respectively; Fig. 6a–c). There were also positive correlation between progression index (PI) scores and Tfh-like, Th-17 and Th1 cells percentages of patients ($r = 0.308$, $p = 0.006$; $r = 0.223$, $p = 0.05$ and $r = 0.255$, $p = 0.02$ respectively; Fig. 6d–f)).

## 4. Discussion

There are many evidences which support the idea that the Th1/Th17 axis is controlled by cytokines that are involved in Th17 biology [31]. IL-21 is one of the cytokines required for differentiation of naive activated CD4+ T cells into Th17 cells which have pivotal role in the immunopathogenesis of the MS disease [21]. Moreover, IL-21 can enhance proliferation of lymphoid cells, differentiation of plasma cells and in synergy with IL-15 can increase cytotoxicity of CD8+ T cells and natural killer cells [18]. Tfh and B cell activation also have been

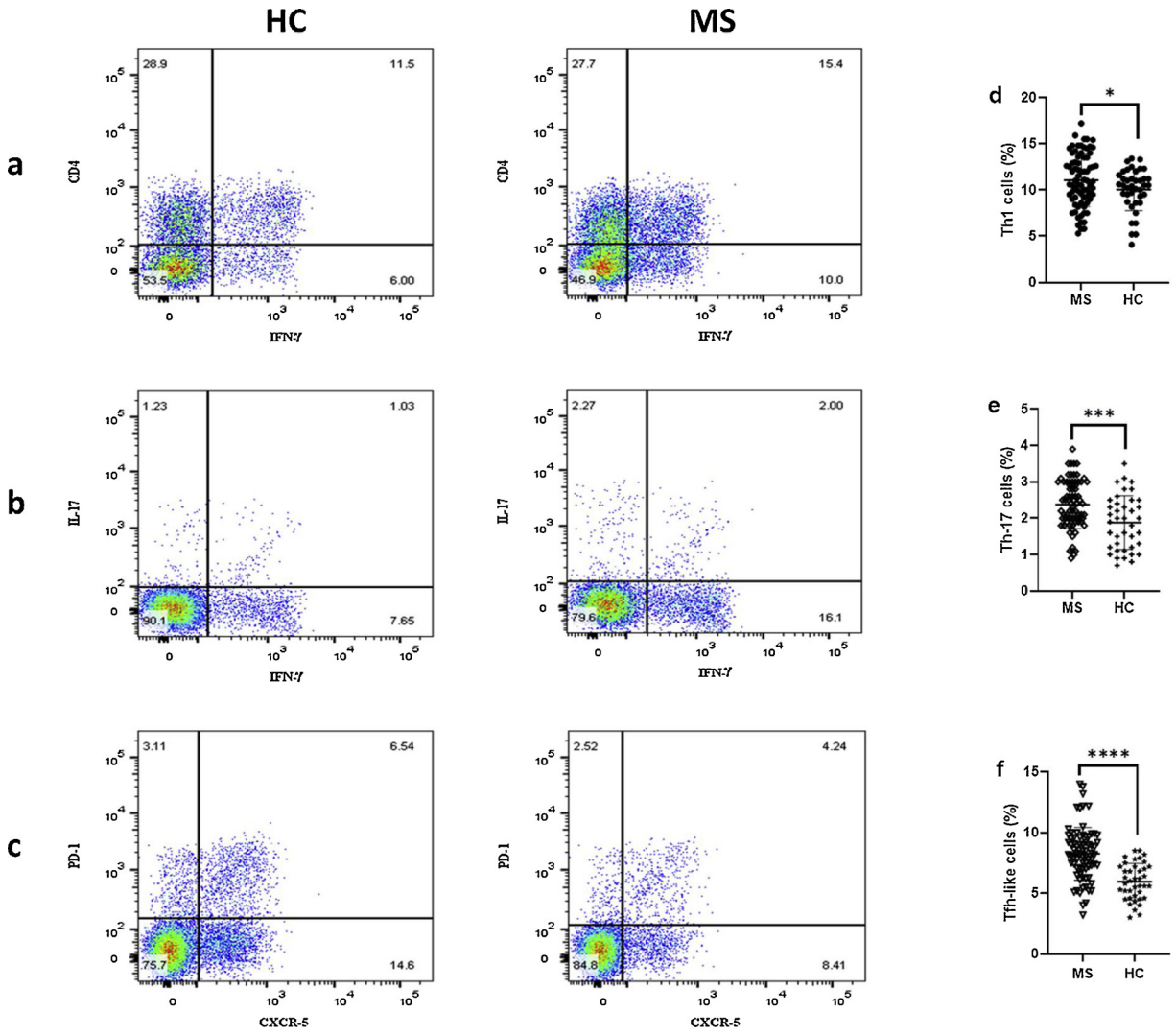


**Fig. 4.** IL-21-producing T cell subpopulations were increased in MS Patients. Representative flow cytometry results of Th1 cells (a), Th-17 cells (B) and CD3 + CD4 + CXCR5 + PD-1 + Tfh-like cells (c) in healthy controls (HCs) and relapsing remitting MS patients. Numbers in the upright corner illustrated the percentage of Th1, Th-17 and Tfh-like cells in CD4 + T cells which were increased in MS patients compared with healthy controls. Graphs d,e and c show statistically significant increase of Th1, Th-17 and Tfh-like cells in MS patients compared with healthy controls. ($p < 0.0001$, $p = 0.0003$ and $p = 0.04$ respectively).

reported to correlate with MS progression and Tfh activation marker IL-21 has been shown to be decreased in MS patients treated with mitoxantrone [42]. Thus, it is reasonable to speculate that the IL-21 plays an important role in pathogenesis of MS.

In the present study, we showed an increase of IL-21-producing T cell subpopulations and IL-21 expression in PBMCs and its protein levels in serum of MS patients compared with healthy individuals. Moreover, we showed that the elevated IL-21 mRNA, protein levels and the percentage of IL-21-producing Tfh-like, Th-17 and Th1 cells are positively correlated with MS severity and progression confirming the pro-inflammatory role of IL-21 and IL-21-producing T cells in the pathogenesis of MS. As our data showed that IL-21 expression, production and release was increased in blood of MS patients, it is supposed that IL-21 also can be produced at a higher level in the inflamed lesions in CNS of patients. Tzartos et al. investigated IL-21 and IL-21R expression in MS lesions using in situ hybridization and immunohistochemistry [40]. In consistent with our results, they detected IL-21 expressing CD4 + T cells mainly in acute and chronic active white matter MS lesions.

Studies have shown that Th-17, TfH, and B cells cooperatively drive disease activity in MS and EAE. It was suggested that CNS infiltrating TfH cells act with Th-17 cells to contribute to an inflammatory B cell response in neuroinflammation [43]. Circulating CD3 + CD4 + CXCR5 + PD1 + Tfh-like cells are thought to be essential for supporting B-cell antibody responses [44]. In EAE mice, Tfh-like cells, together with B cells, were found in the ectopic lymphoid structures in spinal cords. Moreover, Tfh-like cells promote the antibody production *via*IL-21/IL-21R and CD40 ligand/CD40 interaction and the synergy effect of STAT3 and non-canonical NF-κB signaling pathway inside B cell [27].

Christensen et al. have reported increased expression of IL-21, IL-21R and ICOS in $CD4^+$ T-cells obtained from blood of progressive MS patients [28]. They found that activation of IL-21 producing Tfh and Th-17cells along with B-cells correlated with disease progression in Secondary Progressive MS (SPMS), highlighting the role of systemic inflammation in SPMS. These results are in agreement with our finding which showed positive correlation of IL-21-producing Tfh-like, Th-17 and Th1 cells with MS severity and progression. They also reported that treatment of SPMS patients with mitoxantrone decreased Tfh cells and IL-21 mRNA and serum level leading to disease alleviation suggesting that inhibition of Tfh function and IL-21 production may be an effect of mitoxantrone in SPMS.

Other groups have reported that IL-21 serum levels were decreased following treatment of MS patients with alemtuzumab and secondary autoimmunity following treatment with this drug can be predicted by the high levels of IL-21 in serum of patients [45–47]. These findings are

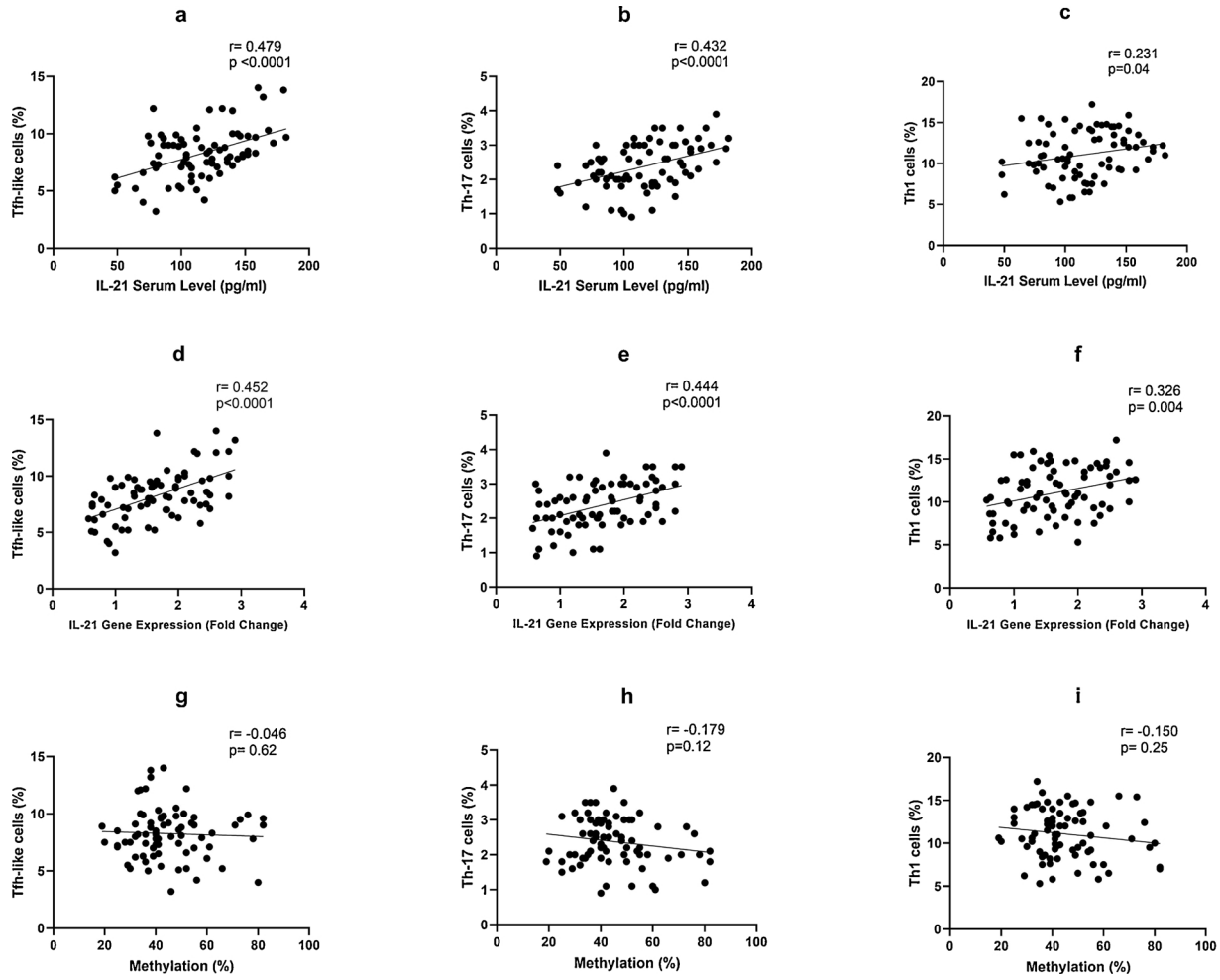


**Fig. 5.** Correlation of IL-21-producing T cell subpopulations percentage with IL-21 gene expression, promoter methylation and serum levels in MS patients. a,b and c, IL-21-producing Tfh-like, Th-17 and Th1 cells percentage were positively correlated with IL-21 serum level in MS patients. Graphs d, e and f show that the percentage of Tfh-like, Th-17 and Th1 cells in MS patient also are positively correlated with IL-21 gene expression level. g, h and i, there was also a partially negative correlation between IL-21 promoter methylation percentage and the percentage of Tfh-like, Th-17 and Th1 cells in patients.

consistent with our results which collectively demonstrate the role of IL-21 in MS pathogenesis supporting the treatments targeting IL-21 and IL-21-producing Tfh and Th-17 cells in the treatment of MS. Moreover, our results show that IL-21 mRNA and serum level has relative potency to predict the rate of progression and outcome of MS disease, suggesting IL-21 as a useful biomarker for MS progression.

While IL-21 has been shown to be one of the cytokines required for Th-17 cells differentiation it can inversely inhibit pro-inflammatory activities of granulocyte-macrophage colony-stimulating factor (GM-CSF) such as DC activation and maturation. It has been shown that, in the presence of IL-21, GM-CSF-induced DCs differentiated into functionally and phenotypically altered DCs characterized by reduced major histocompatibility complex class II (MHCII) expression and low stimulatory capacity for T-cell activation in vitro [48].

Moreover, IL-21 has been demonstrated to promote IL-10 secretion but inhibit IFN-γ and GM-CSF production in developing Th-17 cells, preventing the generation of Th1/17 effector cells. IL-21 also can selectively inhibit T-bet upregulation and GM-CSF production in Th-17 memory cells [49]. These results are in controversy with our findings and these conflicting results regarding IL-21 role in MS show that further investigations in this issue are required to obtain a reliable conclusion.

Several other studies have reported the increased levels of IL-21 mRNA and serum levels in some other autoimmune diseases. He et al. have reported increased serum IL-21 levels in patients with psoriasis compared with healthy controls which were positively correlated with PASI scores [50]. Moreover, increased mRNA and/or serum IL-21 levels also has been reported in some other autoimmune and inflammatory diseases such as Chronic Rhinosinusitis (CRC) [51], atopic dermatitis [52], dermatomyositis and polymyositis [53], and liver transplant patients [54], indicating significant role of IL-21 in pathogenesis of autoimmune and inflammatory diseases.

We also evaluated the methylation status of IL-21 promoter region and found that the level of methylation (% M) was decreased in patient group compared with the control group. We also found a weak negative correlation between methylation level of IL-21 gene promoter and IL-21 mRNA level, serum level, EDSS scores and PI in patients. The main feature of transcriptionally active genes is lower promoter methylation, and our finding is compatible with this notion that promoter methylation is important in regulation of IL-21expression and could contribute to the production control of this cytokine according to our results. However, our data showed a weak correlation between IL-21 methylation level and gene expression. Nevertheless, the partially moderate negative correlation between IL-21 promoter methylation level and PI (r= -0.430) may implies that hypomethylation of IL-21 promoter may contribute to the risk of development and progression of MS, highlighting the role of epigenetic mechanisms in pathogenesis of this disease.

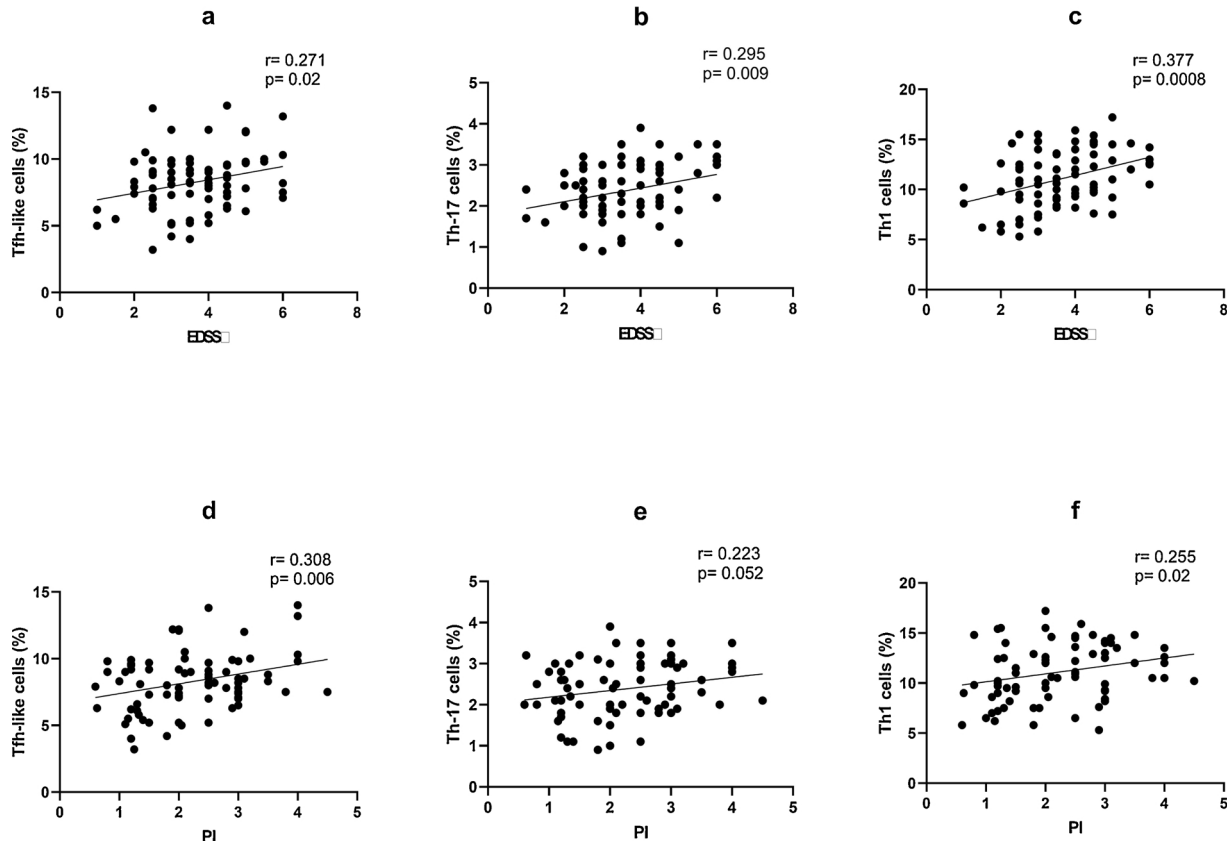


**Fig. 6.** IL-21-producing T cell subpopulations percentage are correlated with patients clinical status. Graphs a, b and c show that IL-21-producing Tfh-like, Th-17 and Th1 cells percentages were positively correlated with EDSS score of MS patients. There were also positive correlation between progression index (PI) and Tfh-like, Th-17 and Th1 cells percentages of patients (graphs d, e and f).

## 5. Conclusion

Collectively, the results of our study suggest a pro-inflammatory and booster role for IL-21 and IL-21 producing cells specially Tfh-like cells and Th17-cells in the pathogenesis of MS. Moreover, our data suggest a role for altered methylation status of IL-21 gene promoter in disease progression. Correspondingly, these results support the treatments targeting IL-21 and IL-21 producing Tfh-like and Th17 cells in the treatment of MS. Moreover, these results support the use of hypermethylation agents such as *S*-adenosylmethionine (SAM) for the treatment of MS and EAE. Finally, since the fate of any immunopathological processes such as autoimmunity is determined by a complex cytokine network present in specific tissue microenvironment, any conclusion on the role of a cytokine in pathogenesis of MS disease demands further investigations.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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