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# Methotrexate treatment efficacy in sarcoidosis might be related to TNF-α polymorphism: real life preliminary study

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**Abstract.** *Introduction:* Methotrexate therapy improves lung function in selected sarcoidosis patients. Variation in TNF gene was associated with response to treatment. *Aim:* To determine the predictive role of-308 G/A, -857C/T, -863 C/A and -1031 T/C *TNF*-α polymorphism in the efficacy of MTX for progressive pulmonary sarcoidosis. *Material and Methods:* Twenty-eight sarcoidosis patients treated with MTX (6-24 months) were genotyped for *TNF*-α polymorphisms: -1031 T/C, -857C/T, -308 G/A and -863 C/A. Pulmonary function test (PFT) were performed every 6 months to determine treatment response, until the drug withdrawal. *Results:* No correlation between the initial clinical presentation of sarcoidosis and *TNF* α polymorphisms was found, neither for every allele nor for combined genotypes distribution. According to PFT evaluation we have discovered 3 types of response to MTX: early (ER), late (LR) and No-response (NR). *TNF*-α-308 A allele carriers have got significantly higher chance to be LR, p=0.02, RRI:83%. *TNF*-α-308 GG genotype transferred the 3-fold higher probability of early vs late response to MTX, p=0.02. Combined genotyping allowed to distinguish LR from ER and NR groups. ER and NR patients are genetically similar (-857CC-308GG). LR are "genetically" different group of patients (-857C/T-308GG or -857CC-308A/G) with 5-fold greater probability to be LR than *TNF*-α-857CC-308GG patients, p=0,005 sensitivity 85%, specificity: 43%, PPV 58%, NPV 75%. *TNF*-α-308GG-857CC patients have significantly lower chance to be LR comparing to other response type p=0.03 OR=0,075 95% CI=0.07-0.08. *Conclusion:* Two types of positive response to MTX therapy (early and late) in chronic respiratory sarcoidosis are associated with polymorphic changes in TNF gene. *(Sarcoidosis Vasc Diffuse Lung Dis 2019; 36 (4): 261-273)*

**Key words:** MTX, *TNF*-α polymorphism, sarcoidosis, treatment

## 1. Introduction

Sarcoidosis, a systemic inflammatory disease affecting predominantly respiratory system, is characterized by granuloma formation progressing in certain cases to irreversible fibrosis of affected organs. Majority of patients do not require treatment as the disease may resolve spontaneously. In some individuals the deterioration of pulmonary functionality may lead to respiratory failure responsible for 75% of deaths caused by sarcoidosis (1). Steroids remain the first line drugs of choice but their clinical efficacy is unpredictable and some patients are refractory to these medications. Additionally, this therapy is associated with multiple adverse effects (AE) that worsen the prognosis in individual cases. Methotrexate (MTX), the most commonly used disease-modi-

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fying anti-rheumatic drug (DMARD) in rheumatologic conditions, is considered as second line agent for sarcoidosis patients (2). We observed significant improvement in PFT in our previously described cohort of pulmonary sarcoidosis patients treated with MTX (3), however almost half of enrolled subjects hasn't responded to the therapy despite optimal dosing regiments. Considering the potential severe AE of MTX, and limited evidence on how to guide the treatment, the identification of responders' profile could optimize MTX administration. Multiple factors, such as genetic profile, heterogeneity and severity of disease manifestations and comorbidities, can influence treatment response to different medications (4).

Various determinants of the production of TNFα, a crucial cytokine in the pathogenesis of granulomatous diseases (5,6), might contribute to the phenotypic heterogeneity of sarcoidosis, including the response to treatment. The aim of our study was to evaluate if assessing TNF α gene polymorphism in chronic progressive pulmonary sarcoidosis might be helpful in predicting the type of clinical response to MTX therapy.

## 2. Methods

### *Study population*

We have analyzed data from a single center real life open prospective study on 53 patients treated with MTX and folates. The initial study was done by Anna Goljan Geremek and published in Advances in Respiratory Medicine 2014 (3). Patients were recruited from those seen by the authors for symptomatic, chronic pulmonary sarcoidosis at Respiratory Department of National Tuberculosis and Lung Diseases Research Institute between JAN 2004 and DEC 2013. All patients met standard criteria for the diagnosis of sarcoidosis in accordance with the guidelines of the World Association of Sarcoidosis and Other Granulomatous Diseases (WASOG) (7). Organ involvement was diagnosed according to the criteria proposed in the A Case Control Etiology of Sarcoidosis Study (ACCESS) (8). Methotrexate was proposed to patients who were previously unsuccessfully treated by systemic corticosteroids and, as a first line therapy, to patients who were not treated by GCS due to contraindications or due to refusal to steroids because of possible adverse effects (AE). The decision to begin systemic MTX therapy in each case was taken collectively, by a team of investigators (the same clinicians for all of the patients). The therapeutic decision was based on clinical criteria (2, 3, 7): presence of disabling or distressing symptoms of sarcoidosis (dyspnea, cough, wheezing, chest pain) associated with a 10% or more decline of FEV1, or FVC, or TLC or a 15% or more decline of DLCO for two consecutive measurements prior to enrollment, or any impairment of PFT parameters at the initial examination, and presence of progressive pulmonary, parenchymal disease with signs of pulmonary fibrosis, confirmed by chest X ray (CXR), high-resolution computed tomography (HRCT) and documented by retrospective analysis of the imaging records by two independent radiologists. Prior to the administration of MTX (visit 0-initial evaluation, recruitment) all patients were followed-up for at least 6 months with no therapy. At the baseline visit (visit 1) medical records were collected for: BMI, previous treatment, disease duration –from establishing the diagnosis of sarcoidosis to the baseline visit. Then the indications for MTX were confirmed. Patients received MTX according to our own protocol. The therapy was planned for 24 months. Initial standard dose was set at 10 mg per week. In individual cases the higher dose of 15 mg weekly was proposed, according to the recommendations adapted from rheumatic diseases and from sarcoidosis experts' opinion (2, 9). Additionally, all patients used 5 mg folic acid every day (excluding the day of MTX administration). Toxicity of therapy was assessed every 4-6 weeks by the treating physician during regular medical visit and additional diagnostic procedures were performed if necessary. Pulmonary function (PF) was assessed by spirometry, plethysmography and diffusion capacity. All spirometric and plethysmographic measurements were performed using the Jaeger Master Screen Body, version 4.65, according to ERS/ATS 2005 statement. Measured parameters were presented as percent of predicted value. The primary outcome were the pulmonary function parameters (FEV1, FVC, TLC, DLCO) assessed at 6 months intervals. We considered improvement of ≥10% of FEV1, FVC, TLC or ≥15% of DLCO from the initial value (before starting MTX) to be significant for clinical improvement of lung function.

Clinical assessment at follow-up visits included chest X-ray image, extrapulmonary involvement and exercise capacity evaluation. 6MWT was performed on a treadmill. A heart rate (HR), blood pressure (BP), pulse oximetry with maximal (at rest) and minimal (during exercise) oxygen saturation (SaO2min) were recorded, as well as the walking distance (6MWD) and the dyspnea Borg score before and after exercise. Study flow is presented in figure 1.

First evaluation of MTX effectiveness was made 6 months after initiation of the therapy, as suggested by other authors (10). At this point the decision for the continuation of treatment or withdrawal of the medication was made by a physician. In case of good treatment tolerance and improvement in at least one of analyzed clinical parameters, the medication was continued. At every 6 months interval patients underwent clinical and functional assessment in order to decide about the continuation or withdrawal of the therapy.

The MTX therapy was stopped before planned 24 months in patients with severe adverse events related to MTX therapy and/or subjective intolerance of the drug and/or sarcoidosis' worsening documented by radiological or functional evidence and/or no improvement in any of analyzed clinical parameters: radiological, functional (measured by PFT and 6MWT), extra thoracic sarcoidosis confirmed at 2 consecutive visits every 6 months.

The response to MTX therapy was categorized in three groups:

Early responders (ER): if significant improvement in any of PFT parameters at first 6 months of MTX therapy was documented.

Late responders (LR): if significant improvement in any of PFT parameters was documented at the end of therapy, but not at the first 6 months of treatment.

Patients who had no changes or experienced deterioration in the PFT parameters during treatment were classified as "MTX No-responders" (NR).

The study was performed in accordance with the Declaration of Helsinki and its amendments. The protocol was approved by the Medical Ethics Board of National Tuberculosis and Lung Diseases Research Institute in Poland (KE-31/2004). The study was approved by internal review board (N402 052 32/1592; KE-18/2006). Written informed consent for participation in this study was obtained for all subjects.

*Determination of TNFα genotypes*

DNA was extracted from peripheral leukocytes by standard techniques, using NucleoSpin®Quick Pure (Macherey-Nagel), according to manufacturer instruction. With the NucleoSpin® Plasmid method peripheral leukocytes were resuspended (Buffer

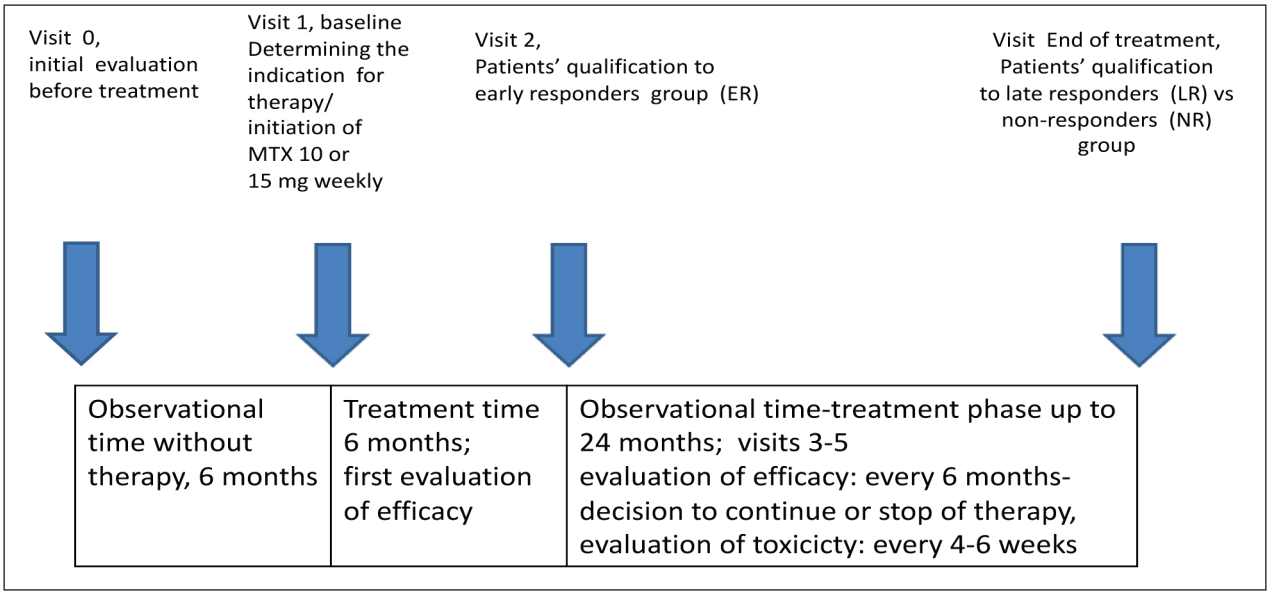


**Fig. 1.** Various types of Iranian cities climate (From IMO website: http://www.irimo.ir)

1) and plasmid DNA was liberated by SDS/alkaline lysis (Buffer A2). Buffer A3 neutralized the resulting lysate and created appropriate conditions for binding plasmid DNA to silica membrane of the NucleoSpin® Plasmid Quick Pure Column. Kit contaminations (nucleases, soluble macromolecular cellular components) were removed by single washing step with Buffer AQ. Pure plasmid DNA was eluted under low ionic strength conditions with alkaline Buffer AE (5mM Tris/HCL, pH 8,5). Purified DNA was collected and stored at –20°C until use. SNP at positions: rs1799964 (-1031T/C), rs1799724 (-857C/T), rs1800629 (-308G/A), rs1800630 (-863 C/A) of the TNFα gene were determined by TaqMan® SNP Genotyping Assays, Applied Biosystems, according to manufacturer instruction(11). Molecular analyses were conducted using the TaqMan® assay (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and were analyzed using the OpenArray® NT Genotyping system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The *TNF-α* polymorphisms were analyzed using a polymerase chain reaction (PCR)- restriction fragment length polymorphism (RFLP) assay. The four polymorphic regions were amplified using ABI PRISM 7000 Sequence Detection System (SDS), according to manufacturer's instruction. The single genotyping reaction included: 2µl genomic DNA, 1,25 µl TaqMan® SNP Genotyping Assay, 12,5 µl TaqMan® Genotyping Master Mix,9,25 µl distilled water. The following PCR thermocycler conditions were used: a single cycle of 96°C for 10 minutes, followed by 40 cycles of amplifications of 92°C for 15 seconds and 60°C for one minute. The fluorescence signal was visualized and analyzed using the ABI Prism 7000 SDS Software.

*Statistical analysis*

Statistical analysis was performed with R environment.

Patients' characteristics and quantitative measurements are presented as mean ±SD. A p value of <0.05 was considered statistically significant. Quantitative data were tested for normality with Shapiro-Wilk in each analyzed group. Homogeneity of variance was tested using Bartlett's test. Two-tailed T-Student test and ANOVA were performed to compare data with normal distributions and equal variances in two and multiple groups respectively. Cochran-Cox test was used if variances in two compared groups with normal distributions were not equal. In other cases, non-parametric tests were used: U Mann-Whitney test for two groups and Kruskal-Wallis ANOVA for multiple comparisons. For statistically significant ANOVA and Kruskal-Wallis ANOVA results Tukey test and Nemeny'i test were performed as post-hoc tests respectively. Logistic analysis was performed, and odds ratios (OR) with 95% confidence intervals (95%CI) were computed. On the basis of ORs and conditional probabilities relative risks (risk ratio, RR) were calculated. Cross tables and $\chi^2$ test were used to compare the observed percentages with each genotype between groups of patients differing in the MTX response. Correspondence analysis between *TNFα* promoter diplotypes (-1031 TT, T/C, CC; -857 CC/, C/T, TT; -308 GG, G/A, AA; -863 CC, C/A, AA), the allelic status (-308A, -1031C, -857T, -863A absent or present) and treatment response was performed. Analysis of correspondence (CA) is a method based on chi square test for larger than 2x2 contingency tables. It assumes a presence of hidden variables, that describe relationships between different states of input variables, i.e. - type response to MTX (ER, LR, NR) and genotype. To present the results of this analysis, a two-dimensional graph is constructed (fig. 2). Each state is represented by one point, and relationships are illustrated by distance between them. Percentage of explained variance is one of calculated parameters

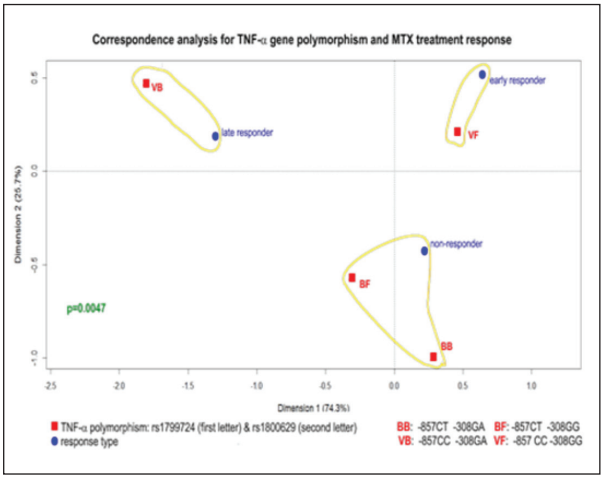


**Fig. 2.** Correspondence analysis for TNF-α gene polymorphism and MTX treatment response

for X and Y axes. The distance on this axis between points is closer, the more important and significant are relations. X-dimension distances explain almost 75% of variance in the whole cohort, so only this dimension should be considered.

## 3. Results

*Clinical characteristics of sarcoidosis patients treated with MTX*

The whole sarcoidosis patient group treated with MTX in our department consisted of 53 patients. 28 patients completed the study, including genotyping for *TNF α* polymorphism and were eligible for the further analysis (table 1).

Of the 28 patients, 15 patients fulfilled the criteria of response to MTX therapy (53,5%).

At first 6 months interval 60% of patients (N=9/15) were classified as ER, according to PFT improvement criteria. At the end of MTX therapy another 6/15 (40%) patients were classified as LR.

In 13 patients (46,5%) MTX therapy didn't improve PFT (NR).

Initial clinical characteristics of the total studied population (N=28) and within groups of ER, LR and NR are summarized in table 2.

Females represented 39% (11/28) of the cohort. The mean age at the onset of sarcoidosis was 37±10 years and did not differ between groups. The mean age at start of MTX treatment in the total cohort was 44±10 years. There was no significant difference in the mean age of patients between groups of ER, LE and NR.

The mean time of disease duration at the initiation of MTX treatment was 7.1±5.3 years. In 23 cases (82%) MTX was administered to patients previously treated by systemic corticosteroids because of progressive pulmonary sarcoidosis. 11 patients received one course of GCS treatment (2 years of

**Table 1.** *TNF α*-308, -857, -863, -1031 polymorphisms in whole cohort, N=28

| Patient, N=28 | *TNFα*-308 AA, GG, G/A | *TNFα*-857 CC, TT, C/T | *TNFα*-863 CC, AA, C/A | *TNFα*-1031 TT, CC, T/C | Early responder: ER / Late responder: LR/ Non-responder: NR |
|---|---|---|---|---|---|
| 1 | GG | C/T | CC | TT | NR |
| 2 | GG | CC | CC | TT | NR |
| 3 | G/A | CC | C/A | TT | LR |
| 4 | GG | CC | AA | TT | ER |
| 5 | GG | C/T | CC | TT | NR |
| 6 | GG | C/T | CC | TT | LR |
| 7 | GG | CC | CC | TT | ER |
| 8 | GG | CC | AA | TT | NR |
| 9 | GG | CC | CC | CC | ER |
| 10 | GG | C/T | CC | TT | NR |
| 11 | GG | C/T | AA | TT | NR |
| 12 | GG | CC | CC | TT | LR |
| 13 | GG | C/T | CC | T/C | NR |
| 14 | GG | C/T | C/A | TT | LR |
| 15 | GG | CC | CC | T/C | NR |
| 16 | GG | CC | CC | TT | ER |
| 17 | GG | CC | CC | TT | ER |
| 18 | G/A | CC | CC | T/C | LR |
| 19 | GG | CC | CC | TT | ER |
| 20 | G/A | C/T | CC | TT | NR |
| 21 | GG | CC | CC | TT | NR |
| 22 | GG | CC | C/A | T/C | NR |
| 23 | G/A | CC | CC | TT | LR |
| 24 | GG | CC | CC | TT | ER |
| 25 | GG | CC | CC | T/C | NR |
| 26 | GG | CC | CC | T/C | ER |
| 27 | GG | CC | CC | TT | NR |
| 28 | GG | CC | C/A | T/C | ER |

**Table 2.** Baseline characteristics of treatment cohort in relation to MTX effectiveness

| Variable Mean ±SD | Total cohort, N=28 | Early responders N=9 | Late responders N=6 | Non-responders N=13 | p |
|---|---|---|---|---|---|
| Gender, female, N (%) | 11 (39%) | 4 (44%) | 1 (17%) | 6 (46%) | 0.439 |
| Age at onset of sarcoidosis (yr.) | 37±10 | 37±10 | 37±9 | 38±10 | 0.987[a] |
| Previous prednisone therapy, N (%) | 24 (86%) | 9 (100%) | 5 (83%) | 10 (77%) | 0.309 |
| Age at start of MTX therapy (yr.) | 44±10 | 42±9 | 43±13 | 46±9 | 0.654[a] |
| Disease duration until start of MTX therapy (yr.) | 7.1±5.3 | 5.1±3.5 | 6.6±4.7 | 8.6±2.3 | 0.411[b] |
| BMI (kg/m2) | 27.8±4.0 | 27.6±3.17 | 28.8±3.9 | 27.5±4.7 | 0.801[a] |
| Bronchiectases, N (%) | 11 (39%) | 2 (22%) | 4 (67%) | 5 (42%) | 0.228 |
| Ground glass opacities, N (%) | 6 (22%) | 3 (33%) | 1 (17%) | 2 (17%) | 0.617 |
| **PFT%predicted** | | | | | |
| FEV1 | 70.3±19.1 | 62.9±18.9 | 83.2±7.1 | 69.4±20.4 | 0.129[a] |
| FVC | 78.3±15.4 | 72.6±14.9 | 87.6±10.1 | 77.9±16.6 | 0.186[a] |
| TLC | 80.9±12.4 | 76.8±12.0 | 84.7±11.1 | 81.9±13.4 | 0.458[a] |
| DLCO | 64.1±13.9 | 59.9±12.1 | 71.4±13.0 | 63.7±15.0 | 0.301[a] |
| FEV1%FVC | 72.1±11.9 | 70.1±15.5 | 77.4±4.1 | 71.0±11.4 | 0.557[b] |
| Airflow limitation, N (%) | | | | | |
| **6MWT** | | | | | |
| Sa02 min (%) | 91±3 | 90±4 | 92±3 | 90±4 | 0.716[a] |
| ΔSa02 (%) | 6±3 | 6±4 | 6±3 | 6±3 | 0.97 |
| Distance (m) | 528±115 | 565±121 | 514±137 | 509±103 | 0.533[b] |
| Dyspnoea (Borg score, points) | | | | | |
| Before exercise | 0±0 | 0±1 | 0±0 | 0±0 | 0.16[b] |
| After exercise | 1±2 | 2±2 | 2±2 | 1±1 | 0.906[b] |
| Extrapulmonary involvement, N (%) | 21 (75%) | 8 (89%) | 3 (50%) | 10 (77%) | 0.229 |
| Skin, N (%) | 4 (14%) | 1 (11%) | 6 (100%) | 3 (23%) | 0.388 |
| Lymph nodes, N (%) | 4 (14%) | 2 (22%) | 0 (0%) | 2 (15%) | 0.478 |
| Liver N (%) | 5 (18%) | 4 (44%) | 0 (0%) | 1 (8%) | 0.07 |
| Spleen, N (%) | 13 (46%) | 6 (67%) | 3 (50%) | 4 (31%) | 0.247 |
| Others, N (%) | 3 (11%) | 1(11%) | 0 (0%) | 2 (15%) | |
| Multiple (at least 2 organ) involvement, N (%) | 4 (14%) | 3 (33%) | 0 (0%) | 1 (8%) | 0.223 |

[a]ANOVA, [b]Kruskal-Wallis ANOVA

therapy), 12 patients received more than one course (total duration of GCS therapy >2 years) and were refractory after GCS withdrawal. MTX was proposed to 8 patient who didn't experienced any significant improvement after GCS and to 15 patients who were responsive to GCS but experienced inacceptable AE after GCS therapy (N=13) or refused to be treated with GCS again (N=2). In 9 patients MTX was started 6 months after GCS withdrawal. In 12 patients time to start MTX therapy after GCS withdrawal ranged from 7 months to 24 months. In 2 patients MTX was started more than 2 years after GCS withdrawal.

In 5 cases MTX (18%) was proposed as a first line therapy to patients who refused steroids because of potential AE or because of contraindication for such treatment.

At the enrollment the initial clinical severity of sarcoidosis (described by PFT parameters, exercise ability measured by 6MWT, radiological image and presence of extrapulmonary involvement) didn't differ between groups. There was no significant correlation between the initial clinical status of sarcoidosis and *TNF α* single nucleotide polymorphism (SNP) of -308 G/A, -857C/T, -1031 T/C and -863 C/A, neither for single allele nor for combined genotypes distribution. Table 3represents baseline clinical characteristics of total cohort in relation to *TNF α*-308 and -857 genotypes distribution.

*Genotype frequencies of TNF-α -1031 T/C, -857 C/T, -863 C/A, -308 G/Ain relation to MTX response type*

The comparison of single allele frequency in alleles -1031C, -857T, -863A (absent or present) between the ER, LR and NR groups, showed no significant distribution differences (p-value in chi square test NS for all three SNPs).

**Table 3.** Baseline characteristics of total cohort in relation to *TNF α*-308 and -857 genotypes distribution

| TNF-α | -308 | | | -857 | | |
|---|---|---|---|---|---|---|
| Variable Mean ±SD | GG N=24 | G/A N=4 | p | CC N=20 | C/TN=8 | p |
| Gender, female, N (%) | 9 (38%) | 2 (50%) | | 7 (35%) | 4 (50%) | 0.760 |
| Age at onset onset of sarcoidosis (yrs) | 38±10 | 34±10 | 0.443 | 38±10 | 35±9 | 0.406 |
| Previous prednisone therapy, N (%) | 21 (88%) | 3 (75%) | 1 | 18 (90%) | 8 (75%) | 0.669 |
| Age at start of MTX therapy (yr) | 44±10 | 46±10 | 0.720 | 45±9 | 42±11 | 0.440 |
| Disease duration until start of MTX therapy (yr) | 6±4 | 12±9 | 0.114[b] | 7±4.2 | 7.2±7.7 | 0.475[b] |
| BMI (kg/m2) | 28±3 | 28±7 | 0.853[c] | 28.8±3.7 | 25.2±3.8 | 0.040 |
| MTX total dose (mg) | 697±434 | 817±271 | 0.394[b] | 752±426 | 608±376 | 0.435 |
| Dose/BMI (mg/kg/m2) | 26±16 | 28±4 | 0.414[b] | 26.5±14.9 | 27.7±14.1 | 0.860 |
| Duration of treatment (weeks) | 58±36 | 77±34 | 0.357[b] | 63±34 | 56±40 | 0.652 |
| Bronchiectases, N (%) | 9(38%) | 2 (50%) | 1 | 7 (37%) | 4 (50%) | 0.836 |
| Ground glass opacities, N (%) | 5 (22%) | 1 (25%) | 1 | 5(26%) | 1(13%) | 0.778 |
| **PFT%predicted** | | | | | | |
| FEV1 | 70±20 | 72±11 | 0.845 | 67±17 | 79±22 | 0.119 |
| FVC | 79±16 | 76±13 | 0.731 | 75±13 | 86±18 | 0.096 |
| TLC | 81±12 | 78±17 | 0.624 | 79±11 | 85±16 | 0.302 |
| DLCO | 64±16 | 64±10 | 0.985 | 63±13 | 66±17 | 0.699 |
| FEV1%FVC | 71±12 | 78±4 | 0.200[b] | 71±13 | 75±10 | 0.334[b] |
| **6MWT** | | | | | | |
| Sa02 min (%) | 90±4 | 92±1 | 0.0504[c] | 90±3 | 91±4 | 0.906 |
| ΔSa02 (%) | 6±3 | 5±0.5 | 0.691[b] | 6±3 | 6±4 | 0.845 |
| Distance (m) | 535±113 | 484±136 | 0.417 | 519±123 | 552±95 | 0.506 |
| Dyspnoea (Borg score, points) | | | | | | |
| Before exercise | 0 | 0 | | 0 | 0 | |
| After exercise | 1±2 | 1±2.5 | 0.715 | 1±2 | 1±1 | 0.203 |
| Extrapulmonary involvement, N (%) | 18 (75%) | 3(75%) | 1 | 14(70%) | 7(87%) | 0.629 |
| Skin, N (%) | 4 (17%) | 0 (0%) | 0.912 | | | |
| Lymph nodes, N (%) | 4(17%) | 0 (0%) | 0.912 | 4(20%) | 0 (0%) | 0.442 |
| Liver N (%)[a] | 5(21%) | 0 (0%) | 0.331 | 4 (20%) | 1 (12%) | 0.865 |
| Spleen, N (%) | 11 (46%) | 2(50%) | 1 | 9 (45%) | 4(50%) | 0.91 |
| Multiple (at least 2 organ) involvement, N (%) | 4 (22%) | 0 (0%) | 0.91 | 3 (21%) | 1 (14%) | 1 |

[a]Histologically confirmed, [b]U Mann-Whitney test, [c]Cochran-Cox test

**Table 4.** *TNFα*-308 genotype distribution in relation to MTX response

| TNFα-308 | G/A (%) | GG (%) |
|---|---|---|
| ER | 0 | 9 |
| LR | 3 | 3 |
| NR | 1 | 12 |
| All patients, N=28 | 4 (14) | 24 (86) |

Pearson chi square test, p=0,02

Table 4 presents the *TNF-α*-308 genotype distribution for the total population and for the subgroups defined by MTX response. In the total population, 86% of patients carried GG variant of *TNF-α*-308allele (24 out of 28 patients) and 14% of patients (4/28 patients) carried G/A variant. No AA carriers were found. Out of 4 patients with G/A genotype 3 were late responders, 1 – non-responder and there was no early responder patient within this group. Patients carrying *TNF-α*-308 A allele have got significantly higher chance to be LR than those without TNF-α-308 A allele (GG diplotype), Pearson chi square test, p=0.02. Relative risk increase (RRI)to be LR vs other response type to MTX, associated with the presence of *TNF-α*-308 A allele was83%. When we compared single allele frequency between the ER, LR and NR groups we have found that LR patients have the 21-fold greater probability of having the allele A in the TNF-α -308 SNP comparing to other type of response (ER or NR patients), p=0.021 OR=21; 95% CI:1.614-273.3 (table 5). Comparing the distribution of TNF-α GG genotype in patients with good (ER+LR) and no response (NR) to MTX we have found the same distribution of genotypes (12 patients vs 12 patients, table 4). When we looked at ER vs LR group the GG genotype transferred the 3-fold higher probability of early response vs late response (9 patients vs 3 patients, table 4).

**Table 5.** The association of *TNF-α*-308 and-857 polymorphism (including combined genotypes) and types of response to MTX treatment

| Examined trait | Types of response | Probabilities of response first type | P value | OR | -95%CI | +95%CI | RR (95%CI) |
|---|---|---|---|---|---|---|---|
| | | | -308G/A | | | | |
| Allele A presence (G/A vs GG)[a] | **Each response vs no response** | **75% vs 50%** | **0,370** | **3** | **0,272** | **33,086** | **1,5 (0,43-1,94)** |
| | ER vs NR | 0% vs 43% | 0,998 | $1,4 *10^{-8}$ | 0 | Inf | $2,46 *10^{-8}$ |
| | ER vs LR | 0% vs 75% | 0,997 | $1,17*10^{-9}$ | 0 | Inf | $4,68*10^{-9}$ |
| | **LR vs NR** | **75% vs 20%** | **0,060** | **12,0** | **0,897** | **160,411** | **3,75 (0,92-4,88)** |
| | ER vs other | 0% vs 38% | 0,996 | $1,19*10^{-8}$ | 0 | Inf | $1,76*10^{-8}$ |
| | **LR vs other** | **75% vs 12,5%** | **0,020** | **21** | **1,614** | **273,307** | **6 (1,50-7,8)** |
| | | | -857 C/T | | | | |
| Allele T presence (C/T vs CC)[a] | **Each response vs no response** | **25% vs 65%** | **0,068** | **0,18** | **0,03** | **1,14** | **0,38 (0,08-1,04)** |
| | ER vs NR | 0% vs 56% | 0,996 | $3,71*10^{-9}$ | 0 | Inf | $8,48*10^{-9}$ |
| | ER vs LR | 0% vs 69% | 0,997 | $2,34*10^{-9}$ | 0 | Inf | $7,6*10^{-9}$ |
| | **LR vs NR** | **25% vs 36%** | **0,601** | **0,58** | **0,08** | **4,39** | **0,69 (0,11-1,97)** |
| | ER vs other | 0% vs 45% | 0,996 | $4,37*10^{-8}$ | 0 | Inf | $7,95*10^{-8}$ |
| | **LR vs other** | **25% vs 20%** | **0,771** | **1,33** | **0,19** | **9,27** | **1,25 (0,23-3,5)** |
| | | | -308G/A -857C/T | | | | |
| BB group (-308G/A & -857C/T) vs other | Each response vs no response | 0% vs 56% | 0,997 | $1,40*10^{-8}$ | 0 | Inf | $3,16*10^{-8}$ |
| | ER vs NR | 0% vs 43% | 0,998 | $1,4*10^{-8}$ | 0 | Inf | $2,46*10^{-8}$ |
| | ER vs LR | 0% vs 60% | 1 | 1 | 0 | Inf | 1 |
| | LR vs NR | 0% vs 33% | 0,997 | $3,82*10^{-8}$ | 0 | Inf | $5,73*10^{-8}$ |
| | ER vs other | 0% vs 33% | 0,998 | $2,11*10^{-8}$ | 0 | Inf | $3,16*10^{-8}$ |
| | LR vs other | 0% vs 22% | 0,997 | $6,68*10^{-8}$ | 0 | Inf | $8,59*10^{-8}$ |
| VB group (-308G/A -857CC) vs others | Each response vs no response | 100% vs 48% | 0,995 | $7,86*10^{8}$ | 0 | Inf | 2,08 (0-Inf) |
| | ER vs NR | 0% vs 41% | 1 | 1 | 0 | Inf | 1 |
| | ER vs LR | 0% vs 75% | 0,997 | $1,17*10^{-9}$ | 0 | Inf | $4,68*10^{-9}$ |
| | LR vs NR | 100% vs 19% | 0,997 | $8,54*10^{8}$ | 0 | Inf | 5,33 (0-Inf) |
| | ER vs other | 0% vs 36% | 0,996 | $1,70*10^{-8}$ | 0 | Inf | $2,65*10^{-8}$ |
| | LR vs other | 100% vs 12% | 0,097 | $2,32*10^{9}$ | 0 | Inf | 8,33 (0,Inf) |
| BF group (-308GG -857C/T) vs others | **Each response vs no response** | **29% vs 62%** | **0,14** | **0,25** | **0,04** | **1,58** | **0,46 (0,09-1,16)** |
| | ER vs NR | 0% vs 53% | 0,996 | $5,09*10^{-9}$ | 0 | Inf | $1,08*10^{-8}$ |
| | ER vs LR | 0% vs 69% | 0,997 | $2,34*10^{-9}$ | 0 | Inf | $7,6*10^{-9}$ |
| | **LR vs NR** | **29% vs 33%** | **0,830** | **0,8** | **0,10** | **6,10** | **0,86 (0,15-2,26)** |
| | ER vs other | 0% vs 43% | 0,996 | $5,45*10^{-9}$ | 0 | Inf | $9,54*10^{-9}$ |
| | **LR vs other** | **29% vs 19%** | **0,597** | **1,70** | **0,24** | **12,17** | **1,5 (0,28 – 3,89)** |
| VF group (-308GG -857CC) vs others | **Each response vs no response** | **59% vs 45%** | **0,49** | **1,71** | **0,37** | **7,92** | **1,29 (0,52-1,91)** |
| | ER vs NR | 56% vs 0% | 0,996 | $2,69*10^{8}$ | 0 | Inf | $2,69*10^{8}$ |
| | ER vs LR | 90% vs 0% | 0,997 | $4,27*10^{9}$ | 0 | Inf | $4,27*10^{9}$ |
| | **LR vs NR** | **12,5% vs 45%** | **0,15** | **0,17** | **0,02** | **1,91** | **0,275 (0,028-1,350)** |
| | ER vs other | 53% vs 0% | 0,996 | $4,32*10^{8}$ | 0 | Inf | $4,32*10^{8}$ |
| | **LR vs other** | **6% vs 45%** | **0,03** | **0,075** | **0,007** | **0,081** | **0,13 (0,01 – 0,89)** |

[a]There were nor homozygotes -308AA, nor homozygotes -857TT in our data set

*Combined genotypes frequencies of TNF-α polymorphism and MTX response.*

To attain more comprehensive information about the association between the TNF-α gene polymorphism and MTX response we have analyzed different combinations of genotypes: -857 CC/, C/T, TT, -308 GG, G/A, AA, -863 CC, C/A, AA, -1031 TT, T/C, CC, in relation to the treatment outcome. Only combined-857 and -308 TNF-α polymorphisms were related to the type of response to MTX. All patients in the ER group were homozygous for

**Table 6.** Distribution of *TNF-α* -857 and -308 combined genotypes in sarcoidosis patients in relation to MTX response

| *TNFα* | -857C/T -308G/A | -857C/T -308GG | -857CC -308G/A | -857CC -308GG |
|---|---|---|---|---|
| ER | 0 | 0 | 0 | 9 |
| LR | 0 | 2 | 3 | 1 |
| NR | 1 | 5 | 0 | 7 |
| All patients, N=28 | 1 | 7 | 3 | 17 |

p=0,0047, $chi^2$=64%, phi=0,595

-308GG combined with -857CC (table 6). The *TNFα* -308GG-857CC genotype (fig. 2) was associated with significantly lower probability of late response to MTX comparing to other response types, p=0.03, OR =0.075; 95%, CI 0.007-0.8 (table 5). The absence of -308A variant allele combined with the absence of -857T variant allele are associated with the early response to MTX, p=0.0047. Power of the correspondence analysis was 63% with effect size measured by phi=0.595 (fig. 2). The effect size of discovered correspondences is above medium range (significantly increased) but the power is low as the studied population is relatively small.

## 4. Discussion

We have recently proved that MTX is a safe and valuable second line option in patients with pulmonary sarcoidosis (3). However almost 50 % of patients did not improve on MTX treatment. There are no definite recommendations about the length of MTX therapy in sarcoidosis patients (2). The decision - how long the patient should be treated is individual, bases on personal experience of the physician, observation of clinical effectiveness, subjective tolerance and AE of therapy. As this was a real-life open study not all patients completed the planned 24 months of therapy. Earlier withdrawal was related to AE, subjective intolerance of MTX or because no improvement in any clinical parameters was observed at 6-month interval. This explains the differences in the therapy duration between enrolled subjects. Variability in the clinical pattern of sarcoidosis creates a real challenge for clinicians to identify patients at risk for progressive respiratory failure or disabling systemic disease. To evaluate the severity of the disease and clinical outcome we have assessed different clinical variables: duration of the disease, need for previous therapy, changes in thorax computed tomography, extrapulmonary involvement, functional capacity (measured by PFT and 6MWT) (table 2 and 3) (3).As the progressive respiratory failure is responsible for around 75% of deaths attributable to sarcoidosis (1) candidates for systemic therapy in our study were selected from patients with progressive respiratory disease, documented by deterioration in PFT prior to study enrollment (3).The reliable monitoring of pulmonary disease severity is problematic (12, 13).The heterogeneity of patterns of pulmonary function impairment in pulmonary sarcoidosis justify selection of different pulmonary function variables (FEV1, FVC, TLC and DLCO) based upon their degree of impairment, for routine monitoring of disease progression/regression (13, 14). Respiratory symptoms of sarcoidosis may be minimal until pulmonary reserve is seriously compromised. These symptoms include cough, exertional dyspnea, chest discomfort, and fatigue. None of them reliably reflects the severity of underlying pulmonary disorder. Due to a variability of pulmonary sarcoidosis phenotypes (including obstructive vs restrictive form of disease) no single PFT should be viewed as the primary measure of a change in disease severity in all sarcoidosis patients (15, 16, 17). The individual selection of PFT variables should be considered to reflect the disease outcome. For example, in a patient with severe airflow limitation, changes in $FEV1_1$ are probably most accurate measure of the disease progression or regression. This explains our decision to designate multiple PFT variables as primary end points of the study and also to adopt only selected parameters when assessing the efficacy of MTX and allocating patients to ER/LR/NR group. Threshold values for a "significant change" are drawn from the reproducibility of individual variables to two standard deviations of change. On repeated measurement spirometry values ($FEV_1$, FVC) differed by less than 10% in 95% of normal individuals. For DLCO an improvement or deterioration of 15% from baseline is needed to exclude confounding factor by measurement's variation. In our cohort the amelioration of 10% from predicted value of $FEV_1$ and/or FVC and/or TLC and/or of 15% of DLCO was defined as significant improvement of PFT parameters after MTX therapy (3) and served as a criteria to distinguish responders from non-responders. Assessment of lung function every 6-month of MTX therapy revealed

that there are two types of positive response to treatment: early, that manifests at first 6 months and late, that occurs only in prolonged therapy. This original finding has not been reported previously and implicates further debate on the selection of candidates for continuation of MTX therapy over 6 months to achieve lung function improvement.

We presumed that treatment efficacy may be related to the pathogenesis of sarcoidosis and the intensity of underlying inflammatory process. Cytokines release by different inflammatory cells promotes granuloma formation and influences the clinical course of the disease (5, 18, 19). Some of them have been proved to be accurate markers of disease activity and may be regarded as key mediators of progressive irreversible fibrotic response in lungs. As the main goal of the therapy is to prevent the development of irreversible pulmonary damage, it is warranted to test if genetic variations of key inflammatory cytokines are associated with the response to MTX therapy(4).

TNFα, a crucial immunoregulatory cytokine released by alveolar macrophages involved in the pathogenesis of granulomatous reactions, is the most extensively studied factor in different inflammatory diseases, including sarcoidosis (20). It is assumed that phenotypic traits of sarcoidosis can be attributable to modifying capability of promoter polymorphism of TNFα as a factor regulating the gene expression (4, 21).

At position -308 in the promoter region of the TNFα gene a biallelic polymorphism has been proved to be associated with disease phenotypes. In sarcoidosis the less common TNFA2 allele seems confer good prognosis in Loefgren's Syndrome (18,22). Polymorphism of the TNFα gene have been found to be strongly correlated with the polymorphic MHC 8.1 (A1B8DR3) ancestral haplotype (18,20), connected with multiple immunopathological diseases and variation in TNFα release. HLA-DR3 is associated with favorable prognosis, predisposition to spontaneous remission and good outcome(23).

Japanese authors showed that the -857C/T polymorphism may affect susceptibility to cardiac sarcoidosis, while the -1031T/C polymorphisms to ocular manifestation of the disease(24). In our group of patients, we didn't find any significant correlation between analyzed TNF polymorphisms and clinical characteristics of sarcoidosis.

In 28 patients with progressive pulmonary sarcoidosis we examined whether the significant functional improvement at first 6 months and at the end of therapy could be predicted by the TNFα promoter genotype at position -1031T/C, 857C/T, 308G/A, 863 C/A. Studies of TNFα genotype represent a novel approach to discovering predictive biomarkers of treatment response. The selection of analyzed TNF αpolymorphisms was based on previous studies' results showing its association with clinical course of sarcoidosis and/or predicting treatment response (4). Several authors found that -308 TNFα genotyping could be a useful tool for predicting effectiveness of anti-TNFα therapy combined with MTX in rheumatic diseases (25-28). In rheumatoid arthritis (RA) non-responders to therapy carried -308 AA or G/A genotypes. In our group of patients, the presence of A allele in -308 locus was a significant predictor oflate response to MTX. The limitations of our study include the small sample size. Despite these limitations, this study supports the assumption on using -308 TNFα genotyping in patients for whom the decision to continue MTX treatment for longer that 6 months should be made. The prognostic influence of 308 A allele should be further validated and confirmed in prospective studies.

In RA patients the -308 GG genotype was associated with good response to anti-TNFα therapy and that finding was confirmed by several authors (25, 26). In sarcoidosis a recent study by Wijnen et al showed that sarcoidosis patients -308 GG genotype had a three-fold higher response to TNF inhibitors (adalimumab or infliximab) (29)

In our study the distribution of -308GG genotype was similar in responders (including early and late responders, N=12) and non-responders group (N=12) (table 4). But when differentiating between early and late response to MTX subgroups we have found that most of ER were carriers of -308 GG genotype (9/12, 75%). Only 3/12 (25%) of –308 GG patients were LR (p=0,02). The -308 GG genotype was associated with early response to MTX therapy in responders group. We can hypothesize that if a patient with -308 GG genotype doesn't improve after 6 months of therapy there is high probability that will not respond at all (as the other 50% of our cohort with GG genotype were in NR group). Our findings suggest that, in -308 GG carriers MTX should be discontinued if significant amelioration in PFT is

not observed at first 6 months of treatment. As previously stated such recommendation should be validated on independent and large cohort of patients.

A significant association of TNF -857T allele with the occurrence of sarcoidosis was found in British and Dutch sarcoidosis populations but these studies didn't evaluate its link with the outcome of the therapy (30). In RA it has been confirmed that patients with the T allele of *TNF* -857C/T SNP respond better to therapy (etanercept) than homozygotes for the C allele (31). In our study *TNF* -857C/T polymorphism proved to play a role in determining the type of response to MTX only in combination with-308 G/A. Combined genotypes that might disclose risk profiles of individual patients has been studied in various conditions(32). In a large European cohort of patients with RA haplotype reconstruction of the *TNF* locus revealed that the GGC haplotype (-238G/-308G/-857C) in a homozygous form (i.e. present in more than half of the patients) was significantly associated with a lower American College of Rheumatology response (ACR50) to Adalimumab (ADA) at 12 weeks (34% vs. 50% in patients without the haplotype) (p=0.003; pa=0.015). This effect was more pronounced in the subgroup of patients concomitantly treated with MTX. Authors concluded that a single *TNF* locus haplotype (-238G/-308G/-857C), present on both chromosomes is associated with a lower response to ADA, mainly in patients treated with ADA and MTX(33).

Ali et al investigated the frequency and distribution of DRB1 and DQB1 alleles in patients with RA and analyzed the relationship between clinical response to MTX and the HLA-DR and HLA-DQ genotypes. HLA-DRB1*03 was significantly-more common among non-responders to MTX. Authors concluded that another gene, involved in MTX metabolism, might be in linkage disequilibrium with HLA-DRB1*03 in the Pakistani population, making such individuals non-responsive to MTX-therapy (34). TNFα gene is strongly associated with the polymorphic MHC 8.1 (A1,B8, DR3) ancestral haplotype(35). The disease phenotype associated haplotype was also confirmed in sarcoidosis showing the significantly higher representation of combined TNFA2 and the HLA-DR3 in the group of patients with Loefgren's syndrome (18).

The frequency of combined genotypes in enrolled patients was following: carriers of-857CC, -308 GG represented 61% (17/28) of total cohort, -carriers of 857C/T-308GG: 25% (7/28) of total cohort, -carriers of 857CC -308G/A: 11% (3/28) of total cohort (table 6). Most of 857C/T-308GG patients (71%, 5 out of 7) were classified as non-responders to MTX, thus this genotype might predict inefficacy of MTX therapy. All patients with -857CC-308G/A genotype belonged to LR group. If we presume that this genotype is associated with late response to MTX, in this specific group of patients, MTX therapy should be continued over 6 months to achieve PFT improvement. Only one patient was heterozygous for both SNPs: -857C/T, -308G/A and he belonged to NR group. A study on a large group of patients is warranted to confirm that this genotype might be associated with poor response to MTX therapy. The genotype most frequently found (17 out of 28 patients, 61%) was: -857CC-308GG. Homozygous patients: -857CC and -308GGhad the best response to MTX, almost 60 % (10 out of 17) responded to the drug. Most of responders (90%, 9 out of 10) experienced PFT improvement after 6 months of therapy (early response), and only 1 out of 10 patients was classified as LR. All patients in ER group were homozygous for -308 GG and -857 CC combined genotype, p=0.005. The absence of -857T variant allele combined with the absence of allele -308 A predetermined the early response to MTX. Accordingly, the continuation of MTX for longer than 6 months would not be beneficial for-308 GG and -857 CC carriers (belonging to ER and NR groups). Opposite, in patients with -857C/T-308GG or -857CC-308A/G genotypes (LR patients) the therapy with MTX over 6 months would be justified. The figure 2 reflects genetical similarity of ER and NR: 53% of -857CC -308GG patients were ER and 41% were NR. On the other side, LR are "genetically" quite different group of patients, and as presented at table 6 are mostly characterized by the presence of at least one variant allele (-857C/T-308GG or -857CC-308G/A). Patients with -857C/T-308GG or -857CC-308G/A genotypes have 5 times fold greater probability to be LR than patients with -857CC-308GG genotype (table 6). According to this analysis the combined TNF-α -857C/T-308A/G polymorphism allowed to discriminate LR from ER and NR patients.

In the light of small sample size, there is possibility of selection bias. Presented data should be further

confirmed to make clinically valid recommendations. However, we have proved that there are two types of positive response to MTX therapy (early and late) in chronic progressive pulmonary sarcoidosis and that they may have different genetic background. Our preliminary data suggest that the *TNF-α* genotyping could serve as a tool to tailor the individual length of MTX therapy in sarcoidosis patient if validated on large cohort of individuals.

## Conclusion

There are two types of positive response to MTX therapy in chronic pulmonary sarcoidosis: early, that is expected within 6 months of therapy and late, if a patient improves after prolonged treatment. In our group of patients, *TNF- α* gene polymorphism -308 G/A and -857 C/T allowed to discriminate between those two types of positive therapy response. In the new era of personalized medicine, the *TNF* genotyping deserves attention as a part of treatment algorithm for chronic, progressive disease. Furthermore, we postulate that characterization of genetic profiles, as signatures of different clinical phenotypes, will provide a basis for the revision of sarcoidosis classification. This may also offer insights into molecular pathogenesis driving phenotype expression and possibly create new options for patient-tailored therapy in sarcoidosis. This concept is still not largely emphasized in scientific publications.

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