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Original Research Article

# Folate status, regulatory T cells and *MTHFR* C677T polymorphism study in allergic children

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ABSTRACT

*Purpose:* This study aimed to investigate early-life folate serum concentrations in children with food, inhalant or mixed type allergy. The influence of folate levels on the $FoxP_3$ expression in Treg (regulatory T) cells in the studied children, taking into account the *MTHFR* (5,10-methylenetetrahydrofolate reductase) genotypes was also analyzed.

*Material and methods:* The study was performed in 83 allergic children (study group) and 49 healthy children (control group), aged 2–72 months. Medical history of each child was obtained and laboratory tests (serum folic acid concentrations and *MTHFR* C677T polymorphism) were carried out. The percentage of Treg cells was evaluated in almost a half of the examined subjects (48.5%).

*Results:* Significantly higher serum folate levels in the group of children with food allergy than in those with inhalant allergy was confirmed ($P = 0.037$). In the study group the TT homozygotes were characterized by significantly lower folate concentrations than CC homozygotes ($P = 0.045$). A negative correlation was demonstrated between the $FoxP_3$ expression in $CD4^+CD25^{high}FoxP_3^+$ peripheral blood lymphocytes and serum folic acid concentrations. The correlation was more pronounced in the group of allergic children and it was statistically significant ($r = -0.339$, $P < 0.05$).

*Conclusions:* The results of the study indicate a possibility of some effects of folate status on Treg cells, thus suggesting their potential role in the development and course of allergy in children.



## 1. Introduction

The onset of allergic disease occurs most often already in the infantile period or in early childhood. The occurrence or the course of allergic disease in genetically predisposed persons may be indirectly induced by a number of agents [1–3]. It has been noted over the recent years that folic acid as a dietary methyl donor may control functions of the immune system via epigenetic modifications. Probably, a specific influence of folates on the DNA of naive lymphocytes takes place during prenatal development and in early childhood [3–5].

Folic acid belongs to B group vitamins and is a chemical substance, necessary for the proper functioning of cells. The metabolically active forms of folates, indirectly participate in the synthesis of nucleic acids and in the reactions of DNA and RNA methylation that plays an important role in the control of gene expression [3,6,7]. The activity of biochemical processes in cells depends not only on the concentrations of folates but also on the activity of the enzymes such as MTHFR (5,10-methylenetetrahydrofolate reductase) which take part in their metabolic transformations [6,8]. The gene encoding enzymatic protein occurs on chromosome 1 (1p36.3) and is characterized by genetic polymorphism. Cytosine transition into thymine in locus 677 (C677T) is the most commonly described polymorphism in the *MTHFR* gene. This transition results in the replacement of alanine by valine in locus 222 and less active variant of MTHFR protein. Homozygotes (TT) are characteristic of a decreased activity of the enzyme (by 70%) and an increased concentration of homocysteine in blood serum [6,9,10].

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The results of some studies indicated that both increased and decreased folate concentrations as well as disturbances of their metabolism in children may exert certain effects on their immune system, increasing the risk of allergic diseases [5,11]. These relationships were observed in the offspring of the mothers taking folic acid during pregnancy, especially when folates were taken after 1st trimester of pregnancy and in dosages higher than recommended (>0.4 mg per day) [3,11]. The results of the study performed in an animal model indicated that too high concentration of serum folic acid promotes DNA methylation, which in turn suppress the transcription factors responsible for differentiation of CD4 lymphocytes in particular in the direction of Treg (regulatory T) cells [4].

Based on these observations we made a hypothesis that folate status in children may influence peripheral Treg cells, $FoxP_3$ expression in lymphocytes and it correlates with an incidence of sensitization. The goal of the study was the evaluation of the effects of folate status and *MTHFR* C667T gene polymorphism on regulatory T cells in allergic and healthy children. The examination of early-life folate serum concentration in children depending on the allergy type was also investigated.

## 2. Material and methods

### 2.1. Study population

The study was performed in 132 children aged 2–72 months admitted to the Gastroenterology, Allergology and Pediatric Department of Polish Mother's Memorial Hospital – Research Institute in Lodz during the years 2011–2014. The study group comprised 83 allergic children: 23 infants (27.7%), 33 children at the 2nd and the 3rd year of life (39.8%) and 27 aged 4–6 years (32.5%). Almost a half of them suffered from food allergy (47.0%), while in 28.9% mixed type allergy (food-inhalant) was confirmed and in 24.1% of the children inhalant allergy was identified. The diagnosis of allergy was based on medical history and physical examination, confirmed by DBPCFC (the double-blind, placebo-controlled food challenge) or open-food challenge and/or positive results of other allergic blood tests: sIgE (specific IgE) and/or SPT (skin prick tests). 49 healthy children (without any allergy incidents in the past as well as any symptoms of allergic diseases during physical examination) matched for age and sex was the control group. Intake of folic acid was not accepted either as medicine or as dietary supplement in all examined groups.

The physical examination of the patients focusing on signs consistent with an allergic reaction or disorder often associated with allergic conditions was performed.

For the purpose of conducting the study, formal written consent of parents and the approval of the Local Ethics Committee were obtained (37/2011).

### 2.2. Biochemical, genetic and immunologic tests

In all the studied children some biochemical tests were performed such as assays of serum folic acid concentrations as well as genetic tests of *MTHFR* C677T gene polymorphism. In almost a half of the studied children (48.5%) the percentage of selected lymphocytes ($CD4^+$, $CD4^+FoxP_3^+$, $CD4^+CD25total$, $CD4^+CD25totalFoxP_3^+$, $CD4^+CD25^{high}$, $CD4^+CD25^{high}FoxP_3^+$) in peripheral blood was assessed and the expression of the $FoxP_3$ in the lymphocytes was examined and given as MFI.

#### 2.2.1. Assessment of serum folate

Plastic EDTA blood collection tubes were used to collect peripheral blood samples from each case and control subject. Upon collection of the whole blood, the samples were maintained for 30 min at room temperature in order to form a clot. The clot was removed by centrifuging (2000 × *g*, 10 min, room temperature). The samples were stored at −20 °C. Serum folic acid concentration was assayed by the Elecsys Folate electroluminescence method (a Cobas gear), using a natural folate binding protein (FBP) specific for the folic acid (Folate III kits, Roche, Switzerland).

#### 2.2.2. Assessment of MTHFR C677T variants

Genomic DNA was extracted from blood samples stored at −20 °C, using Genomic Mini AX Blood (A&A Biotechnology, Poland) in accordance with the manufacturer's protocol. *MTHFR* C677T genotype was analyzed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method. The primers for amplification were: forward 5′-TGA-AGG-AGA-AGG-TGT-CTG-CGG-GA-3′ and reverse 5′-AGG-ACG-GTG-CGG-TGA-GAG-TG-3′. The obtained product of 198 bp (base pairs) was subsequently digested with HinfI (Invitrogen, USA) and subjected to electrophoresis on a 3% agarose gel. While C allel showed no restriction site for HinfI, T allel was detected by the appearance of two fragments: 175 bp and 23 bp. To verify PCR-RFLP method, selected samples were genotyped using direct DNA sequencing.

#### 2.2.3. Treg lymphocyte subpopulations assessment

Blood samples were collected into tubes containing lithium heparin (Monovette Li-Heparin tubes/2.7 ml/, Sarstedt, Germany). Lymphocytes were isolated using Lymphocyte Separation Medium density: 1.077 g/ml (PAA, Austria). The following BD Pharmingen (USA) products were used for Treg cells detection: Alexa Fluor 488 Mouse anti-Human $FoxP_3$, 259D/C7 clone, IgG1 isotype; APC Mouse anti-Human CD4, RPA-T4 clone, IgG1,κ isotype; PE Mouse anti-Human CD25, M-A251 clone, IgG1,κ isotype; Human $FoxP_3$ buffer set, 259D/C7 clone; Isotype control: APC Mouse IgG1,κ, MOPC-21 clone; PE Mouse IgG1,κ, MOPC-21 clone; Alexa Fluor 488 Mouse IgG1,κ, MOPC-21 clone, Stain Buffer.

First, 100 μl of PBMCs ($1 \times 10^6$ cells/ml) were dark-incubated with anti-CD4 (20 μl) and anti-CD25 (20 μl) for 30 min at room temperature, and then washed with the Stain Buffer (2 ml, 250 × *g*, 10 min, room temperature). To fix the cells, 2 ml of human $FoxP_3$ buffer A was added and dark-incubated for 10 min at room temperature. Upon incubation, the cells were centrifuged (500 × *g*, 5 min), the fixative was removed and the cells were washed with the Stain Buffer (2 ml, 500 × *g*, 5 min, room temperature). Next, the cells were permeabilized with $FoxP_3$ buffer C (0.5 ml of diluted buffer, 30 min, room temperature, protected from light), washed with the Stain Buffer, incubated with anti-$FoxP_3$ (30 min, room temperature, protected from light) and double-washed again with the Stain Buffer. Afterwards, the cells were fixed by means of a diluted Fix Buffer and stored at 4 °C. For each analyzed sample a control test was performed using isotype control serums. The analysis was performed with the FACSCalibur flow cytometer using the Cell Quest software, with the Dot-Plot tool.

### 2.3. Statistical analysis

For continuous features, minimum, maximum, arithmetic mean ($\bar{x}$) and standard deviation (SD) were calculated. Normality of distribution was checked by Shapiro Wilk test ($P = 0.05$). For comparison of average values Student's *t*-test or Mann–Whitney *U*-test was used. Correlations between quantitative variables were studied by Pearson's or Spearman's test. The coefficient of regression equation was calculated. For the studied groups, the observed genotype frequencies of the *MTHFR* C677T polymorphism were assessed for Hardy–Weinberg equilibrium using $\chi^2$ test. The presented results were significant at $P < 0.05$.