CHILDHOOD ADVERSITIES ARE NOT A PREDICTORS OF SSTR4MET IN ALCOHOLICS

1. Introduction

Methylation of the cytosine bases in DNA (CpG islands) is an important epigenetic mechanism of gene transcription regulation in the central nervous system [1]. Methylated sites can be targeted by methylated DNA-binding proteins, which results in chromatin condensation and transcriptional repression [1]. Thus, gene promoter methylation generally results in lower gene expression. DNA methylation has been indicated as an important epigenetic mechanism in the modulation of synaptic plasticity in the adult brain by the regulation of neurogenesis in the adult hippocampus [2–4]. The status of an individual genome methylation is shaped during the ontogenesis, from the stadium of zygote till the young adulthood [5]. Among the multiple factors indicated as potentially influencing genome methylation, a variety is listed: from diet, to childhood trauma, and activity of enzymes connected with DNA methylation [6,7]. Alterations in DNA methylation were reported in depression, schizophrenia and neurodegenerative disorders (Alzheimer’s disease, Huntington’s disease, Parkinson’s disease) [8,9]. However the etiology and clinical translation of genome methylation in this disorders is still studied [8,9]. Adverse childhood experiences (ACEs) are one of factors postulated as potentially influencing DNA methylation [10,11]. Noteworthy, this influence can be bimodal. On the one hand, such experiences may increase DNA methylation and decrease the expression of genes that regulate synaptic plasticity and neurotransmission promoting lower adaptive skills after trauma [12]. On the other hand, there is an evidence that stress may decrease the DNA methylation of specific genes involved in stress responses resulting in silencing the harmful inflammatory serum response to stress [12]. Adverse events may influence epigenetic changes that allow for the establishment of long-term genetic programs that control learning and memory [13]. Roth et al. (2009) used a rat model of infant maltreatment to assess the possibility of lasting influences of early-life adversity on DNA methylation [14]. Tissues were obtained from the prefrontal cortices and hippocampal of two groups of neonatal rats that were either exposed to stressed-abusive mothers or care-giving mothers for assessments of brain-derived neurotrophic factor (BDNF) levels and DNA analyses. These authors revealed that infant maltreatment results in the methylation of gene BDNF (BDNF) throughout the lifespan into adulthood and that this result is paralleled by reduced BDNF expression in the adult prefrontal cortex [14].
Patients with AD are known to report significantly higher rate of ACEs than the general population [15]. In our opinion, it can be due to several reasons. Alcohol abuse may be an invalid strategy to deal with unresolved trauma in vulnerable subjects or may be connected with coping health-harming habits from dysfunctional households or may be partially genetically warranted, associated with family alcoholism. In the National Survey of Adolescents, approximately 25 percent of physically assaulted or abused adolescents reported lifetime substance abuse or dependence [16]. In 587 adults assessed by Khoury et al. (2010), physical abuse correlated with the use of all substances examined (alcohol included) [17]. Nationally representative surveys of USA and GB have shown that ACEs have prolonged adverse effects on somatic and mental health [15,17]. In the USA national survey, persons reporting at least 4 ACE categories were 7 times more likely to suffer from alcoholism when compared with persons who reported none ACE category [15,18]. However, not all siblings who grow up in dysfunctional alcoholic households [16,17] develop AD, and among monozygotic twins, frequently only one develops depression or other mental problem, including AD [19]. These findings indicate that neither environmental nor genetic factors exclusively increase the risk of alcoholism; rather, the complete environmental-epigenetic-genetic interaction should be considered.

Somatostatin is a peptide hormone that has been found to act as a neurotransmitter and a neuromodulator of other neurotransmitters [20]. The role in cognitive function is complex and still remains to be determined [21]. One of its receptor, somatostatin receptor subtype 4 (sst-4), is expressed at the highest levels in the fetal and adult lung and brain, particularly in the CA1 hippocampal region [22]. There is a very scarce data on a potential role of the sst-4 in memory formation. Gastambide et al. (2009) found that intra-hippocampal injections of an sst-4 agonist (L-803.087) dramatically and dose-dependently impair place memory formation, but agonists of somatostatin receptor subtypes 1, 2, and 3 have no effects [23]. Kim et al. (2010) found that the SSTR4 rs2567608, sst-4 gene functional polymorphism, T allele carriers display reduced sst-4 activity [24]. There are other studies on genes connected with neuroplasticity and memory formation (BDNF, SLC6A4) and childhood trauma but this is the first to assess SSTR4 in patients with AD severely affected with ACEs [14, 25,26]. We hypothesize ACEs are associated with the SSTR4 promoter region methylation. Since gene promoter methylation is known to silence gene expression, SSTR4 promoter methylation would additively diminish sst-4 activity in T allele carriers and buffer higher sst-4 activity in C allele carriers. This is the first study to verify: 1. The SSTR4 promoter region methylation frequency in patients with AD, 2. If selected environmental (current age, gender, diet, childhood trauma, term and kind of labor, cigarette smoking, alcohol drinking) and genetic (SSTR4 rs2567608) factors are associated with SSTR4 promoter methylation in patients with AD.

2. Subjects and Methods

This is a study based on retrospective and self-reported data, performed in Poland between the years 2013 and 2015.

2.1. Subjects

A total of 209 consecutive patients with AD who were admitted to psychiatry wards for a course of AD psychotherapy or a treatment of alcohol withdrawal syndrome and gave informed consent were involved into the study. Patients were informed in the study informed consent that they have right to withdraw the consent at any step of the study without giving any reason. Of 209 patients, 33 did not undergo further analysis because of incomplete data (giving the questionnaire back without all the answers completed) or consent’s withdrawal during the study (mainly, when finding the questions too personal/intimate or deciding not to undergo buccal smear). The study analyzed 176 inpatients with AD (134 males and 42 females) aged 43.4±10.5 (mean ± SD years). Each patient received a consensus diagnosis of AD by 2 psychiatrists according to the ICD-10 (F10.2) [29]. The period from the most recent alcohol intake was at least one week. Patients with AD scored 27.2±7.5 (mean ± SD points) out of the possible 40 points on the AUDIT interview (Alcohol Use Disorders Identification Test) [30]. The exclusion criteria were: 1. age < 18; 2. a history of a significant psychiatric comorbidity according to the ICD-10 [29]; 3. ever having received chemotherapy consisting of drugs that influence DNA methylation, i.e., 5-azacytidin and decitabine, since these drugs are known to influence genome methylation [31].

The controls were initially 140 healthy volunteers who gave informed consent. Controls were informed in the study informed consent that they have right to withdraw the consent at any step of the study without giving any reason. Of them, 13 did not go further analysis because of incomplete data (giving back the questionnaire with incomplete data) or meeting any from below listed exclusion criteria. The study analyzed 127 healthy volunteers (96 males and 31 females) aged ≥18 [39.4±12.0 (mean ± SD years). Exclusion criteria for controls were: 1. ever been diagnosed with a mental disorder according to the ICD-10 [29] in their lifetimes; 2. ever attempted suicide or self-mutilated; 3. reaching the AUDIT scoring [30] indicating alcohol abuse (F10.1 according to the ICD-10) [29] or possible AD (F10.2 according to the ICD-10) [29]; 4. ever having received chemotherapy consisting of drugs that influence DNA methylation, i.e., 5-azacytidin and decitabine [31]. Controls were introduced to the study to assess the difference between non-clinical subjects and patients with AD according to the history of ACEs’ and SSTR4 rs2567608 allele and genotype frequency.

Patients with AD and the controls were age and sex matched and they were native, unrelated inhabitants of Central Poland.

2.2. Data collection

This study used a structured self-reported questionnaire that had been designed for the study to measure the sociodemographic and clinical characteristics of the study participants. The study participants were ensured confidentiality of the obtained data. The researcher remained present during the completion of the questionnaires in order, to address the participants’ questions and to
make sure the respondents understood all of the items.

The term “age at alcohol initiation” means age at first take of any amount of alcohol. The term “age at onset of problem drinking” (AOPD) sign the age the patient esteemed he had lost control of drinking which influenced adversely his occupational and family life. Respondent was signed as a cigarette smoker while he had given “Yes” answer for question: “Have you smoked at least 100 cigarettes during your lifetime?”.

ADs and controls were asked about proper nutritional health habits with a module of Catalogue of Healthy Behavior (CHB) [27], comprising following items:

- “I eat a lot of fruit and vegetables”
- “I limit a consumption of such products as animal fat and sugar”
- “I care about proper diet”
- “I avoid foods containing preservatives”
- “I avoid eating salt and heavily salted dishes”
- “I eat wholemeal bread”.

Their task was to specify on a 5-point scale how often had they performed a certain action over the last year (1-almost never, 2-rarely, 3-sometimes, 4-often, 5-almost always). Thus, it was possible to score between 6 to 30 points.

The Alcohol Use Disorders Identification Test (AUDIT) [28] with a Cronbach’s alpha index of 0.85 was applied to characterize alcohol intake severity during the past year in patients with AD and to exclude healthy volunteers with alcohol abuse (F10.1 according to the ICD-10) [29] or suspected AD (F10.2 according to the ICD-10) [29].

The ACEs were measured with a tool designed for this study that was named the ACE (13) Score. The first 10 questions were developed by Kaiser Permanente and the Centers for Disease Control and Prevention and evaluated exposure to abuse and family dysfunction occurring during the first 18 years of life (ACE Study Score) [15]. These 10 questions focus on chronic physical, verbal, and sexual abuse; neglect; the loss of one or both parents for any reason (i.e., divorce, separation, or death); exposure to domestic violence; and growing up in a household with mental illness, alcohol abuse, drug abuse, or incarceration. The 3 additional questions concern events that also took place in one’s life under the age of 18 and included: witnessing a family member’s suicide attempt; witnessing a family member’s death due to any cause; and witnessing a stranger’s death due to any cause (e.g., traffic accident). The details of our statistical analysis, allow for our results to still be still comparable with studies based on the ACE Study Score.

In order to address the possible bias connected with the participant’s intentional attempt to present him or herself in either a better or worse mental and general condition, the researcher who remained present during completion of the questionnaires listed above was not involved in the patients’ therapy. The recall bias was still possible during ACE (13) Score completion, which was listed among the limitations of the study.

2.3. Ethics

All of the participants gave written informed consent for their participation in the study. The study was approved by the Local Bioethics Committee: No. RNN/467/13/KB and KB/843/13/P. The study was carried out in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

2.4. Laboratory testing

2.4.1. Endothelium collection

The laboratory work was carried out in the Central Scientific Laboratory of the Medical University in Lodz. Buccal smears were obtained by rubbing the buccal mucosal with a sterile, DNA-free set of forensic swabs (Sarstedt, Nürnberg, Germany, product no 80.629). The buccal smears were obtained by a trained personnel and then stored in accordance with the manufacturer’s instructions until the laboratory analysis. The buccal smears were obtained at least 2 hours after eating, tooth brushing, cigarette smoking, or gum chewing. Choosing buccal smears for the genetic and epigenetic analysis let us to avoid procedures that violate the continuity of the skin, cause pain, or carry a risk of blood disorder transmission, to maintain high level of cooperation with the subjects and to ensure a safety of participation in the study.

Epigenetics has evoked increasing interest in the last decades, the methodologies utilized in original papers vary, and further studies on the role of epigenetics in disorders pathology are necessary [30]. Methylation patterns are known to be tissue specific but change with age and under variety of environmental conditions [31]. The question remains, what is the proper tissue selection to evaluate the role of epigenetic modification in mental disorders. In this area, animal studies rely on hippocampal tissue (i.e., 32,33). Olsson et al. (2010) selected the buccal endothelium to assess the serotonin transporter gene (SLC6A4) promoter methylation in 150 patients with depression [34]. They emphasized that it is necessary for epigenetic studies on mental disorders, to use a peripheral tissue that shares a sufficiently similar exposure history with central nervous system. A good approach might be the selection of a peripheral cell type that is derived from the same embryonic origin as neurons. Buccal cells are derived from the ectoderm; thus, the epigenetic profiles of buccal cells may be more similar to those of neurons than lymphocytic cell lineages if environmentally associated epigenetic changes occur early in development. However, a common embryonic origin does not guarantee that promoter methylation is not altered during other periods of development. It is not clear whether local exposure to psychoactive substances influences promoter methylation in the buccal endothelium and thus decreases the similarity of the promoter methylation of this tissue with that of the central nervous system. There is a little number of epigenetic studies based on buccal mucosa, and our study attempts to fill this gap. Olsson et al. (2010) found no associations of alcohol, tobacco or cannabis consumption with buccal cell methylation, which is crucial for the methodology of our study [34]. Lowe et al. (2013) proposed that the endothelial cells of the mouth are better suited for epigenetic studies than peripheral blood leucocytes [35]. During the enrolment of the controls into our study, 4 males were excluded due to reaching the AUDIT scoring [28] indicating alcohol abuse (F10.1 according to the ICD-10) [29] or possible AD (F10.2 according to the ICD-10) [29]. None of these subjects exhibited STR4 rs2567608 promoter methylation.
An additional exclusion criterion for patients with AD and controls was a history of ever having received chemotherapy consisting of drugs that influence DNA methylation, i.e., 5-azacytidin and decitabine, because these drugs are known to influence genome methylation [36]. Methylation is shaped from the period of zygote till the young adulthood by multiple environmental factors. Here we took into consideration the following variables that may be involved in DNA methylation: 1. current age; 2. gender; 3. variables related to alcohol drinking: AUDIT scoring, age at alcohol initiation, age at onset of problem drinking; 4. cigarette smoking; 5. term and kind of labor; 6. nutritional habits assessed with the CHB module 7. SSTR4 rs2567608 genotype and allele. Since our study relies on self-reported questionnaire, particular data on periconceptual and intra-uterine development, labor and early development (i.e., Apgar scale score, birth weight, prescribed or over-the-counter drugs used by mother during pregnancy) – they could not be obtained with confidence, were not known or forgotten. We generalized that any inequity at that time might have led to pre- or post-term labor or labor disturbances that required surgical interventions (here the term “surgical labor” comprise cesarean sections, forceps and vacuum lift deliveries).

2.4.2. DNA isolation
Genomic DNA was isolated from the buccal swabs using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer’s protocol. DNA was eluted in 100 µl Elution Buffer and quantified using a PicoDrop spectrophotometer (PicoDrop Limited). The quality of the DNA samples was analyzed by measuring the ratio of absorption at 260/280 nm. The purified total DNA was immediately used for PCR reactions or stored at −20°C.

2.4.3. The SSTR4 rs2567608 SNP genotyping
The SSTR4 rs2567608 was analyzed using the commercially available Pre-made TaqMan SNP Genotyping Assay (Applied Biosystems, ID: C_3206279_1). The assay consisted of PCR primers and reporter probes that were labeled with a quencher (MGB) and either 6-carboxyfluorescein (FAM) or VIC (Applied Biosystems’ proprietary dye with λex = 488 nm and λem = 552 nm). Amplification of the probe-specific product causes cleavage of the probe, generating an increase in reporter fluorescence.

The amplification was performed according to the manufacturer’s standard PCR protocol. Briefly, 10 ng total DNA was mixed with 10 µl TaqMan Genotyping PCR Master Mix and 0.5 µl TaqMan Assay to a final volume of 20 µl. The PCR thermal cycling was as follows: initial denaturing at 95°C for 10 min; 40 cycles of 92°C for 15 sec; and 60°C for 1 min. Thermal cycling was performed using a GeneAmp PCR System 9700 (Applied Biosystems). Each 96-well plate contained 92 test samples and 4 reaction mixtures without DNA template (no-template control).

The end-point fluorescence intensities of each probe were monitored using the ABI7900HT Real-Time PCR System (Applied Biosystems). The genotypes were determined automatically and then visually verified based on the dye component’s fluorescence emission data depicted in the X-Y scatter-plot of the Sequence Detection System 2.3 Software.

2.4.4. DNA methylation analysis
The DNA methylation status study was performed via the use of a specific and sensitive method involving chemical modification of cytosines to uracils via bisulfite treatment [37]. To detect the methylation status, the altered DNA was then amplified via methylation-specific PCR (MSP) with selective amplification of the methylated and unmethylated alleles and the analyzed PCR products.

The bisulfite conversion technique, which involves the treatment of the DNA with bisulfite, was utilized employed. The conversion of unmethylated by not methylated, cytosines into uracil was performed using the commercially available EZ DNA Methylation-Gold™ bisulfite conversion kit according to the manufacturer’s protocol (Zymo Research). The methylated cytosines remain unchanged during the treatment. DNA denaturation and bisulfite conversion were performed in a one-step reaction.

DNA (100-500 ng) in a volume of 20 µl was sodium-bisulfite-modified and subsequently denatured with heat and subjected to a CT-conversion reaction in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The DNA was bisulfite-treated along with positive and negative controls (Human Methylated & Non-methylated DNA Set, Zymo Research).

The reaction conditions were as follows:
- for the stage I PCR: SSTR4-fwFor: 5’-TTGAAATAGGTTTGAAGGA SSTR4-rwRev: 5’-AAAAATCAAAAAATACCCA - for the stage II PCR: SSTRM_3For: 5’-TTGGGATGTGGCGCGGC SSTRM_3Rev: 5’-AAATACGGACGGCGAATCCTAACACGC SSTRM_4For: 5’-TTTTTGGTGTAGGGAGGTGGTG SSTRM_4Rev: 5’-ATACCAACACAAAAACACTAACAC

The primers used in the stage I PCR recognized the bisulfate-modified template but did not distinguish between methylated and unmethylated alleles, whereas the primers used in the stage II PCR selectively bound and annealed the unmethylated or methylated sequences of SSTR4.

The methylation-specific stage I PCR was performed in a final volume of 20 µl using 1 U GoTaqG2 Flexi DNA Polymerase (Promega), 1x Enzyme Buffer, 1.2 mM MgCl2,
10 pmol of each primer, 7,5 nmol dNTPs and 1 µl of target DNA.

Amplification was performed with a GeneAmp PCR System 9700 (Applied Biosystems).

The stage I PCR conditions were as follows: initial denaturing at 94°C for 10 min; by 35 cycles of denaturing at 94°C for 45 s, annealing at 54°C for 45 s, extension at 72°C for 45 s; and final extension at 72°C for 10 min.

The stage II PCR was performed in a final volume of 20 µl using 1U GoTaqG2 Flexi DNA Polymerase (Promega), 1x Enzyme Buffer, 1,2 mM MgCl₂, 10 pmole of each primer (methylated or unmethylated), 7,5 nmole dNTPs, and 4 µl of the PCR products from the previous reaction.

The stage II PCR protocol for detecting methylated and unmethylated SSTR4 sequences was as follows: an initial denaturing at 94°C for 10 min, 30/35 cycles of denaturing at 94°C for 45 s, annealing at 65°C/59°C for 45 s, extension at 72°C for 45 s; and a final extension at 72°C for 10 min. The methylated/unmethylated status of SSTR4 was analyzed with a Microchip Electrophoresis System MCE®-202 MultiNA (Shimadzu).

### 2.5. Statistical analysis

Differences in analyzed parameters between groups were evaluated with the Chi² test (nominal variables) and the Student-t test or U Mann-Whitney test (continuous variables), according to the results of Shapiro-Wilk test. Bonferroni correction for multiple testing was applied. Afterwards parameters that differed significantly between patients with or without SSTR4 promoter methylation were included into the logistic regression analysis. Statistical analysis was performed with Statistica 12.0 (StatSoft Polska, Crakow, Poland). A p-value <0.05 was considered significant.

SSTR4 SNPs were evaluated for deviation from Hardy–Weinberg equilibrium using Michael H. Court’s (2005–2008) online calculator (http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls).

### 3. Results

#### 3.1. Study sample characteristic

The comparison between patients with AD and controls is depicted in the Table 1. The patients with AD and controls did not differ significantly according to gender and age (Table 1). Patients with AD reported significantly higher number of ACE categories experienced before the age of 18 within both, ACE Study Score and ACE 13 Score (Table 1); scored significantly lower in CHB module for proper nutritional habits (Table 1); were significantly more frequent cigarette smokers (Table 1). There was no significant difference between patients with AD and controls according to the term and kind of labor (Table 1).

The characteristic of particular clinical variables in patients with AD with comparison between female patients with AD and male patients with AD is presented in Table 2. Male and female patients with AD did not differ significantly according to current age, age at alcohol initiation, and AOPD (Table 2). Female patients with AD scored significantly lower in AUDIT than male patients with AD (Table 2).

#### 3.2. SSTR4 promoter region methylation and SSTR4 rs2567608 allele and genotype frequencies in patients with AD and controls

The genotype and allele frequencies of SSTR4 rs2567608 in patients with AD and controls are shown in Table 3.
polymorphism, the distribution of genotypes within the patients with AD and controls was in Hardy–Weinberg equilibrium (Chi²=3.629; df=1; p=0.056 for ADs; Chi²=0.044; df=1; p=0.833 for controls). There were no significant differences in genotype and allele frequencies between the patients with AD and controls (Table 3) nor between the males and females in each group (p=0.920 for genotypes and p=0.932 for alleles frequencies in patients with AD; p=0.080 for genotypes and p=0.082 for alleles frequencies in controls; data not shown).

Patients with AD had significantly more frequently their SSTR4 promoter region methylated than controls (Table 3). There was no statistical significance according to the frequency of the SSTR4 promoter region methylation between male and female patients with AD (methylation in 20.2% of AD males vs. 26.2% of AD females, p=0.5383; data not shown).

3.3. The comparison of variables potentially significant for DNA methylation in the study subjects (patients with AD and controls) with methylated and unmethylated SSTR4 promoter region

We chose following variables that may be associated with DNA methylation and compared them between study subjects (patients with AD and controls) with and without SSTR4 promoter region methylation: group affiliation, gender, current age, age at alcohol initiation, AOPD, AUDIT scoring, number of reported ACE categories, smoking cigarettes, SSTR4 genotype, SSTR4 allele, term and kind of labor, and scoring in a CHB module for proper nutritional habits. We found study subjects with SSTR4 promoter region methylation to be significantly more frequently alcohol dependent and score significantly higher in AUDIT (which obviously mirror higher frequency of SSTR4 promoter region methylation in ADs), to report significantly higher number of ACE categories (Table 4).

3.4. The logistic regression model for SSTR4 promoter region methylation

Variables that differed significantly between study subjects (patients with AD and controls) with and without SSTR4 promoter region methylation (Table 4) were included into the logistic regression model. The model was significant (Chi² = 32.8; p<0.0001). Only the group affiliation appeared to be a significant predictor of SSTR4 promoter region methylation in the whole study sample of patients with AD and controls (Table 5).

Table 2. Comparison of selected characteristics of alcohol dependence between male patients with AD (n=134) and female patients with AD (n=42)

| Clinicopathological features          | Female patients with AD (n=42) | Male patients with AD (n=134) | p-value | Patients with AD - general (n=176) |
|--------------------------------------|-------------------------------|-------------------------------|---------|-----------------------------------|
| Age (Mean ± SD years)                | 43.5 (11.2)                   | 42.5 (10.1)                   | 0.042¹  | 43.4 (10.5)                      |
| Age at alcohol initiation (Mean ± SD years) | 17.4 (6.2)                   | 15.1 (3.5)                   | 0.013¹  | 15.6 (4.4)                      |
| AOPD (Mean ± SD years)               | 32.0 (14.2)                   | 25.4 (9.7)                   | 0.021¹  | 26.9 (11.2)                     |
| AUDIT (Mean ± SD points)             | 24.2 (7.2)                    | 28.2 (7.4)                   | 0.002²  | 27.3 (7.5)                      |

¹ The U Mann-Whitney test - bold values mean a statistical significance according to Bonferroni correction (p<0.01)
AD – alcohol dependence; AOPD – age at onset of problem drinking; AUDIT - Alcohol Use Disorders Identification Test; p-level of statistical significance; SD – standard deviation

Table 3. Comparison of SSTR4 rs2567608 genotype and allele frequencies and SSTR4 promoter methylation between patients with AD (n=176) and controls (n=127)

| SSTR4 rs2567608 genotype | Patients with AD (n=176) n (%) | Controls (n=127) n (%) | Chi² | p-value |
|---------------------------|---------------------------------|------------------------|------|---------|
| CC                        | 31 (17.6)                       | 28 (22.1)              | 2.1126 | 0.348¹  |
| CT                        | 100 (56.8)                      | 62 (48.8)              |      |         |
| TT                        | 45 (25.6)                       | 37 (29.1)              |      |         |
| SSTR4 rs2567608 allele    |                                 |                        |      |         |
| T (wild)                  | 190 (54.0)                      | 141 (54.23)            | 0.0039 | 0.950¹  |
| C (variant)               | 162 (46.0)                      | 119 (45.8)             |      |         |
| SSTR4 promoter methylation |                                 |                        |      |         |
| Met                       | 38 (21.6)                       | 3 (2.3)                | 22.328 | <0.001¹ |
| unMet                     | 138 (78.4)                      | 124 (97.7)             |      |         |

¹ The Chi² test - bold values mean a statistical significance according to Bonferroni correction (p<0.01)
AD – alcohol dependence; SSTR4 Met – somatostatin receptor subtype 4 gene promoter region methylated; SSTR4 unMet – somatostatin receptor subtype 4 gene promoter region unmethylated; p-level of statistical significance; SD – standard deviation
### Table 4. Comparison of variables potentially associated with DNA methylation in subjects (patients with AD n=176 and controls n=127) with methylated and unmethylated SSTR4 promoter region

| Variable                                           | SSTR4 promoter methylation | p - value |
|----------------------------------------------------|----------------------------|-----------|
|                                                    | Met  | unMet  |
| Group affiliation [number, %] (Patients with AD n=176 and controls n=127) | 38 (21.6) | 138 (78.4) | <0.0001\(^1\) |
|                                                    | 3 (2.3) | 124 (72.7) |
| Gender [number, %] (Patients with AD n=176 and controls n=127) | Females | 12 (29.3) | 64 (24.4) | 0.48034\(^1\) |
|                                                    | Males | 29 (70.7) | 198 (75.6) |
| Current age (Mean±SD years) (Patients with AD n=176 and controls n=127) | 43.1 (9.6) | 41.5 (11.5) | 0.39842 |
| Age at alcohol initiation (Mean±SD years) (Patients with AD n=176) | 15.9 (2.8) | 15.6 (2.9) | 0.5593\(^3\) |
| AOPD (Mean±SD years) (Patients with AD n=176) | 27.6 (11.1) | 26.7 (11.3) | 0.6015\(^3\) |
| AUDIT (Mean±SD points) (Patients with AD n=176 and controls n=127) | 24.8 (9.6) | 15.7 (13.3) | 0.0001\(^1\) |
| ACE Study Score (Mean±SD points) (Patients with AD n=176 and controls n=127) | 2.7 (2.3) | 1.6 (2.3) | 0.0001\(^1\) |
| ACE 13 Score (Mean±SD points) (Patients with AD n=176 and controls n=127) | 2.8 (2.4) | 1.7 (2.4) | 0.0001\(^1\) |
| Cigarette smoking (Patients with AD n=176 and controls n=127) | Yes | 33 (88.5) | 202 (77.1) | 0.77769\(^1\) |
|                                                    | No | 8 (19.5) | 66 (22.9) |
| SSTR4 rs2567608 Genotype (Patients with AD n=176 and controls n=127) | C/C | 7 (11.9) | 52 (88.1) | 0.76685\(^1\) |
|                                                    | C/T | 24 (14.7) | 139 (82.3) |
|                                                    | T/T | 10 (11.9) | 74 (88.1) |
| SSTR4 rs2567608 Allele (Patients with AD n=176 and controls n=127) | C | 38 (13.5) | 243 (86.5) | 0.9337\(^7\) |
|                                                    | T | 44 (12.3) | 287 (86.7) |
| CHB module scoring for proper nutritional habits (Mean±SD points) (Patients with AD n=176 and controls n=127) | 15.9 (5.9) | 17.8 (5.9) | 0.0341\(^1\) |
| Term of labor (Patients with AD n=176 and controls n=127) | Preterm | 36 (13.3) | 235 (86.7) | 1.000\(^1\) |
|                                                    | Term | 4 (12.9) | 27 (87.1) |
| Kind of labor (Patients with AD n=176 and controls n=127) | Natural | 37 (12.9) | 248 (87.0) | 0.50164\(^1\) |
|                                                    | Surgical | 4 (19.1) | 17 (81.0) |

\(^1\) The Chi² test; \(^2\) The t-Student test; \(^3\) The U Mann-Whitney test - Bold values mean a statistical significance according to Bonferroni correction (p<0.003)

AD – alcohol dependence; AOPD – age at onset of problem drinking; AUDIT - Alcohol Use Disorders Identification Test; ACE – adverse childhood experience; CHB – Catalogue of Healthy Behavior; SSTR4 Met – somatostatin receptor subtype 4 gene promoter region methylated; SSTR4 unMet – somatostatin receptor subtype 4 gene promoter region unmethylated; p – level of statistical significance; SD – standard deviation

### Table 5. Variables included into the logistic regression model for SSTR4 promoter methylation (38 patients with AD and 3 controls had methylated SSTR4 promoter region)

| Variable                                           | B-coefficient (SE) | Confidence interval | p - value |
|----------------------------------------------------|--------------------|---------------------|-----------|
| Group affiliation                                  | -3.095 (0.86)      | -4.785 - 1.405      | <0.001    |
| AUDIT                                              | 0.018 (0.03)       | -0.032 0.069        | 0.467     |
| ACE 13 Score                                       | -0.080 (0.07)      | -0.225 0.066        | 0.283     |
| Proper nutritional habits (CHB module)             | -0.045 (0.037)     | -0.117 0.027        | 0.224     |

Bold values mean a statistical significance; AD – alcohol dependence; AUDIT - Alcohol Use Disorders Identification Test; ACE – adverse childhood experience; CHB - Catalogue of Healthy Behavior; p – level of statistical significance;
4. Discussion

This is the first study to analyze a possible influence of selected environmental inequities on SSTR4 promoter methylation in human sample of patients with alcohol dependence. Due to an exploratory nature of our results, it requires further investigation in future studies.

Our female patients scored significantly lower in AUDIT than male patients with AD. It stays in line with data presented by World Health Organization (WHO) in 2014 [38], WHO found in all WHO regions that females drank less on average and engage less often in heavy episodic drinking. WHO showed that total alcohol per capita consumption in 2010 among male and female alcohol drinkers in Poland was on average 31.5 litres and 14.0 litres of pure alcohol, respectively [38].

The adverse influence of ACEs on mental and physical health in the adulthood have been widely assessed and confirmed in national representative surveys [15,18]. The previous study by Berent et al. (2017) showed ACEs to rise significantly the risk of suicide attempt in patients with AD [39]. Not all siblings who grow up in dysfunctional alcoholic households [25,26] develop AD, and among monozygotic twins, frequently only one develops depression or other mental problem, including AD [19]. These findings indicate that neither environmental nor genetic factors exclusively increase the risk of alcoholism, rather the complete environmental-epigenetic-genetic interaction should be considered.

The mean number of self-reported ACE categories were 2.9 in patients with AD and 0.5 in the controls (Table 1). Among the controls, 3 carriers with a methylated SSTR4 promoter included one female who reported 8 ACEs, one male who reported 1 ACE, and a second male who reported no ACE. Number of self-reported ACE categories was not a significant predictor of SSTR4 promoter region methylation in our study sample of patients with AD and controls (Table 5). Patients with AD had their SSTR4 promoter region significantly more frequently methylated than controls (Table 3). We made an attempt to verify if SSTR4 promoter region methylation in our whole study sample of patients with AD and controls is associated with factors, proposed in the relevant literature as possibly significant for DNA methylation [6,7,34,36]. When individuals with methylated and unmethylated SSTR4 promoter region compared (patients with AD and controls), individuals with methylated SSTR4 promoter region were significantly more frequently patients with AD, scored significantly higher in AUDIT, and reported significantly higher number of ACEs’ categories. However, only the group affiliation was found to be a significant predictor for SSTR4 promoter methylation (Table 5). These findings suggest that SSTR4 promoter methylation in here studied individuals may be either a primary epigenetic change (inherited) or a secondary modification but neither to alcohol drinking severity nor to childhood trauma. Roth et al. (2009) found that altered methylation can be passed from one generation to the next [14]. If the methylation of the SSTR4 promoter region in our study sample was inherited, it could be proposed as a possible primary molecular background for vulnerability to childhood trauma, maybe due to better memorizing of ACEs. However, there is a constellation of personality characteristics that decide about awareness of emotions and strategies to manage strong emotions, which hardly allows us to state that SSTR4 promoter methylation may partially determine individual susceptibility to childhood trauma [40]. Since, evolutionary, it would be unwarranted to pass a harmful characteristic through subsequent generations, it should be taken into account that better memorizing is a potentially harmful characteristic in carriers brought up in dysfunctional households but also potentially advantageous in carriers brought up in supportive households. If the methylation of the SSTR4 promoter region was secondary to ACEs, it would be an acquired factor promoting vulnerability for further adverse and supportive life events. In our opinion, SSTR4 promoter region methylation may act as a buffer that modifies sst-4 activity warranted by SSTR4 rs2567608. SSTR4 promoter region methylation may cause SSTR4 rs2567608 C allele carriers more similar to carriers of the T allele, and similarly, SSTR4 promoter region methylation may enhance the sst-4 activity of the SSTR4 rs2567608 T allele carriers. However the discussion about the role of SSTR4 promoter region methylation would be stronger if the factors responsible for methylation in this genome region were known. There is a little number of studies on DNA methylation and ACEs. Van Ijzendoorn et al. (2010) assessed serotonin transporter gene (SLC6A4) promoter methylation and functional length polymorphisms (i.e., the short (s) versus the long (l) allele) in the serotonin transporter-linked promoter region (5-HTTLPR) in a sample of adopted children [25]. They found that the vulnerability of carriers of the ss variant of the 5-HTTLPR to the development of psychological problems in response to adverse events may be reduced by higher levels of methylation, which may reduce the risk of unresolved loss or trauma in the carriers of the s variant of the serotonin transporter gene and thus entail adaptive value [25]. Further, van Ijzendoorn et al. (2012) conducted a meta-analysis of the potentially 5HTTLPR-mediated association between positive environments and developmental outcomes [26]. In the total set of studies, including studies with mixed ethnicities, these authors found that ss/sl carriers were significantly more vulnerable to negative environments than ll carriers. However, in the Caucasian samples, the ss/sl carriers also profited significantly more from positive environmental input than the ll carriers. The associations between (positive or negative) environment and (positive or negative) developmental outcome were absent in the ll carriers [26].

It is questionable if local exposition of buccal mucosa to psychoactive substances can change promoter methylation in endothelial cells. Olsson et al. (2010) found no associations between alcohol, tobacco or cannabis consumption and buccal cell methylation [34]. Our patients with AD and controls with methylated SSTR4 promoter region did not differ significantly from AD and control individuals with unmethylated SSTR4 promoter according to current age, age at alcohol initiation, AOPD, and cigarette smoking (Table 4). Although the severity of drinking assessed with AUDIT was significantly higher in AD and control individuals with methylated SSTR4 promoter region (Table 4), AUDIT scoring was
Nutrients and bioactive food components were shown to alter genome methylation [6]. Folate, vitamin B-12, methionine, choline, and betaine can affect DNA methylation through altering 1-carbon metabolism. Folate comprises a methyl group that enters the synthesis of S-adenosylmethionine, which is a direct methyl donor for enzymes engaged in DNA methylation. Individual differences in activity of these enzymes are associated with functional polymorphisms for enzymes’ genes and additionally modified by food components, i.e. genistein and tea catechin [6]. The MTHFR (methylmethyltetrahydrofolate reductase gene) 677T allele carriers present reduced enzyme activity [7]. Friso et al. (2002) found additionally that only the TT subjects with low levels of folate account for the diminished DNA methylation [47]. Sinclair et al. (2007) indicated that dietary methyl nutrients during the periconceptional period can change DNA methylation patterns in sheep offspring [48]. Steegers-Theunissen et al. (2009) found the differentially methylated region (DMR) of the insulin-like growth factor 2 gene (IGF2) higher methylated in children of mothers using folic acid periconceptionally than of mothers who did not [49]. According to the possible nutritional influence on genome methylation, it was rather predictable to find SSTR4 promoter less frequently methylated than in controls. First, lower than in controls scoring in CHB module for proper nutritional habits (Table 1), indicated that patients with AD were rather low-methyl donor intakers (had lower folate and fiber intake and diet richer in fat (Western diet: high meat, energy, and alcohol intake and low fruit, vegetable, and fiber intake). Second, alcohol intake, additionally diminishes folate amount, probably because of degradation of folate in the colon by acetaldehyde, the first metabolite of alcohol [47,48]. Nutritional habits assessed with CHB module were not significant predictors of SSTR4 promoter methylation in our study sample of patients with AD and controls (Table 5). Also, Zhang et al. (2011) found no significant difference in global leukocyte DNA methylation in 161 cancer-free participants for diet (intake of dietary folate equivalents and other one-carbon nutrients) [50].

5. Limitations

Due to several limitations, the study results should be interpreted cautiously. The study would provide stronger evidence after stratification for functionally important SNPs in genes involved in folate metabolism.

Because of population stratification, here found SSTR4 rs2567608 allele and genotype frequencies and SSTR4 promoter region methylation frequency are restricted to the sample of polish Caucasian population and can not be generalized for other populations [51]. This is a first study that makes efforts to map SSTR4 promoter region methylation frequency in this study sample of polish Caucasian population (please see the inclusion and exclusion criteria for this study). Population stratification should be controlled for in future studies that report phenotypic associations in samples from different populations [51].

There are periods in human life cycle in which the individual is particularly susceptible to epigenetic influences; these include fertilization, gametogenesis, and early embryo development. DNA methylation is of multiple etiology, hardly possible to be verified in adults.

Proper verification of the association between diet and SSTR4 promoter region methylation require quantitative assessment of the food consumed in the life periods crucial for DNA methylation, i.e., folate and choline [3]. Mehedint et al. (2010) showed that mice maternal choline status deprivation in the late gestational age results in hypomethylation of specific CpG islands in genes controlling cell cycling in fetal hippocampus [52].

Starvation has also been indicated as a possible factor influencing promoter methylation. We did not strictly ask our respondents if they starved during the childhood or adolescence, but one of questions from ACE 13 Score concerns physical neglect that partially include not eating enough (“Did you often or very often feel that You didn’t have enough to eat (…)”). However it is rather far from starvation.

Exposure to heavy metals and other air pollutants, bioflavonoids, and endocrine disruptors, such as bisphenol A and phthalates, has been also shown to affect brain development and epigenetic memory [53,54]. However their long-term effects are unclear at this point and require further studies [53,54].

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Authors’ Disclosures

Regarding research work described in the paper, each one of our co-authors declares that there is no conflict of interest, and conformed to the Helsinki Declaration concerning human rights and informed consent, and followed correct procedures concerning treatment of humans in research.

Here assessed study population of 176 patients with alcohol dependence and 127 controls was analyzed in the previous study by Berent et al. (2017) [39].
Authors’ Contribution

DB designed and coordinated the study, qualified the patients and controls for entry into the study, analyzed and interpreted the results, and wrote the manuscript.

MP performed the statistical analysis and interpreted the results.

ZP coordinated and performed the laboratory testing.

EK performed the statistical analysis.

DK-W performed the laboratory testing.

MM qualified the controls for entry into the study.

ZP coordinated and performed the laboratory testing.

All of the authors approved the final version of this manuscript.

All the authors gave agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of interest

The authors report no declarations of interest.

References

[1] Weber M., Schübel D., Genomic patterns of DNA methylation: targets and function of an epigenetic mark, Curr. Opin. Cell Biol., 2007, 19, 273–280
[2] Levenson J.M., Sweatt J.D., Epigenetic mechanisms: a common theme in vertebrate and invertebrate memory formation, Cell. Mol. Life Sci., 2006, 63, 1009–1016
[3] Miller C.A., Sweatt J.D., Covalent modification of DNA regulates memory formation, Neuron, 2007, 53, 857–869
[4] Choi S.-W., Friso S., Epigenetics: A New Bridge between Nutrition and Health, Adv. Nutr., 2010, 1, 8–16
[5] Ogino S., Wilson R.B., Genotype and haplotype distributions of MTHFR677C>T and 1298A>C single nucleotide polymorphisms: a meta-analysis, J. Hum. Genet., 2003, 48, 1–7
[6] Chen D, Meng L, Pei F, Zheng Y, Leng J. A review of DNA methylation in depression. J Clin Neurosci. 2017 Jun 20. pii: S0967-5868(17)30417-4. doi: 10.1016/j.jocn.2017.05.022
[7] Neidhart M. Chapter 17 – DNA Methylation in Psychiatric Diseases. In: DNA Methylation and Complex Human Disease. Edited by: Neidhart M, Gay S. 2016, Pages 289–314. https://doi.org/10.1016/B978-0-12-420194-1.00017-8
[8] LaPlant Q., Vialou V., Covington H.E., Dumitriu D., Feng J., Warren B.L., et al., Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens., Nat. Neurosci., 2010, 13, 1137–1143
[9] Zhang T.-Y., Hellstrom I.C., Bagot R.C., Wen X., Diorio J., Meaney M.J., Maternal care and DNA methylation of a glucamic acid decarboxylase 1 promoter in rat hippocampus, J. Neurosci., 2010, 30, 13130–13137
[10] Murgatroyd C., Patchev A.V., Wu Y., Micale V., Bockmühl Y., Fischer D., et al., Dynamic DNA methylation programs persistent adverse effects of early-life stress, Nat. Neurosci., 2009, 12, 1559–1566
[11] Cortés-Mendoza J., Díaz de León-Guerrero S., Pedraza-Alva G., Pérez-Martínez L., Shaping synaptic plasticity: the role of activity-mediated epigenetic regulation on gene transcription, Int. J. Dev. Neurosci., 2013, 31, 359–369
[12] Roth T.L., Rubin F.D., Funk A.J., Sweatt J.D., Lasting epigenetic influence of early-life adversity on the BDNF gene, Biol. Psychiatry, 2009, 65, 760–769
[13] Felitti V.J., Anda R.F., Nordenberg D., Williamson D.F., Spitz A.M., Edwards V., et al., Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults. The Adverse Childhood Experiences (ACE) Study, Am. J. Prev. Med., 1998, 14, 245–258
[14] Kilpatrick D.G., Saunders B.E., Smith D.W., Youth Victimization: Prevalence and Implications [Electronic], U.S. Department of Justice, Office of Justice Program, National Institute of Justice, 2003
[15] Khoury L., Tang Y.L., Bradley B., Cubells J.F., Ressler K.J., Substance use, childhood traumatic experience, and Posttraumatic Stress Disorder in an urban civilian population, Depress. Anxiety, 2010, 27, 1077–1086
[16] Bellis M.A., Hughes K., Leckenby N., Hardcastle K.A., Perkins, C., Lowey H., Measuring mortality and the burden of adult disease associated with adverse childhood experiences in England: a national survey. J. Public Health (Oxf.), 2015, 37, 445–454
[17] Zhao J., Goldberg J., Bremner J.D., Vaccarino V., Association between promoter methylation of serotonin transporter gene and depressive symptoms: a monozygotic twin study, Psychosom. Med., 2013, 75(6), 523–529
[18] Patel Y.C., Somatostatin and its receptor family, Front. Neuroendocriniol., 1999, 20, 157–198
[19] Kluge C., Stoppel C., Szinyei, C., Stork O., Pape H.-C. Role of the somatostatin system in contextual fear memory and hippocampal synaptic plasticity, Learn. Mem., 2008, 15, 252–260
[20] Meyer M.A., Highly Expressed Genes within Hippocampal Sector CA1: Implications for the Physiology of Memory, Neurol. Int., 2014, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4077213/
[21] Gastambide F., Viollet C., Lepousez G., Epelbaum J., Guillou J.-L., Hippocampal SSTR4 somatostatin receptors control the selection of memory strategies, Psychopharmacology (Berl.), 2009, 202, 153–163
[22] Kim J.C., Kim S.Y., Cho D.H., Roh S.A., Choi E.Y., Jo Y.K., et al., Genome-wide identification of chemosensitive single nucleotide polymorphism markers in colorectal cancers, Cancer Sci., 2010, 101, 1007–1013
[23] van Ijzendoorn M.H., Caspers K., Bakermans-Kranenburg M.J., Beach S.R.H., Philibert R., Methylation matters: interaction between methylation density and serotonin transporter genotype predicts...
unresolved loss or trauma, Biol. Psychiatry, 2010, 68, 405–407
[26] van Ijzendoorn M.H., Belsky J., Bakermans-Kranenburg M.J., Serotonin transporter genotype 5HTTLPR as a marker of differential susceptibility? A meta-analysis of child and adolescent gene-by-environment studies, Transl. Psychiatry, 2012, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3432188/

[27] [Juczyński Z. [Instruments for measurement in health promotion and health psychology], 2nd ed., Pracownia Testów Psychologicznych, Warsaw, 2012 [in Polish]

[28] Daeppen J.B., Yersin B., Landry U., Pécoud A., Decrey H., Reliability and validity of the Alcohol Use Disorders Identification Test (AUDIT) imbedded within a general health risk screening questionnaire: results of a survey in 332 primary care patients, Alcohol Clin. Exp. Res., 2000, 24(5), 659-65

[29] World Health Organization, The ICD-10 Classification of Mental and Behavioural Disorders: Clinical Description and Diagnostic Guidelines, WHO, Geneva, 1992

[30] Holliday R., Epigenetics: a historical overview, Epigenetics, 2006, 1(2), 76–80

[31] Christensen B.C., Houseman E.A., Marsit C.J., Zheng S., Wrensch M.R., Wiemels J.L., et al., Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context, PLoS Genet., 2009, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2718614/

[32] Weaver I.C.G., Champagne F.A., D’Alessio A.C., D’Ambrosio A.C., Sharma S., Seckl J.R., et al., Epigenetic programming by maternal behavior, Nat. Neurosci., 2004, 7(8), 847–854

[33] Francis D., Diotto J., Liu D., Meaney M.J., Nongenomic transmission across generations of maternal behavior and stress responses in the rat, Science, 1999, 286(5442), 1155–1158

[34] Olsson C.A., Foley D.L., Parkinson-Bates M., Byrnes G., McKenzie M., et al., Prospects for epigenetic research within cohort studies of psychological disorder: a pilot investigation of a peripheral cell marker of epigenetic risk for depression, Biol. Psychol., 2010, 83, 159–165

[35] Lowe R., Gemma C., Beyan H., Hawa M.I., Bazeos A., Leslie R.D., et al., Buckucks are likely to be a more informative surrogate tissue than blood for epigenome-wide association studies, Epigenetics, 2013, 8, 445–454

[36] Klener P., [Epigenetic cancer drugs and their role in anticancer therapy], Vnitřní lékařství, 2013, 59, 463–465

[37] Herman J.G., Graff J.R., Myöhänen S., Nelkin B.D., Baylin S.B., Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands, Proc. Natl. Acad. Sci. U. S. A., 1996, 93, 9821–9826

[38] World Health Organization, Global Status Report on Alcohol and Health. WHO Library Cataloguing-in-Publication Data, WHO, 2014, https://apps.who.int/iris/bitstream/10665/112736/1/9789240692763_eng.pdf

[39] Berent D., Emilien G., Podgórski M., Kusiedł E., Kulczycka-Wojdala D., Szymańska B., Macander M., Pawłowska Z., SSTR4, childhood adversity, self-efficacy and suicide risk in alcoholics, Transl. Neurosci., 2017,8,76-86. doi: 10.1515/tnsci-2017-0013

[40] Powers A., Etkin A., Gyurak A., Bradley B., Jovanovic T., Associations Between Childhood Abuse, Posttraumatic Stress Disorder, and Implicit Emotion Regulation Deficits: Evidence From a Low-Income, Inner-City Population, Psychiatry, 2015, 78, 251–264

[41] Hsing D.T., Marsit C.J., Houseman E.A., Eddy K., Furniss C.S., McClean M.D., et al., Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma: Cancer Epidemiol, Biomarkers Prev., 2007, 16 (1), 108–114

[42] Moore L.E., Pfeiffer R.M., Poscablo C., Real F.X., Kogevinas M., et al., Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case-control study, Lancet Oncol., 2008, 9(4), 359–366

[43] Hou L., Wang H., Sartori S., Gawron A., Lissowska J., Pollati V., et al., Blood leukocyte DNA hypomethylation and gastric cancer risk in a high-risk Polish population. Int. J. Cancer, 2010, 127(8), 1866–1874

[44] Zhang F.F., Cardarelli R., Carroll J., Fulda K.G., Kaur M., Gonzalez K., et al., Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood, Epigenetics, 2011, 6(5), 623–629

[45] Zhu Z.Z., Hou L., Pollati V., Tarantini L., Marinelli B., Cantone L., et al., Predictors of global methylation levels in blood DNA of healthy subjects: a combined analysis. Int. J. Epidemiol., 2012, 41(1), 126–139

[46] Friso S., Choi S.-W., Girelli D., Mason J.B., Dolnikowski G.G., Bagley P.J., et al., A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status, Proc. Natl. Acad. Sci., 2002, 99, 5606–5611

[47] Friso S., Choi S.-W., Girelli D., Mason J.B., Dolnikowski G.G., Bagley P.J., et al., A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status, Proc. Natl. Acad. Sci., 2002, 99, 5606–5611

[48] Sinclair K.D., Allegrucci C., Singh R., Gardner D.S., Sebastian S., Bispham J., et al., DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status, Proc. Natl. Acad. Sci. U. S. A., 2007, 104, 19351–19356

[49] Steegers-Theunissen R.P., Obermann-Borst S.A., Kremer D., Lundemans J., Siebel C., Steegers E.A., et al., Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child, PLoS One, 2009, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2773848/

[50] Zhang F.F., Cardarelli R., Carroll J., Fulda K.G., Kaur M., Gonzalez K., et al., Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood, Epigenetics, 2011, 6(5), 623–629

[51] Vulturaru R., Chiş A., Hambrich M., Kelemen B., Ungureanu L., Miu E., [Instruments for measurement in health promotion and health psychology], 2nd ed., Pracownia Testów Psychologicznych, Warsaw, 2012 [in Polish]

[52] Mehdint M.G., Niculescu M.D., Craciunescu C.N., Zeisel S.H., Choline deficiency alters global histone methylation and epigenetic marking
at the Re1 site of the calbindin 1 gene, FASEB J., 2010, 24, 184–195

[53] Pozharny Y., Lambertini L., Clunie G., Ferrara L., Lee M.-J., Epigenetics in women’s health care, Mt. Sinai J. Med., 2010, 77, 225–235

[54] Brockmeyer S., D’Angiulli A., How air pollution alters brain development: the role of neuroinflammation, Transl. Neurosci., 2016, 7, 24-30. doi: 10.1515/tnsci-2016-0005