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ORIGINAL ARTICLE

# Psychotropic drug use and suggestive depression symptoms associated with *NR3C1* DNA methylation

**Júlia de Assis Pinheiro[a], Aline Ribeiro Borçoi[a], Flávia Vitorino Freitas[a,f], Suzanny Oliveira Mendes[a], Anderson Barros Archanjo[a], Mayara Mota de Oliveira[a], Joaquim Gasparini dos Santos[a], Juliana Krüger Arpini[b], Rafael Assis de Souza[d], Ivana Alece Arantes Moreno[a], Marcele Lorentz Mattos de Souza[a], Dirceu Pereira dos Santos[e], Wagner Miranda Barbosa[f], José Claudio Casali-da-Rocha[g], Bruna Pereira Sorroche[c], Leonardo Oliveira Trivilin[h], Elizeu Batista Borloti[i], Letícia Batista Azevedo Rangel[a], Lidia Maria Rebolho Batista Arantes[c], Adriana Madeira Alvares-da-Silva[a,b]**

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[a]Biotecnologia/Renorbio Programa de Pós-Graduação, Universidade Federal do Espírito Santo, ES, Brasil;

[b]Departamento de Biologia, Universidade Federal do Espírito Santo, Alegre, ES, Brasil;

[c]Centro de Pesquisa em Oncologia Molecular, Hospital de Câncer de Barretos, Barretos – São Paulo, Brasil;

[d]Departamento de Agronomia, Universidade Federal do Espírito Santo, Alegre, ES, Brasil;

[e]Instituto Federal Fluminense, Campos dos Goytacazes, RJ, Brasil;

[f]Departamento de Farmácia e Nutrição, Universidade Federal do Espírito Santo, Alegre, ES, Brasil;

[g] A.C. Camargo Câncer Center, São Paulo, SP, Brasil;

[h] Departamento de Medicina Veterinária, Universidade Federal do Espírito Santo, Alegre, ES, Brasil;

[i] Programa de Graduação em Psicologia, Universidade Federal do Espírito Santo, Vitoria, ES, Brasil.

**Corresponding author**
adriana.m.silva@ufes.br

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**Abstract**

**Introduction:** Psychiatric disorders have become a global problem that leads millions of people to use psychotropic medications, especially benzodiazepines. The effects of these substances are widely known regarding tolerance and chemical dependence, however, from epigenetics perspective, there are still little known.

**Objective:** To evaluate the association between psychotropic drug use, NR3C1 gene methylation and its relation with symptoms suggestive of depression in adult individuals assisted in the public health system.

**Methods:** 385 adult volunteers (20-59 years) users of the Brazilian Unified Health System were recruited to evaluate socioeconomic, health, lifestyle conditions in a cross sectional study. BDI-II evaluated symptoms suggestive of depression and pyrosequencing evaluated NR3C1 DNA methylation. Bivariate and multivariate Poisson regression model with robust variance ($p < 0.05$) evaluated the association between psychotropic drug use and NR3C1 gene methylation.

**Results:** Specific depressive symptoms such as irritability, insomnia and fatigability were associated with psychotropic drug use. Symptoms of past failure, indecision and loss of appetite were associated with hypermethylation patterns in CpGs 40 to 47 of NR3C1 gene. Moreover, psychotropic drug use is associated with 50% reduction in NR3C1 gene methylation, through model adjusted with socioeconomic, health and lifestyle confounding variables.

**Conclusions:** Psychotropic drug use and depressive symptoms was associated with changes in NR3C1 DNA methylation. In this context, epigenetic modification resulting from psychotropic drug use and depressive symptoms could be considered, mainly in population studies with epigenetic evaluation, where these factors may be influencing the findings of future studies.

**Key words:** Methylation, NR3C1, psychotropic drugs use, depressive symptoms.

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**Authors summary**

**Why was this study done?**
Epigenetics is the link between environment and genetics. Epigenetic alterations in stress-related genes have been associated with mental disorders such as depression and post-traumatic stress. The use of psychotropic drugs are among the most prescribed medications in the world, however little is known about the association between epigenetic modifications and psychotropic drug use.
**What did the researchers do and find?**
The present cross-sectional study assessed 385 individuals aged 20-59 years for drug use, depressive symptoms, socio-economic, health and lifestyle conditions. DNA was extracted after blood sample collection for post bisulfite conversion DNA pyrosequencing. In multivariate regression analysis, symptoms of feeling of failure, indecision, and loss of appetite were associated with NR3C1 gene hypermethylation, and psychotropic drug use is associated with a 50% reduction in NR3C1 gene methylation, by means of a model adjusted with socioeconomic, health, and lifestyle confounding variables.

**What do these findings mean?**
Psychotropic drug use and specific depressive symptoms are associated with changes in NR3C1 gene methylation, which demonstrates that the increased use of psychiatric drugs may epigenetically interfere with genes involved with the stress response. Furthermore, it demonstrates the importance of these variables being considered as confounding variables in future epigenetic research.

**Highlights**
Psychotropic drug use is associated with NR3C1 gene hypomethylation.
Suggestive depression symptoms are associated with NR3C1 gene hypermethylation.
Alterations in NR3C1 methylation are associated with stressful life events and specific symptoms of depression.

## INTRODUCTION

According to the World Health Organization (WHO), in its action plan on mental health 2013-2020, one in ten people suffer from some mental health disorder in the world[1], which main diseases are depression, bipolar affective disorder and schizophrenia[2]. Depression is a global public health concern, as 17% of the world's general population exhibits comorbidities[3,4]. In Brazil, this major depressive disorder has a prevalence of approximately 20% in São Paulo and 17% in Rio de Janeiro[5].

Psychotropic drugs are an important tool in the treatment and control of various psychopathological conditions. Considering the prevalence and relevance of mental disorders, the high use of c is seen in many countries and these drugs are among the most widely prescribed medications[6]. In a study conducted in 2015 in ten European countries, when assessing depressed individuals, it was found that the annual prevalence of psychotropic drug use was 15.1% among women and 8.0% among men[7]. Another study conducted in Brazil with 1999 individuals reported a prevalence of 11.7% of psychotropic drug use, 7.3% among men and 15.8% among women. The most common therapeutic classes were antidepressants (38.2%) and benzodiazepines (24%)[8].

Clonazepam is a benzodiazepine which binds to the gamma-aminobutyric acid receptor (GABA), the main inhibitory neurotransmitter of the Central Nervous System (CNS) which can cause tolerance, dependence and physiological changes in the human body. Its indicated for anxiety, insomnia, muscle relaxation and epilepsy[9].

Studies have shown that epigenetic changes and consequent altered gene expression may result from drugs use[10-12]. A potential epigenome alterations should be considered due to the side effects and impact on treatment[10-12]. Thus, some drugs can act as an additional factor in the modulation of epigenetic mechanisms[13], as antiepileptic drugs that modify histones via direct chemical interaction with histone deacetylase[14], as well as the cannabinoids and opiates that trigger DNA hypermethylation[15,16].

The use of medications for psychiatric disorders treatment has also been linked to epigenetic changes[17], since the role of epigenetic changes in epigenome regulation has been comprehensively addressed worldwide18 with in-depth study of several modifications, including DNA methylation, histone modifications, chromatin remodeling and microRNA[19].

DNA methylation is a regulation mechanism of gene expression widely known[20]. However, it has recently been described as the "modus operandi" of environment adaptation process, a rapid response to exposure events[21]. Methylation is thought to be the most stable form of epigenetic alteration. Typically, it consists in addition of a methyl group at sites where a cytosine nucleotide occurs next to a guanine nucleotide (CpG) and when located in a gene promoter, DNA methylation typically acts to repress gene transcription[22].

Thus, stressful events perceived by the individual may result in the addition or withdrawal of epigenetic marks at specific DNA positions resulting in altered gene expression[22-24].

Stressful events in humans or in animal models have been related to hypermethylation at specific positions in the DNA at the glucocorticoid receptor (GR) promoter region, which has hypothalamic regulation function of the stress on neuroendocrine Hypothalamic-Pituitary-adrenal axis (HPA) via cortisol production[25,26]. However, the literature reports that among other events or conditions, stressors have already been described as related to hypomethylation in the same GR region[27]. Animal studies have evaluated methylation events directly in the hypothalamus[28,29], while human studies have evaluated blood methylation events by their homology observed in different tissues with equivalent expression[30].

The glucocorticoid receptor gene belongs to the subfamily of Nuclear Receptor 3, Group C, Member 1 (NR3C1), which encodes the human glucocorticoid receptor and it is located on chromosome 5q31-3231. This gene consists of eight coding exons numbered 2–9 and nine non-coding first exons referred to as A–J (excluding "G")

which are thought to act as alternate promoters[32]. Exons 1D, 1J, 1E, 1B, 1F, 1C and 1H are located within a CpG island covering 3 kb along the proximal promoter region of NR3C1 gene[32,33].

The non-coding exons in the NR3C1 gene promoter region contains multiple CpG dinucleotide sequences subject to methylation. In 1F region there are 47 CpG sites that have been studied by many authors relating effects of stressing events such as prenatal and early-life stress, post-traumatic stress and depression[26,32,34-43].

Several studies have shown association between methylation and life-stressing events or clinical severity, reporting hyper or hypomethylation (or both) using different methods, including pyrosequencing at different CpG sites of NR3C1 1F region[33]. Furthermore, psychotropic drug use to control stress-induced pathologies, including depression, were related to epigenetic alterations and changes in gene expression. Some reports have shown that antidepressants and mood stabilizers exert their therapeutic effect, at least in part, through epigenetic mechanisms[44-46].

To date, there are no conclusive data on epigenetic changes regarding the methylation patterns of NR3C1 gene and psychotropic drug use. Therefore, the objective is evaluated the association between psychotropic drug use and NR3C1 gene methylation in adult individuals, to assess the role of each variable and its association with symptoms suggestive of depression.

## METHODS

### Study design

This is a cross-sectional observational study.

### Study location and period

The study was conducted between April and June 2017 in the municipality of Alegre-ES, located in the southeastern region of Brazil in the state of Espírito Santo, in the Caparaó Capixaba region.

### Study population and eligibility criteria

The study population was composed of a aleatory sample of 386 individuals living in urban and rural areas.

The inclusion criteria for participation in the study were the following: be 20 to 59 years old, be SUS user and to declare free consent for participation. Exclusion criteria were not be pregnant and not have cognitive conditions that would interfere with answering the questionnaires.

### Data collection

Data were collected through individual interview based on questionnaire that evaluated socioeconomic, health and lifestyle conditions. Low-income was defined by a per capita income/day less than $5 (five American dollars)47. Schooling was categorized into less than 8 years of study, 8 to 11 years of schooling, and university higher education. Gender, self-reported race, age; alcohol and tobacco consumption in the present and the past, leisure activity and physical activity (in weekly frequency); self-perception of health (considering good or bad health), self-reported stress and anxiety; use of medications, grouped in similar classes; were also collected.

The depression were investigated through the application of the Beck Depression Inventory (BDI-II). BDI-II is a self-reporting instrument that assesses the presence and severity of 21 depressive symptoms (sadness, pessimism, past failure, loss of pleasure, guilty feelings, punishment feelings, self-dislike, self-criticalness, suicidal thoughts, crying, irritability, social withdrawal, indecisiveness, worthlessness, loss of energy, changes in sleeping, fatigability, loss of appetite, loss of weight, somatic worries and loss of interest in sex). Every symptom are rated on a 4-point scale ranging from 0 to 3, with higher scores indicating more severe symptoms of depression[48]. The values obtained were adequate to the total scores categorized according to the following regrouping used for primary care use population and non-clinical populations: minimal depression (BDI-II <10), mild depression (BDI-II 10-17) and depression (BDI-II ≥ 18)[3,49,50].

### Blood analysis

For NR3C1 gene methylation analysis, 286 patients' peripheral blood were collected after the person had fasten for at least eight hours. Salting-Out DNA extraction method with saline precipitation was performed in whole blood leukocytes according to Salazar and colleagues[51]. DNA quality and concentration were verified through NanoDrop® at wavelength λ = 260 and 280 nm, and the wavelength ratio, ie 1.8 to 2.0, confirms DNA integrity without contamination.

### Quantitative Pyrosequencing Methylation Assays - PMA

Sodium-bisulfite conversion of 1 µg of DNA was performed using a kit (EpiTect® Bisulfite Kit; Qiagen, Valencia, CA), following the manufacturer's recommendations. Pyrosequencing methylation assays were performed as previously described[52,53]. Briefly incubation of the target DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. Therefore, bisulfite treatment gives rise to different DNA sequences for methylated and unmethylated DNA. The chemistry of cytosine deamination by sodium bisulfite involves three steps: (1) sulphonation; (2) deamination and (3) desulphonation.

Confirmation of PCR product quality and lack of contamination was established on 2% agarose gels using GelRedTM (Uniscience). Pyrosequencing was performed using the PSQ96ID Pyrosequencer (Qiagen, Valencia, CA) with the PyroMark Gold Q96 Reagent Kit (Qiagen, Valencia, CA), according to manufacturer's protocol. All pyrosequencing conditions are available in table 1.

A mean methylation index was calculated from the mean of methylation percentages for CpG sites evaluated in Pyromark Software, using default software settings. In this study, we considered all methylation levels detected in pyrosequencing, in order to classify individuals into unmethylated (hypomethylated) and methylated (hypermethylated), when they presented methylation in any percentage above zero.

A representative scheme of the amplified region of 47 CpGs and the 8 CpGs site-specific analyzed using bisulphite-pyrosequencing assays is shown in figure 1.

**Table 1:** Primers, PCR conditions and analyzed sequences for pyrosequencing reactions

| PCR Primer | | PCR conditions | | |
|---|---|---|---|---|
| Forward | 5'-TTTTTTTTTTGAAGTTTTTTTA-3' | 95ºC | (14'30") | 45 cycles (410bp) |
| Reverse | 5'-BIOTIN-CCCCCAACTCCCCAAAAA-3' | 94ºC | (30") | |
| | | 50ºC | (30") | |
| | | 72ºC | (30") | |
| | | 72ºC | (10') | |
| | | 4ºC | indefinitely | |
| Sequencing primers | | | | |
| 40 to 42 CpG | 5'-AGAAAAGAAATTGGAGAAATT-3' | | | |
| 43 to 47 CpG | 5'-GTTTTAGAGAGATTAGGT-3' | | | |
| Analyzed sequences | | | | |
| Seq 1 | YGGTGGTTTTTTTAAYGTYGTTTTAATCGTGTTGATCAGTCGCTTA | | | |
| Seq 2 | YGGTTTTYGTYGTTGTYGTYGTTAGTCAGTTCAGTCGTAGTCAGTCGTA | | | |

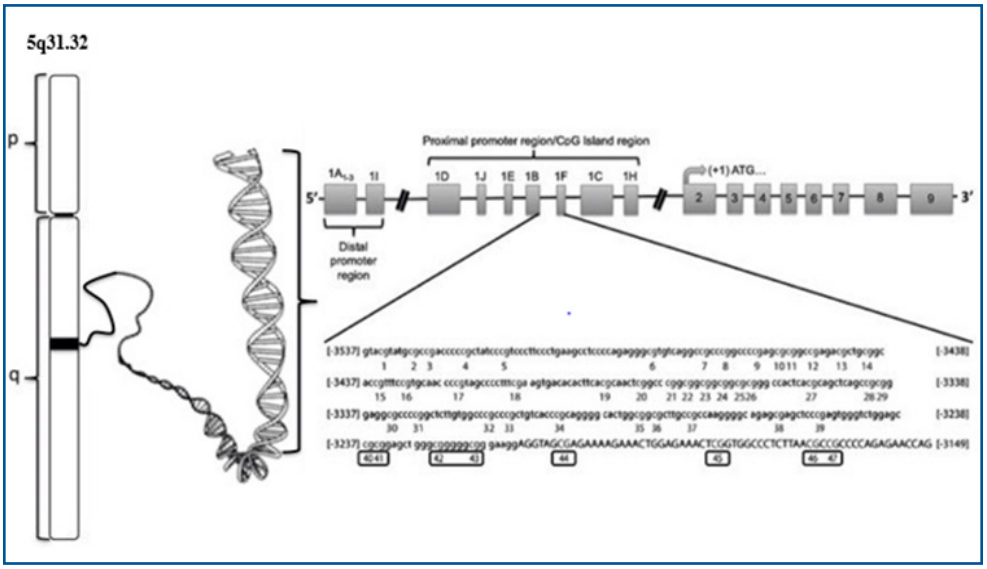


**Figure 1:** NR3C1 1F region containing 47 CpGs. The studied CpGs (40-47) are shown in the bold text box. GenBank (NCBI - Access number: AY436590.1)

## Data analysis

The data were analyzed using the Chi-Square teste contingency table at a 5% significance level for sample characterization. For variables with more than two categories, p values were corrected by Bonferroni[54].

The quantitative analysis of mean methylation values of CpGs 40-47 did not follow the normal distribution, even after exponential conversion. Thus, values were dichotomized as unmethylated (zero values) and methylated (greater than zero) for analysis by Poisson regression with robust variance.

Bivariate and multivariate Poisson regression models with robust variance were performed using psychotropic drug use variable, suggestive symptoms of depression variables and mean methylation values of CpGs 40 to 47 as dependent variables.

Predictive variable in bivariate analysis with a p-value lower than 0.20 ($p < 0.20$) were inserted into the multivariate Poisson Regression model with robust variance. The backward method was used, and those variables with less significance were removed one by one from the model until all variables present in the model had statistical significance $p < 0.05$. Hosmer & Lemeshow test was used to verify the fit of the final model. The prevalence ratio (PR) with 95% confidence interval (95% CI) was used as an effect measure.

To verify the association between psychotropic drug use and methylation, a new modeling was defined for Poisson Regression with hierarchically constructed models. The following order was used: simple model (crude analysis); model 1: analysis adjusted for socioeconomic variables (sex, schooling); model 2, adjusted for socioeconomic and health variables (stress, anxiety, insomnia and self-reported health); and model 3, adjusted for socioeconomic, health and lifestyle variables (tobacco and alcohol consumption, and physical or leisure activities).

For all statistical analysis, a 5% significance level was adopted, performed using SPSS® software (version 13.0 for Windows) and Stata version 11.0.

### Ethical and legal aspects of the research

Individuals participating in the study signed a written Informed Consent Form (ICF) and this study was approved by The Ethics Committee in Research with Humans, of the Universidade Federal do Espírito Santo Health Sciences Center (CEP / CCS / UFES), under number 1,574,160, dated 6/6/2016.

## RESULTS

### Socioeconomic profile

Data obtained through the application of the questionnaires indicated that most participants were women (311 or 80.6%). Of the total population, 62.4% reported being under stress, 66.9% reported anxiety and 52.8% considered their health to be poor. Socioeconomic profile was also traced according to psychotropic drug use. The results showed a higher prevalence of psychotropic drug use among women, individuals aged 41 to 59 years, self-reported stressed and with poor self-perception of health. Psychotropic drug use was verified in 70 of 386 individuals (10.9%). Among the reported drugs are clonazepan, cloxazolam and alprazolam. Bupropion use was verified in 1%, fluoxetine/venlafaxine/paroxetine was observed in 4.7% and 1.6% used amitriptyline (table 2).

**Table 2:** Sample descriptive characteristics by psychotropic drug use and univariate Poisson regression analysis with robust variance

| Characteristic | Psychotropic drug use | | | | | | |
|---|---|---|---|---|---|---|---|
| | total | | yes | | no | | p |
| | n | (%) | n | (%) | n | (%) | |
| Sex | | | | | | | |
| Male | 75 | 19,4 | 27 | 36.0 | 48 | 64.0 | 0.001 |
| Female | 311 | 80,6 | 187 | 60.1 | 124 | 39.9 | |
| Age | | | | | | | |
| 20 to 40 years | 170 | 44.0 | 75 | 44.1 | 95 | 55.9 | 0.001 |
| 41 to 59 years | 216 | 56.0 | 139 | 64.4 | 77 | 35.6 | |
| Race | | | | | | | |
| White | 216 | 56.0 | 120 | 55.6 | 96 | 44.4 | 0.959 |
| Not White | 170 | 44.0 | 94 | 55.3 | 76 | 44.7 | |
| Years of education | | | | | | | |
| < 8 years | 179 | 46.4 | 100 | 46.7 | 79 | 45.9 | 0.874 |
| between 8 and 11 years | 141 | 36.4 | 76 | 35.5 | 65 | 37.8 | |
| Higher Education | 66 | 17.1 | 38 | 17.8 | 28 | 16.3 | |
| Income | | | | | | | |
| Non-low income (≥ $5.00/ day) | 115 | 29.8 | 20 | 28.6 | 95 | 30.1 | 0.805 |
| Low income (< $5.00/ day) | 271 | 70.2 | 50 | 71.4 | 221 | 69.9 | |
| Self-rated stress | | | | | | | |
| No | 145 | 37.6 | 15 | 21.4 | 130 | 41.1 | 0.002 |
| Yes | 241 | 62.4 | 55 | 78.6 | 186 | 58.9 | |
| Self-rated anxiety | | | | | | | |
| No | 127 | 32.8 | 39 | 52.0 | 120 | 38.0 | 0.001 |
| Yes | 259 | 66.9 | 33 | 44.0 | 196 | 62.0 | |
| Depression | | | | | | | |
| BDI-II < 10 | 202 | 52.2 | 22 | 33.3 | 180 | 62.1a | 0.001** |
| BDI-II 10-17 | 77 | 19.9 | 24 | 36.4 | 53 | 18.3b | |
| BDI-II ≥ 18 | 77 | 19.9 | 20 | 30.3 | 57 | 19.7b | |
| Not available* | 31 | 8.0 | | | | | |
| Self-rated health | | | | | | | |
| Good or very good | 204 | 52.8 | 23 | 32.9 | 181 | 57.3 | 0.001 |
| Regular or poor | 182 | 47.2 | 47 | 67.1 | 135 | 42.7 | |

**Continuation - Table 2:** Sample descriptive characteristics by psychotropic drug use and univariate Poisson regression analysis with robust variance

| Characteristic | Psychotropic drug use | | | | | | |
|---|---|---|---|---|---|---|---|
| | total | | yes | | no | | p |
| | n | (%) | n | (%) | n | (%) | |
| Tobacco consumption | | | | | | | |
| No | 355 | 92.0 | 61 | 87.1 | 294 | 93.0 | 0.101 |
| Yes | 31 | 8.0 | 9 | 12.9 | 22 | 7.0 | |
| Alcohol consumption | | | | | | | |
| No | 252 | 65.1 | 39 | 55.7 | 213 | 67.4 | 0.063 |
| Yes | 134 | 34.6 | 31 | 44.3 | 103 | 32.6 | |
| Physical activity | | | | | | | |
| Yes | 127 | 32.9 | 20 | 28.6 | 107 | 33.9 | 0.394 |
| No | 259 | 67.1 | 50 | 71.4 | 209 | 66.1 | |
| Leisure activity | | | | | | | |
| Yes | 179 | 46.4 | 31 | 44.2 | 148 | 46.8 | 0.699 |
| No | 207 | 53.6 | 39 | 55.8 | 168 | 53.2 | |
| Methylation status | | | | | | | |
| Yes | 87 | 22.5 | 9 | 10,3 | 78 | 89,7 | 0,032* |
| No | 299 | 77.5 | 61 | 20,4 | 238 | 79,6 | |
| Total | 386 | 100.00 | | 100.00 | | 100.00 | |

Poisson bivariate regression analysis with robust variance showed that psychotropic drug use was associated with many feelings addressed in the inventory, such as pessimism, past failure, irritability, social withdrawal, fatigability, insomnia, somatic concerns and loss of interest in sex. Moreover, it was found that feeling of failure, self-aversion and loss of appetite were related to NR3C1 hypermethylation, as can be observed in table 3. Methylation distribution across the population studied can be seen in supplementary figure 1 and supplementary table 1.

**Table 3:** Bivariate Poisson regression analysis with robust variance for symptoms suggestive of depression with psychotropic drug use and with methylation analysis

| BDI-II Symptoms (N=356) | Psychotopric Drug Use (N=386) | | | HYPERMETHYLATION (N=286) (Total CpG 40 to 47) | | |
|---|---|---|---|---|---|---|
| | PR | 95% CI | p | PR | 95% CI | p |
| Sadness | 1.20 | 0.99 – 1.44 | 0.051* | 1.24 | 0.86-1.80 | 0.238 |
| Pessimism | 1.24 | 1.03 – 1.49 | 0.019* | 1.17 | 0.80-1.71 | 0.403 |
| Past Failure | 1.22 | 1.01 – 1.47 | 0.031* | 1.52 | 1.05-2.20 | 0.024 |
| Loss of Pleasure | 1.08 | 0.89 – 1.30 | 0.412 | 1.24 | 0.85-1.79 | 0.254 |
| Guilty Feelings | 0.96 | 0.76 – 1.20 | 0.726 | 1.07 | 0.69-1.64 | 0.750 |
| Punishment Feelings | 1.20 | 0.97 – 1.47 | 0.082* | 1.17 | 0.76-1.80 | 0.453 |
| Self-Dislike | 0.99 | 0.81 – 1.21 | 0.955 | 1.53 | 1.06-2.22 | 0.023* |
| Self-Criticalness | 0.96 | 0.79 – 1.17 | 0.740 | 1.23 | 0.85-1.78 | 0.260 |
| Suicidal Thoughts | 1.15 | 0.90 – 1.41 | 0.236 | 1.29 | 0.80-2.10 | 0.289 |
| Crying | 1.19 | 0.99 – 1.43 | 0.061* | 1.26 | 0.87-1.83 | 0.211 |
| Irritability | 1.34 | 1.10 – 1.63 | 0.003* | 1.17 | 0.80-1.71 | 0.402 |
| Social Withdrawal | 1.23 | 1.03 – 1.49 | 0.022* | 1.38 | 0.95-2.01 | 0.083* |
| Indecisiveness | 0.96 | 0.79 – 1.17 | 0.710 | 0.70 | 0.46-1.05 | 0.092* |
| Worthlessness | 1.04 | 0.85 – 1.26 | 0.686 | 1.29 | 0.88-1.88 | 0.182* |
| Loss of Energy | 1.29 | 1.07 – 1.55 | 0.005* | 1.07 | 0.73-1.56 | 0.697 |
| Changes in Sleeping | 1.35 | 1.11 – 1.64 | 0.002* | 1.35 | 0.88-2.07 | 0.156* |
| Fatigability | 1.09 | 0.91 – 1.32 | 0.329 | 1.37 | 0.94-2.00 | 0.096* |

**Continuation - Table 3:** Bivariate Poisson regression analysis with robust variance for symptoms suggestive of depression with psychotropic drug use and with methylation analysis

| BDI-II Symptoms (N=356) | Psychotopric Drug Use (N=386) | | | HYPERMETHYLATION (N=286) (Total CpG 40 to 47) | | |
|---|---|---|---|---|---|---|
| | PR | 95% CI | p | PR | 95% CI | p |
| Loss of Appetite | 0.98 | 0.77 – 1.24 | 0.869 | 1.56 | 1.05-2.31 | 0.027* |
| Loss of Weight | 1.14 | 0.93 – 1.41 | 0.193* | 1.39 | 0.93-2.09 | 0.104* |
| Somatic Worries | 1.29 | 1.07 – 1.53 | 0.007* | 1.11 | 0.76-1.61 | 0.566 |
| Loss of Interest in Sex | 1.21 | 1.01 – 1.45 | 0.038* | 1.21 | 0.83-1.76 | 0.309 |

Depression symptoms by Beck Depression Score (BDI-II). * variables for the multivariate model *Not available (not considered in the statistical calculations). PR: prevalence ratio; 95% CI: confidence interval; p: p-value.

Psychotropic drug use was explained by irritability, difficulty at work and insomnia in multivariate model (table 4). Hypermethylation of NR3C1 gene was associated with symptoms of failure and loss of appetite (table 5). After Hosmer & Lemeshow adjustment, both showed good adherence (p> 0.05).

**Table 4:** Multivariate Poisson regression analysis with robust variance for psychotropic drug use with symptoms suggestive of depression

| Symptoms | Psychotopric Drug Use | | |
|---|---|---|---|
| | PR | 95% CI | p |
| Irritability | 1.22 | 1.00-1.50 | 0.046 |
| Loss of Energy | 1.20 | 1.00-1.45 | 0.049 |
| Changes in Sleeping | 1.25 | 1.03-1.52 | 0.022 |

* PR: prevalence ratio; 95% CI: confidence interval; p: p-value.

**Table 5:** Multivariate Poisson regression analysis with robust variance for hypermethylation e hypomethylation with symptoms suggestive of depression

| Symptoms | HYPERMETHYLATION (Mean value CpG 40 to 47) | | |
|---|---|---|---|
| | PR | 95% CI | P |
| Past Failure | 1.58 | 1.09-2.29 | 0.014 |
| Indecisiveness | 0.63 | 0.41-0.96 | 0.032 |
| Loss of Appetite | 1.52 | 1.03-2.24 | 0.034 |

* PR: prevalence ratio; 95% CI: confidence interval; p: p-value.

The univariate analysis performed by Poisson regression showed that there is an association between psychotropic drug use and methylation patterns in CpGs 40-47 (p=0,032). Thus, psychotropic drug use reduces the risk of hypermethylation by 48% in the evaluated segment.

A model was built with the insertion of confounding variables in a hierarchical way for multivariate analysis and, finally, psychotropic drug use showed an association with methylation patterns (p=0.009). The data showed that psychotropic drug use is associated with 50% reduction in NR3C1 gene methylation.

Psychotropic drug use was associated with hypomethylation when using mean methylation values of CpGs 40 to 47, shown in table 6.

**Table 6:** Association between drug use and mean methylation values of CpGs 40 to 47

| Drug class | | |
|---|---|---|
| | yes (n%) | No (n%) |
| Psychotropic drug use | 70 (18,1) | 316 (81,8%) |

| Univariate Poisson regression Crude analysis | | |
|---|---|---|
| IRR | CI 95% | P |
| 0.52 | 0.27;0.98 | 0.046 |

**Continuation - Table 6:** Association between drug use and mean methylation values of CpGs 40 to 47

| Multivariate Poisson regression adjusted for confounding variables | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Model 1 | | | Model 2 | | | Model 3 | |
| IRR | CI95% | P | IRR | CI 95% | p | IRR | CI 95% | p |
| 0.48 | 0.26;0.91 | 0.025 | 0.43 | 0.22;0.83 | 0.012 | 0.50 | 0.26;0.95 | 0.009 |

Complex sample. Univariate Poisson regression. Significance level of 5% (p <0.05). Dependent variable: Methylation(yes); Independent variables: psychotropic drug use Crude analysis and univariate Poisson regression models hierarchically adjusted for confounding factors: Model 1 - crude analysis adjusted for socioeconomic variables (gender, age and schooling); Model 2 – analysis, adjusted for socioeconomic , and health variables (stress, anxiety, insomnia and health); Model 3 – crude analysis, adjusted for socioeconomic , health , and lifestyle variables (tobacco, alcohol, physical activity and leisure). PR: prevalence ratio; 95% CI: 95% confidence interval; p: p-value.

## DISCUSSION

Modifications in NR3C1 DNA methylation was associated with life-stressing events and stress-induced pathologies or clinical severity[33]. Psychotropic drug use to control depression and other stress-induced pathologies were related to epigenetic alterations[44-46], but there are few data about association between epigenetic changes in NR3C1 gene methylation and psychotropic drug use.

Therefore, we hypothesized that there was an association between psychotropic drug use and NR3C1 gene methylation in adult individuals influenced by symptoms suggestive of depression.

Therefore, psychotropic drug use was associated with 50% reduction in NR3C1 gene methylation in multivariate analysis. Moreover, depressive symptoms such as feeling of failure and loss of appetite were related to NR3C1 DNA hypermethylation.

Methylation analysis and the relationship with psychotropic drug use were evaluated in our study population through bivariate and multivariate analyzes in Poisson regression models. Our data showed a relationship between the use of these drugs and DNA unmethylation. This relationship remained statistically significant in the three models presented, even after addition of confounding factors such as socioeconomic, health and lifestyle variables.

The literature reports an association between psychotropic drug use and changes in methylation levels, pointing to some drugs as DNA demethylating agents in various genes and repetitive regions, demonstrating that there may be global genomic demethylation associated with psychotropics drug use[13]. Cassel and colleagues highlighted GABAergic neurons as the main target cells of Mecp2 expression in response to serotonin elevating agents, and suggests that serotonin signaling increases gene silencing in post-mitotic neurons[55].

The studied population consists of individuals who frequently use the public health system, most composed of women and with a high prevalence of individuals with symptoms of stress, anxiety and depression. In a previous publication with the same casuistic, it was verified that this population is exposed to psychosocial stress, which had a high prevalence of overweight, non-communicable chronic diseases, stress and anxiety[56] and depression[50].

A prevalence of individuals with symptoms of depression assessed in the population studied by the BDI-II inventory, considering a score ≥ 18, was 19.9%. Detailed analysis of obtained scores was 48.8% of individuals with these symptoms, with a cutoff of >10, considered above the population average normally found and frequently used in general population studies of non-psychiatric population[57]. A study published by the World Health Organization in 2017 showed that 5.8% of the Brazilian population was depressed and American data from the general population show that the frequency is 5.9%[1].

In our sample, 18.1% of individuals presented continuous use of psychiatric drugs. Our data showed that psychotropic drug use was related to various depression symptoms assessed by the Beck Depression Inventory. In multivariate analysis, psychotropic drug use was related to irritability, difficulty in working and sleep disorders.

The final multivariate analysis model showed that symptoms of failure, indecision and loss of appetite associated with methylation changes remained in the model. Failure and loss of appetite were related to increase in NR3C1 DNA methylation while indecision was related to unmethylation. Interestingly, methylation and depression may not be a unique disease from the perspective of gene expression regulation, as different symptoms are related to contrary epigenetic events.

In this study samples, NR3C1 gene methylation was relatively low, corroborating to McGowan and colleagues (2009)36. Also, CpGs 16-21 and 37-38 match to transcription factor (NGFI-A) binding site of the NR3C1 1F region, which correspond to exon 17 in rats whose regulatory genomic region was evaluated by Weaver and colleagues[26]. They also demonstrated that low maternal care resulted in increased methylation of NGFI-A binding site in NR3C1 gene in hippocampal of offspring rats, leading to decreased expression[26]. However, Moser and colleagues (2007) showed no differences in methylation in the same region evaluating human healthy controls and individuals with Parkinson, Alzheimer or dementia[58]. Another study with 224 survivors of Rwandan genocide reported lower methylation at NGFI-A binding site of the NR3C1 1F gene associated with post-traumatic stress disorder[59].

The literature provides controversial results on methylation patterns in NR3C1 gene in association with depression, probably due to methodological differences, such as the 1F region chosen for analysis, exclusion or inclusion of individuals using antidepressants, sample size, among others. Na and colleagues in his study with 177 patients, had 45 patients with depression without antidepressants, reported hypomethylation at positions 46 and 47 in NR3C1 gene region 1F as related to major depressive disorder (MDD)[38]. However, Melas and colleagues, in a population-based mental health study

of 1668 individuals, with no exclusion of drug-using individuals, assessed methylation and correlated with childhood depression and adversity by observing increased methylation patterns in CpG 36 of NR3C1 gene 1F region[60]. Oberlander and colleagues, in their work with pregnant women with depression and anxiety, evaluated in two groups, with and without antidepressant use and observed hypermethylation in CpG 4739. Although controversial, these data present sufficient information to assume that different events in depression may alter DNA methylation profile in NR3C1 gene[39].

There are few studies available in the literature that allow us to understand exactly how feelings can specifically alter DNA by promoting hyper or hypomethylation. However, it is already known that specific life situations, such as those described in Argentieri and colleagues review article, might promote changes in specific CpGs for both hypermethylation and hypomethylation, suggesting that the mechanism of gene expression regulation in NR3C1 should be highly refined[30].

This study has some limitations. A cross-sectional study does not allow us to infer causality and direction of effect. The NR3C1 DNA methylation was dichotomized in below or above 0% in order to analyze the association between methylation and other variables by multivariate Poisson regression. This categorization allows some loss of sensitivity in relation to the use of the quantitative variable in percentage of the methylation profile.

## CONCLUSION

Thus, our data associate psychotropic drug use with hypomethylation in the NR3C1 gene promoter, and hypermethylation with symptoms suggestive of depression. In this context, epigenetic modification resulting from psychotropic drug use and depressive symptoms may be considered, mainly in population studies with epigenetic evaluation, where these factors may be influencing the findings of future studies.

Moreover, these findings corroborate to a better knowledge since a large world population consume psychotropic drug use. Studies such as this one, in primary care users, show the need for better attention to these individuals, since from the individual perspective; they have worse quality of life and health. From a collective perspective, because of care demand, they also end up burdening health services due to the high demand for consultations and medicines.

### Author statement

Conceptualization: JAP, FVF, ARB. Methodology: JAP, FVF, ARB, BPS, LMRBA, AMAS. Analysis: FVF. Resources: CLC, JKA, BPS, SOM, ABA, MMO, JGS, RAS, IAAM, DPS, WMB, JCCR, LOT, EBB, LBAR, LMRBA. Data Curation: FVF, BPS. Writing Original Draft: JAP, FVF, ARB, AMAS. Writing - Review & Editing: JAP, FVF, ARB, LMRBA, AMAS. Supervision: AMAS. Funding acquisition: AMAS.

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### Conflict of interest statement

The authors wish to confirm that there is no conflict of interest associated with this publication.

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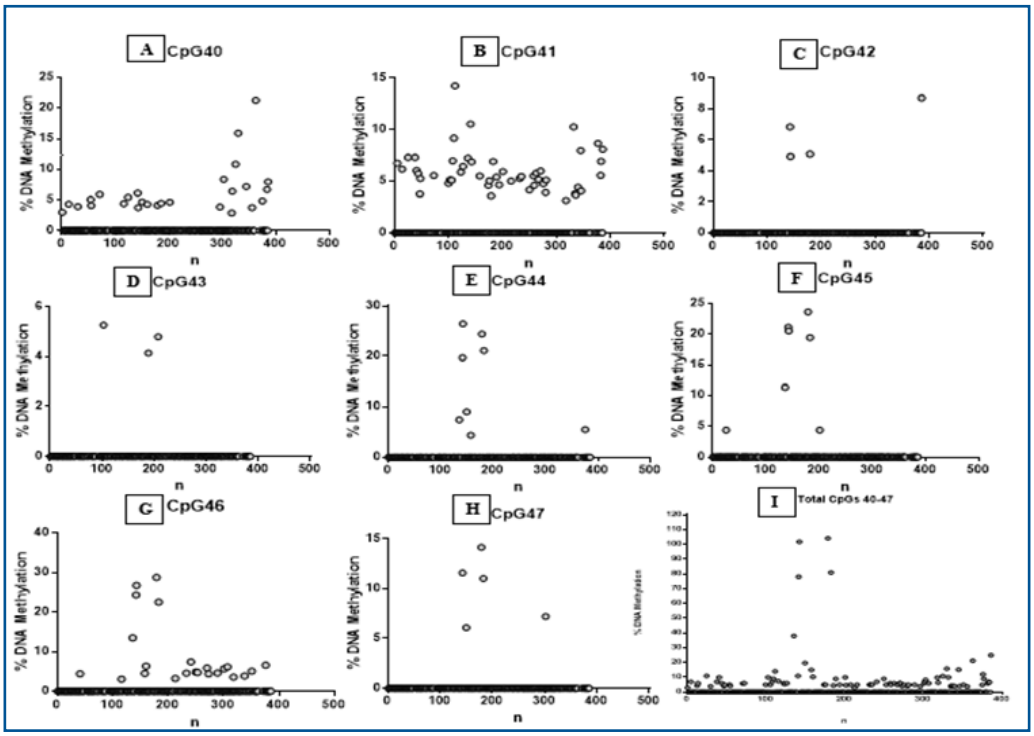


**Supplementary Figure 1.** Scatter plots of the methylation profile from individuals participating in the study. Hypomethylated individuals are adhered to the x-axis (methylation values equal 0) and individuals with methylation percentages above zero are dispersed in the graph with percentage values ranging from 0.4 to 29%. The graphs from A to H show the dispersions by individual CpGs at positions 40 to 47. And graph I shows the dispersion considering the entire segment evaluated. In the composition of the graphs, hypermethylated individuals (n = 87) and hypomethylated individuals (286) were considered.

**Supplementary Table 1:** Methylation pattern for individuals with metyhlation levels above zero

| **CpG (n=286)** | **METHYLATION (CpG 40 to 47)** | | |
|---|---|---|---|
| | **Methylation prevalence % (n)** | **Median percentage of methylation** | **Values: minimum-maximum** |
| CPG 40 | 9.1 (27) | 4.7 | 2.9-21.3 |
| CPG 41 | 17.2 (51) | 5.5 | 3.0-14.0 |
| CPG 42 | 1.4 (4) | 6.0 | 5.0-9.0 |
| CPG 43 | 1.0 (3) | 4.8 | 4.0-5.0 |
| CPG 44 | 2.8 (8) | 14.4 | 4.0-26.0 |
| CPG 45 | 2.4 (7) | 19.4 | 4.0-24.0 |
| CPG 46 | 8.0 (23) | 5.1 | 3.0-29.0 |
| CPG 47 | 1.7 (5) | 11.3 | 6.0-14.0 |
| CPG40-47 | 22.5 (87) | 0.7 | 0.4-12.9 |

**Resumo**

**Introdução:** os distúrbios psiquiátricos tornaram-se um problema global que leva milhões de pessoas ao uso de medicamentos psicotrópicos. Os efeitos dessas substâncias são amplamente conhecidos quanto à tolerância e dependência química, porém, do ponto de vista epigenético, ainda são pouco conhecidos.

**Objetivos:** avaliar a associação entre o uso de drogas psicotrópicas, metilação do gene NR3C1 e sua relação com sintomas sugestivos de depressão em indivíduos entre 20 a 59 anos usuários da rede pública de saúde.

**Método:** 385 voluntários de 20-59 anos, usuários do Sistema Único de Saúde brasileiro foram recrutados para avaliação das condições socioeconômicas, de saúde e de estilo de vida em estudo transversal. O BDI-II avaliou sintomas sugestivos de depressão e o pirosequenciamento avaliou a metilação do DNA de NR3C1. Modelo de regressão de Poisson bivariado e multivariado com variância robusta ($p < 0,05$) avaliou a associação entre o uso de drogas psicotrópicas e metilação do gene NR3C1.

**Resultados:** sintomas depressivos específicos como irritabilidade, insônia e fadiga foram associados ao uso de medicamentos psicotrópicos. Sintomas de fracasso passado, indecisão e perda de apetite foram associados a padrões de hipermetilação nos CpGs 40 a 47 do gene NR3C1. Além disso, o uso de psicofármacos está associado à redução de 50% na metilação do gene NR3C1, por meio de modelo ajustado com variáveis de confusão socioeconômicas, de saúde e estilo de vida.

**Conclusão:** o uso de drogas psicotrópicas e sintomas específicos depressivos foram associados a alterações na metilação do DNA de NR3C1.

**Palavras-chave:** Metilação, NR3C1, uso de drogas psicotrópicas, sintomas depressivos.