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JÚLIA DE ASSIS PINHEIRO

FATORES DETERMINANTES DA METILAÇÃO DO GENE *NR3C1* EM USUÁRIOS DO SISTEMA ÚNICO DE SAÚDE NO MUNICÍPIO DE ALEGRE/ES

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia (RENORBIO) do Ponto focal da Universidade Federal do Espírito Santo, como requisito parcial para obtenção do título de Doutor em Biotecnologia.

Orientador: Adriana Madeira Álvares da Silva

Coorientador: Flávia Vitorino Freitas

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BANCA EXAMINADORA

Prof.ª Dr.ª Adriana Madeira Álvares da Silva
Universidade Federal do Espírito Santo (UFES)
RENORBIO
(Orientadora)

Prof.ª Dr.ª Flávia Vitorino Freitas
Universidade Federal do Espírito Santo
(Coorientador)

Prof.ª Dr.ª Flávia de Paula
Universidade Federal do Espírito Santo (UFES)
RENORBIO
(Examinadora Interna)

Prof.ª Dr.ª Flavia Imbroisi Valle Errera
Universidade Federal do Espírito Santo (UFES)
RENORBIO
(Examinadora Interna)

Prof.ª Dr.ªLidia Maria Rebolho Batista Arantes
Hospital de Câncer de Barretos
(Examinador Externo)

Prof. Dr. Heberth de Paula
Universidade Federal do Espírito Santo
(UFES)
(Examinador Externo)

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LISTA DE ABREVIATURAS

5-meC: 5-metilcitosina
ACTH: Hormônio Adrenocorticotrópico
BDI-II: Inventário de Depressão de Beck-II
CCS: Centro de Ciências da Saúde
CEP: Comitê de Ética em Pesquisa com Seres Humanos
CH_3: grupo metil
CpG: Citosina-p-Guanina
CRH: Hormônio Liberador de Corticotropina
DNA: Ácido desoxirribonucleico
DNMTs: DNA metiltransferases
GR: Receptor de Glicocorticoide
HPA: eixo Hipotálamo-Pituitária-Adrenal
IMC: Índice de Massa Corporal
miRNA: micro Ácido ribonucleico
NF-κB: Fator nuclear kappa B
OMS: Organização Mundial da Saúde
PCR: Reação em Cadeia da Polimerase
PPSUS: Projeto para o Sistema Único de Saúde
PTSD: Transtorno de Estresse Pós-Traumático
RENORBIO: Rede Nordeste de Biotecnologia
RP: Razão de prevalência
SAH: S-adenosina-L-homocisteína
SAM: S-adenosina-L-methionina
SFC: Síndrome da Fadiga Crônica
SISVAN: Sistema de Vigilância Alimentar e Nutricional
SUS: Sistema Único de Saúde
TCLE: Termo de Consentimento Livre e Esclarecido
UFES: Universidade Federal do Espírito Santo

RESUMO

O gene *NR3C1* está relacionado ao estresse psicossocial e possui a região promotora regulada por metilação frente à exposição ambiental. A literatura se refere a metilação neste gene como relacionada à fome gestacional, estresse de guerra e depressão. Assim, este estudo teve como objetivo avaliar as alterações epigenéticas de metilação na região promotora do receptor do glicocorticoide, nas CpGs 40 à 47 da região 1F por pirosequenciamento, em usuários do SUS e correlacionar com características socioeconômicas, de saúde, cortisol, vitamina D, estilo de vida e depressão. Os resultados revelaram uma correlação entre hipermetilação do *NR3C1* região 1F com depressão, e hipometilação com status nutricional, cortisol elevado, uso de álcool e uso de medicamentos psicotrópicos nos 386 indivíduos avaliados. Os resultados indicam que a relação ambiente e epigenética é de grande complexidade e que a relação entre genótipo-ambiente e o desfecho fenotípico pode ser mais refinada do que se pensava; ela depende não somente do evento estressor, mas também do tipo de evento, e pode resultar em consequências clínicas diversas. Nesse sentido, a presença ou ausência de metilação não deve ser vista apenas como fator de risco à saúde ou de proteção, mas como resultado e resultante da interação do indivíduo com o meio ambiente, no sentido da sua adaptação às condições à qual está exposto.

Palavras-chave: Metilação do DNA; receptor do glicocorticoide, pirosequenciamento, Sintomas sugestivos de depressão, estilo de vida.

ABSTRACT

The *NR3C1* gene is related to psychosocial stress and has the promoter region regulated by methylation in the face of environmental exposure. The literature refers to methylation in this gene as related to gestational hunger, war stress and depression. Thus, this study aimed to assess epigenetic methylation changes in the promoter region of the glucocorticoid receptor, in CpGs 40 to 47 in region 1F by pyrosquencing, in SUS users and to correlate with socioeconomic, health, cortisol, vitamin D characteristics. lifestyle and depression. The results revealed a correlation between hypermethylation of *NR3C1* region 1F with depression, and hypomethylation with nutritional status, elevated cortisol, use of alcohol and use of psychotropic drugs in the 386 individuals evaluated. The results indicate that the relationship between environment and epigenetics is of great complexity and that the relationship between genotype-environment and the phenotypic outcome may be more refined than previously thought; it depends not only on the stressful event, but also on the type of event, and can result in different clinical consequences. In this sense, the presence or absence of methylation should not be seen only as a risk factor for health or protection, but as a result and result of the individual's interaction with the environment, in the sense of his adaptation to the conditions to which he is exposed

Keywords: DNA methylation; glucocorticoid receptor; pyrosequenching, Symptoms suggestive of depression, lifestyle.

SUMÁRIO

1. INTRODUÇÃO

O papel das alterações epigenéticas na regulação do genoma tem sido abordado de forma abrangente (SHANKAR et al., 2016). Diversas áreas de pesquisa foram estabelecidas para compreender essas modificações epigenéticas, metilação do DNA, modificações histônicas, remodelação da cromatina e microRNA (miRNA)(GAL-YAM et al., 2008).

A definição original de epigenética incorpora fenômenos herdáveis, mas reversíveis, que afetam a expressão gênica sem alterar os pares de bases do DNA (LIEB et al., 2006). Mesmo que nem todos os traços epigenéticos listados acima tenham demonstrado frente à hereditariedade, todos eles podem alterar a transcrição gênica sem modificação da sequência genética subjacente (TAMMEN; FRISO; CHOI, 2013). Como esses padrões epigenéticos também podem ser afetados pelo ambiente de um organismo, eles servem como uma ponte importante entre experiências de vida e os fenótipos (ESTELLER, 2008).

Os padrões epigenéticos podem mudar ao longo da vida, por uma experiência de vida precoce, exposição ambiental ou estado nutricional (LACAL; GHAI; MAHARAJ, 2018). Marcas epigenéticas influenciadas pelo ambiente podem determinar nossa aparência, comportamento, resposta ao estresse, suscetibilidade a doenças e até a longevidade (KADER; GHAI, 2017).

A interação entre tipos de modificações epigenéticas em resposta à fatores ambientais e como as sugestões ambientais afetam os padrões epigenéticos elucidará ainda mais como a transcrição gênica pode ser alterada afetivamente (LEE et al., 2018).

Tendo em vista a carência de resultados sobre as influências dos fatores ambientais e da adaptação dos organismos frente à adversidade e seu impacto no epigenoma, o presente estudo teve como objetivo estudar as alterações epigenéticas de metilação na região promotora do receptor do glicocorticoide em usuários do SUS em relação aos sintomas depressivos, cortisol, vitamina D, estilo de vida, status socioeconômico, status nutricional, condições de saúde, em usuários do SUS no município de Alegre.

2. REVISÃO DE LITERATURA

2.1 Epigenética

Apesar do genoma eucariótico ser o mesmo em todas as células somáticas de um organismo, existem estruturas e funções específicas do genoma que se distinguem em tipos celulares diferentes (TAMMEN; FRISO; CHOI, 2013).

Essas diferenças são devidas aos padrões de expressão de genes específicos dos tecidos e órgãos e são determinadas durante a diferenciação celular na embriogênese, mecanismo conhecido por destinação celular. Desta forma, alguns genes são expressos ou silenciados durante esse período e seguem mantendo o mesmo padrão por toda a vida do indivíduo. No entanto, para outros genes, esses padrões de expressão podem ser afetados pela exposição ambiental ao longo da vida, acarretando mudanças fenotípicas e de expressão (ESTELLER, 2008).

Tanto as assinaturas de expressão de genes específicos de tipos celulares, quanto as mudanças do padrão de expressão mediadas pelo ambiente podem ser explicadas por uma rede complexa de modificações no DNA, proteínas histonas e graus de empacotamento de DNA, chamadas genericamente de “marcas epigenéticas” (FRAGA et al., 2005).

O termo Epigenética surgiu em 1942 e foi definido como mudanças hereditárias na expressão de genes sem alteração da sequência de bases do DNA (LIEB et al., 2006). Padrões epigenéticos podem ser hereditários e preservados durante a divisão celular; no entanto, fatores extrínsecos, hormonais, metabólicos e ambientais contribuem com modificações epigenéticas que podem alterar o risco de algumas doenças (KADER; GHAI, 2017).

Os mecanismos epigenéticos, essenciais na regulação normal da função celular (LEE et al., 2018), têm despertado interesse da ciência nos últimos anos devido a sua relação direta com o estilo de vida, consumo de nutrientes e até com fatores emocionais, que podem influenciar o epigenoma, alterando o risco de doenças. No entanto, também se mostram promissores na elaboração de estratégias de

prevenção e tratamento de doenças baseado no controle de metilação (CHOI; FRISO, 2010).

Embora vários mecanismos epigenéticos tenham sido identificados, existem três principais que são conhecidos por regular a expressão gênica (CHOUDHURI, 2011). Estes incluem: a metilação do DNA com a geração do produto de ligação covalente metil-citosina da sequência do DNA; alteração da estrutura da cromatina pela modificação pós-traducional das proteínas histonas ou não-histonas; e pequenos microRNAs não codificantes (miRNAs) que modulam a expressão gênica inibindo a tradução ou causando degradação de miRNAs específicos (GAL-YAM et al., 2008). O conjunto dessas “marcas epigenéticas” forma o Epigenoma.

2.2 Metilação do DNA

A metilação do DNA foi a primeira modificação epigenética identificada no DNA e é a mais estudada atualmente. São “marcas epigenéticas” determinantes do desenvolvimento normal do genoma humano (KADER; GHAI, 2017).

O papel da metilação do DNA na regulação diferencial da expressão gênica é determinado pelo *imprinting* genético, ou impressão digital do DNA, a forma como ele está expresso, o que é muito particular em cada tecido e diferencialmente herdado dependente da origem materna ou paterna para algumas regiões do genoma. O *imprinting* e o silenciamento de genes têm ajudado a entender as relações entre genótipo e fenótipo (OAKES et al., 2016).

A metilação do DNA ocorre principalmente em dinucleotídeos citosina (C) seguido por uma guanina (G), referidas como Citosina-p-Guanina ou CpG (SCHULTZ et al., 2015; ADAMS, 2019). Embora as sequências CpG estejam distribuídas de forma desigual pelo genoma humano, elas são frequentemente enriquecidas em promotores de genes e muitas vezes chamadas de ilhas CpG (BIRD, 2002).

Existem dentro do genoma humano aproximadamente 30 milhões de dinucleotideos CpG não metilados, hemi-metilados ou abundantemente metilados, variando de acordo com a região do cromossomo, alelos, tipo de célula ou fase de desenvolvimento (REINIUS et al., 2012).

A maioria das ilhas de CpG são metiladas em resíduos de citosina por um grupo de enzimas conhecidas como DNA metiltransferases (DNMTs), entretanto, as ilhas de CpG dentro de promotores de genes tendem a ser protegidas da metilação preservando o padrão de expressão daquele gene naquele tipo celular ou tecido (SHANKAR et al., 2016).

A reação de metilação é mediada por DNMTs, (DNMT1, DNMT3A, DNMT3B, e DNMT3L) (TAMMEN; FRISO; CHOI, 2013) que introduzem, através de uma ligação covalente, um grupo metil (CH_3) derivado da S-adenosina-L-methionina (SAM) na posição C5 do anel de citosina que precede a guanina na sequência dinucleotídica CpG, formando 5-metilcitosina (5-meC) e S-adenosina-L-homocisteína (SAH) (SHANKAR et al., 2016). Evidências sugerem que a hipermetilação das ilhas CpG está associada ao silenciamento epigenético e a hipometilação do DNA promove a transcrição do gene (CHEN et al., 2017).

No controle da expressão gênica por metilação, também pode haver a retirada dos radicais metil promovendo a desmetilação do DNA. Essa remoção da metilação do DNA tem sido observada particularmente na linha germinativa e embriogênese precoce (LI; ZHANG, 2014).

A eliminação dos padrões de metilação permite que o modelo da cromatina reverta para um estado menos diferenciado, caracterizado por baixos níveis de metilação do DNA o que é necessário na reprogramação celular. Desta forma, a partir da embriogênese precoce, ocorre progressivamente a metilação durante a formação dos tecidos e diferenciação celular (HILL; AMOUROUX; HAJKOVA, 2014; LEITCH et al., 2013).

A desmetilação do DNA é um componente importante do “apagamento” ou retirada de “marcas” da impressão epigenética dos pais, porém algumas marcas não podem ser facilmente apagadas sendo transmitidas para as futuras gerações no sentido de “adaptação ambiental” (KAWASAKI et al., 2015).

Existem genes específicos que se utilizam da metilação como forma primária da regulação da expressão gênica e são reconhecidos pela vasta região reguladora rica em CpGs, algumas vezes dispostas de maneira bastante complexa e ainda não completamente conhecidas (MOORE; LE; FAN, 2013).

2.3 Gene *NR3C1* Receptor de Glicocorticoide (GR)

O gene *NR3C1* pertence à subfamília do Receptor Nuclear 3, Grupo C, Membro 1. É um gene de codificação de proteínas, que codifica o receptor de glicocorticoides (GR) que é ativado pelo cortisol e regula a 11β-hidroxiesteróide desidrogenase tipo 2 (11b-HSD-2) (CHEN et al., 2017).

Esse gene tem mais de 150 kb de comprimento e está localizado no cromossomo 5q31-32 (TURNER et al., 2014). Contém 17 exons, oito codificantes, 2 a 9 e nove não-codificantes localizados em uma região de 3 kb no promotor do gene. Sete desses exons não-codificantes são agrupados ao longo de uma mesma ilha CpG e enumerados como 1D, 1J, 1E, 1B, 1F, 1C e 1H, que antecedem a região de início da transcrição +1, localizada no exon 2 (PALMA-GUDIEL et al., 2015).

O gene *NR3C1* codifica uma proteína que está envolvida na regulação do eixo hipotálamo-pituitária-adrenal (HPA) e sua expressão foi encontrada diminuído no cérebro *post mortem* em pacientes com depressão (WEBSTER et al., 2002).

A regulação do *NR3C1* é complexa e fortemente influenciada por fatores ambientais. Em muitos casos, a metilação do DNA está associada ao silenciamento transcricional e inibição do eixo HPA (BRENET et al., 2011).

As adversidades da infância na forma de abuso (MCGOWAN et al., 2009), maus tratos (TYRKA et al., 2012), exposição à depressão materna (OBERLANDER et al., 2008) e separação materna (MCGOWAN et al., 2011; WEAVER et al., 2004) têm sido associadas à hipermetilação de DNA na região 1F do *NR3C1* (MELAS et al., 2013). Alterações de metilação do DNA na região promotora do exon 1F do gene *NR3C1* pode ser um mecanismo potencial decorrente das experiências ambientais (VANGEEL et al., 2018).

A hipometilação de *NR3C1*-1F foi encontrada em células mononucleares do sangue de veteranos de combate com transtorno de estresse pós-traumático (PTSD), que foi adicionalmente associada à resposta do cortisol após testes de administração de dexametasona (YEHUDA et al., 2015).

Além disso verificou-se que a resposta do eixo HPA foi significativamente associada aos níveis de metilação do DNA no *NR3C1*-1F em uma amostra de estudo de 76

pacientes do sexo feminino com Síndrome da Fadiga Crônica (SFC) que apresentaram hipometilação significativa de *NR3C1*-1F no sangue total em comparação com controles femininos saudáveis (VANGEEL et al., 2015).

Além disso, em roedores, mães com comportamento de lambedura dos filhotes alteravam os níveis de metilação do DNA no GR no hipocampo promovendo hipometilação. Por sua vez, isto alterou as respostas ao estresse e reações a novos ambientes na descendência (WEAVER et al., 2004).

Há relatos que evidenciam de alteração na metilação do DNA em estresse pré-natal materno. Assim, em situações de abandono ou morte do cônjuge pode haver o nascimento de crianças com baixo peso, maior risco de doenças psiquiátricas e alterações de padrão de metilação, tanto no recém-nascido quanto na placenta (MUELLER; BALE, 2008).

2.4 Estresse e eixo Hipotálamo-Pituitária-Adrenal (HPA)

O estresse psicossocial é um dos principais contribuintes para a morbidade e mortalidade nas populações atualmente, entretanto não gera apenas custos para o indivíduo, mas também para a saúde pública.

Atualmente o estresse psicológico tem sido relatado como estresse ocupacional, econômico, estresse associado à discriminação racial, ansiedade e depressão (HASSOUN et al., 2015; LIU et al., 2017) e com isso tem sido fator de risco para doenças cardiovasculares, câncer, distúrbios imunológicos e doenças crônicas não transmissíveis (COHEN et al., 2017; FREITAS et al., 2018).

O estresse pode afetar indiretamente a saúde, aumentar a frequência com que indivíduos desenvolvem comportamentos prejudiciais à saúde, como a ausência de alimentação saudável, hábito etilista e tabagista, bem como a diminuição da frequência com que se envolvem em atividades físicas e de lazer (MILAS et al., 2019)

O estresse pode ser subdividido em agudo e crônico e, embora o estresse intermitente agudo possa ser essencial para uma adaptação bem sucedida às mudanças naturais e aos ambientes sociais novos, o estresse excessivo crônico

promove um alto risco de consequências prejudiciais para a saúde (BAKUSIC et al., 2017).

O estresse é uma condição da interação corpo-mente e um fator importante para incidência de doenças, porém estas se manifestam de forma particular nos indivíduos que podem ser mais ou menos suscetíveis ao estresse (MCEWEN, 2006). Os hormônios associados ao estresse protegem o corpo a curto prazo e promovem sua adaptação ao ambiente, mas a longo prazo provocam alterações fisiológicas que podem levar ao aparecimento de doenças (SAPOLSKY, 2000).

O cérebro é o órgão chave do estresse, através dele ocorre a determinação do que é ameaçador e, portanto, estressante. Ele também determina as respostas fisiológicas e comportamentais do organismo. As regiões do cérebro, como o hipocampo, a amígdala e o córtex pré-frontal, respondem ao estresse agudo e crônico por meio de remodelação estrutural, que altera as respostas comportamentais e fisiológicas (SHELINE; GADO; KRAEMER, 2003).

O hipocampo é uma área do cérebro importante na modulação do eixo HPA, nosso principal sistema de resposta a estresse (ANACKER et al., 2011). Após a exposição ao estresse, o núcleo paraventricular do hipotálamo ativa a hipófise anterior que secreta o hormônio liberador de corticotropina (CRH) que age na hipófise anterior para estimular a síntese e a liberação do hormônio adrenocorticotrópico (ACTH) para as glândulas supra-renais, que liberam glicocorticoides como o cortisol (OAKLEY; CIDLOWSKI, 2013).

Nos seres humanos, o cortisol é distribuído sistemicamente e executa uma ampla gama de funções envolvendo os sistemas imunológico, digestivo e endócrino e a auto-regulação do eixo HPA. Como molécula lipofílica, o cortisol atravessa a membrana celular por difusão passiva e liga-se ao receptor do glicocorticoide, ocorre então a ativação do GR, que é a chave para um indivíduo lidar adequadamente com o estresse (PALMA-GUDIEL et al., 2015).

Após essa ligação o complexo cortisol-GR atravessa a membrana nuclear seguindo dois possíveis caminhos, a transativação ou a transrepressão de uma série de genes. A transativação está relacionada à expressão da maioria dos genes da via do eixo HPA, consequentemente promovendo uma “up regulation” da síntese de

proteínas. Já a transrepressão está relacionada à inibição da expressão de genes da via de inflamação regulados pelo fator nuclear kappa B (NFkB).

Desta forma, o complexo do receptor associado ao glicocorticóide age como um fator de transcrição nuclear se ligando aos genes alvo em sítios específicos ativando ou inibindo a expressão genica dos genes regulados pelo eixo HPA. De outro lado ele também age como um anti-inflamatório através da transrepressão do NFkB (ARGENTIERI et al., 2017; MILAGRO et al., 2013)

Um número crescente de estudos identificou a metilação do DNA de genes dentro do eixo HPA como um dos principais mecanismos de regulação à adaptação ao meio ambiente físico e psicossocial estressantes, com consequentes alterações na produção de glicocorticoides (DASKALAKIS; YEHUDA, 2014; NEEDHAM et al., 2015; PALMA-GUDIEL et al., 2015; ZANNAS et al., 2016). O estresse psicossocial crônico tem sido associado com a hipermetilação do gene *NR3C1* (WITZMANN et al., 2012).

CAPÍTULO 1: PSYCHOTROPIC DRUG USE AND SUGGESTIVE DEPRESSION SYMPTOMS ASSOCIATED WITH *NR3C1* METHYLATION

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PSYCHOTROPIC DRUG USE AND SUGGESTIVE DEPRESSION SYMPTOMS ASSOCIATED WITH *NR3C1* METHYLATION

Authors

Júlia de Assis Pinheiro[a], Flávia Vitorino Freitas[a f], Aline Ribeiro Borçoi[a], Catarine Lima Conti[a], Juliana Krüger Arpini[b], Bruna Pereira Sorroche[c], Suzanny Oliveira Mendes[a], Anderson Barros Archanjo[a], Mayara Mota de Oliveira[a], Joaquim Gasparini dos Santos[a], Rafael Assis de Souza[d], Ivana Alece Arantes Moreno[a], Dirceu Pereira dos Santos[e], Wagner Miranda Barbosa[f], José Claudio Casali-da-Rocha[g], 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]**

Affiliations

a Biotechnology/Renorbio Graduate Program, Federal University of Espirito Santo, ES, Brazil.

b Department of Biology, Federal University of Espirito Santo, Alegre, ES, Brazil.

c Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos – São Paulo, Brazil.

d Department of Agronomia, Federal University of Espirito Santo, Alegre, ES, Brazil.

e Fluminense Federal Institute, Campos dos Goytacazes, RJ, Brazil.

f Pharmacy and Nutrition Department, Federal University of Espirito Santo, Alegre, ES, Brazil.

g Hospital Erasto Gardner, Curitiba, PR, Brazil.

h Department of Medicina Veterinária, Federal University of Espirito Santo, Alegre, ES, Brazil.

i Psychology Graduate Program, Federal University of Espirito Santo, Vitoria, ES, Brazil.

***Corresponding Author:** lirebolho@hotmail.com (LMRB Arantes)

** **Corresponding Author**: adriana.biomol@gmail.com (A.M. Alvares-da-Silva)

Address: Alto Universitário s/n

Departamento de Biologia, Alegre – ES, Brazil

Zipcode: 29500000

Abstract

Psychiatric disorders have become a global problem which 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, mainly in populational studies. Therefore, the present study evaluated the association between psychotropic drug use, *NR3C1* gene methylation and its correlation with symptoms suggestive of depression in individuals assisted in the public health system. Our results showed that specific symptoms assessed through Beck's BDI-II inventory, such as irritability, insomnia and fatigability were associated with psychotropic medications use, as well as symptoms of past failure, indecision and loss of appetite were associated with methylation patterns in CpGs 40 to 47 of *NR3C1* gene. In this context, epigenetic changes resulting from psychotropic drug use and depressive symptoms must be considered, as they can culminate in different effects of gene expression, not yet fully known, such as physiological changes and changes in the effect of the medication.

Key words: Stress, Lifestyle, Glucocorticoid receptor (GR), Depressive symptoms

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 (WHO,2017), which main diseases are depression, bipolar affective disorder and schizophrenia (GOMES et al., 2019). Depression is a global public health concern, as 17% of the world's general population exhibits comorbidities (KANG et al., 2018). In Brazil, this major depressive disorder has a prevalence of approximately 20% in São Paulo and 17% in Rio de Janeiro (RIBEIRO et al., 2013).

Considering the prevalence and relevance of mental disorders, psychotropic drugs are an important tool in the treatment and control of various psychopathological conditions. 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 (BOYD et al., 2015). 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%) (ESTANCIAL FERNANDES et al., 2018).

Brazil is currently the seventh country most selling psychotropic drugs in the world according to the National Agency of Sanitary Surveillance (ANVISA). Clonazepam was the most selling psychotropic drug in Brazil from 2007 to 2010 (ANVISA).

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 (GRIFFIN et al., 2013).

Studies have shown that epigenetic changes and consequent altered gene expression may result from drugs use (CSOKA; SZYF, 2009; MINUCCI; PELICCI, 2006; YOO; JONES, 2006). A potential epigenome alteration should be considered due to the side effects and impact on treatment (CSOKA; SZYF, 2009; MINUCCI; PELICCI, 2006; YOO; JONES, 2006). Thus, some drugs can act as an additional factor in the modulation of epigenetic mechanisms (LÖTSCH et al., 2013), as

antiepileptic drugs that modify histones via direct chemical interaction with histone deacetylase (GOTTLICHER, 2001), as well as the cannabinoids and opiates that trigger DNA hypermethylation (DOEHRING et al., 2013; PARADISI et al., 2008).

The use of medications for psychiatric disorders treatment has also been linked to epigenetic changes (OVENDEN et al., 2018), since the role of epigenetic changes in epigenome regulation has been comprehensively addressed worldwide (SHANKAR et al., 2016) with in-depth study of several modifications, including DNA methylation, histone modifications, chromatin remodeling and microRNA (GAL-YAM et al., 2008).

DNA methylation is a regulation mechanism of gene expression widely known(CHEN et al., 2017). However, it has recently been described as the "modus operandi" of environment adaptation process, a rapid response to exposure events (VIDAKI; DANIEL; COURT, 2013). 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 (MOORE; LE; FAN, 2013).

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 (KELLER; HAN; YI, 2016; MOORE; LE; FAN, 2013; YANG et al., 2014).

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 (NANTHARAT et al., 2015; WEAVER et al., 2004). However, the literature reports that among other events or conditions, stressors have already been described as related to hypomethylation in the same GR region (TYRKA et al., 2016). Animal studies have evaluated methylation events directly in the hypothalamus (ALT et al., 2010; MCGOWAN et al., 2011), while human studies have evaluated blood methylation events by their homology observed in different tissues with equivalent expression (ARGENTIERI et al., 2017).

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 5q31-32 (TURNER et al., 2014). 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 (PALMA-GUDIEL et al., 2015). 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 (DASKALAKIS; YEHUDA, 2014; PALMA-GUDIEL et al., 2015).

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 (BRENET et al., 2011; BUSTAMANTE et al., 2016; MCGOWAN et al., 2009; MURGATROYD et al., 2015; NA et al., 2014; OBERLANDER et al., 2008; PALMA-GUDIEL et al., 2015; PERROUD et al., 2011; PROVENÇAL; BINDER, 2015; RADTKE et al., 2011; VAN DER KNAAP et al., 2014; WEAVER et al., 2004).

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 (DASKALAKIS; YEHUDA, 2014). Furthermore, psychotropic drug use to control stress and 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 (CHMIELEWSKA et al., 2019; MOLENDIJK et al., 2011; OKADA et al., 2014).

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

Material and methods

Ethics

This is a cross-sectional study carried out with users of the Brazilian Public Unified Health System (SUS) in a southeastern municipality (Alegre-ES) in Brazil. The study population was composed of individuals living in urban and rural areas and

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. Individuals participating in the study signed a written Informed Consent Form (ICF).

Sample and population characteristics

This study was made of a convenient sample of 386 individuals between 20 and 59 years old, users of the Brazilian Primary Health Care Units. Based on SUS individual registration forms, data were collected through individual interviews that evaluated socioeconomic, health and lifestyle conditions. Low-income was defined by a per capita income/day less than $5 (five American dollars) (NERI, 2008).

Factors such as living habits and its relation to alcohol and tobacco consumption in the present and the past, leisure activity and physical activity, in weekly frequency; gender, self-reported race, age and schooling (less than 8 years of study, 8 to 11 years of schooling, and university higher education); self-perception of health, considering good or bad health, self-reported stress and anxiety; besides the use of medications, which were grouped in similar classes.

The symptoms suggestive of depression were investigated through the application of the Beck-II Depression Inventory (BDI-II) (JACKSON-KOKU, 2016). The values obtained were adequate to the total scores categorized according to the regrouping used for non-psychiatric population according to Gomes-Oliveira and colleagues (GOMES-OLIVEIRA et al., 2012) (BDI-II <18) and symptoms suggestive of depression (BDI-II ≥ 18)(WANG; GORENSTEIN, 2013).

Blood analysis

For *NR3C1* gene methylation analysis, were evaluated 286 patients' peripheral blood, collected after the person had fasten for at least eight hours. Whole blood leukocytes DNA extraction was performed using the Salting-Out method with saline precipitation, according to Salazar and colleagues (SALAZAR et al., 1998). NanoDrop® was used to verify DNA quality and concentration at wavelength λ = 260 and 280 nm, 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 (COLELLA et al., 2003; TOST; DUNKER; GUT, 2003). 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.

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

PCR Primer		**PCR conditions**		
Forward	5'-TTTTTTTTTTGAAGTTTTTTTA-3'	95 ºC	(14'30'')	
Reverse	5'-BIOTIN-CCCCCAACTCCCCAAAAA-3'	94 ºC	(30'')	45 cycles (410bp)
		50 ºC	(30'')	
		72 ºC	(30'')	
		72 ºC	(10')	
		4 ºC	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			

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

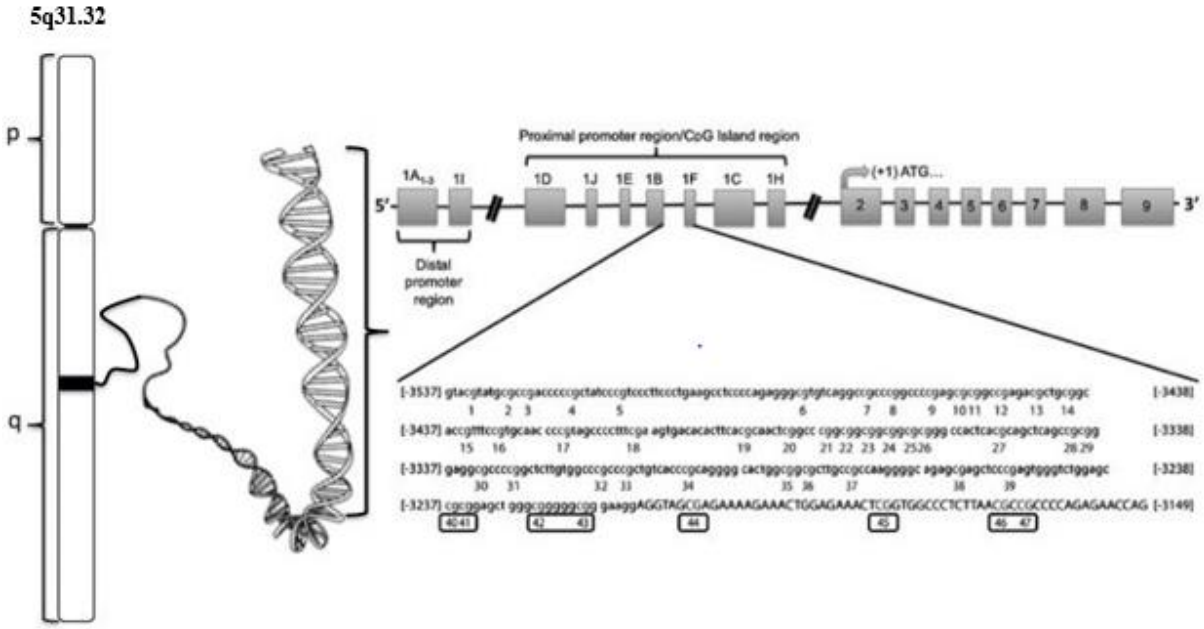

Figure 1. *NR3C1* 1F region containing 47 CpGs. The studied CpGs (40-47) are shown in the bold text box. GenBank (NCBI - Access number: AY436590.1).

Statistical 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 (FIELD, 2011).

The quantitative analysis of methylation did not follow the normal distribution, even after exponential conversion, so that the methylation data were dichotomized, using mean methylation values of CpGs 40-47. Thus, values greater than zero were categorized as methylated and zero values as unmethylated.

Bivariate analyses were performed using Poisson regression models with robust variance, with the dependent variable being psychotropic drug use and, as explanatory variables suggestive symptoms of depression (variables related to the 21 questions in the Beck BDI-II inventory). Then, new bivariate analyzes were performed with mean methylation values of CpGs 40 to 47 as the dependent variable.

Predictive variable 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 (greater p value) were

removed one by one from the model. The procedure was repeated 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.

Variables chosen for univariate Poisson regression models were: socioeconomic variables including sex, schooling (years of study); health variables such as stress, anxiety, insomnia and self-reported health; lifestyle with evaluation of tobacco and alcohol consumption, and physical or leisure activities, in weekly frequency.

To verify the association between psychotropic drug use and methylation, a new modeling was defined for Poisson Regression with hierarchically constructed models as follows: simple model (crude analysis); Model 1: analysis adjusted for socioeconomic variables; Model 2, adjusted for socioeconomic and health variables; and Model 3, adjusted for socioeconomic, health and lifestyle variables.

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

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. Characteristics of the sample by psychotropic drug use

Characteristic	Total		PSYCHOTROPIC DRUG USE				*p*
			Yes		No		
	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	62.1[a]	**0.001****
BDI-II 10-17	77	19.9	24	36.4	53	18.3[b]	
BDI-II ≥ 18	77	19.9	20	30.3	57	19.7[b]	
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	
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**	

Abbreviation: BDI-II: Beck Depression Inventory-II. *Not available (not considered in the statistical calculations). Categorical variables presented in relative (%) and absolute (n) frequencies. * p value: chi-square, at 5% significance (p<0.05); ** p value chi-square corrected by Bonferroni. Different letters in the same column mean statistical difference.

The results of 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. Regarding the association between evaluated symptoms in the inventory and DNA methylation, 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.

Symptoms avaliados no BDI-II (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*
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.

Multivariate models showed that psychotropic drug use was explained by variables: irritability, difficulty at work and insomnia (**Table 4**). Hypermethylation of 40 to 47 CpGs from 1F region of *NR3C1* gene was associated with symptoms of failure and loss of appetite (**Table 5**). Both models presented statistical significance (p=0.002, pseudo r2 of 0.0119 and 0.00238, respectively). 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. In model I, socioeconomic variables (gender, age and schooling) were inserted and the association between psychotropic drug use and methylation remained significant. In model II the variables in model I were maintained and health variables (stress, anxiety, insomnia and health) were inserted and the association remained significant.

Finally, psychotropic drug use in combination with all confounding variables (socioeconomic, health, and lifestyle variables: tobacco, alcohol, physical activity and leisure) 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.

All models analyzed were significant, confirming that 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.

			Univariate Poisson regression			Univariate Poisson regression models adjusted for confounding variables									
Drug class			**Crude analysis**			***Model 1***			***Model 2***				***Model 3***		
	Sim n(%)	**Não n(%)**	**IRR**	**95%CI**	**P**	**IRR**	**95%CI**	**P**	**IRR**	**95%CI**	**p**	**IRR**	**95%CI**	**p**
Psychotropic drug use	70 (18,1)	316(81,8%)	0.52	0.27;0.98	**0.046**	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 and conclusions

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 (FREITAS et al., 2018).

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 (GOMES-OLIVEIRA et al., 2012). 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% (WHO, 2017).

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) (MCGOWAN et al., 2009). 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 (2004) (WEAVER et al., 2004). 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 (WEAVER et al., 2004). 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 (MOSER et al., 2007). 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 (VUKOJEVIC et al., 2014).

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) (NA et al., 2014). 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 (MELAS et al., 2013). 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 47 (OBERLANDER et al., 2008). Although controversial, these data present sufficient information to assume that different events in depression may alter DNA methylation profile in *NR3C1* gene (OBERLANDER et al., 2008).

Methylation analysis and the relationship with psychotropic drug use was 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 (LÖTSCH et al., 2013). 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 (CASSEL, 2006).

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 (ARGENTIERI et al., 2017).

Thus, our data relate psychotropic drug use with hypomethylation in the glucocorticoid receptor gene promoter, and hypermethylation with symptoms suggestive of depression. These findings corroborate to a better knowledge since a large world population consume psychotropic drug use. In addition, studies such as this one, in a non-psychiatric population, but with frequent use of public health system, show the need for better attention to these individuals, since from the individual perspective, they have worse quality of life and health, and from a collective perspective, on account of care demand, they also end up burdening health services due to the high demand for consultations and medicines.

Author statements

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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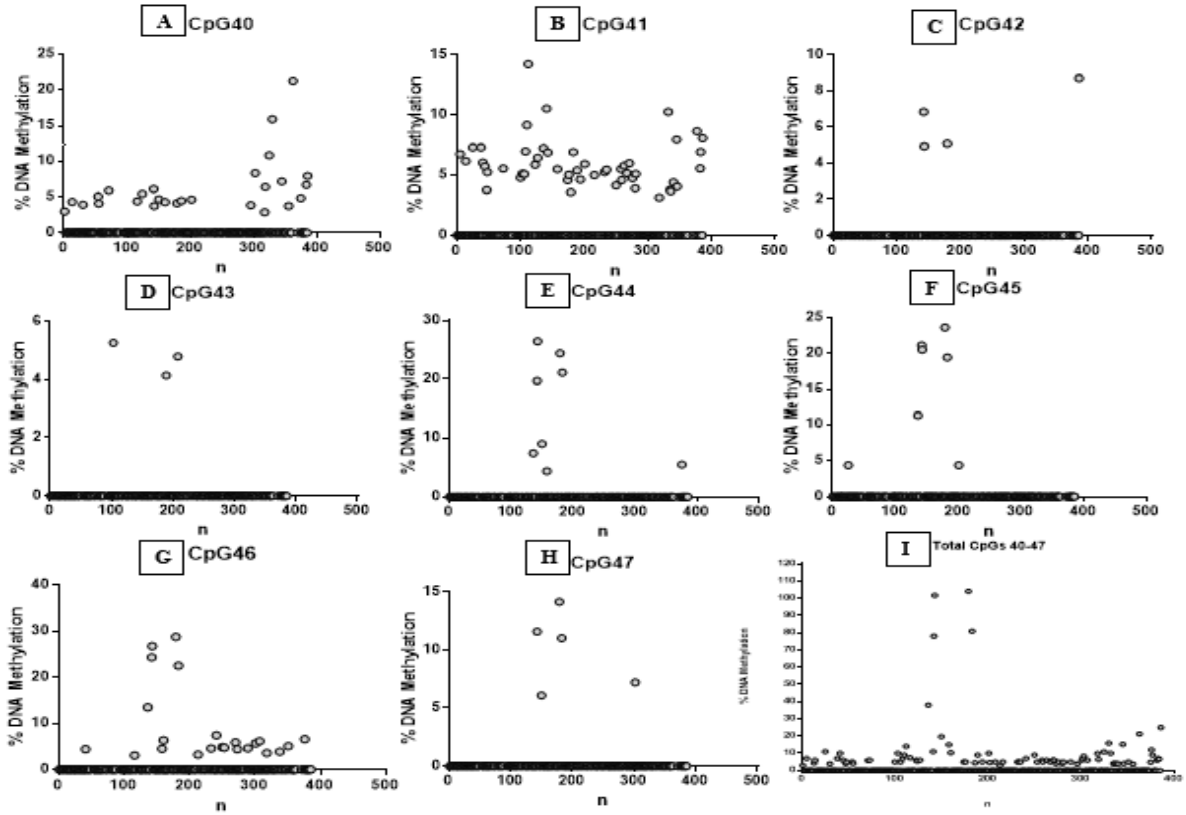

Supplementary Figure 1. Scatter plots of the methylation profile from individuals participating in the study. Hypomethylated individuals are adhered to the x-axis (methylation values equal 0) and individuals with methylation percentages above zero are dispersed in the graph with percentage values ranging 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

CAPÍTULO 2: ALCOHOL CONSUMPTION, DEPRESSION, NUTRITIONAL STATUS AND CORTISOL LEVELS AS DETERMINING FACTORS OF NR3C1 METHYLATION

Artigo submetido: Translational Psychiatry

ALCOHOL CONSUMPTION, DEPRESSION, NUTRITIONAL STATUS AND LEVELS CORTISOL AS DETERMINING FACTORS OF *NR3C1* METHYLATION

Authors

Júlia de Assis Pinheiro[1], Flávia Vitorino Freitas[1,2], Aline Ribeiro Borçoi[1], Catarine Lima Conti[1], Juliana Krüger Arpini[3], Rafael Assis de Souza[4], Dirceu Pereira dos Santos[5], Wagner Miranda Barbosa[2], Suzanny Oliveira Mendes[1], Anderson Barros Archanjo[1], Mayara Mota de Oliveira[1], Joaquim Gasparini dos Santos[1], Bruna Pereira Sorroche[6], José Claudio Casali da Rocha[7], Tamires dos Santos Vieira[1], Leonardo Oliveira Trivilin[8], Elizeu Batista Borloti[1], Iuri Drumond Louro[1], Lidia Maria Rebolho Batista Arantes[6], Adriana Madeira Alvares-da-Silva[1,3*]

[1] Biotechnology/Renorbio Graduate Program, Universidade Federal do Espirito Santo, Alegre, ES, Brazil.

[2] Department of Pharmacy and Nutrition, Universidade Federal do Espirito Santo, Alegre, ES, Brazil.

[3] Department of Biology, Universidade Federal do Espirito Santo, Alegre, ES, Brazil.

[4] Department of Agronomia, Universidade Federal do Espirito Santo, Alegre, ES, Brazil.

[5] Fluminense Federal Institute, Campos dos Goytacazes, RJ, Brazil.

[6] Molecular Oncology Research Center, Hospital do Câncer de Barretos, Barretos, SP, Brazil.

[7] Hospital Erasto Gardner, Curitiba, PR, Brazil.

[8] Department of Medicina Veterinária, Federal University of Espirito Santo, Alegre, ES, Brazil.

* Corresponding author: adriana.biomol@gmail.com (A.M. Alvares-da-Silva)
Alto universitário sem número.
Universidade Federal do Espirito Santo, Departamento de Biologia,
Algre – ES, Brazil
ZipCode: 29500000

Abstract

BACKGROUND: The *NR3C1* glucocorticoid receptor (GR) gene is a component of stress response system, which can be regulated by epigenetic mechanisms. Its gene methylation has been associated with trauma and mental disorders, including depression, posttraumatic stress, anxiety and personality disorder. Literature reports that each event is related to hypermethylation or hypomethylation in specific CpG islands, suggesting that environmental effects on *NR3C1* regulation are complex.

OBJECTIVES: The present study aimed to evaluate the relationship between socioeconomic/health conditions, cortisol levels, vitamin D and lifestyle with *NR3C1* gene methylation.

MATERIAL AND METHODS: This study consisted of 386 individuals' users of the Brazilian Public Unified Health System (SUS), where were evaluated socioeconomic and health conditions, cortisol levels, vitamin D and lifestyle. The data was correlated to *NR3C1* gene methylation, performed using pyrosequencing technique.

RESULTS: The results showed that alcohol consumption, nutritional status and high cortisol levels are related to *NR3C1* hypomethylation, while depression is related to hypermethylation.

CONCLUSION: We suggest that habits, lifestyle and health status may influence *NR3C1* gene regulation via methylation, revealing the complexity of environmental impact on *NR3C1* methylation.

Keywords: Glucocorticoid Receptor (GR), alcohol consumption, depression, nutritional status, hypermethylation

Introduction

DNA methylation is a widely known mechanism of gene expression regulation (CHEN et al., 2017). It has been recently described as the "modus operandi" of environmental adaptation, rapid response to exposure events, which can be passed on to future generations (VIDAKI; DANIEL; COURT, 2013). Imprinting patterns are inherited and preserved during cell division; however, extrinsic or environmental factors contribute to epigenetic changes during the life of an individual (KADER; GHAI, 2017; KADER; GHAI; MAHARAJ, 2018; LACAL; VENTURA, 2018). So, stressful events can result in addition or withdrawal of epigenetic marks at specific DNA locations, resulting in altered gene expression (KELLER; HAN; YI, 2016; MOORE; LE; FAN, 2013; YANG et al., 2014).

Stress events in humans or animal models have been related to epigenetic changes in specific regulatory regions of glucocorticoid receptor (GR), which has the function of regulation hypothalamic stress on the neuroendocrine axis Hypothalamic-Pituitary-Adrenal (HPA) via cortisol production (NANTHARAT et al., 2015; WEAVER et al., 2004). Increased cortisol levels has been previously related with stress and methylation (MCGOWAN et al., 2009; PALMA-GUDIEL et al., 2015; RADTKE et al., 2011). However, other events or conditions, including stressors, have been related to hypomethylation of the same GR region (YEHUDA et al., 2015).

Animal studies have evaluated methylation events directly in the hypothalamus (ALT et al., 2010; MCGOWAN et al., 2011), while human studies have evaluated blood methylation events by their homology observed in different tissues with equivalent expression (ARGENTIERI et al., 2017).

GR belongs to the ligand-dependent nuclear receptor transcription factor superfamily, and in humans it is encoded by the *NR3C1* gene, located in chromosome 5q31-q32 with approximately 140,000 base pairs (OAKLEY; CIDLOWSKI, 2013; STEIGER et al., 2013; TURNER et al., 2014). This gene is composed of 17 exons, eight coding exons (numbered 2 to 9) and nine non-coding exons, which are located in the gene promoter (DASKALAKIS; YEHUDA, 2014; PALMA-GUDIEL et al., 2015). The GR promoter region contains multiple methylation sensitive Cytosine-phosphate-Guanine (CpG) dinucleotide repeats (BRENET et al., 2011; PALMA-GUDIEL et al., 2015; PROVENÇAL; BINDER, 2015), among them the

1F region containing 47 CpG sites (MURGATROYD et al., 2015; OBERLANDER et al., 2008; RADTKE et al., 2011).

Promoter methylation is responsible for different GR protein levels in various tissues (PRESUL et al., 2007; TURNER et al., 2006), such as heart, kidney, lung, liver, skin, especially the hippocampus (CHEBOTAEV; YEMELYANOV; BUDANOVA, 2007; ITO; GETTING; CHARRON, 2006; MCGOWAN et al., 2009; MUELLER et al., 2012; PANAGIOTOU et al., 2018; RICHARDSON et al., 2017; WANG et al., 2017; YAN et al., 1999). Although it is not expressed in T-cells, it is expressed in B-cells and dendritic cells, homologous to the hippocampus, therefore, it can be evaluated in blood under conditions involving HPA axis changes (ARGENTIERI et al., 2017; TURNER; MULLER, 2005).

Associations between methylation and life events, or clinical severity, describing hyper or hypomethylation (or both) at various CpG sites have been evaluated by different methods including Pyrosequencing, MassARRAY EpiTYPER and Clone-based Sanger Sequencing (DASKALAKIS; YEHUDA, 2014).

It is still unclear how different conditions alter CpG methylation and regulate GR, especially in broader and multifactorial systems. Thus, the present study evaluated the association between socioeconomic and health conditions with cortisol levels, vitamin D and lifestyle in relation to *NR3C1* gene methylation in adult individuals.

Materials and methods

Patient samples

This is a cross-sectional study carried out with users of the Brazilian Public Unified Health System (SUS) in a south-eastern municipality (Alegre-ES) in Brazil held from March 2017 to November 2018. The study population was composed of individuals living in urban and rural areas and 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. Individuals participating in the study signed a written Informed Consent Form (ICF).

Population characteristics

This study was made of a convenient sample of 386 individuals between 20 and 59 years old, users of the Brazilian Primary Health Care Units. Based on SUS individual registration forms, data were collected through individual interviews that evaluated socioeconomic, health and lifestyle conditions. Low-income was defined by a per capita income/day less than $5 (five American dollars) (NERI, 2008).

The habits evaluated were alcohol and tobacco use, leisure and weekly physical activity. Marital status, age, working conditions and education (less than 8 years, 8 to 11 years, and higher education) were also analyzed. Self-perceived health was assessed by good or bad health responses. Regarding alcohol consumption, type of drink consumed, weekly dose, if the consumption was in the past (with at least one year of abstinence), in the present or if it never occurred. In addition, quantities of weekly doses were also evaluated, classified into 1 to 7 doses and greater than 7. The level of alcohol dependence was not evaluated, but the amount consumed.

Symptoms suggestive of depression were assessed by the Beck Depression Inventory-II (BDI-II) (JACKSON-KOKU, 2016). Values were categorized according to Gomes-Oliveira et al. (GOMES-OLIVEIRA et al., 2012) considering normal or mild mood disorder (BDI-II <17) and symptoms suggestive of depression (BDI-II ≥ 17).

Nutritional status was determined by an anthropometric assessment carried out by a qualified professional, using the Food and Nutrition Surveillance System (SISVAN) (BRASIL. MINISTÉRIO DA SAUDE., 2011), with weight and height assessment. From the data obtained, body mass index (BMI) was calculated and classified according to the World Health Organization (WHO, 2000).

Blood analysis

For the analysis of vitamin D, cortisol levels and DNA methylation, patients' peripheral blood was collected after fasting for at least eight hours. Blood collection for cortisol levels analysis was performed strictly at 8:00 am, and the rest conditions of the previous night were respected.

Cortisol levels and vitamin D dosages were quantified by chemiluminescence, with reference values for morning cortisol levels of 6.7 to 22.6 µg/dL (SILVA et al.,

2007). Vitamin D deficiency was defined as < 20 ng/mL, insufficiency 20-29 ng/mL and sufficiency > 30 ng/mL (HOLICK, 2007; HOLICK et al., 2011).

DNA extraction was performed according to Salazar et al. (SALAZAR et al., 1998). NanoDrop® was used to verify DNA quality and concentration. For *NR3C1* gene methylation analysis, were evaluated 286 patients' peripheral blood, collected after the person had fasten for at least eight hours.

Quantitative Pyrosequencing Methylation Assays - PMA

Sodium-bisulfite conversion of 1 µg of DNA was performed using EpiTect® Bisulfite Kit (Qiagen, Valencia, CA), following the manufacturer's recommendations. Pyrosequencing methylation assays were performed as previously described (COLELLA et al., 2003; TOST; DUNKER; GUT, 2003).

PCR product quality was checked on 2% agarose gels using GelRedTM (Uniscience). Pyrosequencing was performed using the PSQ96ID Pyrosequencer (Qiagen, Valencia, CA) with 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.

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

PCR Primer		**PCR conditions**		
Forward	5'-TTTTTTTTTTGAAGTTTTTTTA-3'	95 ºC	(14'30'')	
Reverse	5'-BIOTIN-CCCCCAACTCCCCAAAAA-3'	94 ºC	(30'')	45 cycles (410bp)
		50 ºC	(30'')	
		72 ºC	(30'')	
		72 ºC	(10')	
		4 ºC	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			

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

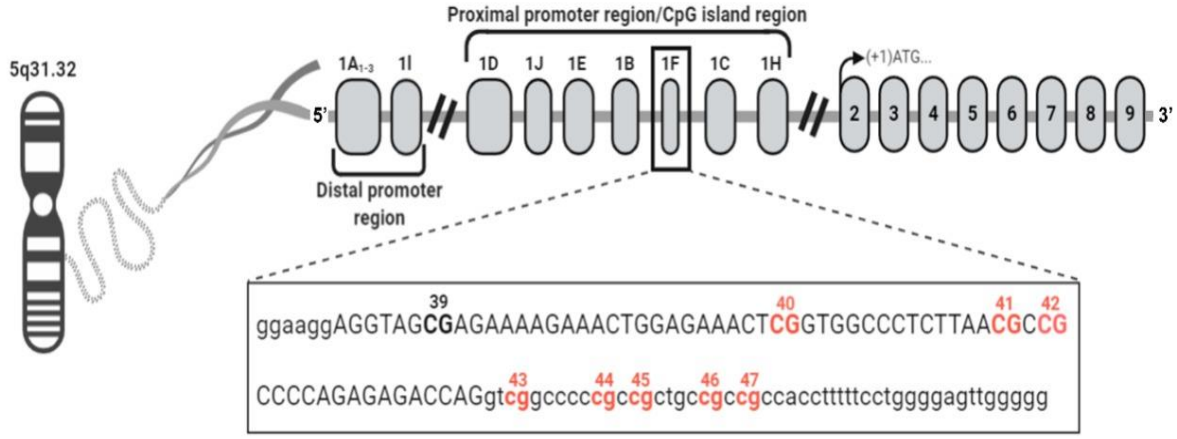

Figure 1. Promoter region of *NR3C1* examined within this study. The CpGs studied (40-47) are represented in red and are also numbered. Lowercase nucleotides represent intronic regions,while upercase nucleotides represent exon 1F. GenBank (NCBI - Access number: AY436590.1).

Statistical Analysis

Data was analyzed using the Chi-square test in a 2x2 contingency table and a significance level of 5%. Quantitative data are expressed as median and interquartile ranges.

Quantitative analysis of methylation did not follow a normal distribution, even after exponential conversion, in this way, the methylation data was dichotomized, the mean of methylation values of CpGs segment from 40 to 47 was calculated for qualitative analysis so that values greater than 0 were categorized as hypermethylated and values equal to zero were categorized as hypomethylated, then applied bivariate analyses were performed using Poisson regression models with robust variance, with the dependent variable methylation of the segment and as explanatory variables socioeconomic aspects, health and lifestyle, as well as

suggestive symptoms of depression. After data characterization, the variables age, education, nutritional status and vitamin D were recategorized dichotomous before being inserted into the multivariate study model. Thus, the variables that were dichotomized for inclusion in the model were: age, education, nutritional status and vitamin D.

Predictive variables that obtained 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 (greater p value) were removed one by one from the model. The procedure was repeated until all the variables present in the model had statistical significance (p <0.05). The 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. For all analyses, the significance level of 5% was adopted. Statistical analyses were performed using SPSS® software (version 13.0 for Windows) and Stata version 11.0.

Results

Socioeconomic Profile

Our results showed that 198 individuals are non-methylated for NR3C1 gene and 87 are methylated, from a total of 285 individuals evaluated. The median methylation level was 0.0 (0.0 - 12.9%). **Table 2** shows the population description, divided into non-methylated and methylated. Individuals were predominantly female (80.6%), 75% lived with a partner and the median age was 42.5 (33.7-52.0) years. Most of them had less than 8 years of formal education (46.4%), low income (70.2%), did not drink, smoke, performed physical or leisure activities.

Table 2. Sample characteristics.

Characteristic	Total		SEX				*p*
			Male		Female		
	N	(%) or Median(min-máx)	N	(%)or Median(min-máx)	N	(%)or Median(min-máx)	
Age							
20 to 30 years	74	19.2	20	26.7	54	17.4	0.312
31 to 40 years	96	24.9	18	24.0	78	25.1	
41 to 50 years	110	28.5	18	24.0	92	29.6	
51 to 60 years	106	27.5	19	25.3	87	28.0	
Marital status							
Single	96	24.9	31	41.3	65	20.9	**0.000**
Not single	290	75.1	44	58.7	246	79.1	
Years of education							
< 8 years	179	46.4	38	50.7	141	45.3	0.706
between 8 and 11 years	141	36.4	25	33.3	116	37.3	
Higher Education	66	17.1	12	16.0	54	17.4	
Working							
Yes	208	53.7	46	61.3	162	52.1	0.149
No	178	46.3	29	38.7	149	47.9	
Income							
Non-low income (≥ $5.00/ day)	115	29.8	32	42.7	83	26.7	**0.007**
Low income (< $5.00/ day)	271	70.2	43	57.3	228	73.3	
Tobacco consumption							
No	355	92.0	68	90.7	287	92.3	0.644
Yes	31	8.0	7	9.3	24	7.7	
Alcohol consumption							
No	252	65.1	30	40.0	222	71.4	**0.000**
Yes	134	34.6	45	60.0	89	28.6	
Weekly drinking							
<7 drinks	102	76.7	28	63.6	74	83.1	**0.012**
>7 drinks	31	23.3	16	36.4	15	16.9	
Physical activity							
Yes	127	32.9	29	38.7	98	31.5	0.236
No	259	67.1	46	61.3	213	68.5	
Leisure activity							
Yes	179	46.4	38	50.7	141	45.3	0.406
No	207	53.6	37	49.3	170	54.7	
Self-rated health							
Good or very good	204	52.8	49	65.3	155	49.8	**0.016**
Regular or poor	182	47.2	26	34.7	156	50.2	
Depression							
BDI-II < 17	271	70.2	63	91.3	208	72.7	**0.001**
BDI-II ≥ 17	84	21.8	6	8.7	78	27.3	
Not available*	31	8.0					
Nutritional Status – BMI							
Low weight	10	2.6	1	1.3	9	2.9	0.186
Eutrophy	114	29.5	26	34.7	88	28.3	
Overweight	127	32.8	29	38.7	98	31.5	
Obesity	135	34.9	19	25.3	116	37.3	
Vitamin D							
Sufficiency	176	45.6	39	52.0	137	44.1	0.302
Insufficiency	182	47.2	33	44.0	149	47.9	
Deficiency	28	7.3	3	4.0	25	8.0	
High cortisol levels							
No	364	94.3	74	100.0	290	97.3	0.154
Yes	8	2.1	0	0.0	8	2.7	
Not available	14	3.6					
Methylation							
No	198	51.3	50	66.7	148	47.6	**0.009**
Yes	87	22.5	10	13.3	77	24.8	
Not available	101	26.2	15	20.0	86	27.7	
Methylation Quantitative							
Median(min-máx)	285	0.0(0.0-12.9)	60	0.0(0.0-12.6)	225	0.0(0.0-12.9)	**0.030**
Total	**386**	**100.00**	**75**	**19.40**	**311**	**80.60**	

Abbreviation: BDI-II: Beck Depression Inventory-II; BFIS: Brazilian Food Insecurity Scale; FNS: Food and Nutrition Security; FNiS: Food and Nutrition Insecurity; BMI: Body Mass Index. *Not available (not considered in the statistical calculations). Categorical variables presented in relative (%) and absolute (n) frequencies. Quantitative variables presented in medians and interquartile ranges (IR), according to normality (Kolmogorov-Smirnov test); * p value: Mann-Whitney U or chi-square, at 5% significance (p<0.05).

Methylation analysis of NR3C1

Univariate analysis showed an association between methylation profile and variables sex (p=0.047), alcoholism (p=0.00), depression (p=0.022), nutritional status (p=0.017) and cortisol levels (p=0.000) (**Table 3**).

Table 3. Bivariate Poisson regression analysis with robust variance for *NR3C1* 1F region methylation.

Characteristic	Methylation (CpG 40 to 47)		
	PR	95% CI	p
Sex			
Male	1.8	1.00 – 3.41	**0.047**
Female			
Age			
20-40 years	1.0	0.70 – 1.47	0.938
41-60 years			
Marital status			
Single	1.6	0.95 – 2.63	0.073
Not single			
Schooling			
Basic education	0.9	0.55 – 1.54	0.779
Higher education			
Working			
Yes	0.9	0.67 – 1.43	0.785
No			
Income			
Non-low income (≥ $5.00/ day)	1.2	0.77 – 1.80	0.443
Low income (< $5.00/ day)			
Tobacco consumption			
No	0.2	0.69 – 1.04	0.058
Yes			
Alcohol consumption			
No	0.3	0.16 – 0.53	**0.000**
Yes			
Physical activity			
Yes	1.1	0.76 – 1.72	0.500
No			
Leisure activity			
Yes	1.2	0.87 – 1.87	0.196
No			
Self-rated health			
Good or very good	1.3	0.90 – 1.91	0.147
Regular or poor			
Depression			
BDI-II < 17	1.5	1.06 – 2.27	**0.022**
BDI-II ≥ 17			
Nutritional Status – BMI			

Not overweight	0.6	0.44 – 0.92	**0.017**
Overweight			
Vitamin D			
Sufficiency	1.2	0.69 – 2.40	0.413
Deficiency			
High cortisol levels			
No	2.2^{-9}	1.11^{-9} – 4.65^{-9}	**0.000**
Yes			

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

For the construction of the multivariate model we used variables considering p≤0.20. A mean methylation index was calculated from CpG sites methylation percentages. Those percentages were dichotomized in hypomethylated (0% methylation) and hypermethylated (values above 0% methylated).

Multivariate model results (**Table 4**) showed that methylation is associated with alcohol, depression, nutritional status and high cortisol levels. As observed in **Table 4**, alcohol consumption was associated to hypomethylation, as well as nutritional status and high cortisol. In contrast, depression has the opposite effect, being directly related to hypermethylation.

The final model was statistically significant (p=0.000), presenting a pseudo r2 = 0.0759 and, after adjustment by Hosmer & Lemeshow, showed good adherence (p=0.99).

Table 4. Multivariate Poisson regression analysis with robust variance for methylation of *NR3C1* 1F region.

Characteristic	**Methylation (CpG 40 to 47)**		
	PR	**95% CI**	**p**
Alcohol consumption			
No			
Yes	0.30	0.16-053	**0.000**
Depression			
BDI-II < 17			
BDI-II ≥ 17	1.55	1.07-2.24	**0.018**
Nutritional Status – BMI			
Not overweight			
Overweight	0.66	0.46-0.95	**0.017**
High cortisol levels			
No			
Yes	0.09^{-5}	3.8^{-7} – 1.9^{-6}	**0.000**

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

Discussion and conclusions

This study present individuals with low income, low education and predominantly female gender. Our goal was to make a broader assessment of factors related to methylation, such as socioeconomic aspects, habits and lifestyle.

We have shown that alcohol, obesity and high cortisol levels are related to DNA hypomethylation, while depression is related to *NR3C1* hypermethylation. Few studies evaluated relationship between methylation patterns and alcohol consumption. Corroborating our findings, Dogan et al. (2016) showed a relationship between alcohol and *NR3C1* gene hypomethylation in 64 patients from the Family and Community Health Study (FACHS) cohort, an aging study (Hannum) and a study on methylation changes associated with alcohol consumption (AlcMeth) (DOGAN et al., 2016).

Xenobiotics, including alcohol, have been reported as capable of altering gene expression through epigenetic events (BROMER et al., 2010). The finding is extremely relevant since alcohol represents a large fraction of drugs consumed by the world population and epigenetic effects of its use are still little known.

Our analysis showed a relationship between symptoms suggestive of depression, evaluated by Beck score ≥17, with increased methylation in *NR3C1* 40-47 1F CpGs sites. Other authors have associate methylation alterations in 1F region CpGs 36-39 sites with depressive status in adolescents (EFSTATHOPOULOS et al., 2018) and in CpGs 36-44 on maternal exposure to gestational stress and depression in children (BRAITHWAITE et al., 2015).

Besides, authors have studied 1F region showing hypomethylation in CpGs 35-47, with hypomethylation specifically in CpG 43 associated to depression (SONG et al., 2014). There are also studies addressing 1F region showing CpGs 35-39 hypomethylation in individuals with depression (NA et al., 2014). However, the present study provides unprecedent data: individuals with depressive symptoms, frequent users of public health system, show hypermethylation of *NR3C1*.

On the other hand, multivariate analysis of risk factors showed that overweight is associated with the hypomethylation, with a prevalence ratio of 0.67 indicating that being overweight reduces the prevalence of methylation by 33%. Chronic stress has been previously related to increased cortisol levels leading to weight gain

(JACKSON; STEPTOE, 2018). Excess weight is also related to chronic inflammation through NFkB pathways, however, no association between overweight and methylation status has been previously reported for *NR3C1* gene (MILAGRO et al., 2013). It is possible that hypomethylation of this region is related to low-grade inflammation, a feature of the overweight state (FREITAS et al., 2018).

Furthermore, high cortisol levels was associated with hypomethylation, however with a very low prevalence ratio (**Table 4**). High levels of cortisol levels have already been related to *NR3C1* gene methylation in maternal and postnatal gestational exposures to childhood stress (HOMPES et al., 2013; OBERLANDER et al., 2008; YEHUDA et al., 2015). In our study, only 8 individuals presented high levels of cortisol levels, while the others had normal or low levels, which may not be very representative.

We present a relatively numerous samples, of which the methylation status was evaluated by pyrosequencing. We suggest that habits, lifestyle and health status may influence *NR3C1* gene regulation via methylation. Argentieri et al. (2017) (ARGENTIERI et al., 2017) showed a series of studies that related hyper or hypomethylation with specific CpG methylation.

In conclusion, the relationship between genotype, environment and phenotypic outcome may be more refined than previously thought, depending on specific stressful events which can result in unique clinical consequences. This study is relevant as it revealed a direct or inverse association between methylation: alcohol consumption, nutritional status and high cortisol levels related to NR3C1 hypomethylation, while depression is related to hypermethylation.

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Declaration of interest statement

The authors wish to confirm that there are no known conflicts of interest associated with this publication.

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CONCLUSÃO GERAL

As análises gerais dos dados nos revelam a complexidade das relações ambientais com a epigenética, indicando que a relação entre genótipo, ambiente e desfecho fenotípico pode ser mais refinada do que se pensava; ela depende não somente do evento estressor, mas também do tipo de evento, e pode resultar em consequências clínicas diversas. Isto está conectado diretamente à discussão sobre risco e proteção à saúde. Questões envolvendo tal complexidade, que abordem os indivíduos como um conjunto complexo de fatores ou variáveis, devem ser consideradas em ações de intervenção em saúde. Logo, a presença ou ausência de metilação não deve ser vista apenas como fator de risco à saúde ou de proteção, mas como resultado e resultante da interação do indivíduo com o meio ambiente, no sentido da sua adaptação às condições à qual está exposto. Desta forma, estudos para a compreensão desses mecanismos ocorrendo em um ser que é multidimensional são importantes para a compreensão real dos fenômenos epigenéticos.

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ANEXOS

ANEXO 1. Termo de Consentimento Livre e Esclarecido – TCLE

ANEXO I – TERMO DE CONSENTIMENTO LIVRE ESCLARECIMENTO

Profa. Dra. Adriana Madeira Álvares da Silva (28) 3552-8624; (28) 99271-9791

Dados de identificação do Voluntário

Nome: _____Sexo: F ( ) M ( )
Data de Nascimento:___/___/___Idade:____ Fumante ( ) Não Fumante ( )
Endereço:_____Bairro:_____
Cidade:_____Estado:____CEP: _____Telefones:(___)_____ /_____

Título do estudo: "Impacto do programa de capacitação de agentes SUS no estado nutricional de vitamina D e sua relação com obesidade, depressão, câncer, metilação do Receptor do Glicocorticoide e avaliação de hipovitaminose D na população da região do Caparaó Capixaba"

Natureza e objetivo do estudo: Você está sendo convidado(a) a participar de um estudo que tem por objetivo avaliar o impacto da implantação de um programa de educação e capacitação dos agentes do SUS quanto ao estado nutricional de vitamina D e sua relação com obesidade, depressão e câncer, bem como investigar a prevalência de hipovitaminose D e suas doenças correlacionadas em grupos de indivíduos da área urbana e rural na região do Caparaó Capixaba, assim como avaliar o status de metilação do gene receptor do glicocorticoide para a possibilidade de utilização como marcador de doenças.

Local do estudo: Esse estudo será realizado no Laboratório de Biotecnologia do Centro de Ciências Agrárias da Universidade Federal do Espírito Santo (CCA/UFES).

Procedimentos do estudo: Para esse estudo você responderá a um questionário sobre os hábitos, ocupação, exposição, presença de depressão, ansiedade e stress. Você terá o **direito** de se recusar a responder as perguntas, se assim achar conveniente. **Serão colhidas amostras:** coleta de sangue através de punção venosa através de utilização de seringa descartável estéril, coletada por profissional habilitado, **com a duração aproximada de 30 minutos**. O material coletado será identificado com código de barras e seu nome não ficará exposto nos tubos e frascos mantendo o **sigilo** de sua identidade, em seguida o material será transportado em gelo e levado ao laboratório de Biotecnologia do CCA/UFES. Depois de utilizadas, as amostras de sangue (soro e plasma) e material genético (DAN e RNA) serão armazenadas no Biorrepositório de Materiais Biológicos do CCA-UFES até o final do estudo.

Riscos e Benefícios: Você terá direito de responder quantas questões quiser do questionário e se você achar que alguma pergunta é ofensiva, poderá deixar de responder ou mesmo se recusar. Você sentirá desconforto na coleta de sangue (uma picada de agulha), poderá ficar com o braço roxo no local da picada e sentir alguma dor. Todo o material coletado será analisado no laboratório sob a responsabilidade da Profa. Dra. Adriana Madeira Álvares da Silva, se você assim concordar. O uso e coleta de sua amostra de sangue não implicarão em riscos adicionais para a sua saúde, nem exigirão que você se submeta a qualquer outro procedimento depois. Você não terá nenhum ônus na participação desta pesquisa. Como benefício você receberá os resultados dos exames realizados, além de informações e orientações quanto ao conteúdo dos exames e encaminhamento para os profissionais qualificados. Esses resultados poderão beneficiar, no futuro, outras pessoas e a ciência.

Confidencialidade dos registros: Nesse estudo, todos os frascos e tubos receberão uma etiqueta de código de barras para manutenção de **sigilo**, em conformidade com Resolução 466/12 do CNS, de forma que você, paciente não seja identificado por seu nome. Caso os resultados do estudo sejam publicados ou apresentados em congresso a confidencialidade das informações serão garantidas e sua identidade não será revelada. **Você receberá uma via desse termo, ficando a outra com o pesquisador.**

Esclarecimento de Dúvidas: Em caso de dúvida ligue para o pesquisador responsável **Profa. Dra. Adriana Madeira Álvares da Silva** nos telefones **(28) 3552-8622, 3552-8624; (28) 99271-9791**. Você poderá retirar o consentimento para pesquisa em qualquer época do estudo. O Comitê de Ética em Pesquisa responsável pela autorização do estudo atende pelo telefone **(27) 3335-7211** e o link na Internet é www.ccs.ufes.br/cep - o e-mail é cep.ufes@hotmail.com. O Comitê de Ética em Pesquisa da UFES fica na Rua Marechal Campos, número 1468, Bairro Maruípe, CEP 29.040-090, Centro de Ciências da Saúde (CCS) - Prédio da Direção, Vitória, ES.

Declaro que fui verbalmente informado e esclarecido sobre o teor do presente documento, entendendo todos os termos acima expostos, como também, os meus direitos, e que voluntariamente aceito participar deste estudo. Também declaro ter recebido uma via deste Termo de Consentimento Livre e Esclarecido assinada pelo(a) pesquisador(a). Autorizo também o uso das informações obtidas na pesquisa em publicações em revista médicas e apresentações em congressos (desde que meus dados de identificação pessoal sejam mantidos em sigilo).

Na qualidade de pesquisador responsável pela pesquisa "Impacto do programa de capacitação de agentes SUS no estado nutricional de vitamina D e sua relação com obesidade, depressão, câncer, metilação do Receptor do Glicocorticoide e avaliação de hipovitaminose D na população da região do Caparaó Capixaba", eu, ADRIANA MADEIRA ÁLVARES DA SILVA, declaro ter cumprido as exigências do(s) item(s) IV.3 e IV.4 (se pertinente), da Resolução CNS 466/12, a qual estabelece diretrizes e normas regulamentadoras de pesquisas envolvendo seres humanos.

_____

(LOCAL/DATA)

_____ _____

Participante da pesquisa **Pesquisador colaborador**

Adriana Madeira A. da Silva

_____

Pesquisador Principal
Profa. Dra. Adriana Madeira Álvares da Silva

ANEXO 2. Questionário aplicado

	QUESTIONÁRIO	Código: |__||__||__||__|						
Data:								
Entrevistador:								
	MÓDULO 1: INFORMAÇÕES GERAIS							
1	Nome:							
2	Telefone:	CEL: _____		Idade: _____				
3	Possui filhos?	( ) sim	( ) não					
4		Quantos filhos? _____						
5	Renda pessoal (mês anterior):	R$ _____						
	ou em salários mínimos	|__| Sem rendimento	|__| Entre 1 e 3 SM	|__| Mais de 5 SM				
		|__| Até 1 salário mínimo	|__| Entre 3 e 5 SM					
6	Renda familiar (mês anterior) (toda família):	R$ _____						
	ou em salários mínimos	|__| Sem rendimento	|__| Entre 1 e 3 SM	|__| Mais de 5 SM				
		|__| Até 1 salário mínimo (SM)	|__| Entre 3 e 5 SM					
	MÓDULO 2: CONDIÇÕES DE SAÚDE							
1	Fuma (cigarro, charuto, etc.)?	|__| Nunca fumou (pule para Q3)						
		|__|Já fumou e não fuma atualmente						
		|__| Fuma atualmente	Quantos cigarros por dia? _____					
2	Qual o tipo de cigarro?	_____						
3	Você consome bebidas alcoólicas?	|__| Nunca bebeu	(pule para Q7)					
		|__|Já bebeu no passado e atualmente não bebe						
		|__| Bebe atualmente						
4	Quantas doses (1 copo americano cheio)	|__|Até 2 doses						
	por semana?	|__|Entre 2 e 7 doses						
		|__| Acima de 7 doses						
5	No ato de beber, acontece de beber grande	( ) não	( ) sim					
	quantidade de uma vez só?							
6	Qual o tipo de bebida alcoólica que você	|__|Cerveja	|__|Destilados em geral					
	consome com maior frequencia?	|__|Cachaça	|__|Outras: _____					
		|__|Vinho						
7	Quais sintomas você tem apresentado?	|__|Irritação ocular	|__|Lacrimejamento	|__|Dor de cabeça				
		|__|Queimaduras na pele	|__|Tonturas/Vertigens	|__|Suor excessivo				
		|__|Náuseas/ânsia de vômito	|__|Tosse	|__|Salivação				
		|__|Catarro	|__|Falta de ar/dispneia	|__|Agitação/irritabilidade				
		|__|Dor abdominal	|__|Visão turva	|__|Formigamento				
		|__|Diarreia	|__|Tremores	|__|Palpitação cardíaca				
		|__|Digestão difícil	|__|Vômitos	|__|Câimbras				
8	Você tem alguma dessas doenças?							
		|__|Leões na pele/Alergia	|__|Hepatite	|__|Depressão				
		|__|Asma	|__|Doenças ósseas	|__|Doenças cardiovasculares				
		|__|Inflamações gástricas	|__|Doenças renais	|__|Câncer				
		|__|Doenças hepáticas	|__|Doença respiratória	|__|Hiperatividade				
		|__|Hipertensão arterial	|__|Déficit de atenção					
		|__|Infertilidade	|__|Abortamentos	( ) hipoglicemia				
		( ) hipotireoidismo	( ) obesidade	( ) diabetes				
		( ) hipertireoidismo	( ) síndrome metabólica					
			( ) doenças auto-imunes					
9	Você faz uso de algum medicamento contínuo?		|__|NÃO	|__|SIM				
10	Qual(is) medicamento(s) de uso contínuo você faz uso? _____							
	Tipo de medicamento:	|__|Medicamento para dormir		|__|Rémedio para coração				
		|__|Rémedio para depressão		|__| Rémedio para diabetes				
		|__|Rémedio para pressão arterial		Outros: Quais? _____				
11	Você faz uso de suplemento de vitamina D?	|__|Não	|__|Sim					
12	Você faz uso de protetor solar?	|__|Diariamente						
		|__|Somente quando exposto ao sol						
		|__|Raramente						
		|__|Não faz uso						
13	Possui atividade de lazer/ recreação?	( ) semanal	( ) mensal					
		( ) quinzenal	( ) não					
14	Pratica atividade atividade física?	( ) sim	( ) não					
15	Pratica atividade atividade física sob	( ) Mais que 2 horas na semana		( ) Em média, 30 minutos na semana				
	sob exposição solar?	( ) Em média, 2 horas na semana		( ) Não pratica				
		( ) Em média, 1 hora na semana						
16	No trabalho, quantas horas, por dia, você é exposto à luz solar?							
		( ) 0 horas	( ) de 1h a 4 horas					
		( ) até 1 hora	( ) a partir de 5 horas					
17	No geral, como você avalia a sua saúde?	( ) muito boa	( ) ruim					
		( ) boa	( ) muito ruim					
		( ) regular						
18	Fez ou faz tratamento com psiquiatra ou teve internação por problema de saúde mental? |__|Sim |__|Não							
19	Usa Plantas medicinais? |__|Sim |__|Não	Se sim, Quais? _____						
20	Usa outras práticas integrativas e complementares? |__|Sim |__|Não							

ANEXO 3. Comprovante de aprovação do Comitê de Ética

CENTRO DE CIÊNCIAS DA SAÚDE/UFES

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Impacto do programa de capacitação de agentes SUS no estado nutricional de vitamina D e sua relação com obesidade, depressão, câncer, metilação do Receptor do Glicocorticoide e avaliação de hipovitaminose D na população da região do Caparaó Capixaba

Pesquisador: ADRIANA MADEIRA ALVARES DA SILVA

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 3

CAAE: 52830216.5.0000.5060

Instituição Proponente: CENTRO DE CIENCIAS AGRARIAS DA UNIVERSIDADE FEDERAL DO

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.574.160

Apresentação do Projeto:

Pesquisador responsável: ADRIANA MADEIRA ALVARES DA SILVA

Instituição: CENTRO DE CIENCIAS AGRARIAS DA UNIVERSIDADE FEDERAL DO ESPIRITO SANTO-CCA-UFES

Finalidade: PPSUS

Curso: Biologia/Programa de Pós-Graduação em Biotecnologia/RENORBIO da UFES

JUSTIFICATIVA: A vitamina D é um hormônio esteroide que atua como fator de transcrição nuclear que exerce papel importante no metabolismo ósseo, com propriedades anti-inflamatórias e moduladoras do sistema imune. Estudos recentes mostram uma correlação entre níveis baixos de vitamina D e o aparecimento de doenças, incidência de câncer e mortalidade, e doenças autoimunes. A fonte mais importante para sua obtenção é o sol, que contribui com 80 a 90% na produção da vitamina, apenas cerca de 10 a 20% provêm dos alimentos. Ocorre que a com descoberta de que a radiação ultravioleta do sol poderia provocar câncer de pele, associada ao estilo de vida e trabalho, em locais sem insolação, levou grande parte da população mundial à

ANEXO 4. Comprovante de submissão de artigo 1

Your co-authored submission

P

Progress in Neuropsychopharmacology & Biological Psychiatry <EviseS upport@elsevier.com>
Seg, 23/03/2020 19:35
Você

Dear Mrs. Assis Pinheiro,

You have been listed as a Co-Author of the following submission:

Journal: Progress in Neuropsychopharmacology & Biological Psychiatry

Title: PSYCHOTROPIC DRUG USE AND SUGGESTIVE DEPRESSION SYMPTOMS ASSOCIATED WITH NR3C1 METHYLATION

Corresponding Author: Lidia Arantes

Co-Authors: Julia Assis Pinheiro, Flávia Vitorino Fretias, Aline Ribeiro Borçoi, Catarine Conti, Juliana Krüger Arpini, Bruna Sorroche, Suzanny Oliveira Mendes, Anderson Barros Archanjo, Mayara Mota de Oliveira, Joaquim Gasparini dos Santos, Rafael Assis de Souza, Ivana Alece Arantes Moreno, Dirceu Pereira dos Santos, WAGNER BARBOSA, José Claudio Casali da Rocha, Leonardo Oliveira Trivilin, Elizeu Borloti, Leticia Rangel, Adriana Álvares-da-Silva

Lidia Arantes submitted this manuscript via Elsevier's online submission system, EVISE®. If you are not already registered in EVISE®, please take a moment to set up an author account by navigating to http://www.evise.com/evise/faces/pages/navigation/NavController.jspx?JRNL_ACR=PNP

ANEXO 5. Comprovante de submissão de artigo 2

manuscripttrackingsystem

Translational Psychiatry

Detailed Status Information

Manuscript #	2020TP000365
Current Revision #	0
Submission Date	7th Apr 20
Current Stage	
Title	Alcohol consumption, depression, nutritional status and cortisol as determining factors for NR3C1 gene methylation
Running Title	Determining factors for NR3C1 gene methylation
Manuscript Type	Article
Corresponding Author	Dr. Lidia Arantes (Barretos Cancer Hospital)
Contributing Authors	Dr. Julia Pinheiro , Dr. Flávia de Freitas , Dr. Aline Borçoi , Dr. Catarine Conti , Dr. Juliana Arpini , Dr. Rafael de Souza , Dr. Dirceu dos Santos , Dr. Wagner Barbosa , Dr. Suzanny Mendes , Dr. Anderson Archanjo , Dr. Mayara de Oliveira , Dr. Joaquim dos Santos , Dr. Bruna Sorroche , Dr. José Claudio Casali-da-Rocha , Dr. Tamires Vieira , Dr. Leonardo Trivilin , Dr. Elizeu Borloti , Dr. Iuri Louro , Dr. Adriana Alvares-da-Silva
Abstract	BACKGROUND: The NR3C1 glucocorticoid receptor (GR) gene is a component of stress response system, which can be regulated by epigenetic mechanisms. Its gene methylation has been associated with trauma and mental disorders, including depression, posttraumatic stress, anxiety and personality disorder. Literature reports that each event is related to hypermethylation or hypomethylation in specific CpG islands, suggesting that environmental effects on NR3C1 regulation are complex. OBJECTIVES: The present study aimed to evaluate the relationship between socioeconomic/health conditions, cortisol, vitamin D and lifestyle with NR3C1 gene methylation. MATERIAL AND METHODS: This study consisted of 386 individuals' users of the Brazilian Unified Health System (SUS), where were evaluated socioeconomic and health conditions, cortisol, vitamin D and lifestyle. The data was correlated to NR3C1 gene methylation, performed using pyrosequencing technique. RESULTS: The results showed that alcohol consumption, nutritional status and high cortisol are related to NR3C1 hypomethylation, while depression is related to hypermethylation. CONCLUSION: We suggest that habits, lifestyle and health status may influence NR3C1 gene regulation via methylation, revealing the complexity of environmental impact on NR3C1 methylation.