Behavioural Brain Research

Epigenetic regulation of DAT gene promoter modulates the risk of Externalizing and Internalizing behaviors on a normative population.

--Manuscript Draft--

Istituto Superiore di Sanità

00161 *Roma*, ...

VIALE REGINA ELENA, 299
TELEGRAMMI: ISTISAN ROMA
TELEFONO: 06 49901
TELEFAX: 06 49387118
http://www.iss.it

Dear Editor,

please find enclosed our communication titled **"Epigenetic regulation of DAT gene pro moter modulates the risk of Externalizing and Internalizing behaviors on a normative popu lation"** which is authored by Carpentieri V, Cerniglia L, Cimino S, Pascale E, Pucci M, D'Addario C, and myself undersigned.

We have been dealing in recent years with the study of two particular motifs found in the 5'- UTR of the DAT gene, very well known for vulnerability to ADHD as well as many other addictive disorders, or compulsive behaviors. We have tried, and report here about, a completely new ap proach to methylation levels: instead of looking at CpGs individually, we think that cross-correla tions may inform about which ones are getting (de)methylated at the same time on the very same DNA strand. We suggest here, for the very first time, diverging profiles for opposite psychopatholo gical risks, as far as epigenetic regulation through CpG methylation is concerned.

POSSIBLE REFEREES ARE:

Lucia Monacis - University of Foggia, Foggia, Italy. lucia.Monacis@unifg.it
Raymond F Anton - Institute of Psychiatry, Medical University of South Carolina, Charleston, USA. antonr@musc.edu

There is 1 item for potential conflict of interest to be disclosed: Adriani W., Laviola G., Pa scale E., D'Addario C. - INVENTORS - "*Metodo per determinare il deficit di attenzione con iperatti vità*" (Method to determine Attention Deficit and Hyperactivity Disorder). Italian Patent Application at no. 102016000129938 (date 22-December-2016); turned into European Patent Application at no. 17830021.6 (date 21-December-2017).

Rome, 07 November 2020

Prof. Walter Adriani,

Center , Behavioral Sciences & Mental Health

Istituto Superiore di Sanità
viale Regina Elena 299, I-00161 Rome, Italy

Epigenetic regulation of DAT gene promoter modulates the risk of Externalizing and
Internalizing behaviors on a normative population.

**Valentina Carpentieri [1], Luca Cerniglia [2], Silvia Cimino [3], Mariangela
Pucci [4], Esterina Pascale [5], Claudio D'Addario [4], Walter Adriani [1, 2]**

1. Center for Behavioral Science and Mental Health - Istituto Superiore di Sanità, Rome, Italy
2. Faculty of Psychology - Uninettuno University, Rome, Italy
3. Department of Dynamic and Clinical Psychology - Sapienza University of Rome, Rome, Italy
4. Faculty of Bioscience and Technology for Food, Agriculture and Environment - University of Teramo, Teramo, Italy
5. Department of Medical-Surgical Sciences and Biotechnologies - Sapienza University of Rome, Rome, Italy

Correspondence to walter.adriani@iss.it

Abstract-Accumulating research addressed epigenetic modifications and their role on behavioral phenotypes. We recently proposed to study methylation dynamics of two CpG motifs within the 5'-UTR of dopamine transporter (DAT) gene. Starting from a normative population sample of young adults, we selected three sub-groups based on their prevalent symptoms: subjects were assigned to Internalizing, Externalizing and Low-risk sub-groups according to elevated scores in specific phenotypic scales. Using a new approach, we calculated three independent matrixes of cross-correlation between CpG methylation levels, one within each phenotypic sub-group, to determine in which dynamics did the sub-groups differ. We found specific cross-correlation patterns in Externalizing (CpG1, 2 and 3, opposite to the methylation at CpG6) and Internalizing individuals (CpG1 methylation opposite to CpG2, 3 and 6), while Low-risk individuals could follow both trends. A possible exploitation of our results is to identify the risk to develop psychopathological symptoms, related to Internalizing or Externalizing phenotypes.

Highlights

1. A community sample of young adults was divided in: Low-Risk, Internalizing and Externalizing problems.

2. Our study focuses on the epigenetic modification of DAT gene promoter, at two 5'-UTR motifs.

3. Externalizing: CpGs 1, 2 and 3 are opposite to CpG6; Internalizing: CpGs 2, 3 and 6 are opposite to CpG1.

4. Cross-correlations between CpG methylation levels is a new approach to epigenetic analysis.

5. We found innovative biomarkers to highlight the risk for psychopathological deviance.

1. Introduction

Complex psychopathological traits result from a combination of genetic factor and environmental influences. Gene–environment (G–E) interactions are thought to play a crucial role in the individual's behavioral phenotypes. Most recent studies linked gene-environment interaction to epigenetics, suggesting that complex behavioral phenotypes depend on epigenetic variations within specific polymorphic genes and their complex interplay with the environmental factor. One of the most common epigenetic variations is methylation, a biological process by which methyl groups are added to the DNA molecule.

Methylation of cytosine represents almost the totality of methylation on eukaryotic DNA and, in mammals, 5-methyl-cytosine is found almost only in the dinucleotide CpG (cytosine followed by guanin) in the regulatory region of a gene. Methylation of CpG is studied on multiple genes to understand also vulnerability to neuropsychiatric disorders in children, adolescents and adults. Recent studies have shown a possible role of DNA methylation in schizophrenia, depression and suicidal behavior (Petronis, 2004; Mill et al., 2008; Autry & Monteggia, 2009). Methylation is also studied to understand the development of externalizing and internalizing phenotypes in the general population.

Methylation of dopamine transporter (DAT1) gene plays a key role for the risk of ADHD (Adriani et al. 2018; et al. 2015), addictive diseases (Hillemacher et al., 2009; Reyes et al., 2010; De Nardi et al., 2020) neurodegenerative disorders (Rubino et al., 2020;). DAT1 function is essential for normal dopaminergic neurotransmission since it terminates the actions of dopamine by rapidly removing it from the synapse. DAT1 expression can be affected by a 40bp variable number of tandem repeats (VNTR) in the 3'-untranslated region (3'-UTR) of the gene. This polymorphic region can be repeated between 3–11 times, with the 9- or 10-repeat being the more frequent alleles. Both in vitro and in vivo studies demonstrated that gene expression was greater for the 10-repeat allele than for the 9-repeat allele.

Our previous work (Adriani et al., 2018) was able to identify an association between ADHD, methylation profiles of two CpG island motifs in the 5'-UTR of DAT1 gene and DAT1 VNTR genotype at the 3'-UTR. More recently, we designed a novel approach to study the dynamics of methylation considering genetic (VNTR at the 3'-UTR of the DAT1 gene) and epigenetic determinants (Cross-correlation of CpGs methylation levels at the 5'-UTR of the DAT1 gene) in various clinical samples (Tonelli et al., 2020; Tafani et al., 2020), however, data about non-clinical sample are yet lacking.

Thus, we aimed at investigating methylation dynamics by simply extending our analysis to a community sample, likely composed of internalizing and externalizing individuals then compared to low-risk ones. In the present study, starting from a normative sample of post-adolescent population, three subgroups were selected. Subjects characterized by aggression, breaking rules and intrusive behavior were grouped as "externalizing". Likewise, subject showing a withdrawn phenotype, anxiety-depression and somatic complaints were grouped as "internalizing". Internalizing symptoms include anxiety, sadness, social withdrawal, and fearfulness while externalizing symptoms include overactivity, poor impulse control, noncompliance, and aggression (Achenbach & Edelbrock, 1981; Werry & Quay, 1971; Parade et al., 2016). The low-risk sample is composed of individuals with very low scores on each of these behavioral scales.

2. Methods

The study gathered 80 young adults of a community sample with a median age of 23 years (M=22.7; SD=2.9). Biological samples were collected by buccal swabs. All individuals were then subjected to the Adult Self Report (ASR) (Achenbach et al., 2003) questionarie. The ASR for ages 18–59 is a self-report, paper-and-pencil survey used to elicit informations regarding psychological functioning. Items are assessed on a three-point Likert scale (0 = not true, 1 = somewhat true or sometimes true, and 2 = very often or very true). For the current study, two narrowband scales were utilized as indicators for the latent constructs of internalizing and externalizing behavior problems, respectively.

Specifically, the indicators of externalizing behavior problems were as follows: Aggressive Behavior (e.g., "I argue a lot" and "I am mean to others"), Rule-Breaking Behavior (e.g., "I destroy my own things" and "I act without stopping to think"), and Intrusive Behavior ("I brag" and "I try to get a lot of attention"). Similarly, indicators of internalizing behavior problems were as follows: Anxious/ Depressed (e.g., "I feel that no one loves me" and "I cry a lot"), Withdrawn (e.g., "I am not liked by other kids" and "I keep from getting involved with others"), and Somatic Complaints (e.g., "I feel dizzy or lightheaded" and "I feel overtired without good reason"). Research has demonstrated good reliability and validity for the ASR scales (Achenbach et al., 2003).

Genomic DNA was prepared from buccal swab samples by using the BuccalAmp™DNA Extraction Kit, following the manufacturer's instructions (Epicentre, USA). The 3'-UTR repeated sequence of the DAT1 gene was amplified by the polymerase chain reaction (PCR). The primer sequences employed were 5'-TGT GGT GTA GGG AAC GGC CTG AG-3' (DAT1-F) and 5'-CTT CCT GGA GGT CAC GGC TCA AGG-3' (DAT1-R). The PCR amplification was carried out in a final volume of 50 μl containing 3 μl of genomic DNA prepared using the Buccal Amp DNA extraction kit, 1.5 mM of MgCl2, 200 μM of dNTP, 50 mM of KCl, 10 mM of Tris–HCl (pH 8.3), 0.25 μM of each primer, and 1 U of Promega Taq DNA polymerase. The PCR amplification was performed for 35 cycles consisting of 94 °C for 45 s, 57 °C for 30 s, and 72 °C for 30 s. The genotype was estimated from the size of the PCR product analyzed by electrophoresis on 6% acrylamide gels stained with ethidium bromide.

The level of methylation for each subject was analyzed using PyroMark Q24 Software (Qiagen, Hilden, Germany), which calculates the methylation percentage (mC/(mC + C)) for each CpG site, allowing quantitative comparisons (mC is methylated cytosine, C is unmethylated cytosine). Methylation status the 5'-UTR DAT1 (ENST00000270349.12) was determined on bisulfite converted DNA isolated by standard method from saliva sample. After extraction, 0.5 μg of DNA from each sample was treated with bisulfite, using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA). Bisulfite treated DNA was amplified by PyroMark PCR Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. PCR conditions were as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and, finally, 72 °C for 10 min. The schematic representation of CpG island in DAT1 is illustrated in Figure 1. 5'-UTR was analyzed using pyrosequencing assay (PM00022064) and all the details on the sequence are available on the Qiagen web site. Two standard human DNA samples, fully methylated (100%) and unmethylated DNA (0%) were purchased from Zymo (Zymo Research; Irvine, CA, USA) and used, respectively, as positive and negative methylation control. They were bisulfite-converted and were run along with the experimental samples. With the buccal swab, we were able to analyze the DNA methylation levels of two CpG island motifs in DAT 5'-UTR. M1 to M7 CpG residues are underscored in the following sequence, being ordered from left to right, at 1,444,717 on chromosome 5 (+713 from TSS): [1]CGG[2]CGG[3]CGGCTTGC[4]CGGAGACT[5]CG[6]CGAGCTC[7]CG. Notably, the CpG residues named M4 do not appear to undergo methylation. M1–M3 represent a

CGGCGGCGG motif, while contiguous M5/M6 represent a CGCG motif. We took pairs of loci namely one position and following one and we considered the four possibilities for each pair, considering that a pair can be demethylated or methylated in each position.

The indexes of probability for these four "situations" were: both methylated, M1-M2; first methylated and following de-methylated M1-D2, (M1) x (100-M2); first de-methylated and following methylated D2-M1, (100-M2) x (M1); both de-methylated D1-D2. For every couple (even across motif, non consecutive loci) we could still identify the same four possible situations (Lambacher et al. 2020). Therefore, we decided to look for all the possible correlations between couples (probability of a given situation in one position *vs* probability of a given situation in any other intra-motif or inter-motif pair of positions). In this way, we aimed at looking which setup was the most probable when considering CpGs dynamics (i.e. correlation couples: one locus *vs* one intra-motif or inter-motif pair of loci, which could each be either methylated or de-methylated).

2.1 What was done to build up the three subgroups.

Grouping the subjects, we took into account the psychometric-scale values for the following emotional-behavioral characteristics, or phenotypic traits: withdrawn, anxiety/depression, and somatic complaints; rules' breaking, aggression, and intrusive behavior. The first three phenotypic traits are internalizing while the three others externalizing, by definition (Achenbach & Edelbrock, 1981).

The psychometric-scale values, obtained from ASR test, were used to build the three subgroups. We considered as Internalizing or Externalizing the subjects with the highest psychometric-scale values for at least two out of the three peculiar phenotypic scales of the subgroup. Our Internalizing subjects present a values range included between 1.6 and 0.6, while between 1.67 and 0.63 for the Externalizing subgroup. Both of these ranges are just below the threshold characterizing pathological individuals on the CBCL scale (Achenbach et al., 2003) and therefore represent individuals who are not overtly pathological but highly vulnerable.

Low-risk individuals were those with overall lowest psi-values in all phenotypic scales so that their risk of developing internalizing or externalizing traits was very low. Every individual presenting low psychometric-scale values (score < 0.57) in all phenotypic scales was accounted as belonging to the low-risk subgroup. We have also excluded from such subgroup all subjects with even just one score > 0.57, i.e. whenever low scores were not shown in the totality of phenotypic scales considered. Following these criteria we ended up with three subgroups (14 subjects for each subgroups). The median age of each subgroup was between 22 and 23 years as in the initial normative population sample.

We thought that subjects showing more elevated scores in phenotypic traits of rules' breaking, aggression, and intrusive behavior, were more likely to represent vulnerability to psychopathologies with prevailing externalizing traits. The same goes for subjects belonging to the Internalizing subgroup. In some cases, we have included, in a given subgroup, even subjects with quite high scores for one or two scales of the opposite subgroup; this, only if the scores in scales of their final subgroup were all higher than those of the other subgroup. Subjects with high scores for scales belonging sometimes to externalizing and sometimes to internalizing subgroups were excluded from our study, because not specific but rather representative of overlapping phenotypic risks.

2.2 What was done to compare the three subgroups.

First of all, in order to get a clear insight into our results, we need to clarify what approach was followed. Three independent matrixes of cross-correlation, one per phenotypic subgroup, were built. Within each matrix, pairwise correlations were run between pairs, defined as above. We limited our analysis to "most important" CpGs (Tonelli et al., 2020; De Nardi et al., 2020): with them, ten pairs can be formed. In these pairs, since each locus can enter as either methylated or de-methylated, we can have a total of 4 situations x 10 pairs = 40 permutations. As such, when running 40 pairwise correlations (one per permutation), the chance to find false positives is high; thus, the P values associated with R values (with 13 degrees of freedom) had to be corrected for multiple comparisons. Within each of the three phenotypic groups, there are 40 pairwise permutations and ten steps between ordered means; since Bonferroni correction was then applied, a significant ($P < 0.05$) correlation was only considered for $R > 0.6411$ while a significant tendency ($0.10 < P < 0.05$) was only considered for $R > 0.6121$ (these pairwise correlations were considered « present » with acceptable risk of a false positive, and are reported as « red » in **Table 1**).

Correlations below these thresholds however are not necessarily absent, as the chance to find false negatives is high as well; therefore, with 40 pairwise permutations and ten steps between ordered means, probability 100% that one is wrong if we always refuse H0 was used as threshold. Hence, pairwise correlations were considered « absent » with acceptable risk of a false negative for $R < 0.3646$ (these correlations are reported as « blue» in **Table 1**). A number of all these pairwise correlations for $0.3646 < R < 0.6121$ were classified as « undetermined » since there was too high a probability to commit either a false positive or a false negative. All correlations are also represented in **Figure 1**.

As a final step, once all pairwise correlations were classified as "present" "absent" or "undetermined" independently in each matrix (one per phenotypic group), we made a comparison across matrixes to see what correlations were "present" in a phenotypic group and "absent" in another phenotypic group.

3. Results

The three subgroups we formed were checked for any unbalance in the distribution of genotypes for DAT1 and age of the subjects **(Table 2)**.
Interestingly, DAT1 genotypes were equally represented in the subgroups.

3.1 What happens in other subgroups when Low-Risk does show a correlation?

A significant positive correlation was found in Low-risk subgroup ($R = 0.63$; $p < .05$) between M2 and M1M6 but not in Internalizing ($R = 0.03$; $.10 < p$) and in Externalizing ($R = 0.29$; $.10 < p$) ones.

A significant positive correlation was found in Low-risk ($R = 0.68$; $p < .05$) and in Internalizing ($R = 0.7$; $p < .05$) subgroups between M2D1 and M3 but not in Externalizing ($R = 0.36$; $.10 < p$) one.

A significant positive correlation was found in Low-risk (R = 0.72; p < .05) and in Internalizing (R = 0.71; p < .05) subgroups between M3 and M2M5 but not in Externalizing (R = 0.36; .10 < p) one.

A significant positive correlation was found in Low-risk (R = 0.64; p < .05) and in Internalizing (R = 0.71; p < .05) subgroups between M3 and M2M6 but not in Externalizing (R = 0.29; .10 < p) one.

A significant positive correlation was found in Low-risk (R = 0.74; p < .05) and in Internalizing (R = 0.67; p < .05) subgroups between M3 and M2 but not in Externalizing (R = 0.1; .10 < p) one.

3.2 What happens in other subgroups when Low-Risk doesn't show any correlation?

3.2.1 When Low-risk and Internalizing subgroups don't show a significant positive correlation while Externalizing subgroup does.

Low-risk (R = -0.36; .10 < p) and in Internalizing (R = 0.17; .10 < p) subgroups don't show a correlation between M5D6 and M3 but Externalizing one does (R = 0.78; p < .05).

Low-risk (R = -0.14; p < .05) and in Internalizing (R = 0.32; .10 < p) subgroups don't show a correlation between M5 and M3 but Externalizing one does (R = 0.76; p < .05).

3.2.2 When Low-risk and Externalizing subgroups don't show a significant positive correlation while Internalizing subgroup does.

Low-risk subgroup (R = 0.33; .10 < p) doesn't show a significant positive correlation between M3 and M5M2 while Internalizing one does (R = 0.79; p < .05). Externalizing subjects are undetermined.

Low-risk (R = 0.28; .10 < p) and Externalizing (R = 0.1; .10 < p) subgroups don't show a significant positive correlation between M5 and M1 while Internalizing one does (R = 0.89; p < .05).

Low-risk (R = 0.09; .10 < p) and Externalizing (R = 0.24; .10 < p) subgroups don't show a significant positive correlation between M5D6 and M1 while Internalizing one does (R = 0.84; p < .05).

3.2.3 Other situations

Low-risk subgroup (R = 0.34; .10 < p) doesn't show a significant positive correlation between M5D6 M7 while Internalizing subgroup (0.71; p < .05) and Externalizing one do (R = 0.76; p < .05).

Low-risk subgroup (R = -0.06; .10 < p) doesn't show a significant positive correlation between M5 and M6M1 while Externalizing subgroup shows a significant negative correlation (R = -0.65; p < .05). Internalizing subjects are undetermined.

Externalizing subgroup (R = 0.66; p < .05) shows a significant positive correlation between M1 M3M2 while Internalizing one doesn't (R = 0.33; .10 < p). Low-risk subjects are undetermined.

4. Discussion

The results issued out of this innovative kind of analysis have shown that specific patterns exist for dynamics of methylation at DAT1 5'-UTR, within subjects showing non-clinical Internalizing and Externalizing symptoms, when compared to the low-risk subgroup. To be noted that this is the first time, for CpG methylation data, that a matrix of pairwise correlations is investigated in a control group (composed of healthy volunteers). So far, the same innovative approach was only applied to clinical groups, composed of either ADHD or Parkinsonian patients and in Internet Addiction (Lambacher et al., 2020; Tafani et al., 2020; De Nardi et al., 2020). As far as the DAT1 5'-UTR is concerned, these clinically-recruited patients were characterized by an overall hypomethylated versus hyper-methylated vs. unchanged condition, respectively.

Starting from (healthy) low-risk individuals, we found a correlation between the trend of methylation in CpG1 and that in CpG6. This leads to the understanding that the trend of both CpG1 and CpG6 is opposite to that of CpG2 and CpG3; therefore, when CpG1 and CpG6 get methylated, apparently CpG2 and CpG3 get de-methylated and vice versa.

This notion was quite unexpected. In our recent study (Tafani et al., 2020), we already found two possible profiles: a clear cross-correlation among CpGs of the first motif (i.e. loci 1, 2, 3) was typical of nearly a half of the subjects, and specifically of those individuals with low levels of methylation at CpG6. Interestingly, the other half of subjects showed instead consistent patterns of cross-correlations between the two motifs (CpG 1 with 3 and CpG 2 with 6), exactly alternative to those found in the present study. To reconcile this apparent inconsistency, we will first discuss the two deviant subgroups. In regard to the Internalizing subgroup, we found that methylation in CpG1 follows an opposite trend compared to CpG2 and CpG3. Specifically, while CpGs 2 and 3 appear to covary with CpG 6, the CpGs 1 and 5 conversely anti-covary with CpG 6. Such a specific dynamic at CpG 1 is substantiated by the positive correlation found in Low-risk and in Internalizing between M2D1 and M3; this denotes that loci 1 and 2 are strictly but inversely related, as the multiplication between M2 and 100-M1 is the correlating term; therefore, if the CpG1 gets methylated, CpG2 and CpG3 get de-methylated. Regarding CpG6, after a careful investigation of data, we propose that covariation dynamics follows specifically two paths: upon locus-6 de-methylation, both loci 2 and 3 get de-methylated while loci 1 and 5 get methylated. An overall de-methylation happens on CpG6 and CpG 2, as M2xM6 index is lowered to 8,42 ±3,10 (Tafani et al., 2020). Therefore, in presence of methylation at CpG1, interestingly CpG2, CpG3 and CpG6 would all get de-methylated. This strikingly resembles what was found previously. The profiles composed of twin trans-motif covariation are consistent with DAT 10\10 genotype Parkinsonian patients; the hypo-methylation at loci 2 and 6 while CpG1 gets methylated was found for severe DAT 10\10 genotype ADHD patients (Adriani et al. 2018).

Compared to Internalizing people, methylation dynamics is completely reverted in the Externalizing subgroup, where trends of correlation do change towards a more simple and consistent fashion: that is, CpG1 assumes the same tendency of CpG2 and CpG3 but opposite to CpG6; consequently, the latter gets methylated if CpG1, CpG2 and CpG3 get demethylated (and vice versa). Intriguingly, this profile is in fact composed of a clear-cut covariation within the first motif, which in turn is opposite to the second motif. Very intriguingly, such profile remembers that found for Parkinsonian subjects with high levels of the M2xM6 index (and preferably one 9-repeat DAT allele, also). As a matter of fact, in subjects with Externalizing symptoms, the M2xM6 index reaches 18,66 ± 3,26 (Tafani et al., 2020). However, since the CpG2 anti-covaries, the overall methylation happens on CpG6! Leaving CpG2 apart, the hyper-

methylation at locus 6 (here, dissociated from locus 2) resembles easy-recovery and 9\x ADHD patients (Adriani et al. 2018).

To summarize: In the internalizing subgroup CpG6, 2 and 3 follow the same trend and locus 1 is opposite. In the externalizing subgroup, CpG1, 2 and 3 follow the same trend, and locus 6 is opposite. Still we shall understand why, for people at low risk, CpG6 is correlated with CpG1, while 2 and 3 are opposite. If this is really the control condition, from such starting point two diverse destinies (paths) may occur if the CpGs 1 and 6 cease(stop) to covary. On the one side, the CpG1 may get linked to loci 2 and 3 (strong inter-motif link; present Externalizing people) and the motifs anti-covary. On the other hand, the CpG6 instead may get linked to loci 2 (and 3, strong intra-motif 2-to-6 link; present Internalizing people) and the extreme loci (1 and 6) anti-covary. However, no clear direction was identified in the LR people, allowing us to propose that these two tendencies persist in a dynamic equilibrium.

4.1 Clinical remarks

In a previous study, we have found that a methylation at CpG1 clearly connotes a severe pathological phenotype in relation to ADHD (Adriani et al. 2018). In that study, methylation at CpG6 appeared as a biomarker for recovery subsequent to a therapy for 6 weeks. In our present study, similarly, an increased vs a reduced methylation at CpG6 appears in those individuals who present two quite opposite tendencies towards phenotypic deviance, therefore the same diagnostic method can be used as well in a normative population. In this present study, methylation at loci CpG1 and CpG6 covaries in low-risk individuals: that is, if in one of the two loci methylation is raising, we expect the other locus should rise too. This prediction should be verified in a single healthy individual followed over time. When methylations in CpG1 and CpG6 begin to have opposite dynamic trends, subjects can become deviant. Such alteration drifts towards internalizing behaviors when trend is presenting hypo methylation at CpG6, towards externalizing profiles in the opposite case.

Therefore we can notice in the externalizing subgroup the same single-locus methylation biomarker that we found in easy-recovery ADHD; conversely, about internalizing subjects we found the same across-motif methylations pattern observed in severe no-recovery ADHD.

In recent studies, it has been seen that relations exist between ADHD and externalizing as well as internalizing phenotype (Cimino et al. 2019). Children with ADHD often suffer from comorbid psychiatric disorders, the more common are depressive disorders and anxiety, usually associated with a poorer prognosis. ADHD is often closely related with disruptive behavior, characterized by psychopathological constructs like aggression, impulsivity, violence, anti-sociality and psychopathy. In another study on ADHD, depression, irritability and anxiety symptoms as well as suicidality (suicidal ideation, attempts or self-harm) were measured: authors highlighted the fact that ADHD is also associated with increased suicidality. Potential mechanisms remain unclear (Levy et al., 2020). The effects of genetic and environmental risks are suggested by an increasing number of evidences (Tistarelli et al., 2020; Retz & Rösler, 2009). Parents of children with ADHD are more common to self report symptoms of ADHD than parents of children without ADHD (Xia et al., 2015).

4.2 Conclusion

DNA methylation is known to be tissue and cell-specific. There are many recent studies on the level methylation in DAT gene promoter, in which non-invasive biological samples such as buccal swab or saliva have been used. Several studies underlined that saliva samples with higher epithelial content were more similar to brain with regard to DNA methylation levels (Braun et al., 2019; Smith et al., 2014). Significant and concordant changes in DNA methylation were found for several genes in both saliva and brain, making saliva a valid source for methylation studies in several diseases (Abdolmaleky et. Al. 2015; Nohesara et al. 2011).

According to our results, we hypothesize that even in a normative population it is possible to identify two distinguishable behavioral profiles on the base of epigenetic modifications.

As mentioned, the environment could well affect DNA methylation in multiple instances, including the 5'-UTR in the DAT gene: therefore, exposure to adverse environmental factors could lead to specific changes in the dynamic methylation patterns. Although further investigation is needed, it is tempting to propose a causal link with expression of a particular phenotype, such as anxious, depressed and even suicidal or conversely rule-breaking, antisocial up to violent behavior. The purpose of this commentary was to provide insights into a new possible approach for the search of epigenetic biomarkers, to identify the risk to develop externalizing or internalizing symptoms.

References

H.M. Abdolmaleky, S. Pajouhanfar, M. Faghankhani, M.T. Joghataei, A. Mostafavi, S. Thiagalingam **Antipsychotic drugs attenuate aberrant DNA methylation of DTNBP1(dysbindin) promoter in saliva and post-mortem brain of patients with schizophrenia and Psychotic bipolar disorder** Am. J. Med Genet. Part B Neuropsychiatr. Genet., 168 (2015), p.p. 687–696

T.M. Achenbach, C.S. Edelbrock **Behavioral problems and competencies reported by parents of normal and disturbed children aged four through sixteen** Monogr. Soc. Res. Child Dev*.,* 46 (1981), p.p. 1-82

T.M. Achenbach, L. Dumenci, L.A. Rescorla **Ratings of Relations Between DSM-IV Diagnostic Categories and Items of the Adult Self-Report (ASR) and Adult Behavior Checklist (ABCL)** Burlington : University of Vermont, Research Center for Children, Youth, and Families (2003)

W. Adriani, E. Romano, M. Pucci, E. Pascale, L. Cerniglia, S. Cimino, R. Tambelli, P. Curatolo, O. Granstrem, M. Maccarone, G. Laviola, C. D'Addario **Potential for diagnosis versus therapy monitoring of attention deficit hyperactivity disorder: a new epigenetic biomarker interacting with both genotype and auto-immunity** European Child & Adolescent Psychiatry, 27 (2018), p.p. 241–252

A.E. Autry, L.M. Monteggia **Epigenetics in suicide and depression** Biol. Psychiatry, 66 (2009), p.p. 812-813

P.R. Braun, S. Han, B. Hing, Y. Nagahama, L.N. Gaul, J.T. Heinzman, A.J. Grossbach, L. Close, B.J. Dlouhy, M.A. Howard III, H. Kawasaki, J.B. Potash, G. Shinozaki **Genome-wide DNA methylation comparison between live human brain and peripheral tissues within individuals** Transl. Psychiatry, 9 (2019)

S. Cimino, L. Cerniglia, G. Ballarotto, E. Marzilli, E. Pascale, C. D'Addario, W. Adriani, A.G.I Maremmani, R. Tambelli **Children's DAT1 Polymorphism Moderates the Relationship Between Parents' Psychological Profiles, Children's DAT Methylation, and Their Emotional/Behavioral Functioning in a Normative Sample** Int. J. Environ. Res. Public Health, 16 (2019)**.**

L. De Nardi, V. Carpentieri, E. Pascale, M. Pucci, C. D'Addario, L. Cerniglia, W. Adriani, S. Cimino **Involvement of DAT1 Gene on Internet Addiction: Cross-Correlations of Methylation Levels in 5'-UTR and 3'-UTR Genotypes, Interact with Impulsivity and Attachment-Driven Quality of Relationships** Int J Environ Res Public Health, 17 (2020)

T. Hillemacher, H. Frieling, T. Hartl, J. Wilhelm, J. Kornhuber, S. Bleich **Promoter specific methylation of the dopamine transporter gene is altered in alcohol dependence and associated with craving** J. Psychiatr. Res., 43 (2008), p.p. 388-392

G. Lambacher, E. Pascale, M. Pucci, S. Mangiapelo, C. D'Addario, W. Adriani **Search for an epigenetic biomarker in ADHD diagnosis, based on the DAT1 gene 5'-UTR methylation: a new possible approach** Psychiatry Res., 291 (2020)

T. Levy, S. Kronenberg, J. Crosbie, R.S. Schachar **Attention-deficit/hyperactivity disorder (ADHD) symptoms and suicidality in children: The mediating role of depression, irritability and anxiety symptoms** Journal of Affective Disorders, 265 (2020), p.p. 200-206

J. Mill, T. Tang, Z. Kaminsky, T. Khare, S. Yazdanpanah, L. Bouchard, P. Jia, A. Assadzadeh, J. Flanagan, A. Schumacher, S.C. Wang, A. Petronis **Epigenomic profiling reveals DNA-methylation changes associated with major psychosis** Am J Hum Genet, 82 (2008), p.p. 696-711

S. Nohesara, M. Ghadirivasfi, S. Mostafavi, M.R. Eskandari, H. Ahmadkhaniha, S. Thiagalingam, H.M. Abdolmaleky **DNA hypomethylation of MB-COMT promoter in the DNA derived from saliva in schizophrenia and bipolar disorder** J. Psychiatr. Res., 45 (2011), p.p. 1432–1438

S.H. Parade, K.K. Ridout, R. Seifer, D.A. Armstrong, C.J. Marsit, M.A. McWilliams, A.R. Tyrka **Methylation of the Glucocorticoid Receptor Gene Promoter in Preschoolers: Links With Internalizing Behavior Problems** Child Development, 87 (2016), p.p. 86-97

A. Petronis **The origin of schizophrenia: genetic thesis, epigenetic antithesis, and resolving synthesis** Biol. Psychiatry, 55 (2004), p.p. 965-70

W. Retz, M. Rösler **The relation of ADHD and violent aggression: What can we learn from epidemiological and genetic studies?** International Journal of Law and Psychiatry, 32 (2009), p.p. 235-243

A. Rubino, C. D'Addario, M. Di Bartolomeno, E. Salamone, N. Locuratolo, F. Fattapposta, N. Vanacore, E. Pascale **DNA methylation of the 5'-UTR DAT1 gene in Parkinson's disease patients** Annals of Neurology, in press (2020)

N. Tistarelli, C. Fagnani, M. Troianiello, M.A. Stazi, W. Adriani **Nature and nurture of ADHD and its comorbidities: A narrative review on twin studies** Neuroscience & Biobehavioral Reviews, 109 (2020), p.p. 63-77

E. Tonelli, E. Pascale, M. Troianiello, C. D'Addario, W. Adriani **DAT1 gene methylation as an epigenetic biomarker in Attention Deficit Hyperactivity Disorder: a commentary** Frontiers in Genetics, 11 (2020)

J.S. Werry, H.C. Quay **The prevalence of behavior symptoms in younger elementary school Children** American Journal of Orthopsychiatry, 41 (1971), p.p. 136-143

A.K. Smith, V. Kilaru, T. Klengel, K.B. Mercer, B. Bradley, K.N. Conneely, K.J. Ressler, E.B. Binder**, DNA extracted from saliva for methylation studies of psychiatric traits: Evidence tissue specificity and relatedness to brain** Am. J. Med Genet. Part B Neuropsychiatr. Genet., 168 (2014), p.p. 36–44

X. Tafani, E. Pascale, F. Fattapposta, M. Pucci, C. D'Addario, W. Adriani **Cross-correlations between motifs in the 5'-UTR of DAT1 gene: Findings from Parkinson's disease** Advances in Biological Regulation, 78 (2020)

Y. Xu, X.-T. Chen, M. Luo, Y. Tang, G. Zhang, D. Wu, Y. B. Yang, D.-Y. Ruan, H.-L. Wang **Multiple epigenetic factors predict the attention deficit/hyperactivity disorder among the Chinese Han children** J. Psychiatr. Res., 64 (2015), p.p. 40–50

W. Xia, L. Shen, J. Zhang **Comorbid anxiety and depression in school-aged children with attention deficit hyperactivity disorder (ADHD) and self reported symptoms of ADHD, anxiety, and depression among parents of school-aged children with and without ADHD** Shanghai archives of psychiatry, *27* (2015), p.p. 356–367

Figure 1

Figure 1
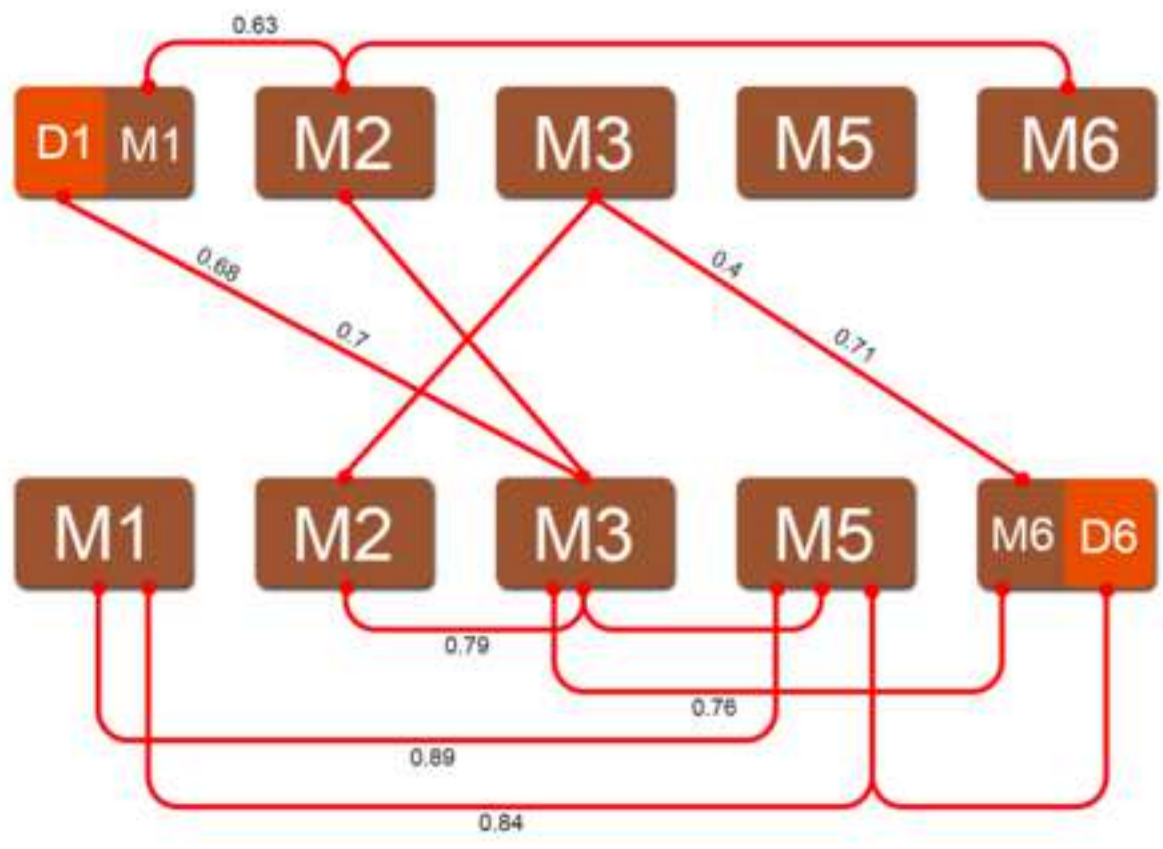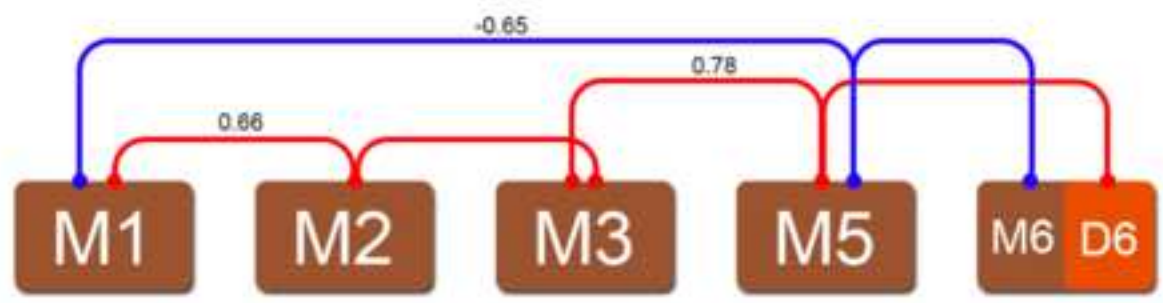
Captions

Click here to access/download
Table
Captions.docx

Table1

Click here to access/download
Table
Tab 1.PNG

Table2

Click here to access/download
Table
Tab 2.png