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Cross-correlations between motifs in the 5′-UTR of DAT1 gene: Findings from Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a neuro-degenerative disorder affecting the striatal motor system, caused by the loss of neuronal cells in the mid-brain, where reduced amounts of dopamine do cause involuntary movements and others symptoms. Alterations of methylome have been reported in PD epigenomic studies, and also human dopamine transporter gene (DAT1, *SLC6A3)* is considered as a candidate risk factor for PD. Since the DNA methylation on DAT promoter may well have a role in the development of this disease, we aimed to further assess the epigenetic control, by focusing on specific CpG sites located in the 5′-untranslated region (5′-UTR) of the DAT1 gene. Significant changes in DAT 5′-UTR methylation were already found in peripheral blood mononuclear cells (PBMCs) of PD subjects (Rubino et al., 2020). Of note, methylation values at the CpG 5 were increased. We run on same data a novel statistical approach: cross-correlation between pairs of loci. CpG 5 was the only always-differing variable but, alternatively, CpGs 2 and 6 or CpGs 1 and 3 were also significantly correlated with CpG 5. Interestingly, this picture emerged for those patients whose M2xM6 index was above-median; loci were rather independent for below-median patients. Present data may shed light into dynamics occurring at 5′-UTR of DAT1, a gene involved in PD but also in many psycho-physiological pathologies.

1. Introduction

The Parkinson's Disease (PD) is a neuro-degenerative disorder that affects the extra-pyramidal tract of the midbrain. The exact cause is still unknown but most of the research points to a combination of genetic and environmental factors. PD symptoms manifest themselves with a loss of neural cells in a well-known part of the midbrain, the substantia nigra, responsible for producing dopamine (DA). The reduced amount of dopamine released in the dorso-striatal forebrain, when the midbrain cells are damaged or dead, causes the involuntary movements and the other symptoms like tremor, slowness of movements and rigidity.

The human dopamine transporter gene (DAT1, symbol *SLC6A3*) is considered as a candidate risk factor for Parkinson's Disease

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(Zhai et al., 2014). Synaptic DA dysfunction may represent an early stage resulting from degeneration of the substantia nigra pars compacta (SNc). Some modifications in PD were reported to occur early on, before the extensive neuronal death has occurred. Interestingly, a trend for reduced expression of DAT1 was detected on idiopathic PD patients in DA neurons from the substantia nigra (Fazio et al., 2018). The dopamine-transporter (DAT) protein would accumulate, otherwise, cytotoxic dopamine within the dopaminergic terminals of neurons. These dopamine levels cannot be measured by imaging techniques: an indirect way to measure them is by looking for the DAT protein as the marker. The DAT-scan technique is used for the confirmation of a Parkinson's diagnosis: it is a specific type of single-photon emission computed tomography (SPECT), used to visualize dopamine-transporter levels in the brain (Costa et al., 2011). The DAT-scan can be used to differentiate PD symptoms from essential tremors or drug-induced PD: in the last two cases, patients don't have any loss of dopamine-transporter sites within their nigro-striatal system. The possible cytotoxic effects, related to DA reuptake, may be conferred by genetic factors like a polymorphism in the DAT gene. The human DAT1 gene contains a 40-bp variable number of tandem repeats (VNTR) polymorphism, found in the 3′-untranslated region (3′-UTR) of the gene. Several groups have studied the associations between this VNTR polymorphism and some neuro-psychiatric disorders such as PD, as well as ADHD, schizophrenia and alcoholism (Vandenbergh et al., 2000; Hahn et al., 2011). Being the VNTR polymorphism located outside the open reading frame, it cannot affect the protein structure but it may affect its expression level, by modulating the structure or degradation of mRNA (Fuke et al., 2001).

Beyond genetics, the promoter-specific DNA methylation may have a role in the development of these diseases: epigenetic mechanisms include any process regulating gene expression without affecting the genome sequence. In most of the cases, a higher methylation leads to repression of a gene (Domcke et al., 2015). DNA methylation has been recently implicated in the development of psychiatric disorders, such as bipolar disorder, depression and schizophrenia (D'Addario et al., 2013; D'Addario et al., 2012). Of note, epigenetic control likely involves the promoter region, well before the start site of mRNA transcription, or the 5′-untranslated region (5′-UTR), namely the portion of the gene which is at beginning of mRNA but before the start of the protein. This portion of DAT1 has been recently investigated by others, for instance in the frame of ADHD (Wiers et al., 2018). We similarly have assessed (Adriani et al., 2018) the epigenetic status of the 5′-UTR of DAT1 gene, but we have been addressing the ^{1}CGG^{2}CGG^{3}CGG and the ^{5}CG^{6}CG motifs on both strands (Tonelli et al., 2020). We found that higher levels of methylation at CpG 1 were serving an index for severity of ADHD; in contrast, higher levels at CpG 6 were correlated with rescue of ADHD symptoms after six weeks of treatment. As a whole, the ADHD patients were characterized by greatly reduced methylation, compared to controls. It was yet to be ascertained what would happen in the case of elevated overall methylation.

Alterations of the methylome have been reported in PD epigenomic studies, and altered pattern of methylation in some genes have been found in both sporadic and familial PD (Eryilmaz et al., 2017; Kaut et al., 2017). Even so, there is no clear report about which methylations do change during the progress of the disease (Wüllner et al., 2016). Therefore, we aimed to assess the epigenetic control of DAT1 in PD (Rubino et al., 2020), by focus on the same specific CpG sites (in the 5′-UTR of DAT1), previously shown to be differentially methylated in ADHD subjects (Adriani et al., 2018). A clear difference between PD patients and controls appeared only when considering the age as factor: methylation values at the CpG sites 2, 3, and 5 were different between the various age groups. Whereas CpG 5 was the only clearly different variable, and can be considered as hyper-methylated, CpG 2 and 3 also changed significantly, depending on the stage of the disease, and were somewhat correlated with CpG5. However, we recently developed a new approach to study dynamic relationships for changes of methylation status (Lambacher et al., 2020; see also Carpentieri et al., 2020). Presently, we aimed at applying such approach on our PD data, because they gave us the unique opportunity to monitor the DAT1 5′-UTR, when a situation of overall hyper-methylation could unveil unsuspected dynamics for interaction among these loci.

2. Method

The original study group (Rubino et al., 2020) consisted of 101 unrelated sporadic PD outpatients, exclusion criteria where the following: signs of atypical parkinsonism; history of neurological diseases other than idiopathic PD. Following initial recruitment, alcohol and substance abuse, family history of movement disorders, any other neurological disorder were also added to the exclusion criteria. Methylation status of the DAT1 5′-UTR sequence was determined using pyro-sequencing of bisulfite-converted genomic DNA (isolated by standard methods from blood cells). The full details of this quantification are reported elsewhere (Rubino et al., 2020).

Out of the original study group, we extracted a subset of 19 patients with "advanced stage of illness" (Rubino et al., 2020). For these, we re-considered all data, not only as individual loci (i.e., value for each CpG site), but also in terms of covariation among them. In first place, we considered not only the level of methylation, calculated by the methylation percentage $\mathbf{(mC/(mC + C)) = M}$ (mC is methylated cytosine and C is unmethylated cytosine), but also de-methylation $\mathbf{(C/(mC + C)) = D}$ i.e. 100-methylation. In second place, for each couple of loci, we considered the four possible situations: both methylated, M1xM2; both demethylated, D1xD2; one methylated and one not, M1xD2 or D1xM2. The latter two situations are particularly important for this new kind of approach (see Lambacher et al., 2020; Carpentieri et al., 2020). Indeed, if a correlation with such an index is found, this sheds innovative insights because it suggests that some loci loose methylation when other loci get new methylation. Cross-correlations analyses were performed in such PD population, by means of Pearson's index between pairs of situations. Being it impossible to consider all permutations of the six CpGs methylations, we restricted our interest to some "quadrants" (this term denotes all the correlations emerging out from one single given situation), where the two loci of the situation under consideration are consecutive and in opposite setup, as far as methylation is concerned (namely: M1D2, D1M2, M5D6, D5M6). Accordingly, the same loci were then also looked for simpler correlations, i.e. individually: M1, M2, M5, M6.

Once the calculations with our new cross-correlation approach were concluded, we realized that the resulting information, depicted in Fig. 1, contained some apparently conflicting profiles. The CpG 2 in particular, could behave in two contrasting ways. On

one hand, it could covary with CpGs 1 and 3; on the other hand, it could covary with CpGs 5 and 6, shown in Fig. 2. To reconcile such contradiction, we hypothesized that either profile could occur in some individuals but not in others.

We therefore aimed at isolating the two possible sub-populations. We followed the reasoning that, when CpGs 2 and 6 covary, they should show both hyper-methylation and this could be detected via a multiplicative index composed of M2xM6 (unpublished observations on data from Adriani et al., 2018). We calculated this index M2xM6 for each subject, then sorted patients according to this index. Based on the median value, patients were split into two halves, above and below the median, namely M2xM6>67 versus M2xM6<67. Once obtained these two sub-populations, we have run on each a simple correlation among loci (Tonelli et al., 2020).

At this point, we inquired whether the two sub-populations obtained could be related in any way to the DAT genotype at 3′-UTR, similarly to what already found (Adriani et al., 2018). Thus, information related to genotype, performed on the 19 patients, was used with Fisher's exact probability test: these 19 patients were seven with 10\10 DAT genotype and twelve with 9\x DAT genotype. If the segregation of genotypes in the sub-populations was entirely casual, the expected frequencies would be 6 vs 6 for the twelve ones with a 9\x DAT genotype and 3.5 vs 3.5 for the seven 10\10-genotype ones.

3. Results

3.1. Cross-correlations among situations

The cross-correlations found in the analysis are too many to be described one by one. Just as an example of how the analysis was conducted, we display below the following ones: few cases of overlapping and\or apparently contradictory findings. In these examples, a same locus is reported twice (i.e. appearing in two situations, both second term of a correlation), either in pair with two different other loci (which are then never returned as pair), or as methylated and de-methylated at once. This is only apparently a strange situation: it has already allowed us to draw interesting insights (Lambacher et al., 2020). All correlations that reach statistical significance can be found in Fig. 1 and are discussed below **(see paragraphs 4.1 to 4.3).**

3.1.1. Quadrant M1-D2

-There is a positive correlation of (R = 0,70768633251) with the situation M3-M5.

-There is a negative anti-correlation of (R = -0,77401026034) with the situation M3-D5.

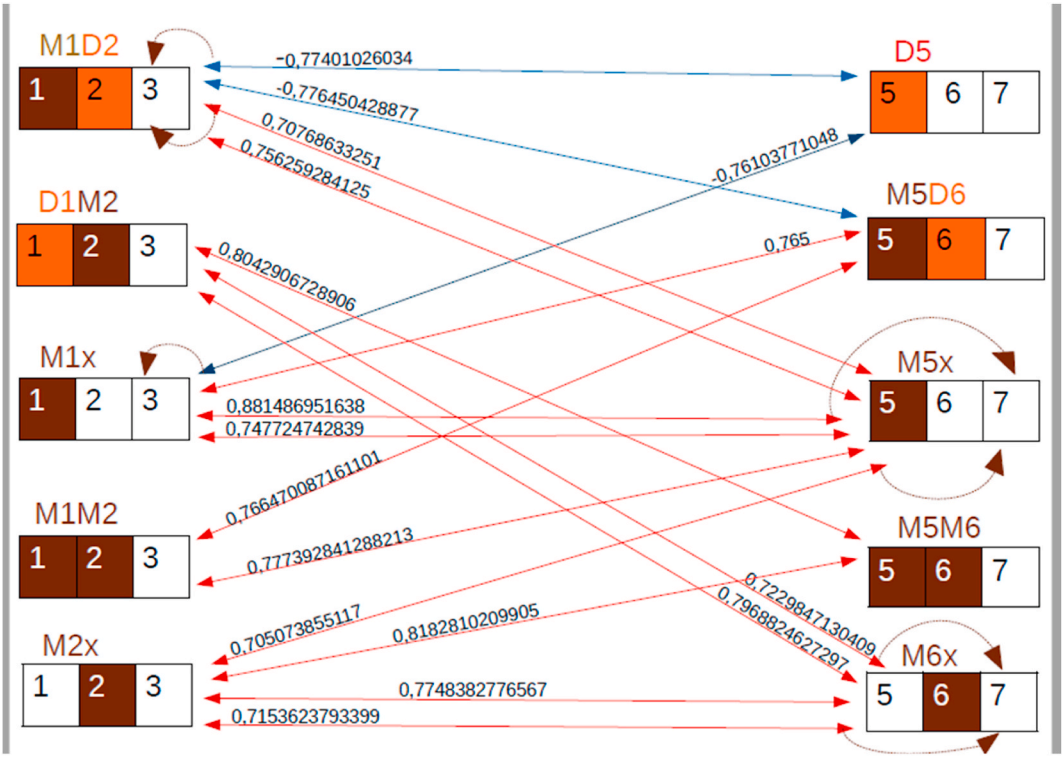

Fig. 1. We present data from cross-correlations in a graphic form. Brown-colour boxes mean the CpGs getting methylated, the orange-colour boxes mean the CpGs getting de-methylated, and the white colours means "not comprised in the pair". *Example: M1-D2 means that position CpG 1 is getting methylated and position CpG 2 is getting de-methylated.* The red arrows stand for any positive correlation and the blue ones for any negative correlation. The little brown arrow, prolonging from a red\blue arrow and indicating the white square, means that such additional CpG (locus number 3 or number 7) is getting methylated.

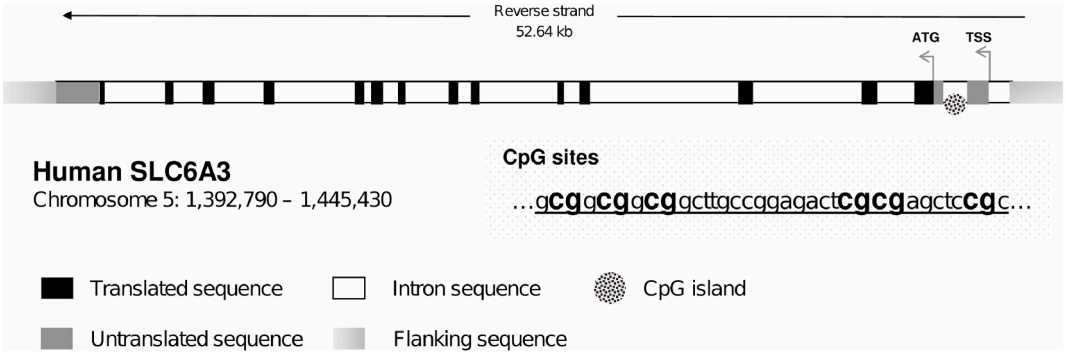

Fig. 2. Schematic representation of human *SLC6A3* gene (DAT1). Position of transcription start site (TSS), translation start code (ATG), exons and introns, are depicted. CpG island which contains the six CpG sites (in bold) is reported from +712 to +746 relative to TSS, well within the first intron, located in the 5′-UTR of the gene. The first "cg" of the shown sequence is the position 1,444,717 (at +713) and 1,444,716 (at +714), on reverse strand of chromosome 5. First motif is CGGCGGCGG (CpGs 1, 2, 3); second motif is CGCG (CpGs 5 and 6).

3.1.2. Quadrant M2-D1

-There is a positive correlation of (R = 0,8042906728906) with the situation M6-M5.
-There is a positive correlation of (R = 0,7229847130409) with the situation M6-M7.

3.1.3. Quadrant M5-D6

-There is a positive correlation of (R = 0,766470087161101) with the situation M1-M2.
-There is a negative anti-correlation of (R = -0,776450428877) with the situation M1-D2.

3.1.4. Quadrant M6-D5

Nothing (in terms of repetitive findings).

3.2. Cross-correlations between loci and situations

The situation is more clear when looking at pairwise correlations between a given CpG and all the possible situations (i.e. pairs of loci, which can be either methylated or demethylated).

3.2.1. Locus M1

- There is a positive correlation of (R = 0,747724742839) on the locus M1 with locus M5; this means very simply that both get methylated together.
- There is an anti-correlation of (R = −0, 76103771049) on the locus M1 with the situation M3-D5; in further specification, it never can happen that CpG 1 gets methylated together with the position CpG 3 whenever there is also a trend towards a de-methylated position CpG 5! In other words, there is *an apparent covariation* among CpGs 1, 3, and 5.
- There is a positive correlation of (R = 0,765) with the quadrant M5D6; in further specification, it can happen that CpG 1 gets methylated together with the position CpG 5 whenever there is a trend towards a de-methylated position CpG 6! As a *quick clue to this situation: CpGs 1 and 6 do go in an opposite fashion !*

3.2.2. Locus M2

- There is a positive correlation of (R = 0,7748382776567) on the locus M2 with the locus M6; this means very simply that both get methylated together.
- There is a positive correlation of (R = 0,8182810209905) with the situation M6-M5; in further specification, it can happen that CpG 2 gets methylated together with the position CpG 6 *whenever there is a trend towards a methylated position CpG 5!*
- There is a positive correlation of (R = 0,7153623793399) with the situation M6-M7.

3.2.3. Locus M5

- There is a positive correlation of (R = 0,777392841288213) with the situation M1-M2.
- There is a positive correlation of (R = 0,756259284125) with the quadrant M1-D2; as further specification, it can happen that CpG 1 gets methylated together with the position CpG 5 *whenever there is a trend towards a de-methylated position CpG 2!* In other words, there is an apparent *opposite* covariation among CpGs 1 and 5 on the one side, and CpG 2 on the other hand. As a *quick clue to this situation: CpG 5 may covary either with CpG 1 or CpG 2, not both !*

- There is a positive correlation of (R = 0,705073855117) with the situation M2-M7.
- There is a positive correlation of (R = 0,881486951638) with the situation M1-M7.

3.2.4. Locus M6

- There is a positive correlation of (R = 0,7968824627297) with the quadrant M2D1; in further specification, it well can happen that CpG 6 gets methylated together with the position CpG 2 *whenever there is a trend towards a de-methylated position CpG 1!* As a *quick clue to this situation: CpGs 1 and 6 do go in an opposite fashion !*

3.3. Simple-correlations between loci

Table 1 shows the Pearson R values for simple correlations among loci in either sub-population. Two intriguing profiles emerge.

For M2xM6<67, the CpG 2 is covarying with CpGs 1 and 3 (Ps < .05); although no direct correlation emerged between CpGs 1 and 3, the overall profile denotes an intra-motif link. The remaining CpGs 5 and 6 were somewhat spared.

For M2xM6>67, conversely, the CpG 2 is covarying with CpGs 5 and 6 while CpG 1 is covarying with CpGs 5 and 3 (Ps < .01); therefore, two inter-motif links emerge, in that the CpG 5 appears to establish two parallel links, one with CpGs 1 and 3 plus one with CpGs 2 and 6.

Interestingly, for these two sub-populations, differing for showing intra- versus inter-motif links, it also appeared to be a role played by DAT genotype.

The segregation of genotypes into sub-populations gave this **2x2 contingency table:**

Genotype \ Sub-population	M2xM6<67	M2xM6>67	Totals by genotype
Group 9\x genotype	4	8	12
Group 10\10 genotype	6	1	7
Totals by sub-population	10	9	19

With Fisher's exact test, the two-tailed P value equals 0.0573 (i.e., a significant tendency). In other terms, the DAT genotype 10\10 is over-represented in the M2xM6<67 population and under-represented in the M2xM6>67 population. For some reason, people who carry the 10\10 alleles are more likely to display only one intra-motif link (see Discussion); vice versa, people who carry at least one 9-repeat allele are more likely to display the two inter-motif profiles. Note that, in ADHD patients with one 9-repeat allele, the high value of the M2xM6 index was related to a recovery of symptoms after therapy (Adriani et al., 2018). This could suggest that inter-motif links in the 5′-UTR methylation dynamics and 9-repeat VNTR polymorphism at the 3′-UTR are somewhat related.

4. Discussion

First of all, the present study was conducted along a recent research line intended to provide new biomarkers, to link the functional dynamics of DAT1 gene with neuro-psychiatric pathologies in which DAT1 is involved. Our first efforts were directed to ADHD (Tonelli et al., 2020; Lambacher et al., 2020) but the question raised whether the emerging profiles were then specific of that condition or could be extended in a more general framework. In such sense, PD appeared to be somewhat "at the opposite corner" compared to ADHD in

Table 1
Bold R-values stay significant (p < .05) on both panels of the Table; the asterisk (*) highlighted R-values are only significant (p < .01) for M2xM6>67 (panel A) and not for M2xM6<67 (Panel B).

Panel A					
M2x6<67 (n=10)	CpG M1	CpG M2	CpG M3	CpG M5	CpG pattern
CpG M2	**0.7779**				1-2-3
CpG M3	0.4277 (*)	**0.6778**			
CpG M5	0.4914 (*)	0.4449 (*)	0.2203 (*)		
CpG M6	0.4999	0.5938 (*)	0.3070	0.5263	
Panel B					
M2x6>67 (n=9)	CpG M1	CpG M2	CpG M3	CpG M5	CpG pattern
CpG M2	**0.6999**				
CpG M3	0.8454 (*)	**0.6742**			1-3-5
CpG M5	0.8499 (*)	0.8841 (*)	0.7915 (*)		2-5-6
CpG M6	0.3568	0.8211 (*)	0.2566	0.5796	

that it is an organic, degenerative condition affecting the elderly as opposed to a more elusive behavioral condition in young developing kids. Additionally, whereas lower overall levels of methylation were typical of ADHD (Tonelli et al., 2020; Lambacher et al., 2020), PD patients showed hyper-methylation at 5'-UTR of DAT1.

In order to get a clear insight into our results, we shall underline that there are four "important" CpGs (i.e. 1, 2, 5, 6): with them, ten pairs can be formed. In this pair, each locus can enter as either methylated or de-methylated (a total of 4 situations x 10 pairs = 40 permutations). Therefore, pairwise correlations of all 40 permutations become $40 \times 40 = 1600$! Note that Fig. 1 shows significance for just a small portion of all theoric relationships.

If however these CpGs varied independently one from each other, there would be no specific relationship emerging as significant from our analysis. Thus, the fact that just some among all these relationships reached significance indicates that specific dynamics exist among the CpGs, and the two motifs, when getting methylated.

If we look well into the graphic, we can easily see that there is no arrow pointing directly between situations M1-M2 and M5-M6; this is a clear indication of the fact that we cannot ever have any dynamic situation with all these 4 positions getting methylated together.

4.1. Dynamics excluded due to negative correlations

If we look into the graphic of Fig. 1 from the up right, we have **D5** who points with a blue arrow to situation **M1-D2** with R value of *-0,77401026034*: this is an anti-correlation. Thus, it can *never* happen that CpG 2 *gets de-methylated together with the position CpG 5 whenever there is a trend towards a methylated position CpG 1!* In other words, the dynamic situation whereby CpG *1 gets methylated, 2 de-methylated* and *5 de-methylated* is impossible.

The same happens with the locus **D5** who points to **M1-M3** with a blue arrow, with R value of *-0,76103771048*: here is another anti-correlation. If the CpG 5 gets de-methylated, this cannot happen when the positions CpG *1 and 3 are getting methylated.* In other words, CpGs 1 and/or 3 shall *get methylated,* if CpG 5 is getting methylated; we don't have any other possible profile of situations.

The situation **M5-D6** that points with a blue arrow to **M1-D2** with R value of *-0,776450428877* is also an anti-correlation (see above). Here, we have a crucial notion about PD patients: there, overall, CpG 5 gets hyper-methylated (Rubino et al., 2020). Even whereby the positions CpG 1 and 5 get together methylated, anyway *it cannot happen* that CpGs 2 and 6 are getting *de-methylated.*

4.2. Dynamics involving correlations of CpG 5 with CpG 1

If we look the red arrows that start from **M1-D2** and point to **M5** with R value of *0,70768633251* plus to **M5-M3** with R value of *0,7562592841 25* (*the little brown arrow means that CpG 3 is methylated*), we will appreciate similarly the locus **M1** who points to **M5-M7** (*see the little brown arrow to CpG 7*), with the R value of *0,881486951638* (all positive correlations). This is a confirmation of the dynamics depicted above: it can happen that the positions CpG 1 and 5 are methylated together, and also CpG 7 *may get methylated.* Moreover, if we have CpGs 1 and 3 methylated together with the CpG 5 *(known to be hyper-methylated),* then it can happen that CpG 2 *alone is getting de-methylated.*

4.3. Dynamics involving correlations of CpG 5 with CpG 6

If we look to the situation **D1-M2** who points to **M6** with R value of *0,7968824627297* and to **M5-M6** with R value of *0,8042906728906* (all positive correlations), we can see how dynamics change when CpG 6 is involved. This can happen whereby CpG 5 *is hyper-methylated*, but the CpGs 2 and 6 are now getting *methylated together !* Interestingly, in such case, we will have the position *CpG 1 (just seen to covary with CpG 5 in one dynamic profile)* who is now getting *de-methylated.*

Accordingly, if we look to the position **M1** who points to **M5-D6**, with R value of *0,765* (see above), a dynamic profile that can easily happen, apparently, is whereby we have CpG 1 getting methylated together with CpG 5 *hyper-methylated* but now CpG 6 *is getting de-methylated*. Once again, CpGs 1 and 6 apparently follow their profiles with reciprocally opposite dynamics.

Conversely, we shall note the quadrant **M1-M2** which points to position **M5** alone with R value of *0,777392841288213* and which points to **M5-D6** with R value of *0,766470087161101* (all positive correlations). This reveals that another dynamic situation, that still can easily happen, is whereby we have CpGs 1 and 2 *methylated together*, when CpG 5 *is hyper-methylated;* and moreover this happens when CpG 6 *gets de-methylated.*

However, in apparent contradiction, position **M2** points to **M5** with R value of *0,705073855117,* to **M5-M6** with R value of *0,8182810209905* and to **M6** with R value of *0,7748382776567* (all positive correlations). There is however the parallel possibility of distinct dynamic profiles, with no contradiction. By looking into all data as a whole, it appears that loci can happen to follow two confirmed dynamics: the position CpG 1 *methylated*, when CpGs 2 or 3 also get methylated and "as opposite" CpG 6 *gets de-methylated;* or, alternatively, CpG 1 *de-methylated*, when CpGs 2 and 6 *get together methylated.*

4.4. Interpretation

Significant changes in DNA methylation were found in peripheral blood mononuclear cells (PBMCs) and brain of PD subjects (Eryilmaz et al., 2017; Kaut et al., 2017; Wüllner et al., 2016). Together with other genes, DAT1 expression is very susceptible to epigenetic modifications which have indeed been proposed for diagnostic and therapeutic approaches (Renani et al., 2019; Hegarty et al., 2016). The role of DNA methylation and its link to sporadic PD is not completely defined nor clearly characterized (Schmitt et al.,

2015; Schulze et al., 2018). By comparing the DAT1 methylation level at individual CpGs within the 5′-UTR, in PD, we recently observed an increased methylation at site CpG 5 (Rubino et al., 2020). On those data, a further analysis was run in the present study: the most important finding is the correlation between the increased methylation of CpG 5 and the distinct dynamics of corresponding change in all other CpGs of the same island.

The increased methylation of CpG5, found in all cases, seems to come along with corresponding de-methylation either at CpG1 or at CpG6, alternatively. This suggest that two distinct patterns, for dynamic changes of methylation, may emerge during the progression of the disease. The alternative patterns of cytosine methylation, indeed, are either CpG 5 with 2 and 6 or CpG 5 with 3 and 1, which in our hands is evidenced for the first time. Such patterns likely can regulate gene expression by affecting the ability of transcription factors to access and bind specific regions in the promoter sequence. It is commonly believed that DNA hyper-methylation could inhibit DAT1 transcription, which in turn may lead to reduced DA uptake: interestingly, however, the exact link between DAT1 expression and CpG methylation is unknown. As just an indirect indication, we found that CpGs 2 and 6 correlated well with the levels of circulating autoantibodies against DAT1 (Adriani et al., 2018), which in turn may reflect the DAT protein expressed in blood cells. If accordingly we take the M2xM6 of Table 1 as an index of DAT expression, we could draw the following hypothesis: patients with low DAT may have a sort of unity among CpGs 1, 2 and 3 (i.e., motif CGGCGGCGG); in patients with high DAT, to allow such increased new methylation at CpG 5, either CpG 1 (and 3) or 6 (and 2) appear to loose methylation. This inter-motif dynamics confirms similar findings on ADHD patients (Tonelli et al., 2020; Lambacher et al., 2020) as wells on healthy controls (Carpentieri et al., 2020).

The role of DNA methylation as a possible mediator that drives or accompanies PD development is starting to be explored. Though these dynamics, PD patients may turn out to display a specific adaptation, attempting to reduce levels of DAT protein in the pre-synaptic membrane of PBMCs; if a similar phenomenon is occurring also within the brain, at the level of nigro-striatal DA neurons, is yet to be ascertained. The small sample here examined cannot allow conclusive evaluations, yet the results support the general theory that epigenetic modifications within the DAT1 5′-UTR could take part in PD progression. The potential methylation dynamics of DAT1 gene promoter as a biomarker in PD, especially during disease progression, warrants further investigations in a larger group of PD patients. The present discovery can be a first, potential step towards a better understanding into the role of DAT1 epigenetic modifications underlying PD pathogenesis.

Within the brain, epigenetic mechanisms dynamically regulate gene expression in response to any environmental influence throughout the life span, enabling adaptive plasticity. Gene expression studies by qRT-PCR in PD dopaminergic neurons showed a trend for reduced expression of DAT (Simunovic et al., 2009). Our previous study (Rubino et al., 2020) showed that dynamic hyper-methylation of the DAT1 5′-UTR may emerge during PD progression. DAT1 gene activity is known to be regulated by the functional variable number of tandem repeats (VNTRs) located in the 3′-UTR (3′-VNTR), with the 9-repeat allele associated with increased level of dopamine transporter protein (van Dyck et al., 2005). Accordingly, DAT1 10\10 patients are under-represented within the subgroup displaying a high M2xM6 value, taken as index of DAT1 expression. Increased levels of dopamine in cytoplasm and its metabolites are neurotoxic and may lead to selective death of neurons within SNc, while treatments that decrease cytoplasmic dopamine levels provide neuro-protection in cultured midbrain neurons (Mosharov et al., 2009). It is hypothesized that depletion of dopamine in nigro-striatal neurons gets increased during PD progression. Thus, a modulation of DAT1 gene expression that leads to a reduction in protein levels could be beneficial: if this phenomenon really occurs in the remaining nigro-striatal neurons, similarly to present evidence in PBMCs, we could unveil an attempt of the midbrain to self-regulate, in order to ensure stable dopamine level in the synaptic gap.

Our results from the cross-correlation analysis, considering methylation levels at the 5′-UTR and the presence of the 9-repeat allele at the 3′-UTR of DAT1 gene, highlighted a possible link between these two regulatory regions. It can be hypothesized that, in PD subjects with advanced state of illness carrying the 9-repeat allele, an increased level of methylation at the specific CpGs of 5′-UTR is necessary in order to decrease DAT1 expression, in an attempt to limit the extent of neuro-degeneration. These preliminary results suggest that gene-expression studies will be mandatory to verify whether the observed hyper-methylation in 9-repeat carriers could represent the dynamic mechanism activated to counteract PD progression.

4.5. Methodological remark and limitations

The correlations were run between pairs of situations, one within first motif (i.e., CpGs 1, 2, 3) and the other within second motif (i.e., CpGs 5, 6, 7) while, in our recent work (Lambacher et al., 2020), we have also considered separately “hybrid” situations for correlation (Fig. 1). Any correlation emerging for a given pair when looking from another pair was interpreted as an inter-motif dynamics, in a cross-correlation strategy (Carpentieri et al., 2020). We are aware that this procedure may appear somewhat complicate. The simplest approach would be to look for simple correlations between couples of individual CpGs (Tonelli et al., 2020); this was already done in the original paper issuing from PD data (Rubino et al., 2020) but no clear dynamics were evidenced. As recently proposed (Carpentieri et al., 2020; Lambacher et al., 2020), we rather investigated pairwise correlations between couples of situations, considering for each member in the situation not only methylation but also corresponding de-methylation levels. Therefore, situations we are correlating are presenting in various set-ups, yielding to 16 combinations: while one given pair is in one state, another pair might be in any of its possible four states (i.e., MM; DD; MD; DM). This innovative approach amplifies the extracted information. Specific pairs of CpGs may well display coordinate changes of their methylation state, compared to other more stable situations (Carpentieri et al., 2020; Lambacher et al., 2020).

Lack of direct brain data is obviously a limitation. Yet, PBMCs are nowadays considered a convenient substitute for cerebral biomarkers (Mosharov et al., 2009; Woelk et al., 2011): they are readily accessible and contain the complete epigenetic machinery also present in neurons (Arosio et al., 2014). PBMCs have been proposed as a valuable tool for early diagnosis in PD studies (Wang et al.,

2019; Mao et al., 2018), and may reflect the molecular processes occurring in the central nervous system (Gladkevich et al., 2004). Furthermore, concordant changes in DNA methylation were found, in several genes, in both PBMCs and brain of both familial and sporadic PD, making PBMCs a valid source for methylation studies (Schmitt et al., 2015; Coupland et al., 2014; Chuang et al., 2017).

5. Concluding remarks

To reconcile the present findings with those published recently on ADHD (Adriani et al., 2018), the simplest observation is that ADHD patients display overall hypo-methylated states, while PD ones do show CpG 5 hyper-methylation. Accordingly, neither ADHD nor normative-population data ever point to CpG 5 (Adriani et al., 2018; Carpentieri et al., 2020). Opposite trends for CpGs 1 and 6, also evident in ADHD, are presently receiving further information: CpG 1 appears related to CpG 5 "through" CpG 3 while CpG 6 appears related to CpG 5 "through" CpG 2 ! Of these two dynamic profiles, interestingly, the second one turned out to be useful as a biomarker in ADHD, while its role in PD deserves further study.

In the end, it is tempting to speculate the hypothesis that present profiles, emerged across different subjects, may be even more evident if looking to a same individual. It may be of interest to follow the same subject repeatedly, for a prolonged period of time, and run similar cross-correlations on these multiple samples. It would become possible to observe whether methylation at these residues would show a similar profile of changes, and demonstrate that such dynamic changes can be coordinated in a yet unsuspected way.

The results of the present cross-correlation study seem to indicate the presence of distinct patterns for dynamic modifications in terms of (de)methylation of specific CpG sites: such levels could affect, through DNA protein binding, DAT1 gene expression hence accounting for variability observed in the course of PD (early or advanced stages). Present data could give insights into the pathogenesis of this disease and\or provide new biomarkers for diagnosis and therapeutic strategies.

Contribution to the field

We have been dealing as a group with the study of two particular motifs found in the 5'-UTR of the DAT gene. We have tried, and report here about, a completely new approach to methylation levels: instead of looking at CpGs individually, we think that cross-correlations may inform about which ones are getting (de)methylated at the same time on the very same DNA strand.

Author contribution

Conceptualization: FF, EP conceived the study; Investigation: EP, MP, CD realized the original CpG-methylation study; Formal analysis: XT, WA described the cross-correlations on the original methylation data; Writing – original draft: XT wrote a first draft; Supervision: XT had close supervision by WA; Visualization: MP contributed to the revision; Writing – review & editing: EP and CD critically commented on the paper.

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There is an item for potential conflict of interest to be disclosed: Adriani W., Laviola G., Pascale E., D'Addario C., inventors: "*Metodo per determinare il deficit di attenzione con iperattività*" (Method to determine Attention Deficit and Hyperactivity Disorder). Currently under interaction with the Examiner; Italian Patent Application at no. 102016000129938 (22-December-2016); turned into European Patent Application at no. 17830021.6 (21-December-2017).

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