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Exploring the role of BDNF DNA methylation and hydroxymethylation in patients with obsessive compulsive disorder

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ABSTRACT

Obsessive-compulsive disorder (OCD) is a clinically heterogeneous neuropsychiatric condition associated with profound disability, whose susceptibility, stemming from genetic and environmental factors that intersect with each other, is still under investigation. In this perspective, we sought to explore the transcriptional regulation of Brain Derived Neurotrophic Factor (BDNF), a promising candidate biomarker in both development and etiology of different neuropsychiatric conditions, in peripheral blood mononuclear cells from OCD patients and healthy controls. In particular, we focused on *BDNF* gene expression and interrogated in depth DNA methylation and hydroxymethylation at gene promoters (exons I, IV and IX) in a sample of OCD patients attending a tertiary OCD Clinic to receive guidelines-recommended treatment, and matched controls. Our preliminary data showed a significant increase in *BDNF* gene expression and a significant correlation with changes in the two epigenetic modifications selectively at promoter exon I, with no changes in the other promoters under study. We can conclude that transcriptional regulation of *BDNF* in OCD engages epigenetic mechanisms, and can suggest that this is likely evoked by the long-term pharmacotherapy. It is important to underline that many different factors need to be taken into account (i.e. age, sex, duration of illness, treatment), and thus further studies are mandatory to investigate their role in the epigenetic regulation of *BDNF* gene. Of note, we provide unprecedented evidence for the importance of analyzing 5-hydroxymethylcytosine levels to correctly evaluate 5-methylcytosine changes.

1. Introduction

Obsessive-Compulsive disorder (OCD) is a condition with frequent early onset and chronic course (Dell'Osso et al., 2013) characterized by recurrent, unwanted, time-consuming obsessive and compulsive behaviors that cause distress and/or impairment (Milad and Rauch, 2012). The World Health Organization classifies OCD as one of the 10 most disabling conditions for decreased quality of life and loss of income, with a lifetime prevalence of 2–3% of the general population (Milad and Rauch, 2012).

Abbreviations: **OCD**, Obsessive-Compulsive Disorder; **BDNF**, Brain Derived Neurotrophic Factor; **SNP**, Single Nucleotide Polymorphism; **5 mC**, 5-methylcytosine; **5hmC**, 5-hydroxymethylcytosine; **TET**, Ten-Eleven-Translocation proteins; **BD**, Bipolar Disorder; **MDD**, Major Depressive Disorder; **PBMCs**, Peripheral Blood Mononuclear Cells; **GAPDH**, Glyceraldehyde 3-phosphate dehydrogenase; **ß-ACT**, beta actin; **BS**, Bisulfite conversion; **oxBS**, oxidative Bisulfite conversion

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OCD diagnosis is complex and several studies investigated its genetic etiology with mixed and sometimes conflicting results mainly addressing serotonergic, glutamatergic and dopaminergic pathways (Carlsson, 1977; Grunblatt et al., 2014; Muneer, 2016).

Currently, the most effective pharmacological intervention for OCD is represented by the selective serotonin reuptake inhibitors (SSRIs) and, due to the delayed onset of their clinical efficacy, downstream molecular targets might be responsible for their therapeutic efficacy (Martinowich and Lu, 2008). One possible mechanism might involve brain-derived neurotrophic factor (BDNF) (Monteggia et al., 2004), well-known for its role in neuronal development, proliferation and survival, as well as in synaptic plasticity (Cattaneo et al., 2016; Duman et al., 2000). Indeed, the association of *BDNF* gene polymorphisms with OCD has been investigated, mainly focusing on the Val/Met polymorphism at codon 66, due to the Single Nucleotide Polymorphism (SNP) at nucleotide 196 (196G/A; rs6265), showing a protective effect of the rarer Met allele (Alonso et al., 2008; Hall et al., 2003; Taj et al., 2018), whereas others reported no association between the polymorphism and OCD susceptibility (Da Rocha et al., 2011; Wendland et al., 2007; Umehara et al., 2016; Wang et al., 2015) in OCD patients.

Despite solid evidence that genetic contribution is important for OCD, environmental factors as well as gene × environment interactions are also involved in disease development (Türksoy et al., 2014; Faravelli et al., 2012; Nestadt et al., 2010). Environmental signals are integrated by epigenetic processes in order to activate or repress gene expression regulating DNA accessibility for transcription factors (Jaenisch and Bird, 2003). The most stable epigenetic mechanism is DNA methylation, well-investigated in different psychiatric conditions (Nestler et al., 2016). DNA methyltransferases are the enzymes responsible of the transfer of a methyl group to the fifth carbon of cytosine residues to form 5-methyl-cytosine (5 mC) usually occurring at CpG dinucleotides (Jurkowska et al., 2011). Presence of 5 mC has been classically associated with gene silencing and formation of repressive chromatin (Schubeler, 2015). Only recently, more attention has been given to the oxidative reaction catalyzed by the ten-eleven-translocation (TET) proteins (Ito et al., 2010; Tahiliani et al., 2009; Zhang et al., 2010) and converting 5 mC into 5-hydroxymethylcytosine (5hmC) (Dahl et al., 2011). This is an intermediary step in the process of DNA demethylation (Guo et al., 2011; Hashimoto et al., 2012; Iqbal et al., 2011) and is generally associated with increased gene expression (Chen et al., 2012; Jin et al., 2011; Song et al., 2010). The relevance of 5hmC in neural function has already been suggested (Szulwach et al., 2011), however, as yet only a few reports have interrogated its role in brain disorders (Feng et al., 2015).

Human *BDNF* gene is located on chromosome 11p13-14, is formed by 11 exons (I-IX, Vh and VIIIh) and 9 promoters (Pruunsild et al., 2007). Alterations of DNA methylation in BDNF promoter exon I and IV have been already observed in peripheral blood mononuclear cells (PBMCs) of patients with different psychiatric conditions such as Schizophrenia (Roth et al., 2009; Ikegame et al., 2013; Kordi-Tamandani et al., 2012), Depressive Disorders (Chen et al., 2010; Lopez et al., 2013; Tadic et al., 2013; Kang et al., 2013), suicidal deaths (Keller et al., 2010), Bipolar Disorder (BD) (D'Addario et al., 2012; Dell'Osso *et al.*, 2014), and Major Depressive Disorder (MDD) (Fuchikami et al., 2011; Kang et al., 2013; Tadić et al., 2013; D'Addario et al., 2013), as well as in female adolescent patients with OCD (Nissen et al., 2016). Moreover, Mill and co-workers studied the coding exon IX, where the CpG SNP rs6265 is located, and reported the association between the polymorphism and DNA methylation at surrounding CpG sites in DNA from frontal-cortex brain tissues of individuals diagnosed with major psychosis (Mill et al., 2008).

We here investigated *BDNF* gene expression and the possible contribution of both 5-mC and 5-hmC at *BDNF* promoter exons I, IV and IX, as well as the role of rs6265, in relation to the pathophysiology of OCD, analyzing DNA extracted from PBMCs of patients and healthy control subjects.

2. Materials and methods

2.1. Subjects, gene expression, methylation analysis and genotyping

35 OCD outpatients followed up at the OCD tertiary outpatient Clinic of the University Department of Psychiatry of Milan, IRCCS Policlinico Hospital, were included in the study. Diagnoses were assessed by the administration of a semi-structured interview based on DSM-5 criteria (SCID 5 research version, RV) (First et al., 2015). In case of psychiatric comorbidity, OCD had to be the primary disorder and illness severity was measured through the Yale-Brown Obsessive Compulsive Scale (Goodman et al., 1989). Exclusion criteria were presence of medical condition and/or drug abuse. All patients were for at least one month on stable pharmacological treatment chosen according to International guidelines in the field (Koran et al., 2007). Control subjects (n = 32), matching for age (31.8 ± 7.4) and sex (Males: 60.7%; Females: 39.3%), were volunteers without any psychiatric disorder, as determined by the nonpatient edition of the SCID and no positive family history for major psychiatric disorders in the first-degree relatives (Maxwell, 1992). All subjects had given their written informed consent to participate in the study, which included the use of personal and clinical data as well as blood drawing for genotyping and methylation analysis. The study protocol had been previously approved by the local Ethics Committee of the IRCCS Ospedale Maggiore Policlinico. Demographic and clinical characteristics for the study sample as well as psychotropics used by OCD subjects are shown in Table 1.

2.2. Analysis of gene expression

Preparation of nucleic acids from PBMCs and analysis of *BDNF* gene expression paralleled methods described in detail elsewhere (D'Addario

Table 1
Social and clinical variables of OCD subjects.

Gender	
Male (N)	16
Female (N)	19
Age at recruitment (years, mean ± SD)	37.5 ± 13.6
Age at onset (years, mean ± SD)	21.8 ± 10.4
Duration of illness (years, mean ± SD)	17.5 ± 10.5
Duration of untreated illness (months, mean ± SD)	56.3 ± 72.0
Education (%)	
Secondary school	15.2
High-school	57.5
University	27.3
Employment (%)	
Employed	39.3
Unemployed	48.5
Student	6.1
Retired	6.1
Married (%)	30.3
Family history of psychiatric disorder (%)	63.6
Psychiatric comorbidity (%)	60.6
Drug naive patients (%)	8.6
Current treatment (%)	
antidepressants	85.7
antipsychotics	57.1
mood stabilizers	34.3
benzodiazepines	42.9
Psychotropic compounds (number, mean ± SD)	2.39 ± 1.6
≥1 psychotropic compounds (%)	74.3
YBOCS (mean ± SD)	23.11 ± 7.95
Presence of comorbidities (%)	61.0

et al., 2012). Briefly, PBMCs were separated by density gradient, total RNA isolated using the Chomczynski and Sacchi's modified method (Chomczynski and Sacchi, 2006) and quantified by means of spectrophotometry. Following reverse transcription abundance of *BDNF* mRNA was assessed by real-time RT-PCR using DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). All data were normalized to four endogenous reference genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-actin (*BACT*), cytochrome C oxydase subunit 6A1 (*COX6A1*) and ribosomal protein lateral stalk subunit P0 (*RPLP0*). Relative expression of different gene transcripts was calculated by the Delta-Delta Ct (DDCt) method and converted to relative expression ratio (2^DDCt) for statistical analysis (Livak and Schmittgen, 2001). Primer sequences are reported in Supplementary Table S1.

2.3. DNA methylation/hydroxymethylation analysis

To study DNA methylation/hydroxymethylation, two aliquots of 500 ng of genomic DNA samples were processed through bisulfite conversion (BS) or oxidative bisulfite conversion (oxBS) (Matsubara et al., 2015; Qu et al., 2015) and amplified by Pyromark PCR Kit (Qiagen) in accordance with the manufacturer's protocol. The sequences were designed to target regions within the CpG islands located upstream *BDNF* exon I (4 Cpg sites) and exon IV (6 CpG sites) as well as in within exon IX (2 CpG sites including the one created by the SNP rs6265) (see Fig. 1 for details). 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. PCR products were verified by agarose electrophoresis. Primers used for DNA methylation/hydroxymethylation analysis are shown in Supplementary Table S2. Data obtained for DNA methylation levels obtained by BS treatment is the combination of 5 mC and 5hmC, while these obtained following oxBS treatment gives the level of 5 mC alone (Booth et al., 2012). The level of methylation was analyzed using PyroMark Q24 Software (Qiagen), which calculates the methylation percentage (mC/(mC + C)) for each CpG site, allowing quantitative comparisons (mC is methylated cytosine, C is unmethylated cytosine). Human fully methylated and unmethylated DNA (Zymo Research (Irvine, CA, USA) were bisulfite-converted and used as controls.

2.4. rs6265 genotyping

SNP rs6265 was genotyped using the pyrosequencing assay and primer for sequencing designed by PyroMark Assay Design (see Table S3 for details). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 10 s, and finally extending 72 °C for 7 min. PCR product, verified by agarose electrophoresis, was tested using the PyroMark Q24 (Qiagen) according to manufacturer's recommendations.

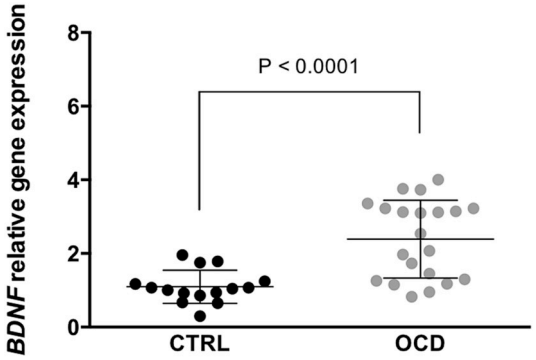

Fig. 2. *BDNF* mRNA levels in PBMCs from patients diagnosed with OCD and control (CTRL) subjects (n = 20 per each group). Scattered plots represent 2^{-DDCt} values calculated by the Delta-Delta Ct (DDCt) method. *p < 0.05 Mann-Whitney test.

2.5. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Data were analyzed using the nonparametric Mann Whitney test, Bonferroni correction for was used for the individual parameters of this model (OCD or control group) and this number will be referred to as corrected P-value in the text. Hardy Weinberg equilibrium for rs6265 was assessed using chi-square test. All samples have been run in duplicate for each assay. Tests were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

3. Results

Significant selective increase of BDNF mRNA levels was observed in OCD patients (2.39 ± 1.06) compared to controls (1.09 ± 0.45) using the primers set exon3/exon4 (P < 0.0001 Mann-Whitney test) (Fig. 2), which is also the more expressed transcript. No alterations between the two sample groups were observed for the other two primer sets used to analyze other possible gene transcripts (Supplementary Fig. S1). We did not observe any difference between OCD patients and control subjects in both 5 mC and 5hmC at gene promoters IV and IX (see Supplementary Tables S4 and S5). However, consistently with the gene expression upregulation, we observed at promoter exon I a reduction in 5 mC levels (OCD: 1.34 ± 0.77; CTRL: 3.11 ± 1.32; p < 0.0001) (Fig. 3), as well as an increase in 5hmC levels (OCD: 2.28 ± 0.93; CTRL: 0.99 ± 0.66; p < 0.0001) (Fig. 3) (Table 2 for details) confirmed by correlation between *BDNF* gene expression and percentage change in 5 mC levels (P = 0.0118, Spearman's r = -0.4464) (Fig. 4a) and 5hmC levels (P = 0.0324, Spearman's r = 0.3851) (Fig. 4b). No differences among OCD subjects were observed in both 5 mC and 5hmC

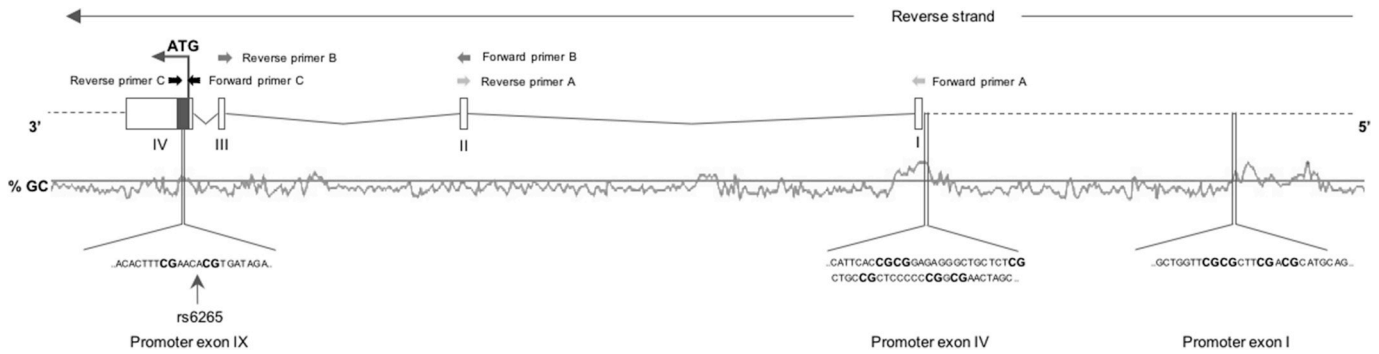

Fig. 1. Schematic representation of human *BDNF* promoter and the 5′ upstream region. ATP is the translation start site. In the lower part are shown the two CpG islands (exon I and IV) with their sequences and the position of the SNP (rs6265) in promoter exon IX. In the upper part are shown the location of primers used for mRNA quantification.

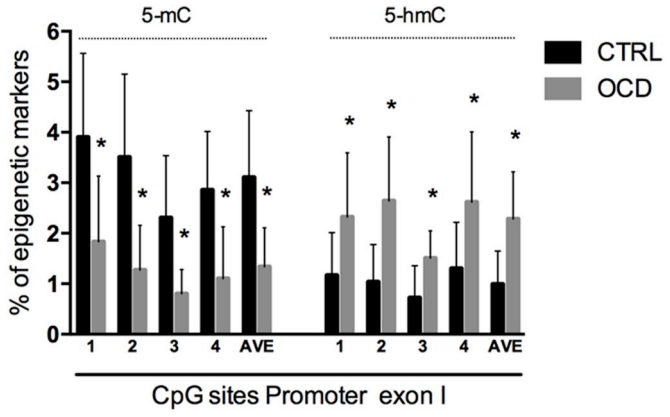

Fig. 3. Comparison of the 5 mC and 5hmC levels in human *BDNF* promoter exon I between OCD and control (CTRL) subjects represented as box plots for individual CpG sites under study (see Fig. 1) as well as of the average (AVE) of the 4 CpG sites. Significant differences are indicated (Bonferroni corrected). *p < 0.05.

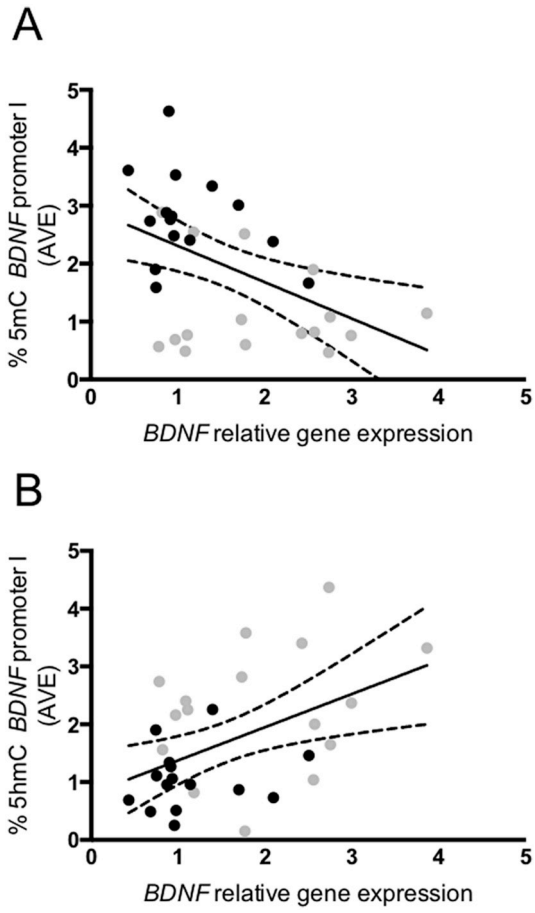

Fig. 4. Correlation between *BDNF* gene expression and % change of (A) 5 mC (P = 0.0279, Spearman's r = - 0.4311) and (B) 5hmC (P = 0.0333, Spearman's r = 0.4552) considering the average of the 4 CpG sites under study in human PBMCs. Gray dots indicate OCD subjects, black dots indicate control subjects. Data were compared by Spearman's rank correlation coefficient.

levels when stratifying data based on gender, age, duration of illness, and pharmacotherapies (see Supplementary Figs. S2 and S3). Achieved Power was 0.96 for gene expression analysis, and 0.99 for DNA methylation analysis. Power calculations were performed using G*Power version 3.1 (Faul et al., 2007). The distribution of genotype data for the SNP was in Hardy Weinberg equilibrium (chi-square = 0.577; Fisher exact test p = 0.593). Incidentally, rs6265 minor allele frequency (MAF) is 0.201 for Caucasian and this is consistent with our population (Supplementary Table S6). Moreover, genotyping rs6265, we failed to observe any association with OCD in subjects carrying the minor allele T as well as any relevant difference when we compared DNA methylation in OCD patients and control subjects with the different genotypes (see Supplementary Tables S7 and S8).

4. Discussion

The first relevant result of this study is the up-regulation in *BDNF* gene expression observed in PBMCs from OCD patients compared to healthy controls.

To the best of our knowledge, this is the first study reporting changes in *BDNF* mRNA levels in blood samples of patients with OCD, even though alterations in BDNF protein plasma levels have been previously documented in patients with OCD (Oliveira-Maia and Castro-Rodrigues, 2015; Şimşek et al., 2016), with studies reporting no differences (Yoshimura et al., 2006) or lower levels (Maina et al., 2010; Wang et al., 2011; Fontenelle et al., 2012) in patients compared with controls.

Indeed, altered *BDNF* gene expression has been reported in patients suffering from other psychiatric disorders showing a down-regulation in depression (Cattaneo et al., 2010, 2013; D'Addario et al., 2013; Pandey et al., 2010) and Bipolar Disorder (D'Addario et al., 2013; Köse Çinar et al., 2016; Lin et al., 2016), consistently with the reduction of BDNF serum levels in these pathologies (Bus et al., 2015; Molendijk et al., 2011; Fernandes et al., 2015) as well as in anxiety disorders (Molendijk et al., 2012). On the other hand, published findings on *BDNF* role in Schizophrenia are more difficult to interpret, with some studies showing brain down-regulation (Durany et al., 2001; Weickert et al., 2003; Hashimoto et al., 2005) as well as up-regulation (Takahashi et al., 2000; Iritani et al., 2003; Cheah et al., 2016) of gene expression and, consistently with the latter, others reporting increased BDNF mRNA in blood of patients with Schizophrenia as well (Kordi-Tamandani et al., 2012). Finally, and of relevance for this study, *BDNF* overexpression was reported in Tourette syndrome (Gunther et al., 2012).

Already in 1878 it was suggested that OC symptoms could be of frequent observation in patients with Schizophrenia (Westphal, 1878) and many studies suggested some clinical overlap between OCD and Schizophrenia (Zink, 2014). Overexpression of *BDNF* has been also associated with working memory deficits, increased anxiety-like traits in preclinical models (Govindarajan et al., 2006; Papaleo et al., 2011) as well as with cognitive problems in clinical samples (Yeom et al., 2016; Sayyah et al., 2009; Taha et al., 2017). Indeed, deficits in working memories and associative learning are commonly encountered in patients with OCD (Chamberlain et al., 2005; Shin et al., 2014). From an animal model perspective, it has been recently reported that hyperactivity of BDNF/TrkB signaling might trigger OCD-like behavior in mice (Ullrich et al., 2017).

It should be also considered that all OCD subjects involved in the study were under long-term treatment with SSRI, and BDNF has been indicated by many reports as a key player in the action of antidepressants (Björkholm and Monteggia, 2016). In particular, we previously reported that in MDD and BD *BDNF* transcriptional regulation was likely not affected by antidepressant treatments (D'Addario et al., 2012, 2013). However, this might not be the case in OCD, whose treatment, according to guidelines, requires higher doses of SSRIs and a longer course of therapy (Koran et al., 2007), compared to MDD, and this might have affected the observed increase in *BDNF*, considered as the main downstream antidepressant treatments mechanism of action (Russo-Neustadt and Chen, 2005). Nonetheless, this hypothesis should be tested by comparing *BDNF* expression in drug free and short term treated OCD patients.

It is also noteworthy to mention that several other clinical variables

collected in our sample, including family history for OCD, early onset, duration of illness, duration of untreated illness, comorbidity with other psychiatric disorders, number of psychotropics, and clinical phenotypes might have somehow influenced the increased *BDNF*; the limited size of our sample, however, likely prevented us to detect any statistical significance in such regard.

Another objective of this study was to evaluate the role of epigenetic mechanisms in regulating BDNF gene expression. Alterations in 5 mC levels and consistent changes in genes expression have been already reported in different psychiatric disorders (Abdolmaleky et al., 2006; Kuratomi et al., 2008; D'Addario et al., 2012, 2013, 2017) including OCD (Nissen et al., 2016). 5 mC can be oxidized to 5hmC and this modification is environment-sensitive (Wu and Zhang, 2011), and highly enriched in the brain (Kriaucionis et al., 2009; Sun et al., 2014; Wang et al., 2014). To date, DNA methylation methods based on sodium bisulfite treatment could not distinguish between 5 mC and 5hmC, and all observed alterations have been attributed to 5 mC only. We here used a recently developed pyrosequencing-based assay that allowed us to discriminate between the two modifications (Matsubara et al., 2015; Qu et al., 2015). The analysis of 5 mC and 5hmC levels was performed at *BDNF* gene promoters' exon I, exon IV and exon IX, and selective alterations were observed only at promoter exon I consistently with the gene expression change.

Of relevance, alterations in DNA methylation at *BDNF* gene exon I promoter have been already observed in depressed and bipolar type II subjects, and were suggested as a possible biomarker for those disorders (Fuchikami et al., 2011; D'Addario et al., 2012, 2013). Another study reported a reduction in DNA methylation at BDNF gene promoter exon I of treatment-resistant MDD patients subjected to electroconvulsive therapy (Kleimann et al., 2014). Selective modulation of DNA methylation in this particular region of *BDNF* gene may thus occur under different pathological conditions and might be driven by different therapies, including pharmacological treatment, as we here described for OCD, and somatic ones (e.g., electroconvulsive in MDD).

No association was observed between rs6265 and OCD susceptibility. However, since the way genetic variations might impact genes DNA methylation pattern is not fully understood, we analyzed possible alterations in the epigenetic marks at all *BDNF* CpG sites under study, based on the presence of the Val or Met allele, which also creates or abolishes a CpG dinucleotide. Again, no relevant alteration was detectable in OCD subjects carrying the minor allele in terms of DNA methylation in any of the CpG sites analyzed. However, the possible interaction between genetic and epigenetic factors is of great relevance and we aim to address this issue in future studies of pathological conditions other than OCD, in line with our recent study on alcoholism and prodynorphin gene regulation (D'Addario et al., 2017a,b).

In conclusion, our preliminary findings suggest the relevance of epigenetic changes in OCD to identify diagnostic and prognostic biomarkers. Moreover, we could hypothesize that pharmacotherapy might be responsible for *BDNF* gene regulation altering DNA methylation status. It would be of great value to investigate DNA methylation changes at early stages of the disease, in order to identify possible gene-environmental risk factors eventually responsible for OCD development, and thus try to revert such changes not only pharmacologically, but through environmental triggers.

4.1. Study limitations and future plans

A limitation of the present study is the small sample size of OCD patients; thus, it is mandatory to confirm reported findings in a large cohort of human subjects, also in order to take into due account different parameters (such as age and gender) and to better weight the effects of alternative pharmacotherapies. It would be also important to analyze drug-naïve patients. Moreover, healthy controls related variables were not collected, therefore a more detailed comparison between groups would be also of relevance.

5. Contributors

CD and BD conceived and designed the experiments; CD, FB, CF, MP, MV, VDC, BB, BG performed the experiments; CD, BD, FB analyzed the data; CD, BD, DG contributed reagents/material/analysis tools; CD, BD, MM wrote the paper, ES revised critically the manuscript.

Statement of interest

All authors declare to have nothing to disclose.

Conflicts of interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2019.04.006.

Table 2. 5mC/5hmC at *BDNF* gene promoter exon I. % of DNA methylation/hydroxymethylation alterations at promoter exon I (results from 4 CpG sites as well as their average) in genomic material from PBMCs of controls (CTRL, n = 28) and OCD subjects (n = 23). Data are expressed as % mean of each CpG site ± SD. Mann–Whitney *U* test for statistic comparison with Bonferroni correction for multiple testing.

5 mC			
CpG site	CTRL	OCD	p value
1	3.9 ± 1.66	1.83 ± 1.30	1,00e-5
2	3.51 ± 1.64	1.27 ± 0.89	1,16e-7
3	2.31 ± 1.23	0.80 ± 0.48	2,69e-5
4	2.86 ± 1.16	1.10 ± 1.02	1,29e-6
Average	3.11 ± 1.32	1.34 ± 0.77	3,88e-7
5hmC			
CpG site	CTRL	OCD	p value
1	1.17 ± 0.84	2.32 ± 1.27	1,81e-3
2	1.04 ± 0.74	2.64 ± 1.26	5,65e-6
3	0.72 ± 0.63	1.51 ± 0.54	3,39e-4

4	1.30 ± 0.91	2.62 ± 1.39	8,84e-4
Average	0.99 ± 0.66	2.28 ± 0.93	3,34e-6

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