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Cytogenetic Screening and Promotor CpG-Island Methylation of Brain-Derived Neurotrophic Factor (BDNF) Gene in Schizophrenia and Parkinson's Disease Patients from South India

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ABSTRACT

Schizophrenia (SCZ) and Parkinson's disease (PD) are two major problems of health worldwide, in which genomic and environmental factors are involved. In both brain disorders there is a severe compromise of the dopaminergic system, which is the target of classical therapeutic intervention. We have studied chromosomal aberrations (CAs) and epigenetic dysfunction in SCZ and PD cases from South India. CAs were found in most cases of SCZ, especially deletions (42.17%), duplications (13.25%) and inversions (44.58%), with an average of 4.15 CAs/patient, affecting Chr. 1, 2, 3, 4, 6,7, 9, 10, 11, 13, 15, 16, 22 and X. In PD, deletions (41.38%), duplications (20.69%) and inversions (37.93%) were present, with an average of 5.8 CAs/patient. As compared to controls, the frequency of CAs was significantly higher in SCZ (5.36 ± 2.46 vs. 0.44 ± 0.83, $p<0.0001$) and PD (5.8 ± 1.73 vs 0.32 ± 0.89, $p<0.001$). No significant differences were found in the distribution and frequency of polymorphic variants in the brain-derived neurotrophic factor (*BDNF*) gene among SCZ, PD and controls. However, CpG islands in promoters I and IV of the *BDNF* gene were found to be hypermethylated in SCZ, but not in PD or in healthy age-matched controls. Significant changes in GABA and transferrin levels were also found in SCZ and PD as compared to control subjects. These results show important cytogenetic anomalies in extensive regions of the genome in both SCZ and PD, and epigenetic changes in the *BDNF* gene in SCZ, with potential repercussions in pathogenesis and pharmacogenetics.

Keywords: BDNF, Biomarker, Cytogenetics, Epigenetics, GABA, Parkinson's disease, Schizophrenia, Transferrin

INTRODUCTION

Schizophrenia (SCZ) is a major mental health problem, and Parkinson's disease (PD) represents the second most important neurodegenerative disorder, after Alzheimer's disease in developed societies. The worldwide prevalence of SCZ ranges between 0.5% and 1%, with the first episode at 21 years of age in men and 27 years of age in women. Approximately one-third of the cases will attempt suicide and, eventually, about 1 out of 10 will take their own lives. Global costs for SCZ are estimated to be over $6 billion in the USA [1]. SCZ is among the most disabling of mental disorders. Several neurobiological hypotheses have been postulated as responsible for SCZ pathogenesis: polygenic/

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multifactorial genomic defects, intrauterine and perinatal environment-genome interactions, neurodevelopmental defects, dopaminergic, cholinergic, serotonergic, gamma-aminobutyric acid (GABAergic), neuropeptidergic and glutamatergic/N-Methyl-D-Aspartate (NMDA) dysfunctions, seasonal infection, neuroimmune dysfunction, and epigenetic dysregulation [2,3]. SCZ has a heritability estimated at 60-90%. Genetic studies in SCZ have revealed the presence of chromosome anomalies, copy number variants, multiple single-nucleotide polymorphisms of susceptibility distributed across the human genome, mitochondrial DNA mutations, and epigenetic phenomena [1,4-9].

PD shows a prevalence ranging from 35.8 per 100,000 to 12,500 per 100,000, with annual incidence estimates ranging from 1.5 per 100,000 to 346 per 100,000 in different countries [10-12]. Several pathogenic risk factors (toxins, drugs, pesticides, brain microtrauma, focal cerebrovascular damage, genomic defects) have been associated with PD. PD neuropathology is characterized by a selective loss of dopaminergic neurons in the substantia nigra pars compacta and Lewy body deposition, with widespread involvement of other CNS structures and peripheral tissues [13,14]. PD is a form of multi-systemic α-synucleinopathy with Lewy bodies deposited in midbrain. Descriptive phenomena to explain in part this neuropathological phenotype include the following: (i) genomic factors, (ii) epigenetic changes, (iii) toxic factors, (iv) oxidative stress anomalies, (v) neuroimmune/neuroinflammatory reactions, (vi) hypoxic-ischemic conditions, (vii) metabolic deficiencies, and (viii) ubiquitin-proteasome system dysfunction [15-21]; all these conditions leading to protein misfolding and aggregation and premature neuronal death.

Mutations in a series of primary genes are known to cause autosomal dominant and recessive forms of PD [19-22]. Mutations in some genes (e.g., *SNCA, PARK2, PINK1, PARK7, LRRK2, BST1, MAPT*) might be causative in familial forms of PD whereas diverse genetic defects in other loci might represent susceptibility loci associated with sporadic PD without family history [20]. Mendelian variants with high penetrance (e.g., *SNCA, LRRK2, PARKIN, PINK1, PARK7* genes), explain less than 10% of familial PD [23]. Over the past decade, several genome-wide association studies (GWAS) have contributed to clarify the contribution of genetic factors to the pathogenesis of PD in the Caucasian population and in other ethnic groups [24-30]. In a recent meta-analysis of PD GWAS with over 7 million variants, 26 loci have shown significant association with PD. Replication studies confirmed 24 SNPs, and conditional analyses within loci showed 4 loci (*GBA, GAK-DGKQ, SNCA, HLA*) with a secondary independent risk variant [31]. Significant associations at different loci (*DLG2, SIPA1L2, STK39, VPS13C, RIT2, BST1, PARK16*) have been found in Asians vs Europeans, together with allelic heterogeneity at *LRRK2* and at 6 other loci, including *MAPT* and *GBA-SYT11* [30]. The expression of PD genes is regulated by the epigenetic machinery (DNA methylation, histone/chromatin modifications, miRNA dysregulation) [32-39].

Dysfunction of the dopaminergic system in the brain is associated with SCZ and PD. Catecholamines are processed by three main nuclei (A8-retrobulbal, A9-substantia nigra pars compacta, A10-ventral tegmental area) arranged in the mesencephalic region where the mesostriatal, mesolimbic and mesocortical pathways are organized [40,41]. Midbrain dopaminergic neurons in the ventral tegmental area and noradrenergic neurons in the locus coeruleus are major sources of dopamine and noradrenaline to the prefrontal cortex, where these amines regulate cognition, behavior, and psychomotor function [42,43]. As a classical concept, brain hyperdopaminergia is associated with SCZ and psychotic disorders in which most neuroleptic drugs exert an inhibitory effect on the hyperactivated dopaminergic system, potentially causing Parkinsonian disorders after long-term treatment [15]; and brain hypodopaminergia is associated with PD, where L-DOPA and other anti-parkinsonian drugs enhance the activity of a deficient dopaminergic system [44-46], chronically leading to potential psychotic disorders in PD. Genomic and epigenomic characterization of these antagonistic biophenotypes, in terms of dopaminergic neurotransmission (hyperdopaminergia vs hypodopaminergia) as well as other neurotransmitters involved in higher activities of the central nervous system and psychomotor function, would help to better understand pathogenesis and implement personalized therapeutic procedures [44-47].

In the present study, our main aims are the following: (i) characterization of cytogenetic aberrations in SCZ and PD; (ii) genetic assessment of brain-derived neurotrophic factor (*BDNF*) variants, especially at promoters I and IV of this gene; (iii) DNA methylation analysis of promoters I and IV of the *BDNF* gene; and (iv) biochemical assessment of GABA and transferrin levels in SCZ and PD. To our knowledge, this is probably the first study on cytogenetics and epigenetics performed in a selected population of SCZ and PD patients from India Tamil Nadu and Kerala States.

MATERIAL AND METHODS

Patients and controls

Four groups of subjects were established: (i) Patients with schizophrenia (SCZ)(N=20); age, 44.8 ± 24.3 years; (ii) age-matched controls for SCZ (CS)(N=20); age, 45.8 ± 21.9 years); (iii) patients with Parkinson's disease (PD)(N=5); age, 53.0 ± 15.1 years; and (iv) age-matched controls for PD (CP)(N=5); age, 52.6 ± 13.2 years. All controls belonged to the same ethnic origin as the recruited patients. The study followed the ethical procedures of the Chaithanya Mental Health Care Center, Cochin, Kerala, India. Informed consent was obtained from the subjects assuring the use of blood for

research purposes alone. The work was carried out in accordance with the ethical standards of the 1964 Declaration of Helsinki.

Sample collection

The inclusion criteria for the classification of SCZ and PD was made by using the Structured Clinical Interview for DSM-IV questionnaire (SCID) and the 39-Item Parkinson's Disease Questionnaire (PDQ-39), respectively. Pedigree charts were drawn to understand the familial background and the inheritance pattern of participants. A blood sample of 10 mL was drawn by venipuncture from an antecubital vein following administration of an anesthetic ointment. The blood was collected in two sterile tubes containing EDTA and sodium heparin. The collected samples were transported to the cytogenetic laboratory within 5 hours and were set aside for 24 hrs at room temperature prior to processing.

Cytogenetic analysis

All chemical reagents were purchased from Sigma Chemical (St. Louis, MO), except for colcemid, which was obtained from Gibco Laboratory (Grand Island, NY). The blood samples were set up to establish cell cultures according to the standard procedures of our laboratory. Briefly, 0.5 mL whole blood was added to 4.5 mL RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% streptomycin-penicillin antibiotics and 0.2 mL reagent-grade phytohemagglutinin, and incubated at 37˚C. After 71 hrs, the cultures were treated with 0.1 µg/mL colcemid to block cells in mitosis. The lymphocytes were harvested at 72 hrs by centrifuging cells to remove culture medium (800-1,000 rpm, 7 min) to which hypotonic solution (KCl 0.075 M) was added at room temperature and incubated for 20 min to swell the cells. The cells were treated twice with fixative (methanol and acetic acid [3: 1 vol/vol]). Cytological preparations were made by placing two to three drops of the concentrated cell suspension onto slides wetted with ice-cold acetic acid (60%) and were carefully dried on a hot plate (56˚C for 2 min). For Chromosomal Aberration (CA) analysis, 100 complete metaphase cells of the first cell cycle were evaluated under a microscope (x100) to identify numerical and structural CA according to the International System for Human Cytogenetic Nomenclature (ISCN). Data were registered on master tables and later transferred to a computer file.

GABA and Transferrin Determination

GABA and transferrin determinations were carried out with an ELISA kit (Abcam, India). 50 µL of derivatized sample and specific antiserum were incubated overnight at 4 - 8°C. After three wash cycles with 300 µL wash buffer, 100 µL enzyme conjugate was incubated in a shaker for 30 min at RT, and the absorbance of 100 µL substrate was measured at 450 nm after the addition of stop solution.

Genotyping

Whole genomic DNA was collected by following kit protocol (Bangalore Genei-Frozen blood DNA extraction kit) and run on 1% agarose at 50V. The ratio of OD 260 and OD 280 was obtained to estimate the purity of DNA. The bisulfate conversion of extracted DNA was performed to change the unmethylated cytosines to uracil but leaving the methylated cytosine residues as such, without any chemical modifications. For the conversion, 1 µg of DNA was denatured by adding it to 50 µL of 0.2M NaOH and incubated for 10 minutes at 37ºC. 30 µL of freshly prepared 10 mM hydroquinone and 520 µL of 3M sodium bisulfite (pH 5.0) was added, mixed and incubated at 50ºC for 16 hrs. Modified DNA was purified and eluded into 50 µL of water. DNA was further subjected to 0.3M NaOH treatment for five minutes followed by ethanol precipitation. The purified DNA was stored in MilliQ water for further use.

Methylation-specific PCR amplification

Primers were specifically designed to amplify the methylated strands of CpG islands of promotor I (F 5'-AGGGAAAGTTGTTGGGCTGG-3'; R 5'-CTCGCTGTTTACGTGACCA-3') and promotor IV (F 5'-ATGACGGTGATAGGCTGCTC-3'; R 5'-TCTCCCAGTTCTGCGTTCAG-3') of the *BDNF* gene using Methprep Tool. The reaction volume used included 4.0 µL of 200 ng template DNA, 1.0 µL of forward and reverse primer each, 12.5 µL of 2X MasterMix for a total reaction volume of 25 µL. An initial denaturing step at 95°C (10 min) followed by 30 cycles of 95°C (30s) denaturation, 60°C (30s) annealing, 72°C (1 min) extension and a final elongation step of 72°C (10 min) was carried out. The PCR products were electrophoresed on 1% agarose gels containing EtBr and viewed under ultraviolet light.

STATISTICAL ANALYSIS

Data were analyzed by using IBM SPSS Statistics 20 and SigmaPlot 10.0 Software. Comparisons between groups were studied by t-Test, Mann-Whitney Rank Sum Test, Chi Square without Yates correction and Fisher exact, and Pearson Correlation Analysis (Nonlinear Regression, Durbin-Watson Statistic, Normality Test, Constant Variance Test, 95% Confidence). All values are expressed as mean ± SD, and the degree of significance is considered when $p<0.05$.

RESULTS

Cytogenetic analysis of SCZ patients revealed that major chromosomal aberrations (MCA)(2.10 ± 0.96 vs 0.30 ± 0.47, $p<0.001$), minor CAs (mCA)(3.10 ± 0.91 vs. 0.15 ± 0.37, $p<0.001$) and total CAs (TCA)(5.20 ± 1.39, $p<0.001$) were significantly more frequent in SCZ than in controls (**Figure 1).** CAs were present in Chr. 1, 2, 3, 4, 6, 7, 9, 10, 11, 13, 15, 16, 22 and X (**Figure 2),** showing deletions (Del)(42.17%; 1.75 Del/patient), duplications (Dupl)(13.25%; 0.55

Dupl/patient), and inversions (Inv)(44.58%; 1.85 Inv/patient), with an average of 4.15 CA/patient. About 45% of CAs affected the short (p) arms of chromosomes, whereas 55% of CAs were identified in the long (q) arms; 54.3% of Del were on p and 45.7% on q arms; 45.45% of Dupl were on p and 54.56% on q arms; and 35.14% of Inv were on p and 64.86% on q arms (**Figure 2**).

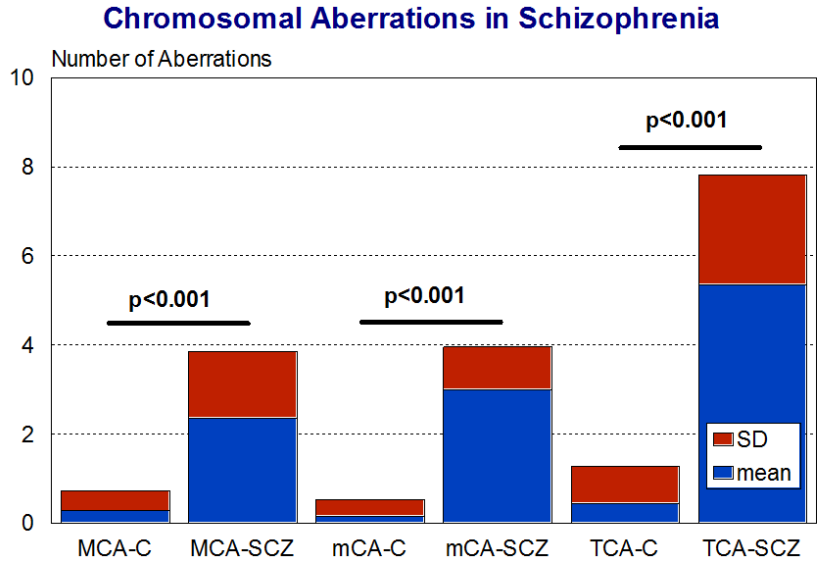

Figure 1. Chromosomal aberrations in schizophrenia.

C: Controls; SCZ: Schizophrenia; MCA: Major chromosomal aberrations; mCA: minor chromosomal aberrations; TCA: Total chromosomal aberrations.

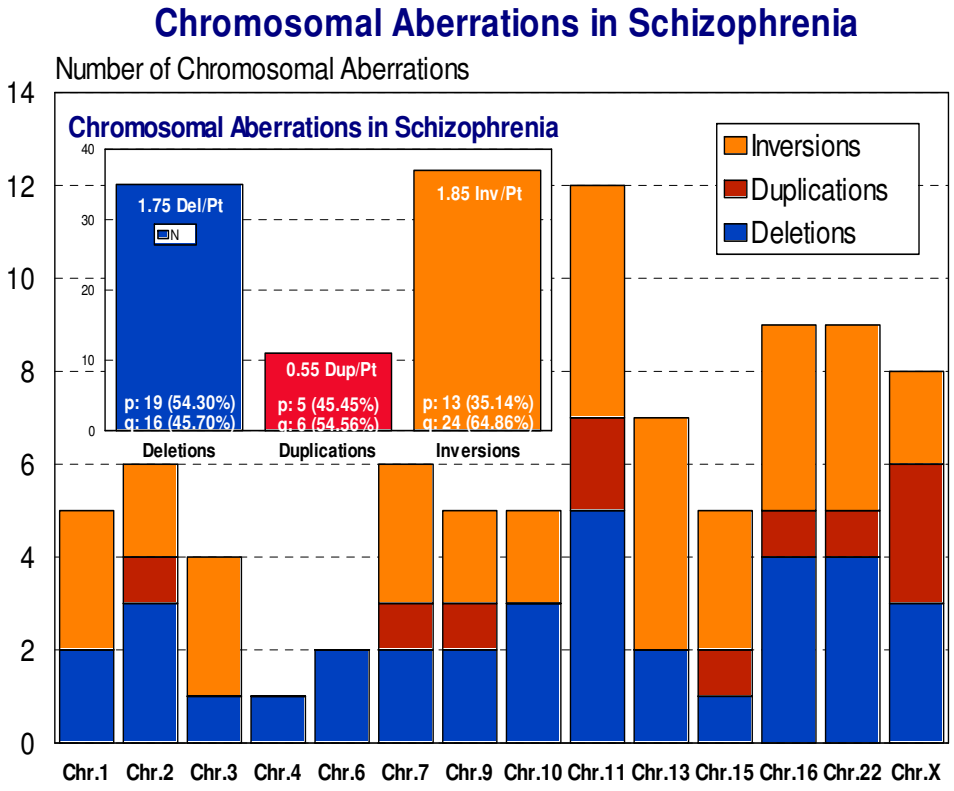

Figure 2. Chromosomal aberrations in schizophrenia

Cytogenetic analysis of PD cases showed that the frequency of MCA (3.40 ± 1.34 vs 0.20 ± 0.44, p<0.001), mCA (2.60 ± 1.14 vs 0.20 ± 0.44, p<0.001) and TCA (6.00 ± 2.12 vs 0.40 ± 0.55, p<0.001) were significantly higher in PD than in controls, with 5.8 CAs/patient (**Figure 3**). Del (41.38%)(2.4 Del/patient) were present in Chr. 1q, 5q, 6q, 8p, 12q, 17p, and 22d; Dup (20.69%) (1.2 Dup/patient) in Chr. 13q and 20p12.3; and Inv (37.93%) (2.2 Inv/patient) in Chr. 1q, 4q, 5q and 17q (**Figure 2 and 4**).

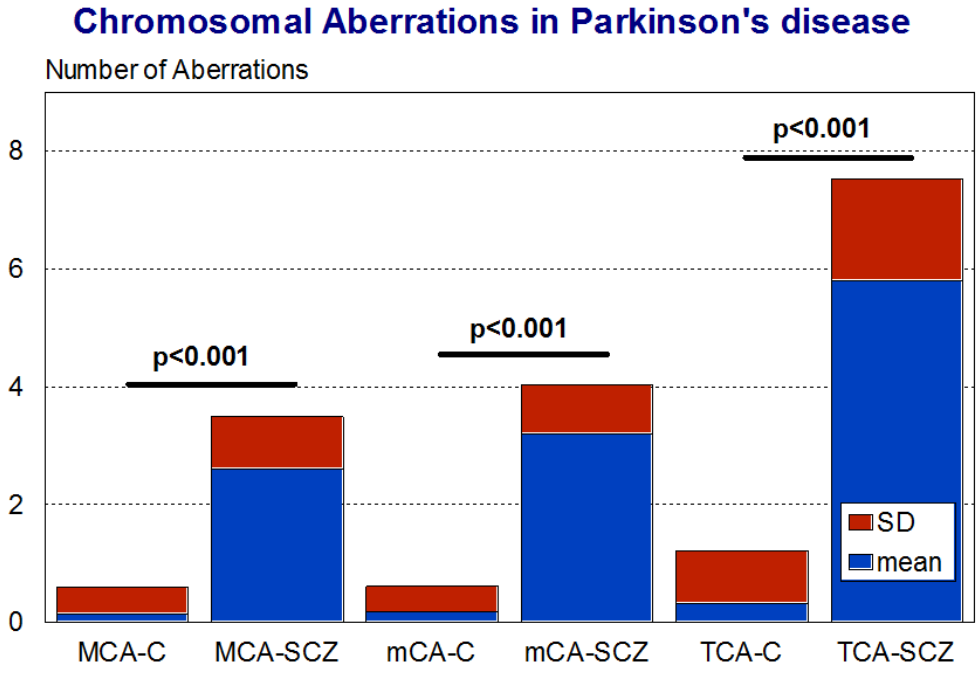

Figure 3. Chromosomal aberrations in Parkinson's disease.

C: Controls; PD: Parkinson's disease; MCA: Major chromosomal aberrations; mCA: minor chromosomal aberrations; TCA: Total chromosomal aberrations.

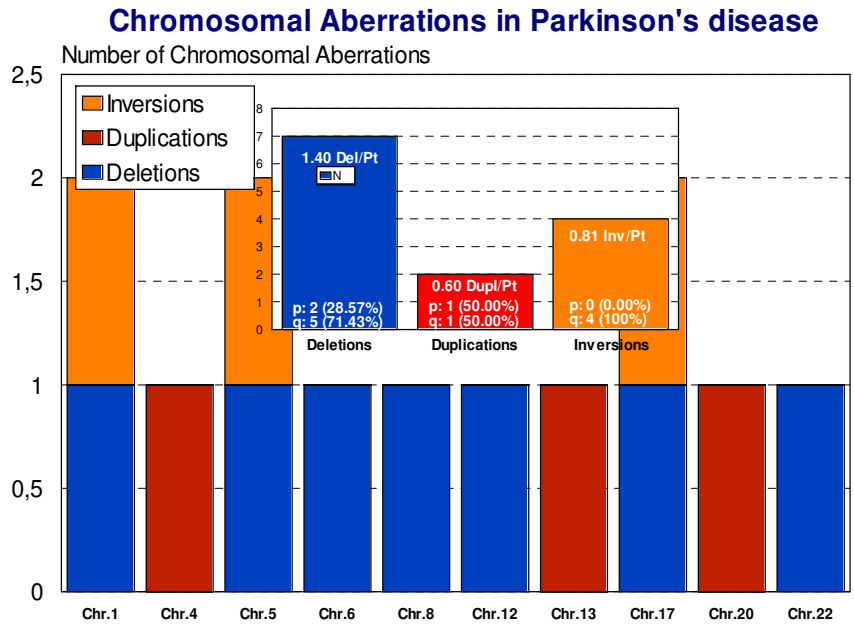

Figure 4. Chromosomal aberrations in Parkinson's disease

Correlation analysis of CAs vs age showed that there is an age-related increase in CAs in SCZ (p=0.04), but not in PD (**Figure 5).**

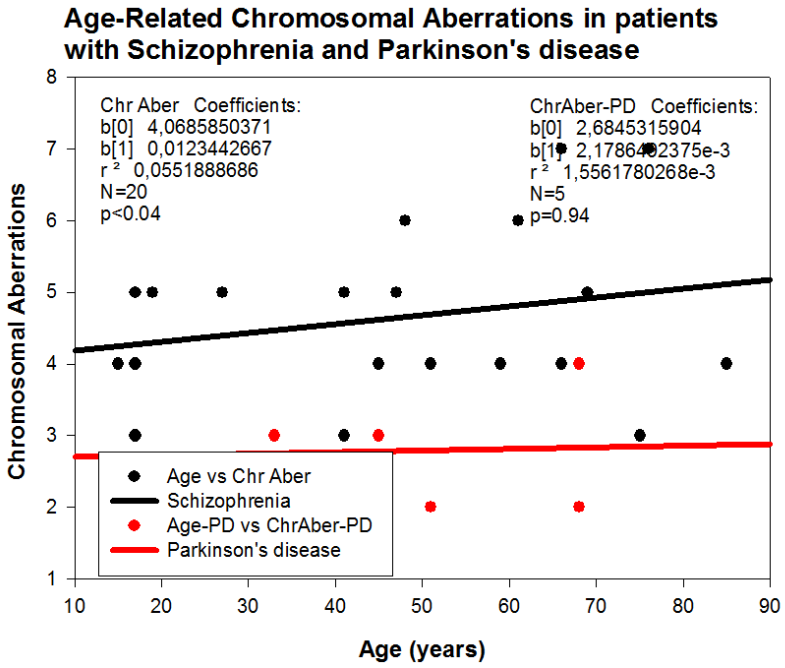

Figure 5. Age-related chromosomal aberrations in patients with schizophrenia and Parkinson's disease.

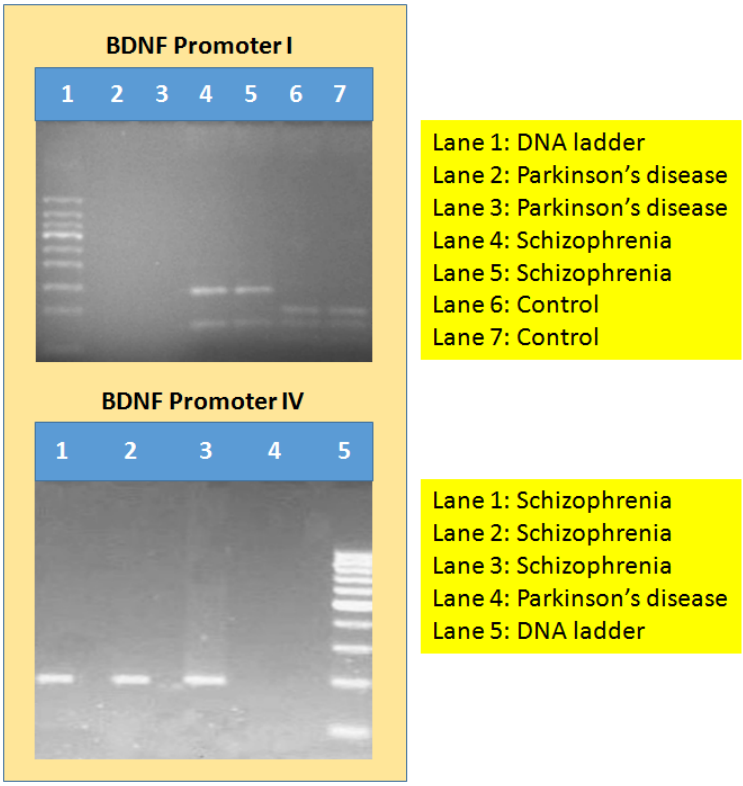

Figure 6. DNA methylation in CpG islands at Promoters I and IV of the *BDNF* gene in schizophrenia and Parkinson's disease.

Upper panel (*BDNF* Promotor I): Image of Agarose gel showing methylation of Promotor I of the *BDNF* gene. Lane 1: DNA ladder. Lanes 2-3: Parkinson's disease (showing no amplification). Lanes 4-5: Schizophrenia (showing amplification of methylated Promotor I CpG island of the *BDNF* gene). Lanes 6-7: Control DNA.

Lower panel (*BDNF* Promotor IV): Image of Agarose gel showing methylation of Promotor IV of the *BDNF* gene. Lanes 1-3: Schizophrenia (showing amplification of methylated Promotor IV CpG island of the *BDNF* gene). Lane 4: Parkinson's disease (showing no amplification). Lane 5: DNA ladder.

No relevant differences have been found in the distribution and frequency of *BDNF* genotypes in SCZ and PD with respect to controls (**Table 1);** however, the promoter I and IV regions of the *BDNF* gene exhibited a hypermethylation profile, which was absent in PD and controls (**Figure 6).**

Table 1. Distribution and frequency of genotypes and alleles in promoters I and IVof the *BDNF* gene in schizophrenia and Parkinson's disease.

***BDNF* Gene**	**Genotype frequency**			**HWE *p***	***p***	**Allele frequency**		***p***	**OR (95% CI)**
Type	**C/C**	**C/T**	**T/T**			**C**	**T**		
Promoter I									
Schizophrenia	10 (0.238)	7.5 (0.465)	2.5 (0.297)	0.12	0.77	9(0.470)	11(0.530)	0.64	1.04
Controls	10 (0.238)	8.5 (0.479)	1.5 (0.301)	0.40		11(0.460)	9 (0.540)		[0.88-1.23]
Promoter IV									
Schizophrenia	10 (0.238)	8 (0.469)	2 (0.245)	0.14	0.74		7(0.480)	0.55	0.95
Controls	10 (0.238)	9 (0.482)	1 (0.227)	0.41		13(0.520)	6 (0.468)		[0.81-1.11]
						14(0.532)			
Promoter I									
Parkinson	5 (0.257)	4 (0.517)	1 (0.297)	0.12	0.69	2(0.442)	3(0.519)	0.71	1.17
Controls	5 (0.257)	3 (0.475)	2 (0.301)	0.40		11(0.460)	9 (0.540)		[0.74-1.13]
Promoter IV									
Parkinson	5 (0.257)	3 (0.421)	2 (0.267)	0.11	0.51	1(0.213)	3(0.540)	0.66	1.21
Controls	5 (0.257)	4 (0.515)	1 (0.312)	0.37		2(0.341)	3(0.540)		[0.63-1.09]

GABA levels were found to be significantly higher in SCZ (149.55 ± 37.84 pmol/mL) and PD (135.20 ± 3.34 pmol/mL) than in controls (CS: 119.60 ± 9.62 pmol/mL; p=0.003; CP: 121.60 ± 8.44 pmol/mL; p=0.01) (**Figure 7),** and these differences were unrelated to age (**Figure 8).** Similarly, transferrin showed higher levels in SCZ (2.67 ± 0.26 mg/mL) than in controls (2.40 ± 0.40 mg/mL; p=0.02); and, to a lesser extent, transferrin levels tended to be higher in PD (2.70 ± 0.18 mg/mL) than in controls (2.30 ± 0.38 mg/mL; p=0.06)(**Figure 9**), with no significant age-related effect among patients, but with a great variability among CS and CP (**Figure 10).**

DISCUSSION

The human genome is enriched in interspersed segmental duplications that sensitize approximately 10% of our genome to recurrent microdeletions and microduplications as a result of unequal crossing-over. Studies of common complex genetic disease show that a subset of these recurrent events plays an important role in autism, SCZ and epilepsy [48]. Diverse cytogenetic abnormalities and over 1,000 genetic defects, reported in about 2,400 studies, have been associated with SCZ during the past two decades [3,8,49]. Structural variations of DNA, such as copy number variations (CNVs), contribute both to normal genomic variability and to risk for SCZ and many other brain disorders. CNVs at genome loci 1q21.1, 2p16.3, 3q29, 15q11.2, 15q13.3, 16p13.1 and 22q11.2 are currently associated with SCZ [50]. Recurrent submicroscopic copy number changes include deletions at 1q21.11, 15q11.3, 15q13.3, and the recurrent CNV at the 2p16.3 neurexin 1 locus [51]. Genome-wide studies of CNVs show replicated associations of SCZ with rare 1q21.1 and 15q13.3 deletions. Complex mutational mechanisms involving rare CNVs elevate risk for SCZ, especially developmental forms of the disease. Most CNVs, including 22q11.2 deletions, appear to account for up to 2% of SCZ cases [52]. In previous studies we found chromosome banding imbalance in several loci at Chr. 1q21.1, 2p24.3, 3q29, 6p, 7q, 9p21.3, 10p, 11q, 13q,15q11.2,16p1.1,22q, and Xp [53]. There are other studies that also implicate the chromosomal region 11q21-22 as containing genes with increased liability for SCZ. Some balanced translocations at q14.3, q21, q22.3 and q25 sites of chromosome 11 were found in SCZ and in other psychiatric disorders [54-56]. In the present study, we replicated previous findings and identified deletions at 1q, 7q, 11q and 22q which meet sufficient and arbitrarily defined criteria as potential susceptibility loci for SCZ [57,58]. In our sample, the rate of deletions and inversions is quantitatively similar in SCZ, whereas the duplication rate is substantially lower

than that of deletions and inversions (**Figure 2).** Recent studies in the Han Southern Chinese population identified four CNVs, including two deletions and two duplications. The 16p11.2 duplication from 29.3 Mb to 29.6 Mb was detected in four cases (0.84%) and one control (0.098%) [59]. Evidence has been confirmed for genome-wide significant associations with SCZ in the Han Chinese population for three loci, at 2p16.1 (rs1051061, in an exon of *VRK2*), 6p22.1 (rs115070292 in an intron of *GABBR1*) and 10q24.32 (rs10883795 in an intron of *AS3MT*; rs10883765 at an intron of *ARL3*) [60]. It has been postulated that CNVs may act to impair inhibitory learning in SCZ, potentially contributing to the development of core symptoms of the disorder [61]. The age-related increase of CAs in SCZ observed in our sample (**Figure 5**) may suggest that some CAs might result from *de novo* formation mutations associated with either the psychotic phenotype and/or the influence of neuroleptic drugs.

CNVs have been reported in major neurodegenerative disorders, including PD [62,63]. Using high-resolution arrays in 16 CNV-PD genes, it has been found that some PD loci are significantly enriched. For instance, *PARK2* is under heavy burden with ~1% of the population containing CNV in the exonic region; and there is a complex interaction of molecules forming a major hub by the α-synuclein, whose direct interactors, *LRRK2, PARK2* and *ATP13A2* are under CNV influence. According to these results, it was hypothesized that CNVs may not be the initiating event in the pathogenesis of PD, remaining latent until additional secondary hits are acquired [64]. In our small sample, deletions (1.40 Del/Pt), duplications (0.6 Dup/Pt) and inversions (0.81 Inv/Pt) (**Figure 4)** were significantly over-represented in PD as compared to controls, indicating that PD-related neurodegeneration is associated with some CAs (**Figure 3);** however, the limited number of cases does not allow any relevant conclusion.

Epigenetic changes (DNA methylation, histone modifications, miRNA dysregulation) have been implicated in neuropsychiatric and neurodegenerative disorders [65-67]. SCZ and other neurodevelopmental disorders are associated with abnormalities in multiple epigenetic mechanisms, resulting in altered gene expression during development and adulthood [66]. Epigenetics-related disruption of the dopamine, NMDA, and GABA signaling pathways are important events in SCZ phenotype [66]. DNA methylation plays a pivotal role in SCZ pathogenesis. Recent studies identified 2,014 CpGs as GWAS risk loci with differential methylation profile in SCZ, and 1,689 hypomethylated genes. Hypermethylated genes include *GNA13*, *CAPNS1, GABPB2, GIT2, LEFTY1, NDUFA10, MIOS, MPHOSPH6, PRDM14* and *RFWD2*. The hypermethylated promoters of a series of pathways (TNF alpha, PDGFR-beta signaling, TGF beta Receptor, VEGFR1 and VEGFR2 signaling, regulation of telomerase, hepatocyte growth factor receptor signaling, ErbB1 downstream signaling and mTOR signaling pathway) suggest that the malfunctioning of these pathways may contribute to SCZ phenotype [9].

In the human genome, DNA methylation occurs almost exclusively at CpG dinucleotides. The cytosine residue of a CpG dinucleotide can be covalently modified by adding a methyl group to its carbon-5 atom resulting in 5-methylcytosine. The methyl group is transferred from S-adenosyl-L-methionine to a cytosine residue via DNA methyltransferases. CpG dinucleotides are unevenly distributed throughout the genome and are generally methylated. Some CpG dinucleotides are clustered in regions known as CpG-islands, which can span hundreds to thousands of base pairs and are generally unmethylated [68]. A direct role of DNA methylation in the regulation of this class of promoters predicts a correlation between their methylation profile and their level of expression. CpG-poor promoters are frequently methylated in the genomes of gametes and cells during early development. As previously stated, these genes are always tissue-specific and show precise expression control during development [69].

Polymorphic variants in the *BDNF* gene (11p14.1), located in a region where CAs accumulate in SCZ, have been associated with obsessive-compulsive disorder [70], eating disorders [71], bipolar disorder [72], and SCZ [73]. The Val66-to-Met polymorphism showed significant association for valine (allele G) with SCZ. Haplotype analysis of the Val/Met SNP and a dinucleotide repeat polymorphism in the promoter region revealed highly significant underrepresentation of the methionine (Met1) haplotype in SCZ [73]. The *BDNF* gene spans 70 kb and contains 11 exons with transcription start site in 9 exons, each of which is associated with a functional promoter [74]. The 3′ exon encodes all or most of the protein, depending on the 5′ exon used. Independent of the 5′ exon usage, two separate polyadenylation signals in exon IX can be utilized in *BDNF* transcripts. *BDNF* gene expression is under the control of at least nine alternative tissue-specific promoters linked to separate 5′ exons. In recent years, epigenetic factors have become an avenue of investigation with some promise [75], with the complex epigenetic regulation of BDNF showing relevance in psychiatric disorders [76].

Increased synthesis of neuronal *BDNF* correlates with a decrease in CpG methylation within the regulatory region of the *BDNF* gene. Increased *BDNF* transcription involves dissociation of the MECP2-histone deacetylase-Sin3A repression complex from its promoter. DNA methylation-related chromatin remodeling is important for activity-dependent gene regulation that may be critical for neural plasticity [77].

In our study, we did not find any significant difference in the distribution and frequency of polymorphic variants at *BDNF* promoters I and IV in SCZ and PD as compared with their respective controls (**Table 1**); however, promoters I and IV of the *BDNF* gene were strongly hypermethylated in SCZ,

but not in PD or controls (**Figure 6).** This hypermethylated profile of *BDNF* might contribute to inhibiting the neurotrophic activity of BDNF on dopaminergic neurons, with a subsequent increase in neurodegeneration.

The methylation status of the *BDNF* promoter CpG island I in our study is in agreement with data previously collected by Matsumoto et al. [69] in Japanese patients. This leads us to hypothesize that the CpG methylation status of promotor I of the *BDNF* gene might represent an epigenetic biomarker of SCZ pathophenotype. In our study, the promoter IV of the BDNF gene was also found to be hypermethylated; however, this finding was not observed in the Japanese cases [69]. This fact might reflect ethnic-related epigenetic variation. A wide range of epigenetic studies on psychiatric diseases have put forth the idea that the epigenetic alterations are not exclusively limited to brain tissues but can also be seen in other peripheral tissues, such as peripheral blood cells [78]. In our study, we detected that both *BDNF* promotors I and IV were hypermethylated in peripheral blood cells from patients with SCZ. It might be possible that the methylation status in brain has a proportional representation of methylation in peripheral blood cells [79].

BDNF is a distinctive activity-dependent neurotrophin which is involved in neuroplasticity and has a role in the differentiation of the neurons. Hence, DNA hypermethylation in this gene might give rise to a wide variety of phenotypic variations [80]. Despite the clear results obtained in our sample, it should be noted that our sample has several limitations. First, as medical history was unavailable for most of the patients, we could not assess the effect of medication on DNA methylation changes. Second, the difference in methylation we identified at CpG I-72 was a nominally significant difference ($p = 0.033$) and could not be detected after multiple testing corrections. Therefore, further validation studies using larger and independent samples will be required. In any case, our data are consistent with previous epigenetic studies, with minor differences [81-84]. Differences may be partly due to ethnic variation, heterogeneity of the sample, therapeutic regime, and disease stage [84]. Third, our analysis focused on a few specific CpG sites in *BDNF* promoters. Although these CpG sites were carefully chosen based on previous studies, levels of DNA methylation at other CpG sites in promoters I and IV (as well as other promoters) in SCZ remain unknown and are worth studying. The bromodomain containing 1 gene (*BRD1*) participates in histone modifying complexes and thereby regulates the expression of a large number of genes. *BRD1* encodes a protein that is essential for embryogenesis and CNS development. Genetic variants in the *BRD1* locus show association with SCZ and bipolar disorder, and risk alleles in the promoter region correlate with reduced *BRD1* expression. The risk allele of the rs138880 SNP in the *BRD1* promoter region correlates with reduced *BRD1* expression [85].

Other important epigenetic factors involved in *BDNF* expression are miRNAs, small noncoding RNAs that post transcriptionally downregulate expression of target mRNAs by inhibiting their translation or causing their degradation. The 3'-UTR of the *BDNF* transcript contains putative binding sites for 26 miRNAs, including MIR30A and MIR195. Quantitative RT-PCR confirmed down regulation of *BDNF* mRNA by MIR30A-5p and MIR195 [86].

The role of GABA in SCZ is still unclear, tough it appears that GABA signaling molecules are critical for both brain development and SCZ pathogenesis [87]. GABA is an inhibitory neurotransmitter which is synthesized from glutamate by glutamic acid decarboxylase (GAD), derived from two genes, *GAD1* and *GAD2*. *GAD1* is expressed as both *GAD67* and *GAD25* mRNA transcripts. *GAD67* mRNA shows a lower expression level in SCZ, and *GAD25* mRNA is expressed in fetal brain, probably regulating neurodevelopment. *GAD25* and *GAD67* gene expression levels are reduced in blood cells, and there is no difference in *GAD25* and *GAD67* gene expression levels between SCZ vs controls [88]. Classical studies, reported by Lindefors [89], demonstrate that the majority of neurons in the striatum (caudate-putamen, dorsal striatum, nucleus accumbens, ventral striatum) and in striatal projection regions (the pallidum, the entopeduncular nucleus and substantia nigra reticulata) use GABA as a neurotransmitter and express glutamic acid decarboxylase (GAD), the rate-limiting enzyme in the synthesis of GABA. Brain GAD is present in two isoenzymes, *GAD65* and *GAD67*, a dual system for the control of neuronal GABA synthesis. Inhibition of dopaminergic transmission in the striatum by lesion of dopamine neurons or by neuroleptic treatment is followed by an increased release of GABA and increased expression of *GAD67* mRNA in a subpopulation of striatal medium-sized neurons which project to the globus pallidus, and increased striatal GAD enzyme activity. Increased dopaminergic transmission by anti-Parkinsonian drugs is followed by decreased striatal GABA release and decreased *GAD67* mRNA expression in a subpopulation of medium-sized neurons in the striatum. GABA neurons in the striatum seem to be under tonic dopaminergic influence. The majority of these GABA neurons are under inhibitory influence, whereas a small number seem to be stimulated by dopamine. Specific changes in activity in subpopulations of striatal GABA neurons probably mediate the dopamine-dependent hypokinetic syndrome seen in PD and following neuroleptic treatment [89]. Hyde et al. [90] examined the expression of transcripts derived from three genes related to GABA signaling [*GAD1 (GAD67* and *GAD25*), *SLC12A2* (*NKCC1*), and *SLC12A5* (*KCC2*)] in the prefrontal cortex (PFC) and hippocampal formation of a large cohort of nonpsychiatric control human brains across the lifespan and in patients with SCZ, and found that development and maturation of both the PFC and hippocampal formation are characterized by progressive switches in expression from GAD25 to GAD67

and from *NKCC1* to *KCC2*, the former leading to GABA synthesis, and the latter regulating the switching from excitatory to inhibitory neurotransmission. In the hippocampal formation, *GAD25/GAD67* and *NKCC1/KCC2* ratios are increased in SCZ, indicating potential immaturity of GABA neurotransmission. These mechanisms may justify the increased levels of GABA in SCZ and PD (**Figure 7).** Mutations in GABA receptors, especially in gamma-aminobutyric acid type A receptor alpha1 subunit (*GABRA1*, 5q34) and gamma-aminobutyric acid type A receptor delta subunit (*GABRD*, 1p36.33), are mainly associated with epilepsy [91-93]. However, Balan et al. [94] studied the association of 375 tagged SNPs in genes derived from the GABAergic system, such as *GABAA* receptor subunit genes, and GABA-related genes (glutamate decarboxylase genes, GABAergic-marker gene, genes involved in GABA receptor trafficking and scaffolding) in Japanese SCZ case-control samples, and found nominal association of SNPs in nine GABA receptor subunit genes and the *GPHN* gene with SCZ. Two SNPs located in the *GABRA1* gene, rs4263535 and rs1157122, showed top hits, followed by rs723432 in the *GPHN* gene. Haplotypes containing associated variants in *GABRA1* but not *GPHN* were significantly associated with SCZ.

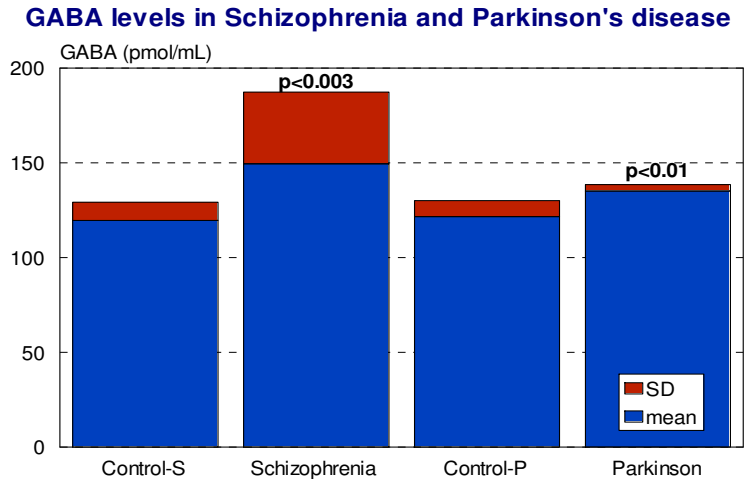

Figure 7. GABA levels in patients with schizophrenia and Parkinson's disease.

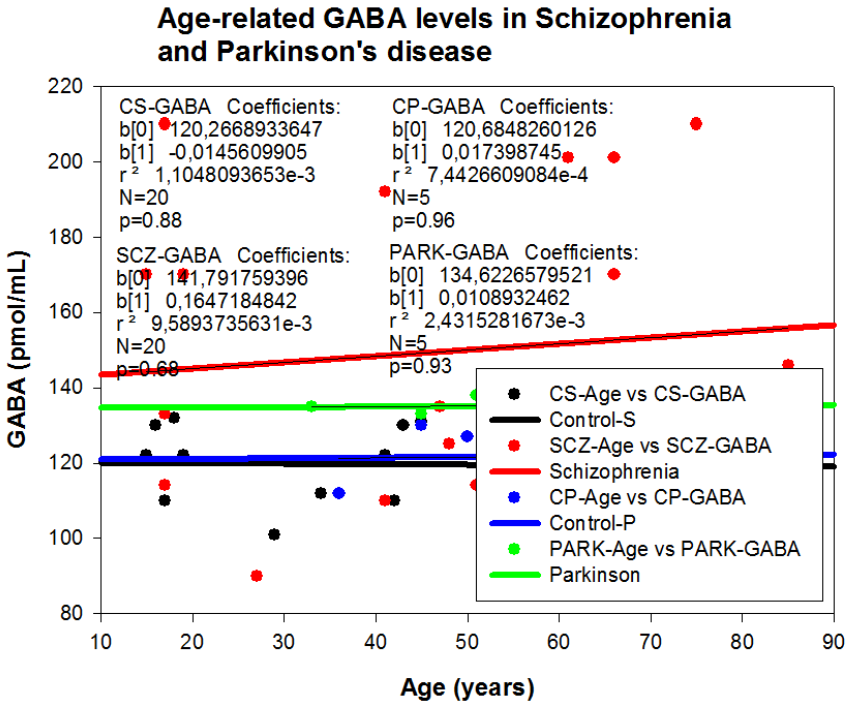

Figure 8. Age-related GABA levels in schizophrenia and Parkinson's disease.

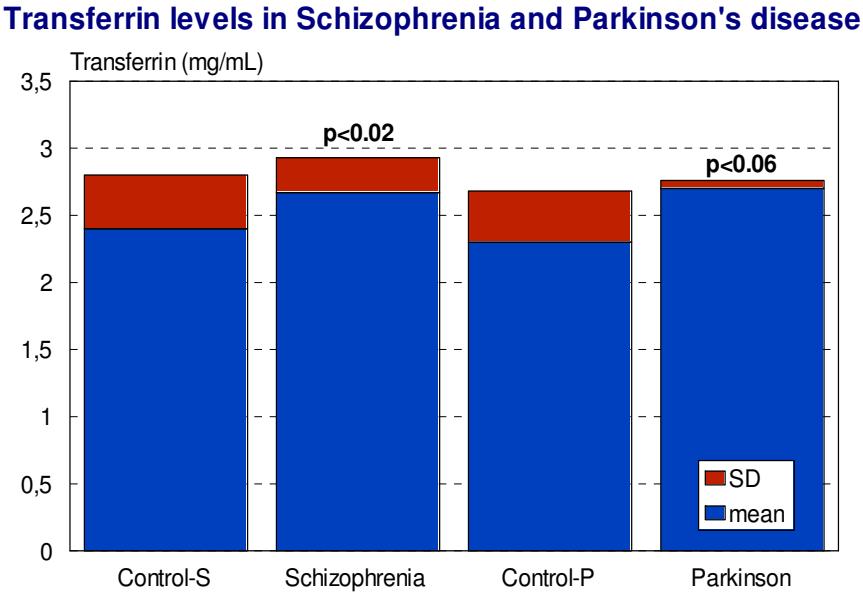

Figure 9. Transferrin levels in patients with schizophrenia and Parkinson's disease.

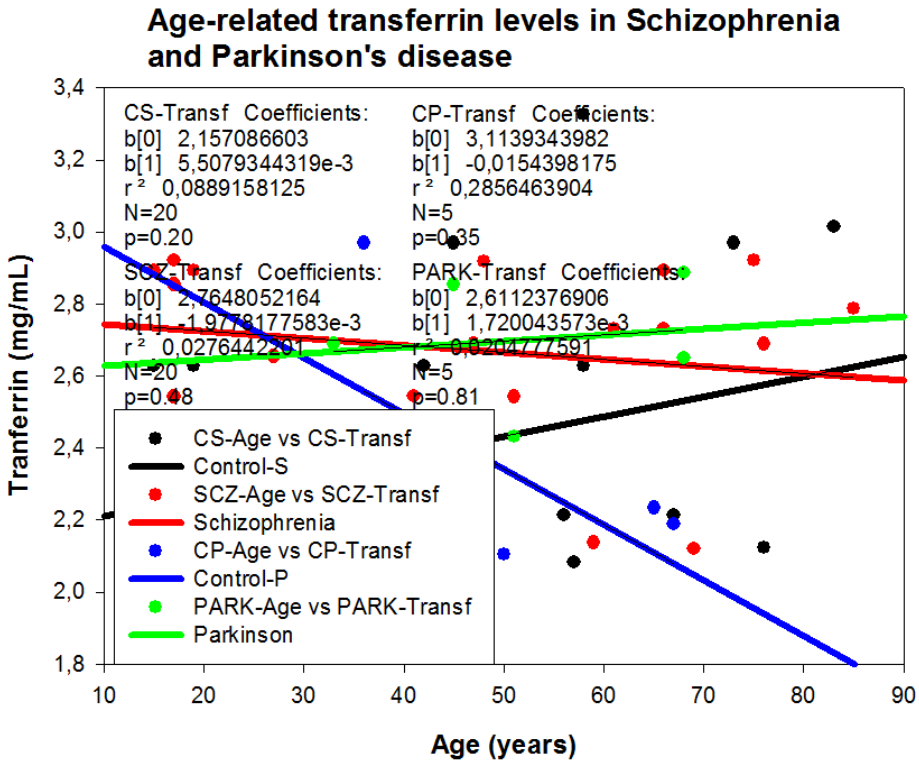

Figure 10. Age-related transferrin levels in schizophrenia and Parkinson's disease.

Finally, high levels of transferrin (siderophilin) in SCZ and PD may reflect mild iron metabolism dysfunction. This iron transport 678 aa protein is a β-globulin with high binding affinity for ferric ions and which enters cells by receptor (CD71)-mediated endocytosis. *HFE* mutations and transferrin *C1/C2* polymorphic variants do not appear to represent risk factors for either SCZ or PD [96]. However, neuroleptic treatment may result in altered glycosylation of serum proteins, compromising transferrin levels. For

instance, olanzapine treatment results in increased levels of a disialylated biantennary glycan and reduced levels of a number of disialylated bi- and triantennary glycans on whole serum glycoproteins [97]. Iron is essential for brain function, and it is not infrequent to detect iron metabolism abnormalities in neurodegenerative disorders, such as PD and Alzheimer's disease, which contribute to accelerating neurodegeneration. Iron entry into the brain is regulated by the blood-brain barrier (BBB). NHE9 (SLC9A9) is an endosomal cation/proton antiporter which regulates this transport system. Ectopic expression of NHE9 in BBB endothelial cells without external cues induces up regulation of the transferrin receptor (TfR) and down regulation of ferritin, leading to an increase in iron uptake [98]. NHE9-GFP localizes to recycling endosomes, where it significantly alkalinizes luminal pH, elevates uptake of transferrin and the neurotransmitter glutamate, and stabilizes surface expression of transferrin receptor and GLAST transporter. Loss-of-function mutations in *NHE9* (L236S, S438P and V176I) may contribute to autistic phenotype by modulating synaptic membrane protein expression and neurotransmitter clearance [99]. Physical and mental fatigue is a very common non-motor symptom in PD. It has been hypothesized that serotonergic dysfunction and abnormal iron metabolism are involved in mental fatigue in PD. The levels of serotonin, iron and transferrin in cerebrospinal fluid have been found increased in Chinese patients with PD; in contrast, serum serotonin and transferrin levels were found to be diminished in these patients [100]. In our PD cases, serum transferrin levels tended to be higher than in controls; however, the limited number of cases in our sample is insufficient to reach any conclusion. On the other hand, α-synuclein, the principal protein involved in the pathogenesis of PD, is expressed widely in the neuroretina, and facilitates the uptake of transferrin-bound iron (Tf-Fe) by retinal pigment epithelial cells that form the outer blood-retinal barrier. Absence of α-synuclein results in down-regulation of ferritin in the neuroretina, indicating depletion of cellular iron stores. Retinal iron dyshomeostasis due to impaired α-synuclein function may contribute to PD-related visual symptoms [101]. Consequently, iron metabolism dysfunction in SCZ and PD cannot be neglected in the clinical setting.

CONCLUSIONS

From the results obtained in this study, we can conclude the following: (i) extensive regions of the human genome show cytogenetic anomalies (deletions, duplications, inversions) in SCZ and in PD; chromosomal aberrations tend to increase with age in SCZ; (ii) epigenetic changes in the *BDNF* gene are represented by hypermethylation of CpG islands in promoters I and IV in patients with SCZ, but not in PD; (iii) serum GABA levels are significantly increased in both SCZ and PD cases; and (iv) transferrin levels are significantly higher in SCZ than in controls and show a tendency to increase in PD, reflecting mild iron metabolism dysfunction in both CNS disorders.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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