

Molecular characteristics of Human Endogenous Retrovirus type-W in schizophrenia and bipolar disorder

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Epidemiological and genome-wide association studies of severe psychiatric disorders such as schizophrenia (SZ) and bipolar disorder (BD), suggest complex interactions between multiple genetic elements and environmental factors. The involvement of genetic elements such as Human Endogenous Retroviruses type 'W' family (HERV-W) has consistently been associated with SZ. HERV-W envelope gene (*env*) is activated by environmental factors and encodes a protein displaying inflammation and neurotoxicity. The present study addressed the molecular characteristics of HERV-W *env* in SZ and BD. Hundred and thirty-six patients, 91 with BD, 45 with SZ and 73 healthy controls (HC) were included. HERV-W *env* transcription was found to be elevated in BD ($P < 10^{-4}$) and in SZ ($P = 0.012$) as compared with HC, but with higher values in BD than in SZ group ($P < 0.01$). The corresponding DNA copy number was paradoxically lower in the genome of patients with BD ($P = 0.0016$) or SZ ($P < 0.0003$) than in HC. Differences in nucleotide sequence of HERV-W *env* were found between patients with SZ and BD as compared with HC, as well as between SZ and BD. The molecular characteristics of HERV-W *env* also differ from what was observed in Multiple Sclerosis (MS) and may represent distinct features of the genome of patients with BD and SZ. The seroprevalence for *Toxoplasma gondii* yielded low but significant association with HERV-W transcriptional level in a subgroup of BD and SZ, suggesting a potential role in particular patients. A global hypothesis of mechanisms inducing such major psychoses is discussed, placing HERV-W at the crossroads between environmental, genetic and immunological factors. Thus, particular infections would act as activators of HERV-W elements in earliest life, resulting in the production of an HERV-W envelope protein, which then stimulates pro-inflammatory and neurotoxic cascades. This hypothesis needs to be further explored as it may yield major changes in our understanding and treatment of severe psychotic disorders.

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Introduction

Schizophrenia (SZ) and bipolar disorder (BD) are severe psychiatric disorders involving complex interactions between genetic and environmental factors.^{1,2} Environmental factors, such as winter birth, urban environment and maternal infection during pregnancy, in particular caused by Influenza virus, Herpesviruses or *T. gondii*, are associated with an increased risk for SZ and for BD.^{3–5} Particular viruses or parasites have been thought to have a role in the pathogenesis of SZ or BD but, as most studies were based on serology that essentially detects an 'immunological scar' represented by the presence of specific immunoglobulin G antibody, the

period of infection is debated and may occur at variable times, as reviewed.^{6,7} Nonetheless, association of BD or SZ with infectious agents quite always represent subgroups of patients and may, therefore, play different or additional roles, or may constitute different etiological causes or contributors as suggested by various studies.^{8–10} Genetic studies revealed an overlapped involvement of loci involved in the inflammatory/immune pathways including the major histocompatibility complex region in both SZ and BD,^{11–14} among other candidate genes.^{15–17} Structural genomic studies also highlighted significant modifications in psychotic patients,

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including copy number variations and deletions.^{18–21} Nonetheless, the mechanisms possibly underlying interactions between genetic and environmental risk factors contributing to the clinical onset and/or to the progression of psychotic disorders remain to be understood.¹⁶

The involvement of atypical genetic elements, such as Human Endogenous Retroviruses (HERVs), has also been reported in SZ and BD.^{22–32} In schizophrenia (SZ), a sequence homologous to an endogenous retrovirus (ERV) identified in MS and named 'Multiple Sclerosis-associated Retroviral element' (MSRV)³³ was first identified from differential DNA amplification in homozygote twins discordant for the disease.²³ MSRV sequences later permitted to unravel a yet unknown HERV family, now named HERV-W.^{34–37} Furthermore, though detected in different brain areas than in MS, many studies have now evidenced white matter and myelin impairment and/or inflammation predominantly in BP but also in SZ.^{38–44} Thus, the expression of the pro-inflammatory envelope protein of MSRV in MS brain microglia within demyelinating lesions⁴⁵ should now be considered in parallel with myelin alterations in the vicinity of activated microglia in BD and SZ brain.

HERVs have properties of mobile genetic elements causing genetic rearrangements^{21,46,47} potentially influenced by their interactions with microbial agents from environment.^{48,49} Coincidentally, important structural modifications in the major histocompatibility complex locus were associated with such characteristics of HERVs.⁵⁰ For these reasons, HERVs may provide a missing link between environmental factors, genetic modifications and pathogeny with downstream neuroinflammation and neurotoxicity in psychotic disorders with such features.^{21,38,51–54}

HERVs are components of the genome that can be transmitted to subsequent generations through gametes, but have evolved differently from other host genes. They have significant inter-individual copy number variations within the genome of healthy humans from different ethnic origins⁵⁵ or among subjects with distinct phenotype for example, complement C4 factor.⁵⁶ These multicopy families have retained characteristics of retroviruses.²¹ In fact, HERVs, which represent 8% of the human genome,⁵⁷ are part of the superfamily of repeated and transposable elements (transposons, retrotransposons and ERVs) representing about 42% of the human genome.⁵⁸ They probably had a role in inter-individual gene transmission, as well as in intracellular gene retrotransposition or recombination, and may undergo changes under selective environmental pressure.^{21,59} In certain conditions undisrupted HERV sequences may be expressed and display viral protein properties.⁴⁹ Although most of the contemporary copies of HERVs are inactivated by mutations or deletions or silenced by epigenetic modifications,⁶⁰ their plasticity and potential responsiveness to environmental triggers are of particular relevance for gene–environment interactions.²¹ In the case of the HERV-W family and its MSRV element, it has now consistently been shown that certain infectious agents can trigger activation of certain copies.⁶¹ In particular, HERV-W elements have been reported to be activated by *T. gondii*,⁶² as well as by Influenza virus⁴⁸ in human cell lines. Such pathogenic activation of HERV-W, mainly focusing on MSRV-type elements in

experimental or clinical studies,^{45,63–65} may result in the production of its envelope protein (HERV-W Env) that strongly stimulates a pro-inflammatory cascade through the TLR4 receptor pathway⁶⁶ and displays potential neurotoxicity.²⁵ We, therefore, considered that these highly relevant features place HERV-W elements at the forefront of gene–environment interactions underlying complex diseases such as SZ.^{49,51}

In the present study, we have further investigated the genetic features and the *ex-vivo* transcriptional activity of HERV-W envelope copies, as reflected in appropriate blood cells, in patients with SZ and BD in comparison with healthy controls (HC). Moreover, as MSRV has now specifically been shown to have detectable and abnormal expression in the peripheral mononuclear cells (PBMC, representing of lymphocytes and monocytes) of patients with MS,^{45,67} the same technical approach was applied here. The cellular RNA and genomic DNA copies were thus quantified in PBMC from patients with BD, with SZ and from HC, using an established real-time PCR technique targeting the MSRV subtype of HERV-W family.⁶⁷

Patients and methods

Participants. Patients fulfilling DSM-IV criteria (American Psychiatric Association, 1994) for SZ or BD were recruited during hospitalization or follow-up visits in two university-affiliated psychiatric departments (Paris, France). Inclusion criteria for study participation were: age between 18 and 65 years, no history of alcohol or drug abuse/dependence, no history of mental retardation, no previous head trauma with loss of conscience. Patients were interviewed with the French version of the 'Diagnostic Interview for Genetic Studies'.⁶⁸ Patients with SZ were evaluated with the Positive And Negative Syndrome Scale⁶⁹ and with the Calgary scale⁷⁰ measuring the depressive symptoms. Patients with BD were screened with the Young Mania Rating Scale⁷¹ searching for manic symptoms, and with the Montgomery and Asberg Depression Rating Scale⁷² for depressive symptoms.

HC without any personal and family history of SZ or BD were enrolled through a clinical investigation center. Patients and controls had negative serology for human immunodeficiency viruses (HIV1 + 2), Hepatitis A, B and C viruses, no known inflammatory, auto-immune or neurological disorder. All subjects gave written informed consent for their participation in this study with ethical committee approval.

Serum collection. One tube (7 ml dry tube B&D, Meylan, France) of blood from each subject was treated within 2 h after collection. The clotted blood was centrifuged for 10 min at 2800 *g* at +4 °C. Clear serum (hemolytic sera were rejected) was collected and stored at –20 °C.

Serological analyses. Immunoglobulin G antibodies were measured as previously described⁷³ in the Stanley Division of Developmental Neurovirology (Baltimore, USA). (1) For viruses of the Herpesvirus family: HSV-1, HSV-2, CMV, EBV, HHV-6 and VZV, (2) for *T. gondii*. Assays for HSV-1 and HSV-2 used envelope glycoproteins gG1 and gG2, respectively.

Quantitative HERV-W PCR. PBMCs were tested according to a previously described quantitative PCR (qPCR) and reverse transcriptase PCR (RT-PCR) specific for MSRV subtype of HERV-W family⁶⁷ further adapted as detailed.

Quantification of MSRV-env RNA in PBMC by real-time RT-PCR. All steps, from the extraction of RNA to the interpretation of the final results, were conducted in blinded conditions and according to a standard operation procedure (SOP, Geneuro-Innovation, Lyon, France). Briefly, PBMCs were washed with phosphate-buffered saline and total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was treated with Turbo DNA-free kit (Applied Biosystems, Foster City, CA, USA) before assessment of its quality and concentration by spectrophotometer; then sample volumes were adjusted to add 50 ng of RNA per reaction. For each sample, the expression (calculated as the cycling threshold or Ct) of MSRV-env and the housekeeping gene *GUS B* encoding the glucuronidase beta was analyzed in triplicate by RT-PCR (Thermal Cycler C1000—CFX96 Real-Time System, Bio-Rad).

Specific sets of primers and probes for MSRV-env (according to Mameli *et al.*⁶⁷) and *GUS B* (Taqman gene expression assay -*GUS B*, ref. 4331182/Hs99999908_m1, Applied Biosystems) were used to perform a One-Step RT-PCR (iScript One-Step RT-PCR for Probes, Bio-Rad) with an initial step of 50 °C for 10 min and 95 °C for 5 min incubations followed by 45 cycles of 95 °C for 10 s and 60 °C for 1 min.

For each sample a control without reverse transcriptase was analyzed to detect possible DNA contamination. Assays were considered acceptable if: (a) amplification of the housekeeping gene was detected in all the extracted samples; (b) no amplification was detected without reverse transcriptase; (c) the efficiency of the PCR was 90–110% ($3.6 > \text{slope} > 3.1$); (d) variability among replicates was $< 5\%$.

Results, representing standardized MSRV RNA expression levels, are expressed as MSRV-env relative expression to the reference and stably expressed *GUS B* RNA for each subject, defined as $2^{E(\text{Ct } GUS B - \text{Ct } MSRV-env)}$ (the ΔCt method using a reference gene, Real-Time PCR Application Guide, Bio-Rad Laboratories).

The normal copy number of transcript reflecting the background transcriptional activity of various HERV-W copies, mostly defective as described for ERVs,²¹ is determined from the HC.

A threshold (cutoff value of the technique) above which an elevated transcriptional activity of HERV-W becomes significant, was, therefore, determined from the mean of the HC population plus twice its s.d. ($M + 2 \text{ s.d.}$). Outliers corresponding to HC individuals with values above the mean plus its s.d. ($M + \text{s.d.}$) are not included in the calculation of this 'cutoff' value.

MSRV-env genomic DNA copy number in total blood leukocytes by real-time PCR. DNA was extracted from peripheral blood leukocytes using a salting out procedure. DNA concentration and quality were assessed using Qubit[®] double stranded DNA BR Assay Kits and Qubit[®] 2.0

Fluorometer (Life Technologies, Carlsbad, CA, USA) and volumes were adjusted to add 20 ng of DNA per reaction.

Relative DNA quantification was performed using the same MSRV primers⁶⁷ in parallel with a reference standard consisting in a monocopy human gene encoding the hydroxymethylbilane synthase.

PCR reactions were performed in a final volume of 20 μl , containing 20 ng of DNA, 0.5 μM of forward and reverse primers, 0.2 μM of fluorescently labeled specific probe and 1 \times of TaqMan Universal Mastermix (Applied Biosystems), and run in a Mastercycler[®] ep realplex2S (Eppendorf, Hamburg, Germany). PCR cycle parameters were 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Common threshold fluorescence for all the samples was set into the exponential phase of the amplification and determined the Ct, corresponding to the number of amplification cycles needed to reach this threshold. All reactions were performed in triplicate and the mean value of Ct was used for subsequent analysis.

Results, representing the MSRV DNA copy number per diploid human genome, were obtained by calculating the ratio between HERV-W/MSRV copies and hydroxymethylbilane synthase copies in the same sample.

When samples did not allow RNA and/or DNA extraction in sufficient quantity or quality, they were not tested and corresponding patients not analyzed in the test series.

PCR product sequencing. MSRV-Env DNA and RNA amplicons were cloned according to the manufacturer's instructions for the TOPO-cloning kit (TOPO[®] TA Cloning[®] Kit, Invitrogen, Carlsbad, New-Mexico, USA) followed by capillary sequencing (Applied Biosystems[®]), by Fasteris SA Plan-Les-Ouates (Switzerland). Sequences were analyzed using Mac Vector[®] v11.1.1 software (MacVector Inc., Cambridge, NC, USA). Multiple sequence alignments were performed using clustalW (v1.83) multiple alignment algorithm. Phylogenetic analysis was performed using a neighbor joining bootstrap with 1000 repeats and Kimura 2-parameters method for graphical 'tree' presentation.

Statistical analysis. Figures from BD, SZ and HCs groups were compared using χ^2 -test for categorical data and the Kruskal–Wallis or Mann–Whitney test for nonparametric data.

All statistical analyses were performed using R software package version 2.13.0 (Stanford University Social Science Data and Software, Stanford, CA, USA).

Results

Demographical characteristics. The study group consisted of 136 patients (91 with BD and 45 with SZ) and 73 HC. Half of them (52.2%) were men (47.3% for BD, 75.6% for SZ and 43.8% for HC) with a mean age at interview of 38.02 (± 12.7) years. Patients with BD were significantly older than patients with SZ and HCs (43.81 ± 12.4 , 34.75 ± 11.1 and 37.33 ± 12.8 years, respectively, $P < = 0.0001$).

For patients with BD, the mean scores of depression (measured with the Montgomery and Asberg Depression

Rating Scale) and of mania (measured with Young Mania Rating Scale) were 6.54 ± 8.9 and 4.92 ± 7.4 , respectively. In the sample of patients with SZ, mean scores of depression (measured with the Calgary) and of psychotic symptoms (measured with the Positive And Negative Syndrome Scale) were of 8.45 ± 6.7 and 65.46 ± 17.5 , respectively. Thus, patients with BD were mostly euthymic, and patients with SZ were not in an acute phase. The majority of them were patients with long-duration disorder: mean of 16.91 ± 11.2 years for BD and 10.93 ± 9.1 years for SZ.

The majority of patient with BD received a mood stabilizer (lithium in 35.4%, valproate in 20.9%, atypical antipsychotics in 4.1%, a combination of two mood stabilizers in 12.5%, a combination of a mood stabilizer and an atypical antipsychotic in 21.9% of the cases). Patients with SZ received atypical antipsychotics in 70.4% of the cases, typical antipsychotics in 7.4% and a combination of a mood stabilizer and an atypical antipsychotic in 22.2% of the cases.

HERV-W RNA expression. As presented in Figure 1a, the levels of HERV-W RNA transcription determined by qPCR were significantly elevated in PBMC of patients with BD and

patients with SZ, compared with HC ($P < 10^{-4}$ for BD and $P = 0.012$ for SZ). When comparing the two groups of patients, BD group had a significantly higher expression than SZ group ($P < 0.01$). When patients with SZ or BD were compared with HC with a C-reactive protein below the normal threshold (C-reactive protein < 5 ; 'C-' subgroup), the statistical significance remained unchanged for all comparisons, or even increased for SZ versus C- ($P = 0.007$).

In Figure 1b, the distribution of HERV-W RNA levels is presented for all clinical categories. A different distribution was observed between patients and controls, with the expected distribution in HC peaking at the lowest value.

Patients were further stratified according to the transcriptional levels of HERV-W RNA in PBMC: patients with values above the cutoff (mean + 2 s.d. of HC; see Patients and methods) were considered to have significantly elevated values (positives), whereas patients with values below this threshold were considered to have normal RNA levels (negatives).

As presented in Table 1a, we found no correlation between HERV-W RNA 'positives' or 'negatives', with respect to age at onset of the disorder, duration of illness, number of prior

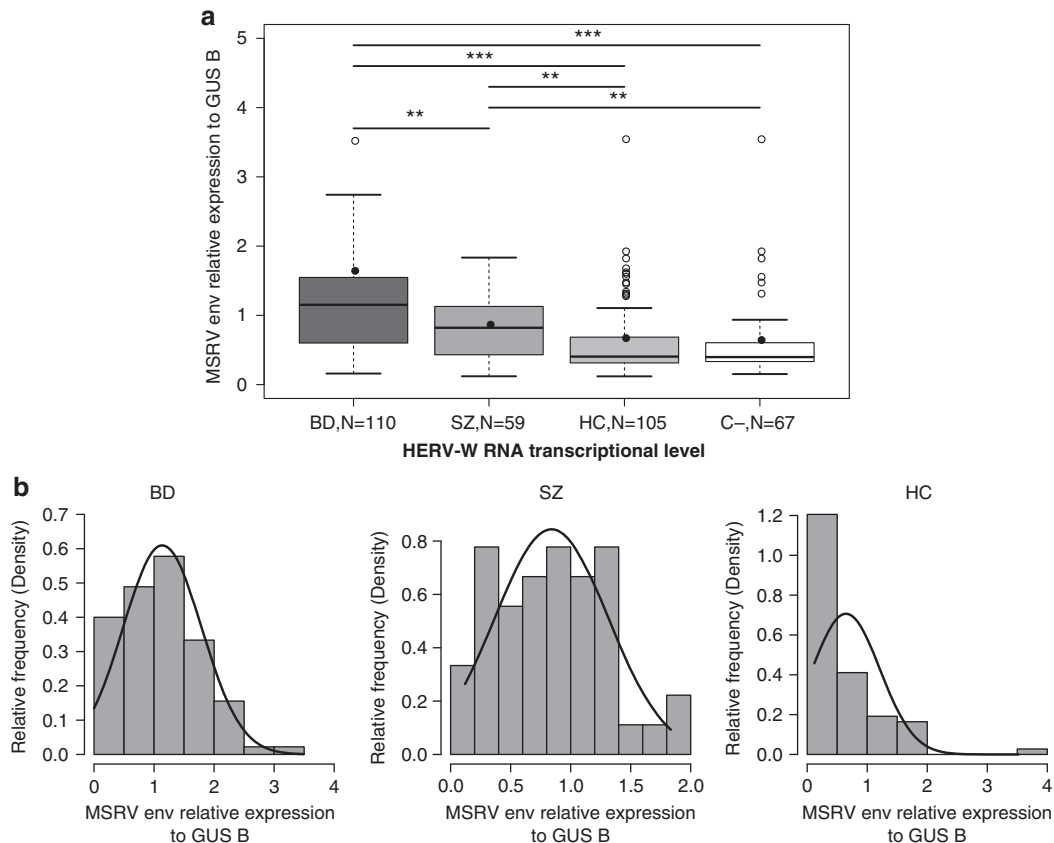


Figure 1 Human Endogenous Retrovirus type-W (HERV-W) RNA transcriptional level. **(a)** HERV-W env RNA expression in patients and control groups. The relative transcription of Multiple Sclerosis-associated Retroviral element (MSRV)-env RNA to the housekeeping gene *GUS B* is indicated on the y axis; it measures the standardized and relative transcriptional activity in all peripheral mononuclear cells samples defined as $2^{E(Ct\ GUS\ B) - Ct\ MSRV\ env}$ (See Patients and methods). Patients and control groups are indicated on the x axis: BD, bipolar disorder; SZ, schizophrenia; HC, healthy controls without psychiatric history; C-, subset of HC group with C-reactive protein below the normal threshold in serum. ** $P \leq 0.01$ *** $P \leq 0.0001$. **(b)** Distribution of HERV-W RNA expression levels in patients and control groups. y axis represents the density of corresponding HERV-W env RNA relative expression values on the x axis, giving a description of their distribution in each group (BD, SZ, HC). Density curve: the area under a density curve (between the curve and the horizontal axis) is always defined as 1 unit. The area under the density curve between two values is the proportion of observations in the data set that fall between those two values. Histogram: the distribution frequency of results between two values is represented by bars.

Table 1a Clinical and serological status according to HERV-W RNA transcriptional level (HERV + and HERV -)

	BD				SZ			
	N used (±)	HERV+ (N = 51)	HERV- (N = 40)	P-value	N used (±)	HERV+ (N = 17)	HERV- (N = 28)	P-value
Age at onset (years ± s.d.)	48/39	25.83 (8.6)	27.28 (11.6)	0.931	16/26	24.06 (10)	23.65 (5.8)	0.773
Age at interview (years ± s.d.)	49/40	44.55 (12)	42.9 (13.2)	0.723	16/25	33.93 (11.1)	35.28 (11.3)	0.749
Duration of illness (years ± s.d.)	49/39	18.30 (11.9)	15.17 (10.1)	0.220	16/25	9.88 (6.4)	11.6 (10.5)	0.952
Number of episodes	43/39	8.62 (6.3)	8.33 (7.8)	0.270	15/24	4.10 (3.1)	4.12 (3)	0.898
MADRS (mean scores)	49/40	6.18 (9.1)	6.98 (8.9)	0.792				
YMRS (mean scores)	49/39	4.50 (7.1)	5.43 (7.8)	0.727				
PANSS (mean total scores)	45/35	37.13 (11.5)	38.94 (13.3)	0.896	15/20	65.93 (20.9)	65.1 (15.1)	0.469

Abbreviations: BD, (patients with) bipolar disorder; HERV-W, Human Endogenous Retrovirus type-W; MADRS, Montgomery and Asberg Depression Rating Scale; PANSS, Positive And Negative Syndrome Scale; SZ, (patients with) schizophrenia; YMRS, Young Mania Rating Scale. HERV-W expression status among patients with BD and SZ. HERV-W 'Positives' have a transcriptional level with RNA copy number significantly elevated, compared with background transcription in healthy controls; 'Negatives' have RNA copy number below the threshold (see Patients and methods). As few samples could not be tested for all parameters (insufficient volume, quality control not passed, and so on) the numbers effectively tested and used for calculations presented in Table 1a are indicated in columns with 'N used (±)'.
HERV-W 'Positives' have a transcriptional level with RNA copy number significantly elevated, compared with background transcription in healthy controls; 'Negatives' have RNA copy number below the threshold (see Patients and methods). As few samples could not be tested for all parameters (insufficient volume, quality control not passed, and so on) the numbers effectively tested and used for calculations presented in Table 1a are indicated in columns with 'N used (±)'.

Table 1b Clinical and serological status according to HERV-W RNA transcriptional level (HERV + and HERV -)

Variables, number of tested sera (N used: HERV +/HERV -)	All patients, SZ and BD			BD			SZ		
	HERV+ (68)	HERV- (68)	P-value	HERV+ (51)	HERV+ (40)	P-value	HERV+ (17)	HERV- (28)	P-value
	Positive serology (% pos)			Positive serology (% pos)			Positive serology (% pos)		
<i>T. Gondii</i> , (63/56)	53 (84.1%)	38 (67.9)	0.036	42 (84)	25 (69.4)	0.109	11 (84.6)	13 (65)	0.216
HSV-1, (63/56)	41 (65.1%)	31 (55.4)	0.279	33 (66)	22 (61.1)	0.641	8 (61.5)	9 (45)	0.353
HSV-2, (63/56)	14 (22.2%)	12 (21.4)	0.917	14 (28)	10 (27.8)	0.982	0 (0)	2 (10)	0.239
CMV, (63/56)	36 (57.1%)	25 (44.6)	0.173	30 (60)	17 (47.2)	0.24	6 (46.2)	8 (40)	0.727

Abbreviations: BD, (patients with) bipolar disorder; CMV, Cytomegalovirus; HERV-W, Human Endogenous Retrovirus type-W; HSV-1 or -2, Herpes Simplex Virus Type 1 or 2; SZ, (patients with) schizophrenia. HERV-W expression status among patients with BD and SZ. HERV-W 'Positives' have a transcriptional level with RNA copy number significantly elevated, compared with background transcription in healthy controls; 'Negatives' have RNA copy number below the threshold (see Patients and methods). As few samples could not be tested for all parameters (insufficient volume, quality control not passed, and so on), the numbers effectively tested and used for calculations presented in Table 1b are indicated in columns with 'N used (±)'.

episodes, frequency of hospitalizations, Positive and Negative Syndrome Scale scores for patients with SZ and Young Mania Rating Scale or Montgomery and Asberg Depression Rating Scale scores for BD. As valproic acid (valproate) was found to be associated with elevated HERV transcription in a previous study,⁷⁴ we compared patients with BD treated or not with valproic acid. We observed that patients with BD treated with valproic acid had higher RNA levels ($P = 0.035$) than valproate-free patients. Therefore, we excluded all patients treated with valproic acid and analyzed valproate-free subgroups of patients with BD and SZ. A significant difference was still observed between HC and BD or patients with SZ ($P < 0.0001$ and $P = 0.007$, respectively).

The BD and SZ groups, in which a great majority of patients presented chronic and/or stable disease as above described, showed no significant correlation with the other tested clinical parameters (Table 1a).

Further, patients with BD or SZ did not differ from controls for seropositivity (immunoglobulin G antibodies) to *T. gondii*,

HSV 1 and 2, and for CMV. Similarly, SZ did not differ from BD. Nevertheless, the seroprevalence of *T. gondii* was significantly higher in patients with BD and SZ, when merged as a single group, than in HC ($P < 0.036$; Table 1b).

HERV-W DNA genomic copy number. As presented in Figure 2, HERV-W MSR-type DNA copy number was significantly lower in DNA of leukocytes from patients with BD and SZ as compared with HC ($P = 0.0016$ and $P < 0.0003$ for BD and SZ, respectively; Supplementary Information). However, no significant difference in the genomic HERV-W DNA copy number was found between BD and SZ, nor between HC and C- (C-reactive protein-negative controls; $p > 0.5$). In Figure 2b, the distribution profile of HERV-W DNA copy number is presented for all categories, with figures of HC peaking at the center (normal distribution). Even after excluding patients treated with valproic acid, the difference remained unchanged and statistically significant (Supplementary Information).

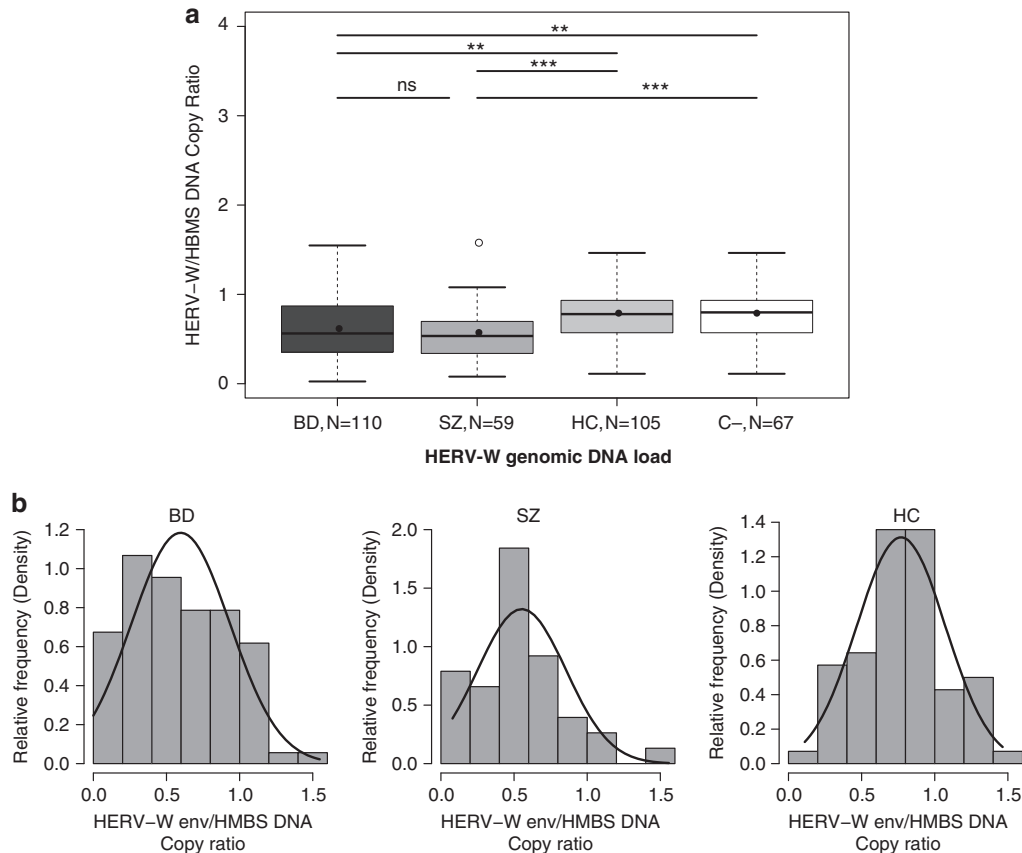


Figure 2 Human Endogenous Retrovirus type-W (HERV-W) genomic DNA load. (a) Relative HERV-W DNA copy number in patients and controls. The ratio between the DNA copy number of HERV-W *env* gene Multiple Sclerosis-associated Retroviral element (MSRV)-subtype and the hydroxymethylbilane synthase housekeeping gene is indicated on the y axis. This measures the relative quantification of copy numbers from the MSRV subtype, detected by the MSRV-*env* probe with the quantitative PCR technical conditions used here on total blood leukocytes. Patients and control groups are indicated on the x axis: BD, bipolar disorder; SZ, schizophrenia; HC, healthy controls without psychiatric history; C-, subset of HC group without C-reactive protein above the normal threshold in serum. ** $P \leq 0.01$ *** $P \leq 0.0001$. (b) Distribution of HERV-W *env* DNA copy number in patients and controls. y Axis represents the frequency and the density of corresponding HERV-W *env* DNA copy values on the x axis, giving a description of their distribution in each group (BD, SZ, HC). Density curve: the area under a density curve (between the curve and the horizontal axis) is always defined as 1 unit. The area under the density curve between two values is the proportion of observations in the data set that fall between those two values. Histogram: the distribution frequency of results between two values is represented by bars.

Sequence analyses of HERV-W PCR amplicons. The PCR products were cloned and sequenced to address the specificity of the qPCR technique, as well as eventual qualitative difference.

The PCR products from randomly selected individuals (three HC, three patients with BD and four with SZ; Table 2) provided a representative panel of DNA and RNA amplicons from each category. In order to avoid mistakes in reassembling irrelevant fragments with overlapping sequences by deep sequencing of short nucleotide stretches within a complex mixture of variants, PCR products were cloned. Inserts were sequenced on both strands and then aligned with the probe used for qPCR. Sequences showing significant alignment, here with a maximum of two mismatches (see Patients and methods), correspond to the amplicons that determine the copy number measured by this probe in qPCR.

Table 2 shows that the highest percentage of clones identical to the reference probe was obtained for RNA from SZ (87%, 33/38). Conversely, in RNA from BD, the majority of

clones had one or two nucleotide mismatches (74%, 26/35). Finally, in RNA from HC, all sequences presenting mismatches (12/34) were absent in RNA of either patients with SZ (0/38) or BD (0/47). This difference was significant when compared with either BD or SZ group (χ^2 : $P < 0.001$).

Corresponding sequence alignments are shown in Supplementary Information.

Detailed analysis was performed with HERV-W reference clones (MSRV-*env*, ERVWE1 encoding Syncytin on chromosome 7) and a distant envelope gene from another HERV family (HERV-K113-*env*) followed by phylogenetic analysis (Supplementary Information and Patients and methods). Significant homology with HERV-W *env* sequence obtained from retroviral RNA within purified extracellular virions^{33,75} was found for most sequences, which emphasizes the optimized selectivity of the present PCR conditions for this subtype of HERV-W family and explains the limited number of copies detected within human DNA.

These qualitative differences in the frequency of variants among BD, SZ and HC are additional discriminating features

Table 2 HERV-W *env* clones obtained from PBMC by standard PCR with primers used in the quantitative PCR

Status	DNA PCR	DNA PCR	RNA RT-PCR	RNA RT-PCR
	Number of clones with insert	Number of sequences aligned with probe	Number of clones with insert	Number of sequences aligned with probe
SZ	32	3 (0); 3 (1); 19 (2)	38	33 (0); 4 (1); 0 (2)
BD	36	5 (0); 5 (1); 18 ^b (2)	47	9 (0); 20 (1); 6 (2)
HC	36	10 (0); 8 (1); 16 (2)	34	20 (0); 11 ^a (1); 1 ^a (2)

Abbreviations: BD, bipolar disorder; HC, healthy controls; HERV-W, Human Endogenous Retrovirus type-W; PBMC, peripheral mononuclear cell; RT-PCR, reverse transcriptase PCR; SZ, schizophrenia.

Clones were obtained in PBMC from three HC, three BD and four SZ individuals. According to software analyses (Mac Vector V.11.1.1, Cambridge, NC, USA) amplicons with a maximum of two nucleotide substitutions could hybridize with the probe used in the quantitative PCR assay. The number of clones are indicated followed by the corresponding number of substituted nucleotides between brackets for 0, 1 and 2 possible substitutions.

Detailed sequences with alignments are presented in Supplementary Information.

^aNucleotide substitutions never found in RNA from SZ, but in 19 clones from BD RNA. This sequence is nonetheless present in DNA: one clone from SZ, three clones from BD and two clones from HC. ^bClones with two nucleotide substitutions from BD DNA are all identical, but never found in clones from RNA (BD, SZ or HC). They are present in 14/19 clones from SZ DNA and 14/16 clones from HC DNA.

to the observed differences in RNA and DNA copy numbers (Figures 1a and 2a) and in their distribution (Figures 1b and 2b).

Discussion

The present molecular study is consistent with previously reported association between HERV-W expression and SZ,^{6,51} but also unravels the existence and specificities of an analogous association between BD and HERV-W *env*.

Moreover, transcriptional levels of HERV-W *env* RNA from patients with BD here appear significantly higher than among patients with SZ, with additional qualitative differences in nucleotide sequences. This is obviously not attributable to treatment as most patients were treated and no correlation between a given treatment and results was evidenced. In particular, valproic acid had no impact as previously suggested,⁷⁴ as excluding patients treated with this mood stabilizer yielded similarly significant differences with HC. It therefore cannot account for the quantitative and qualitative molecular findings in patients with BD or with SZ.

The significance of an elevated HERV-W *env* RNA expression in SZ and BD groups was also confirmed by comparison with a subgroup (C-) excluding controls with elevated C-reactive protein, a nonspecific marker of inflammation⁷⁶ indicative of risk for various diseases in apparently healthy individuals.⁷⁷

The influence of disease state could also be debated as previous studies on RNA only included patients with SZ during first or acute psychotic episode, or patients with BD during acute mood episode. For the first time, our present populations of patients with BD, who were mostly euthymic, and of patients with SZ, most of whom were not in an acute phase, evidence that this HERV-W expression is not simply linked to, or a mere consequence of, active episodes of these diseases.

Importantly, it can now be assumed that elevated HERV-W/MSRV RNA expression appears constitutive in these patients.

Nonetheless, this does not preclude more elevated RNA expression that might peak during active and highly symptomatic periods of patients with BD or SZ. Moreover, only a subset of patients (with first onset or symptomatically active) have been shown to be positive for HERV-W *env* mRNA expression in independent studies,^{22,24,25,50} which is also the case with the present study on stable patients (62% – 28/45 of positives for SZ; 56% – 51/91 of positives for BD). Therefore, eventual transiently ‘negative’ RNA expression cannot be excluded during the clinical course of these illnesses. Alternatively, this association with HERV-W may only be relevant in a subgroup of patients with SZ and BD, which would point to different pathogenic associations in ‘HERV-W negative’ groups. BD and SZ disease entities are only defined by clinical criteria and might comprise subgroups with different etiopathogenic factors, but future longitudinal follow-up studies are now needed to address such important questions.

The unexpected low DNA copy number of HERV-W *env* in genomes of patients with SZ or BD (as compared with HC) is also accompanied by qualitative differences both in the distribution of copy numbers and in the nucleotide sequences within the probe region. In addition, despite similar DNA copy numbers, patients with SZ or BD show differing distributions of RNA transcription levels and of nucleotide sequences.

Altogether, these data confirm a consistent association with SZ and BD of this MSRV-type of HERV-W family that was first isolated in MS.³³ However, the patterns of association between MS or SZ/BD appear quite different for the DNA, as well as for nucleotide sequence features of both RNA and DNA.

Results of the present study could, therefore, suggest that differences in HERV-W panel (number and nature of DNA copies) may preexist in the genome of newborns diagnosed later in their life with SZ or BD. An atypical transcriptional potency could result from inherited HERV-W variants with low copy number. Alternatively, *de novo* acquired modifications of such ‘mobile genetic elements’ cannot be totally excluded, as reported in other conditions.²¹

In animals, analogous situations are encountered with families of Endogenous Retroviruses (ERV), in which pathogenic ERV copies are unevenly distributed in species genomes. The presence of particular and/or numerous ERV ‘defective’ copies can be protective against pathogenic ERV strains, while their absence may be detrimental.^{16,51,52} The hypothesis of *de novo* acquisition of genetic alterations could also be consistent with numerous studies associating intra-uterine/perinatal infections or inflammatory events as potential triggers in the development of BD or SZ later in life.⁵¹ Genetic modifications could affect the HERV-W copy numbers as insertion/deletion phenomena have been reported in association with HERVs,⁵⁶ but could also affect the detection of modified HERV-W copies by the present PCR primers and probe. This alternative explanation may thus shed light on the well-described genetic rearrangements in SZ or BD,¹⁶ with small to large deletions and/or copy number variations in the genome of affected individuals, as HERV rearrangements are also known to involve neighboring genes.⁵⁶

Although the mechanisms are not yet elucidated, activation of HERV-W elements epigenetically silenced in differentiated cells was put forward.⁴⁹ Here, the only environmental agent showing some degree of association with enhanced HERV-W RNA expression is *T. gondii*. It nonetheless required large numbers with the merged SZ and BD groups to reach statistical significance and appeared relevant in a subgroup of patients. The fact that this parasite often had increased prevalence in cohorts of patients with BD or SZ⁷⁸ would then be consistent with a role as a pathogenic co-factor, which can be replaced by other co-factors displaying analogous effects. Alternatively, it might have a role in association with particular symptomatology, as suggested by a recent study.⁹ Interestingly, *T. gondii* is known to transactivate HERVs, including HERV-W elements, when infecting tumor cells,^{62,79} which would also fit with a co-factor triggering HERV-W activation with subsequent genetic and inflammatory effects related to HERV-W envelope. This points to an indirect role of such infectious agents through 'epigenetically susceptible' HERV elements, as it is known that tumor cells have extensive DNA hypomethylation,^{80,81} which occurs physiologically in embryonic cells.⁸² Thus, *T. gondii* could induce a targeted activation of HERV-W elements creating a risk for SZ or BD in individuals carrying an HERV-W 'pathogenic element'. Such HERV-W elements appear here to fit with the MSRV subtype detected under the form of circulating retrovirus-like particles in a low proportion (8.9±6.2%) of studied Caucasian populations.⁸³ Thus, requiring the coincidence of both triggering and responding entities for the genesis of HERV-W pathogenicity, environmental pathogens other than *T. gondii* may not yield significant results with our present numbers and patient selection, due to their variability as co-factors and to their less frequent prevalence in our cohorts.

We thus hypothesize that, at a particular vulnerable developmental stage (for example, when the panel of HERV-W genes is hypomethylated and/or with low epigenetic control), an environmental trigger such as an infection favors cell lineage-specific genetic modifications in these elements (retrotransposition, gene rearrangements, and so on) establishing altered/variable patterns of neurodevelopment. Later in life, each of these patterns would respond differently to subsequent environmental triggers and be translated into distinct clinical phenotypes such as SZ, BD. Quite different nature and conditions of interactions with HERV-W elements (for example, no association ever found with *T. gondii* nor with perinatal infection), as well as temporal differences in triggering events during lifetime, could then lead to MS.^{49,51}

The emerging concept involving HERVs in human medicine²¹ highlights the importance such gene-environment interactions in a number of multifactorial diseases with poorly understood etiology,⁸⁴ including cancer.⁸⁵

Conflict of Interest

Hervé Perron, Raphaël Faucard, Alexandra Madeira, Ingrid Burgelin, Guillaume Ollagnier, and Corrine Bernard are employed by Geneuro. All other authors declare no conflict of interest.

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