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NO DEFINITE EVIDENCE FOR HUMAN ENDOGENOUS RETROVIRAL HERV-W AND HERV-H RNAS IN PLASMA OF LATVIAN PATIENTS SUFFERING FROM MULTIPLE SCLEROSIS AND OTHER NEUROLOGICAL DISEASES

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Multiple sclerosis (MS) is a neurological disease of unknown aetiology. Several research groups reported an increased level of human endogenous retroviruses HERV-W and HERV-H RNAs in cerebrospinal fluid, plasma and supernatants of cell cultures from MS individuals. To quantify the abundance of extracellular virion-associated HERV, RNAs in blood, plasma samples from Latvian MS patients, patients with other neurological diseases (OND), as well as blood donors (BD), were retrospectively studied by using both our previously published and newly developed quantitative Real-time reverse transcription PCR assays (QPCRs) targeting different polymerase (pol) gene regions of HERV-W and HERV-H. Unspecific signals due to incomplete removal of DNA were monitored by running the assays with and without reverse transcription (RT±) in parallel. According to our score, a few MS, OND and healthy controls gave borderline signals simultaneously with both newly developed HERV-H and HERV-W QPCRs, but the rest were negative. All borderline positive samples also had small amounts of non-retroviral cellular mRNA with possible origin from cell-free circulating RNA fragments, apoptotic bodies or exosomes, which can mimic the previously described virus-like particles. The results do not confirm the previous reports on prevalence of HERV-H or -W virion-associated RNA in plasma of MS patients.

Key words: *endogenous retrovirus, multiple sclerosis, plasma, real-time PCR, RNA.*

INTRODUCTION

Multiple sclerosis (MS) is a progressive chronic inflammatory demyelinating disease of the central nervous system of still unknown etiology, where genetic and environmental factors (including infectious agents as a component), as well as autoimmunity, may be involved (Compston and Coles, 2008). In Latvia, there are no recent internationally published data on the MS prevalence rate, albeit the last study showed it was the highest among other Baltic States and rather close to the epidemiology within a "Fennoscandian focus" of high MS prevalence (Kurtzke, 2000; Pugliatti *et al*., 2006). It has been suggested that human endogenous retroviruses (HERVs) could trigger the aetiopathogenesis of MS, see e.g. (Blomberg *et al*., 2005). So-called "virus-like particles" containing reverse transcriptase and retroviral RNA have been reported by several research groups in supernatants from cultured monocytes, B, T and leptomeningeal cells, as well as in cerebrospinal fluid and sera or plasma from MS patients (Perron *et al*., 1989; 1991; Haahr *et al*., 1994; Munch *et al*., 1995; Christensen *et al*., 1997; Perron *et al*., 1997; Garson *et al*., 1998; Christensen *et al*., 1999; 2000; Serra *et al*., 2001; Christensen *et al*., 2002; Dolei *et al*., 2002; Nowak *et al*., 2003; Christensen, 2005; de Villiers *et al*., 2006; Arru *et a*l., 2007). However, in some studies they were also detected in blood from non-MS individuals (Garson *et al*., 1998) including patients with

other neurological diseases (OND) (Dolei *et al*., 2002). According to these data the detected retroviral RNAs preferably belong to the HERV-W or HERV-H groups (Perron *et al*., 1997; Komurian-Pradel *et al*., 1999; Christensen *et al*., 2000; Perron *et al*., 2000; Christensen, 2005). Nevertheless, the real nature and functions of the observed particles are still in need of clarification (Froussard, 1995). The goal of this paper was to check the abundance of extracellular HERV-W and HERV-H RNAs in blood circulation of MS patients versus controls. We used both previously published (Forsman *et al*., 2005) and newly developed sets of broadly targeting, highly sensitive, quantitative reverse transcription real-time PCRs (QPCRs).

MATERIALS AND METHODS

Special methodological precautions. There are many methodological "pitfalls" in HERV molecular studies, which in conjunction with highly sensitive techniques (e.g., QPCRs), may easily cause false-positive results. Even a single human DNA molecule is a rich source of amplifiable HERV sequences (Tristem, 2000; Gifford and Tristem, 2003). Thus, to avoid any reagent and sample contamination with exogenous human DNA from laboratory personnel and environment we observed strict anti-contamination routines throughout the entire pre-PCR and PCR steps, which were performed in physically separated rooms within UV-protected laminar and PCR hoods (Lo and Chan, 2006; Tamariz *et al*., 2006). All working surfaces and equipment were frequently decontaminated with UV irradiation (Ou *et al*., 1991) and DNA AWAY™/RNase AWAY® solutions (Molecular BioProducts Inc., USA) or sodium hypochlorite (bleach).

Clinical samples. Thirty-seven patients with MS (25 females, 12 males), 20 patients (8 females, 12 males) with acute or chronic inflammatory demyelinating polyneuropathies (IDP), 6 patients (3 females, 3 males) with other non-inflammatory neurological disorders of the CNS (ONIND), 17 patients (9 females, 8 males) with non-inflammatory peripheral neuropathies (majority of them spondylogenic radiculopathies) (NIPN) and 25 randomly selected blood donors (BD) (13 females, 12 males) were included in this investigation (Table 1). The 43 IDP, ONIND and NIPN patients were collectively called OND (other neurological diseases). The mean age of the patients with MS was 37 years (16–59 years), of the patients with IDP — 34 years (15–61), of the patients with ONIND — 29 years (15–68), of the patients with NIPN — 40 years (15–54) and of the blood donors — 37 years (18–60). The cohorts were established with the approval of the Ethics Committee of Rīga Stradiņš University and all participants gave informed consent before the examination. A clinical diagnosis of MS was established according to the revised criteria of McDonald (Polman *et al*., 2005). The patients were considered to have acute inflammatory lesion (positive Gd-enhancing lesions) when areas of hyperintensity, compared with surrounding brain parenchyma, were recorded on a T1-weighted MRI scan after i.v. injection of gadolinium-DTPA (0.1 mmol/kg body weight). Active disease was defined as a clinical exacerbation and the presence of active inflammatory lesions on MRI (positive Gd- enhancing lesions); quiescent (inactive) disease phase was defined as a clinical remission in the relapsing/remitting MS and as relative stability in the secondary progressive MS. All patients of the IDP group met the criteria of Ashbury and Cornblath for the Guillain-Barré syndrome and for chronic IDP (Asbury and Cornblath, 1990). The type of nerve damage was estimated by nerve conduction studies.

Table 1

PERSONS INCLUDED IN THE EXAMINATION

Patients with multiple sclerosis:	MS	37
1) Relapsing/Remitting	MSRR	29
a) in relapse		26
b)in remission		3
2) Secondary Progressive	MSSP	4
a) in relapse		2
b) in relative stability		2
3) Primary Progressive	MSPP	4
Other non-demyelinating neurological disorders of CNS	ONIND	6
Inflammatory demyelinating polyneuropathy:	IDP	20
1) Acute inflammatory demyelinating polyneuropathy		14
2) Chronic inflammatory demyelinating polyneuropathy		6
Non-inflammatory peripheral neuropathy	NIPN	17
Blood donors	BD	25

CNS, central nervous system

In order to obtain virtually cell-free plasma all EDTA-anticoagulated blood samples were centrifugated at 1600 × g for 10 min at 4 °C and supernatants were collected. This was followed by a second centrifugation at 16 000 × g for 10 min at 4 °C to completely remove any cellular debris. The clarified plasma samples were further treated with 0.4 μg/μl recombinant ribonuclease A (RNase A)(Ambion, UK) for 10 min at 37 °C to avoid any possible contamination by cellular RNA-containing endogenous retroviral sequences (Dolei *et al*., 2002).

RNA purification. The expected viral RNAs were extracted from 250 μl of plasma samples by using the Trizol®LS Reagent (Invitrogen Life Technologies, USA), as described by the manufacturer, with some modifications. Briefly, to remove high molecular weight DNA, after addition of a sample to Trizol, an additional centrifugation at 12 000 × g for 10 minutes at 4 °C was performed. The cleared solution was transferred to a clean tube and 100 μl of 1-bromo-3-chloropropane (BCP) (Sigma-Aldrich, USA), instead of chloroform, was added (Chomczynski and Mackey, 1995). Prior to precipitation of the RNA with isopropanol, 15 μg of GlycoBlue (Ambion, UK) as a carrier

was added to the aqueous phase. Finally, the RNA pellet was dissolved in 20 µl of RNase-free water. For all preparations 100 000 copies of an exogenous internal control (EIC) pAW109 RNA (Applied Biosystems, USA) were doped to the Trizol® LS reagent to monitor the efficiency of RNA extraction, possible RNA degradation during DNase treatment and presence of RT-PCR inhibitors. To eliminate possible DNA contamination, all RNA preparations (20 µl) were rigorously treated with hyperactive, recombinant DNase according to the manufacturer's recommendations for the TURBO DNA-free™ kit (Ambion, UK).

RT-PCR assays. 10 µl of each plasma RNA sample was reverse transcribed (RT+) in a volume of 25 µl containing 50 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol (Promega, USA), 75 mM KCl, 3 mM $MgCl_2$, 800 µM of each dNTP (Amersham Pharmacia Biotech, Sweden), 265 ng of random hexamer oligonucleotides (Amersham Pharmacia Biotech, Sweden), 40 U of recombinant RNase Inhibitor (Promega, USA) and 25U of StrataScript reverse transcriptase (Stratagene, USA) at 25 oC for 10 min, 42 ^{o}C for 60 min and 95 ^{o}C for 5 min followed by chilling on ice. Controls without reverse transcription (RT-) to check for cellular DNA contamination were included for every sample. For all subsequent QPCR reactions 2 µl of the cDNA sample was used per 25 µl PCR reaction. Thus, to estimate the number of HERV RNA copies per ml of plasma (viral loads), the number of copies per PCR reaction had to be multiplied with 100.

Prior to QPCRs 2 µl of each RT+ preparation was amplified with a primer pair DM151/152 (Applied Biosystems, USA) spanning an IL-1α region of synthetic EIC, as described by the manufacturer. Additionally, all samples were analysed by a "PAN-retrovirus" seminested PCR system with human DNA as a positive amplification control (Perron *et al*., 1997; Tuke *et al*., 1997). The resulting RT-PCR products were respectively analysed by 1.5% or 2.5% agarose gel electrophoresis with ethidium bromide staining.

The human tissue RNA panel from ClonTech (Human total RNA master panel II, catalogue number K4008-1) was also used for evaluation our new QPCRs. As described by the manufacturer, each tissue RNA sample was pooled from several persons, and thus represents an average. For each RNA preparation, 2 µg RNA was used to produce 50 µl of cDNA, as described above. In each subsequent QPCR reaction, 2 µl of cDNA was used.

QPCRs. Two reverse transcriptase (RT) motif-based HERV-H and -W group-specific QPCRs used in this study were described earlier (Forsman *et al*., 2005) and two novel confirmatory assays, targeting conserved portions of the carboxyl- (HERV-W) and aminoterminal (HERV-H) portions of the evolutionary conserved polymerase *(pol)* gene, were developed and characterised according to the MIQE guidelines (Bustin *et al*., 2009). Briefly, four consensus primers and two TaqMan® (hydrolysis) dual-labeled probes (Table 2) theoretically detecting the expression of a majority of HERV-H and -W loci were designed from the multiple alignments of HERV-W (Fig. 1) and HERV-H (Fig. 2) *pol* genes generated by the ClustalW programme (Thompson *et al*., 1994). The included proviral sequences were obtained from the GenBank database using the BLAST network server (Altschul *et al*., 1997), as also derived from the RetroTector© bioinformatics programme (Sperber *et al*., 2007; 2009) and the literature (Perron *et al*., 1997; Tristem, 2000; Kim, 2001; Jern *et al*., 2004; 2005).

The specificity of the selected oligonucleotides was verified by BLAST searching, cloning of PCR products amplified from genomic DNA and sequencing of a number of randomly selected clones. The cloning frequencies of the amplimers relative to target abundance in the human genome did not indicate predominant amplification of some HERV loci against others (D. Uzameckis, unpublished results). The quantification was made by interpolation in standard curves of dilutions of synthetic, entire *pol* gene-containing HERV-H (GenBank accession no. AF026252, nucleotide positions 41-245) (Lindeskog *et al*., 1998) and HERV-W (GenBank accession no. AF009668, nucleotide positions 1680-2150) (Perron *et al*., 1997) plasmids (Integrated DNA Technologues Inc., USA), as well as from a previously constructed histone 3.3C plasmid control (Andersson *et al*., 2005). All QPCR reactions were performed in duplicates in a Corbett Rotorgene (Corbett Research, Australia) real-time thermocycler in a total volume of 25 µl, containing 1 × TaqMan PCR Universal Master Mix, No UNG with 0.05U of HK™-UNG Thermolabile Uracil N-Glycosylase (EPICENTRE, USA) (Longo *et al*., 1990; Pruvost *et al*., 2005) and 2 µl of each cDNA preparation. Reagent or non-template controls were performed be-

Table 2

DESIGNED QPCRS OLIGONUCLEOTIDES

HERV group	pol motif	Sequence$^{\Psi}$ (5'→3')	Degeneracy	Optimal concentrations (nM)
HERV-H	integrase	Fw: gaYaagIcttacaRRttagttca	8	300
		Rev: ttKggcaccaYRgggt	8	300
		Probe: tctRYgYctt*atcaaccaaattgtttt	8	200
HERV-W	integrase	Fw: taYctagtcctccatgcc	2	300
		Rev: ctaatRRcttcctgatgKttgata	8	300
		Probe: aatatggagagaaagggaat*Kcctaacttc	2	100

QPCRs, Real-time reverse transcription polymerase chain reaction assays; HERV, human endogenous retroviruses; $^{\Psi}$IUPAC ambiguity codes: Y = C/T, R = A/G, K = G/T, I = Inosin; *position of internal dark quencher

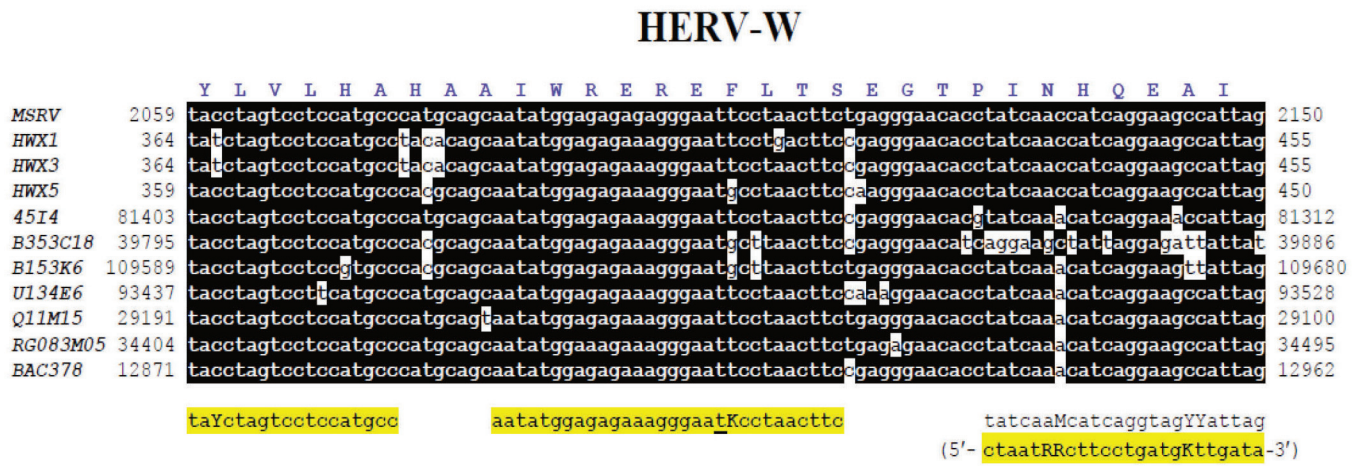

Fig. 1. Alignment of a conserved portion of the integrase domain at the carboxy terminus of the polymerase genes of selected representatives of the HERV-W group. Sequences were obtained from the literature (Kim, 2001; Perron *et al*., 1997) and our own BLAST searches in Genbank. Genbank accession numbers: MSRV (AF009668) (translation was derived from it), HWX1 (AB021919), HWX3 (AB021920), HWX5 (AB021921), 45I4 (AL023581), B353C18 (AC004066), B153K6 (AC005187), U134E6 (Z83850), Q11M15 (AF045450), RG083M05 (AC000064), BAC378 (U85196). HERV, human endogenous retroviruses.

fore and after each clinical sample, as well as a corresponding negative RT- control for each sample was included.

Optimal concentrations of the selected primers and TaqMan® probes were determined according to the TaqMan® PCR Universal Master Mix guidelines (Applied Biosystems, USA) (data not shown). For subsequent QPCR reactions final concentrations of 300 nM of each primer and 100 nM (HERV-W) or 200 nM (HERV-H) of the probe were used (Table 2).

For HERV-W QPCR, the lowest threshold cycle (Ct) and highest reduced normalised fluorescence (ΔR_n) were observed at 50 °C (data not shown). An optimal annealing temperature of 50 °C was therefore chosen to broadly amplify many HERV-W loci. Final QPCR parameters were 50 °C for 3 min, 95 °C for 10 min, followed by 60 cycles of 95 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s. During the optimisation, gradient PCR reactions were performed at different annealing temperatures from 50 °C to 60 °C with an increment of 1 °C. The same amount of human genomic DNA (30 ng per PCR reaction) was used as a template during all optimisation experiments.

For HERV-H QPCR, no significant difference in the threshold cycles (Cts) or ΔRn was observed at different annealing temperatures (data not shown). Final QPCR parameters were 50 °C for 3 min, 95 °C for 10 min, 60 cycles of 95 °C for 15 s and 60 °C for 1 min. The PCR optimisation procedure was otherwise the same as described for HERV-W. The linearity and sensitivity of the developed assays were ascertained by generation of a standard curve with serial plasmid DNA dilutions (10^0–10^7 copies/reaction with 10-fold increment). Although an exact quantification through these real-time PCRs cannot be expected due to measurement of DNA rather than RNA and frequent mutations in primer and probe target sequences of members of these two HERV groups, we subsequently refer to them as quantitative PCRs (QPCRs). Sensitivity of both new assays was 1–10 nucleic acid equivalents of plasmid DNA standards or down to 100 fg of human genomic DNA per PCR reaction (data not shown), which corresponds to a detection limit for the whole system of approximately 100–1000 viral RNA copies per mL of plasma, conservatively counting one HERV DNA copy equal to one HERV RNA copy. For simplicity we use the word "RNA equivalents" for clinical samples even if the available standards were DNAs and the QPCR reactions were preceded by a reverse transcription step (two-step RT-PCR). Lacking a more precise quantification, these correspond to "cDNA copies".

The efficiencies of the developed HERV-H and HERV-W QPCRs were 98% (slope –3.37, $r^2 > 0.99$) and 95% (slope –3.45, $r^2 > 0.99$), respectively.

The obtained results were scored as:

(1). negative: if a RT+ signal lower than 10 nucleic acid equivalents per PCR reaction was observed, or signals of similar strength occurred in the RT+ and the RT- reactions;

(2). borderline: if the RT+ signal was from 10 till 99 nucleic acid equivalents per PCR reaction and the RT- signal was negative or less than 1/10 of the RT+ signal;

(3). clearly positive: if the RT+ signal was 100 nucleic acid equivalents per PCR reaction or more, and the RT- signal was negative or less than 1/10 of the RT+ signal.

In spite of a much lower RT- signal, there is a considerable uncertainty in the borderline zone, both due to the stochastic nature of the signal in this range and the somewhat uncertain contribution of cellular DNA or cDNA to the signal. As a confirmatory test, all QPCR products were electrophoretically resolved in 2% preparative agarose gels and stained with ethidium bromide. The PCR results were obtained in Uppsala before the identity of the samples was revealed from Latvia.

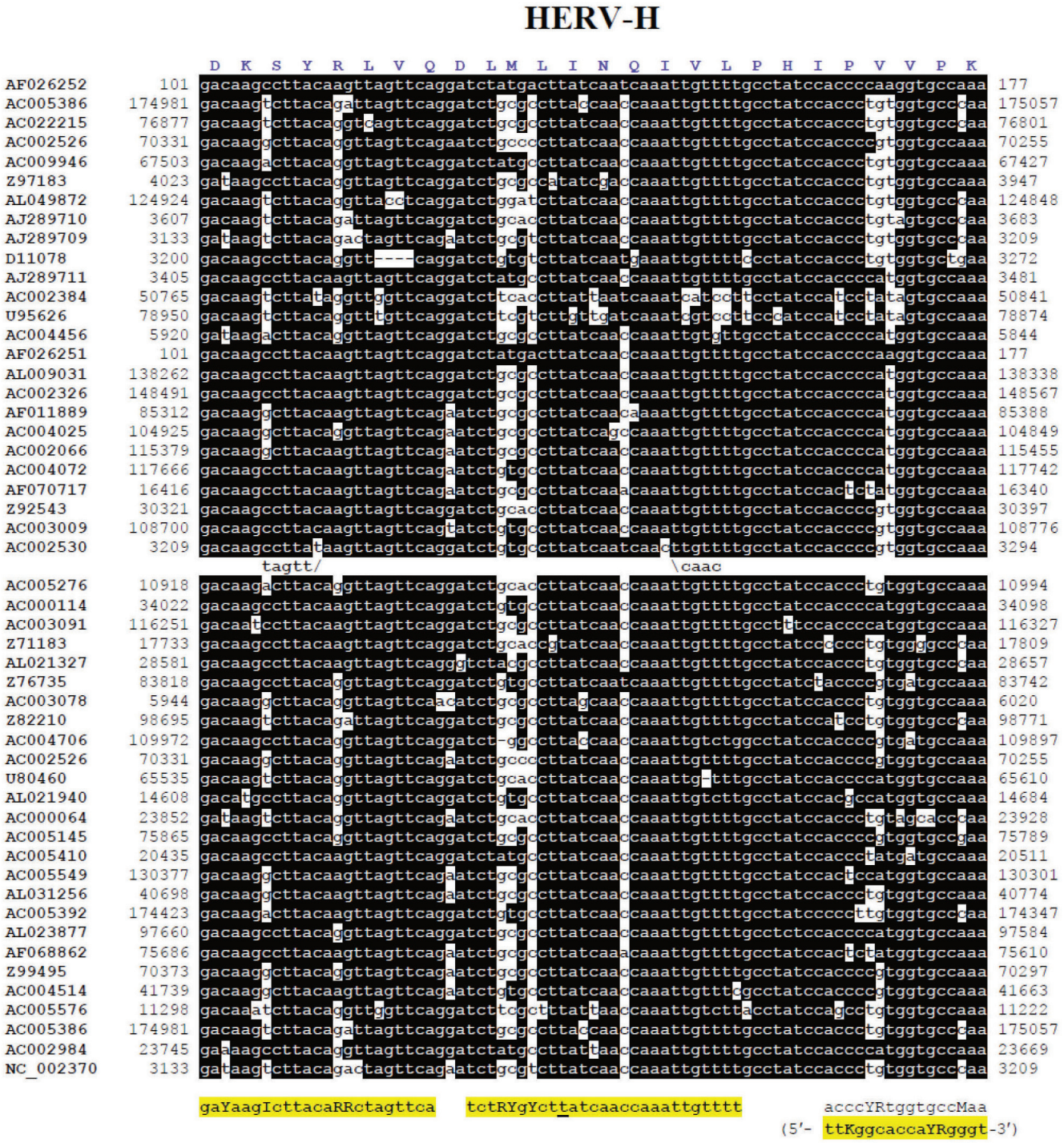

Fig. 2. Alignment of amino termini of HERV- H sequences. GenBank entries were partially obtained from Tristem (2000) and our own BLAST searches in GenBank. HERV, human endogenous retroviruses.

The QPCRs were also used to study HERV-H and HERV-W expression in twenty different human tissues. No RNA reference was used. In other studies, we measured histone 3.3 C (Andersson *et al*., 2005), as also UBC, HPRT, and GAPDH (Vandesompele *et al*., 2002) housekeeping genes mRNA expression levels in the same tissue RNA panel samples. The most evenly expressed gene per µg of RNA was histone 3.3 C (data not shown). The relative expression pattern became essentially the same with and without normalisation against histone 3.3 C. In this paper, we therefore chose not to perform the normalisation.

Statistical analysis. The Freeman-Halton extension of two-tailed Fisher's exact test was used to analyse differences in categorical variables (Freeman and Halton, 1951; Soper, 2015), as well as the nonparametric Kruskal-Wallis analysis was implicated to compare the viral loads between MS and control groups. Statistical differences were considered significant when $p < 0.05$.

RESULTS

The PCRs. Clearly visible 308-bp internal control amplicons with similar band intensities were obtained from all RT+ samples (data not shown) excluding any troubleshooting in RNA processing. All clinical samples included in the study were negative with the PAN primers; however, the human DNA controls included in the assay were highly positive (data not shown).

The QPCRs. Despite the extreme sensitivity of our HERV QPCR assays down to few nucleic acid targets per PCR reaction, almost all outcomes of these PCRs for plasma samples were either negative or borderline. Eight of 37 MS (~21.6%) patients, one of 43 OND patients (~2.3%) and 3 of 25 blood donors (12.0%) were borderline reactive simultaneously with both our new HERV QPCRs and yielded negative or significantly lower signals in RT- than in RT+ reactions, which were roughly equivalent to 11–28 HERV-H copies per PCR reaction (1100–2800 viral copies per mL of plasma) and 39–74 HERV-W copies per PCR reaction (3900–7400 viral copies per mL of plasma), respectively (Table 3). The borderline positive sample in the OND patients group with NIPN was from a patient with spondylogenic radiculopathy, who underwent spinal surgery due to spinal stenosis one year before the investigation. All other plasma samples were negative according to our scores. In summary, 12 of 80 patient samples (15.0%) were borderline reactive and 8 of them (10.0%) were in the MS group. However, histone 3.3C QPCR (Andersson et al., 2005) was reactive in all of the HERV positive plasma samples, showing the presence of certain cellular RNA transcripts. All borderline reactive QPCR samples also showed weak bands of sizes appropriate for the studied HERVs on agarose gels. Moreover, all clearly negative samples were absolutely negative in gel electrophoresis analysis (data not shown). The uncertain nature of the borderline reactivity, as also the presence of histone 3.3 C positivity, make it hard to draw definite conclusions from the results regarding the presence of retroviral particles.

In contrast with plasma samples, the tissue specific expression of HERV-H and HERV-W was characterised by strong QPCR signals and varied much. In concordance with our previous studies, HERV-H showed the highest expression in whole brain and testis, while the HERV-W group was most expressed in whole brain, uterus, trachea, adrenal gland, thyroid gland, prostate, spleen and placenta (Fig. 3) (Forsman *et a*l., 2005).

For all PCR assays, no contamination was observed in any of the reagent control samples.

DISCUSSION

Our "story" with the controversial findings is suspiciously similar to the reports of HRV-5, SV40 and XMRV "epopees" when these viral sequences were false-positively detected in clinical samples and misassociated with certain human diseases (Griffiths *et al*., 2002; Forsman *et al*., 2003; Shah, 2007; Elfaitouri *et al*., 2011; Arias and Fan, 2014). In all these cases laboratory contamination was proposed as a source of doubtful weakly positive results. However, the situation with HERVs is even more "dramatic", because the human genome contains over 3000 of amplifiable HERVs copies (Lander *et al*., 2001). Recent ancient DNA studies have shown the huge difficulties in creating a laboratory virtually free of human DNA (Cooper and Poinar, 2000; Knapp *et al*., 2012). Our very stringent precautions against human and HERV DNA contamination may be the explanation of the true-negative or borderline PCR results. Additionally, frequent presence of animal and human DNA contamination of different molecular biology reagents (including those for PCRs) and laboratory consumables from different vendors has been reported (Cooper and Poinar, 2000; Leonard, 2007; Gefrides *et al*., 2010). As an example, the native reverse transcriptases (e.g., ALV, MuLV), which were used in some previously published studies, in contrast to recombinant enzymes (in our case), can contain the retroviral sequences and cause false-positive RT-PCR results with broadly amplifying primers (Perron *et*

Table 3

QPCRS RESULTS FOR PLASMA SAMPLES

	MS patients (n = 37)					OND patients (n = 43)			BD (n = 25)
	RR (n = 29)		SP (n = 4)		PP (n = 4)	IDP (n = 20)	NIPN (n = 17)	ONIND (n = 6)	
	relapse (n = 26)	remission (n = 3)	relapse (n = 2)	stability (n = 2)					
HERVs:	4/26*	2/3*	1/2*	0/2	1/4*	0/20	1/17*	0/6	3/25*
Total:	8/37* (~21.6%)*					1/43* (~2.3%)*			3/25* (12.0%)*
HERV-H: (copies/mL)	2019 ± 409					1100 ± 100			1917 ± 417
HERV-W: (copies/mL)	5788 ± 850					3900 ± 100			5733 ± 867

QPCRs, Real-time reverse transcription PCR assays; HERV, human endogenous retroviruses; see abbreviations also in Table 1; *Histone 3.3C positivity

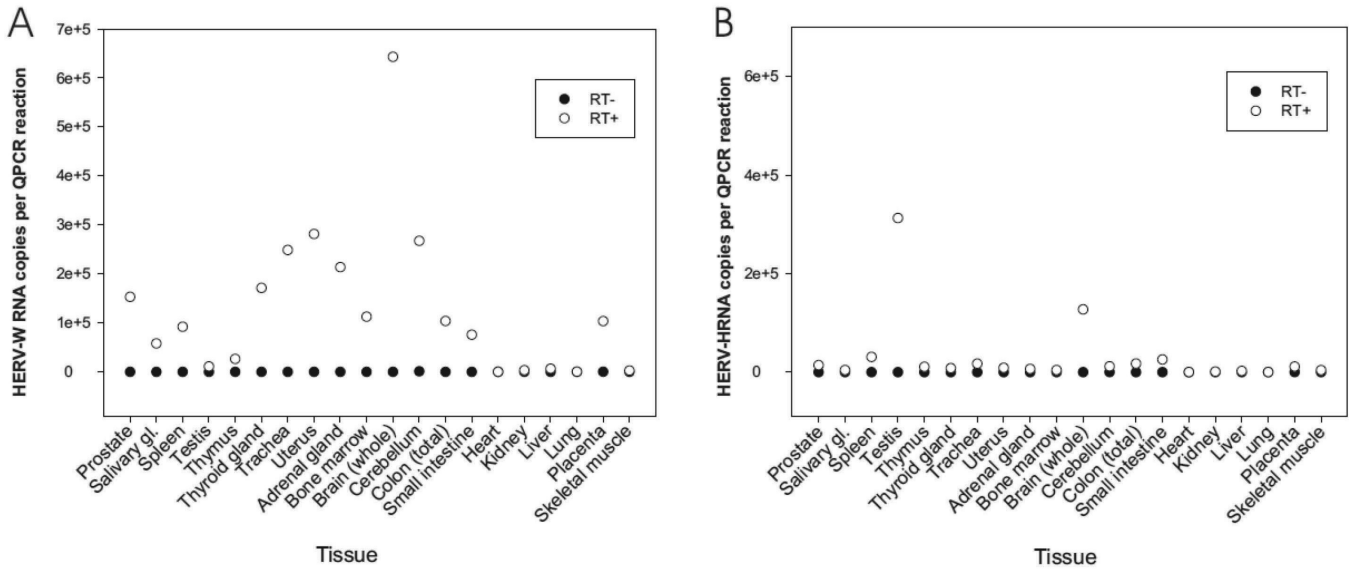

Fig. 3. Expression of HERV-H and HERV-W in twenty human tissues studied with new HERV-W (A) and HERV-H (B) QPCRs. HERV, human endogenous retroviruses. QPCRs, Real-time reverse transcription PCR assays.

al., 1997; Tuke *et al*., 1997). The weakness or negativity of the observed QPCR signals can be also due to the usage of a novel, hyperreactive variant of DNase I (TURBOTM DNase), thus, according to the manufacturer's data, the removal of possible DNA contamination prior to cDNA synthesis was up to 50-times more effective than in the previous studies where conventional, wild-type enzyme was used.

It is also known that, in spite of high activity of RNases in human body fluids, free circulating extracellular RNAs somehow are protected from degradation and may occur in cell-free plasma of both healthy and diseased humans (Kamm and Smith, 1972; Wieczorek *et al*., 1985; 1987). Thus, the borderline HERV QPCR signals detected in plasma even after extensive RNase treatment may contain a contribution from this RNA type, of which HERV transcripts may be a normal, but not the major or obligatory component. We also propose that the apoptotic bodies from dying, aged or damaged cells (Hasselmann *et al*., 2001; El-Hefnawy *et al.*, 2004), as well as other extracellular vesicles (e.g., exosomes and microvesicles), which are also known to occur in plasma and serum (El-Hefnawy *et al*., 2004), may mimic retroviral particles in which HERV RNA could be protected from ribonucleases. Thus, artefactual PCR detection of HERV RNA in "virions" may explain the previous reports on HERV RNA in "retroviral particles". Unfortunately, in spite of the strong evidence about highly elevated levels of circulating DNA and main microRNAs (miRNAs) in MS plasma (Liggett *et al*., 2010; Siegel *et al*., 2012), there are no adequate data about the cell-free circulating RNA (cfcRNA) fraction (Unger *et al*., 1985) and its HERV component(s) in MS at the moment. Nevertheless, the release of microvesicles enriched in HERV nucleic acids and RT was found in the plasma of some cancer patients, as also media supernatants from certain cultured tumour cells (Bronson *et al*., 1979; Seifarth *et al*., 1995; Contreras-Galindo *et al.*, 2008; Balaj *et al.*, 2011). One more explanation of our negative or borderline QPCR results also could be due to occasional absence of detection because of mismatch(es) between the cDNA targets and primers or hydrolysis (TaqMan®) probes due to frequent mutations in HERV-H and HERV-W pol genes (Jern *et al*., 2004). However, the agarose gel electrophoresis analysis of all QPCR products, as well as highly positive, reproducible results with human tissue RNAs do not confirm this assumption (Fig. 3). The EIC also controlled for inadequate RNA extraction, degradation of isolated RNA during treatment with DNase, presence of reverse transcription and/or PCR inhibitors, as well as negligence or serious technical errors. Our findings do not exclude the presence of low amounts of retroviral RNA-containing particles (e.g., exosomes) in systemic circulation of MS patients, but advocate that this disease does not involve a systemic intense virus-like particle- or non-particle-associated HERV RNAemia. In this case, the concentration of virus-like particles on columns or gradients from a much more large volume of plasma would be very useful methodological approach. Additionally, some noteworthy data do not confirm the transcriptional activity of HERV-W in MS (Antony *et al*., 2006; Laufer *et al*., 2009; Schmitt et al., 2013) and even existence of so intensively studied Multiple Sclerosis Associated Retrovirus (MSRV) *in vivo* (Flockerzi *et al*., 2007; Laufer *et al.*, 2009; Schmitt *et al*., 2013). Indeed, an absence of HERV-W or/and HERV-H RNA detection for MS plasma and CSF was reported (Antony *et al*., 2007; Alvarez-Lafuente *et al*., 2008). Moreover, in one study (Alvarez-Lafuente *et al*., 2008) our previously published (also used in the present work) QPCR assays were used (Forsman *et al.*, 2005). It is hard to exclude that the negativity of the same PCR methods in our study could be due to the absence of detection of certain HERV loci. In literature, there is no convincing evidence for HERV capability to cause "virionemia" and the term "retrovirus-like particles" is overused. However, our study does not allow us to exclude that a low

number of microvesicles from human biological fluids may indeed be of retroviral origin (Muralidharan-Chari *et al.*, 2009).

In agreement with previous data our study also shows some upregulation of HERV-H and -W RNAs in MS plasma (21.6%) versus controls (5.9%) ($p = 0.02$, Fisher Exact test), but the control group was too heterogeneous, which complicates the interpretation. The "retroviral loads" of the samples which yielded a positive result were 2019 ± 409 vs 1713 ± 513 copies/mL (MS vs controls) for HERV-H and 5788 ± 850 vs 5275 ± 1075 copies/mL (MS vs controls) for HERV-W. They were not significantly different with $p < 0.05$, using the Kruskal-Wallis test. The HERV signals were likely of cellular origin. It is natural that cellular debris, apoptotic blebs or exosomes occur in an autoimmune disease, which inherently involves an increased frequency of cell death. No difference in the frequency of HERV-W and HERV-H RNA between different forms and stages of MS was observed (data not shown). In spite of the simultaneous detection of both HERVs in all positive clinical samples, the similar QPCRs efficiencies and detection limits, as well as the higher HERV-H versus HERV-W proviral loci number (660 and 115 copies per haploid genome, respectively) (Tristem, 2000), their "viral loads" were different (HERV-W > HERV-H, 5617 ± 926 vs 1917 ± 436 copies/mL). Possible explanations of this phenomenon may be lower transcriptional activity of HERV-H loci, a broader range for the new HERV-W QPCRs, or preferable incorporation of HERV-W transcripts into viral particles or extracellular vesicles. We speculate that the previously reported "retroviral particles" were exosomes, due to their similar size (100 nm) and buoyant density in a sucrose gradient (Thery *et al*., 2006), albeit some data posit the presence of these particles and reverse transcriptase activity in different fractions (Froussard, 1995). Thus, to clarify the origin of HERV RNA transcripts in diseased and healthy human plasma, cfcRNA, miRNAs, retrovirus-like particles (probably exosomes) and microvesicles should be individually isolated and carefully analysed for their retroviral components. Theoretically, we also cannot exclude the existence of real HERV virions co-packaged with cell RNAs (Greijer *et al*., 2000; Sciortino *et al*., 2001) and even the formation of complex chimeric virions containing a "cocktail" of multiple retroviral RNAs (e.g. HERV-H and -W together) with cellular transcripts *in vivo*. However, in our opinion, the low "viral loads" and simultaneous detection of both HERVs and nonretroviral RNAs in the MS samples makes it rather hard to attribute the QPCRs findings to the production of virions. We may speculate that the previous findings were experimental artifacts or drafts due to laboratory flaws. Even in our hands, while we rigorously removed DNA from RNA preparations and very carefully verified the absence of DNA contamination in all QPCR experiments, it is nevertheless very difficult to remove all DNA traces from RNA preparations. One therefore cannot formally exclude the presence of some residual DNA in the RNA used for cDNA generation. Moreover, the effect of interindividual variations (Nellaker *et al*., 2009), ethnical (Arru *et a*l., 2007), age- and therapy-specific factors (Diem *et al*., 2012; Liu *et al*., 2013; Arru *et al*., 2014; Balestrieri *et al*., 2015), as also MS clinical forms and disease severity (Garcia-Montojo *et al*., 2013; 2014) on the expression and release of HERV RNAs into the bloodstream cannot be excluded. Thus, one of the falsification criteria for one of the models for retrovirally caused disease in MS may be fulfilled (Blomberg *et al*., 2005). The optimised methods can be useful for understanding of the pathobiology of HERV-H and HERV-W. The study also illustrates the methodological difficulties of getting and interpreting reliable HERV PCR results, especially in patients with cytopathic diseases. In our opinion, to further elucidate the possible physiopathological functions of HERVs, there is a need for several carefully designed, preferably prospective, studies methodologically harmonised between all investigators.

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NAV APSTIPRINOŠU PIERĀDĪJUMU PAR ENDOGĒNO RETROVĪRUSU HERV-W UN HERV-H RNS SASTOPAMĪBU LATVIJAS PACIENTU AR MULTIPLO SKLEROZI UN CITĀM NEIROLOĢISKĀM SLIMĪBĀM ASINS PLAZMĀ

Multiplā skleroze (MS) ir neiroloģiska slimība ar nezināmu etioloģiju. Dažas pētnieku grupas ir ziņojušas par divu cilvēka endogēno retrovīrusu HERV-W un HERV-H paaugstinātu RNS līmeni cerebrospinālajā šķidrumā, asins plazmā un šūnu kultūru supernatantā, kas iegūti no MS pacientiem. Abu HERV ekstracelulāro vīrusspecifisko RNS kvantitatīvai noteikšanai Latvijas MS pacientu un pacientu ar citām neiroloģiskām slimībām asins plazmas paraugos tika lietotas gan mūsu iepriekš publicētas, gan arī jaunizstrādātas kvantitatīvas reālā laika apgrieztās transkripcijas PCR (*polymerase chain reaction*, polimerāzes ķēdes reakcija) metodes (QPCRs) ar praimeriem un zondēm, kas ir komplementāras dažādiem HERV-W un HERV-H polimerāzes (*pol*) gēna rajoniem. Lai izslēgtu nespecifisku HERV signālu esamību, kas rodas sakarā ar nepilnīgu šūnu DNS degradēšanu RNS paraugos, paralēli tika veiktas QPCRs ar un bez apgrieztās transkripcijas posma (RT±). Atbilstoši mūsu kritērijiem, daži MS un kontroles grupu paraugi bija robežpozitīvi vienlaikus abās jaunizstrādātajās HERV-H un HERV-W QPCRs, bet pārējie bija negatīvi. Visi šie robežpozitīvie paraugi saturēja nelielu šūnu iRNS daudzumu ar iespējamu izcelsmi no asinīs cirkulējošas šūnām brīvas RNS, apoptotiskiem ķermenīšiem vai ekzosomām, kas var mimikrēt iepriekš aprakstītās vīrusiem līdzīgās daļiņas. Iegūtie rezultāti neapstiprina iepriekš publicētos datus par HERV-H un HERV-W vīrusasociētās RNS sastopamību MS pacientu asins plazmā.