Soluble hexameric form of pathogenic HERV-W envelope in MS active brain lesions and sera

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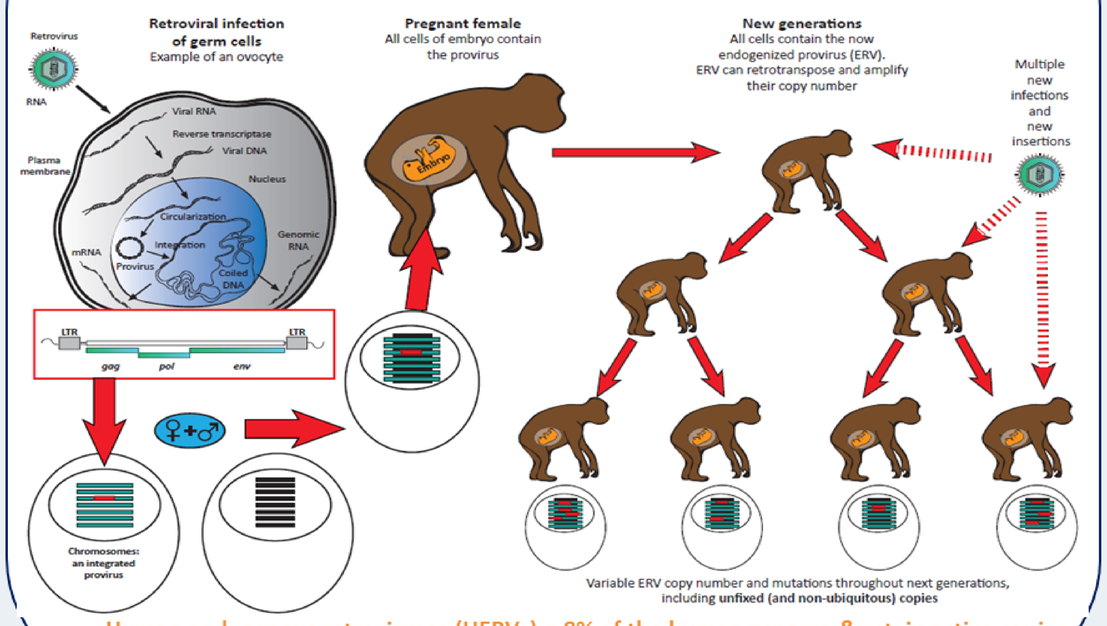
Endogenized retroviruses in genomes of species



Human endogenous retroviruses (HERVs) = 8% of the human genome & retain active copies

Küry et al., 2018 *(Trends in Molecular Medicine, Cell Press Reviews)*

RATIONALE:

Human Endogenous RetroVirus sequences (HERVs) are usually non-coding genetic elements but **still functional copies may be activated by environmental triggers** to express ancestral retroviral proteins. **HERV-W was first identified from virion-like particles produced by leptomeningeal or B-lymphocyte cultures** (*Perron et al., 1997*). Its envelope glycoprotein **pHERV-W-Env (previously MSRV-Env), *in vivo*, promoted immune-mediated inflammation and autoimmunity as observed in EAE** *(Perron et al., 2013)* **and, *in vitro*, impaired (re)myelination by oligodendrocyte precursor cells** (*Kremer et al., 2013 ; 2014*). Immunohistochemistry studies on >75 multiple sclerosis (MS) cases from 7 different centers and countries, independently **confirmed the presence of pHERV-W-Env within demyelinating areas of MS lesions** where it was mainly expressed by microglia but also observed in lymphoid cells in the vicinity of active lesions (van Horssen et al., 2016); Cf. global review in Küry P. et al., 2018, Trends in Molecular Medicine.

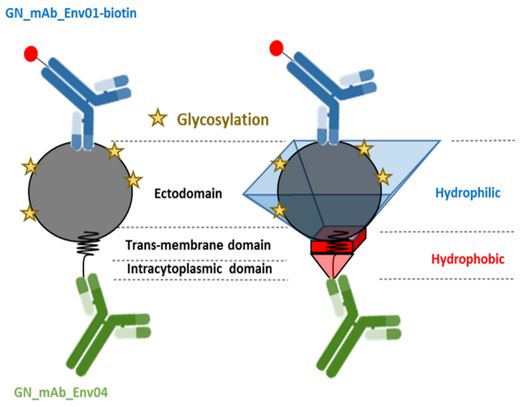
Having identified a conserved site of trimerization on its sequence, we have analyzed the macromolecular organization of its envelope protein in active MS lesions and further sought for a characterized soluble hexameric form in sera from MS patients.

METHODS:

- **Transfected cells samples with reference plasmids :** HEK-293T cells were transfected with expression plasmids, using

pCMV-GFP (control protein), pCMV-HERV-W-Env (MS isolate; GenBank no. AF331500.1) or pCMV-SYN1 (placenta syncytin-1, a "physiologically adopted" copy; GenBank no. AF072506.2).

- **Brain samples:** Snap frozen necropsy brain samples were obtained from the Dept. of Pathology, VU University Medical Center, Amsterdam, NL); **5 from active MS lesions, 4 from MS normal appearing white matter (NAWM) and 5 from non-MS controls**.
- **Sera from MS and controls:** Sera were obtained from the Nehme and Therese Tohme Multiple Sclerosis Center, American University of Beirut, Lebanon. 5 from Primary Progressive MS, 43 from Relapsing-Remitting MS, 5 from Secondary Progressive MS patients, 3 from CIS and 20 from non-MS patients.
- **Protein extraction:** Protein extraction was performed in Fos-Choline 16 (1%) - RIPA buffer during 2h at 25°C.
Protein extracts were centrifuged and protein detection was performed on the soluble fraction.
- **Automated capillary western blot – WES (Protein Simple)**: WES analyses were performed on 2mg/mL of total protein Extract using GN_mAb_Env01-biotin or GN_mAb_Env04 primary antibodies (GeNeuro).
Results were analyzed using Compass software.



Biochemical forms of pHERV-W-Env antigen *(transfected cells with reference plasmids)*

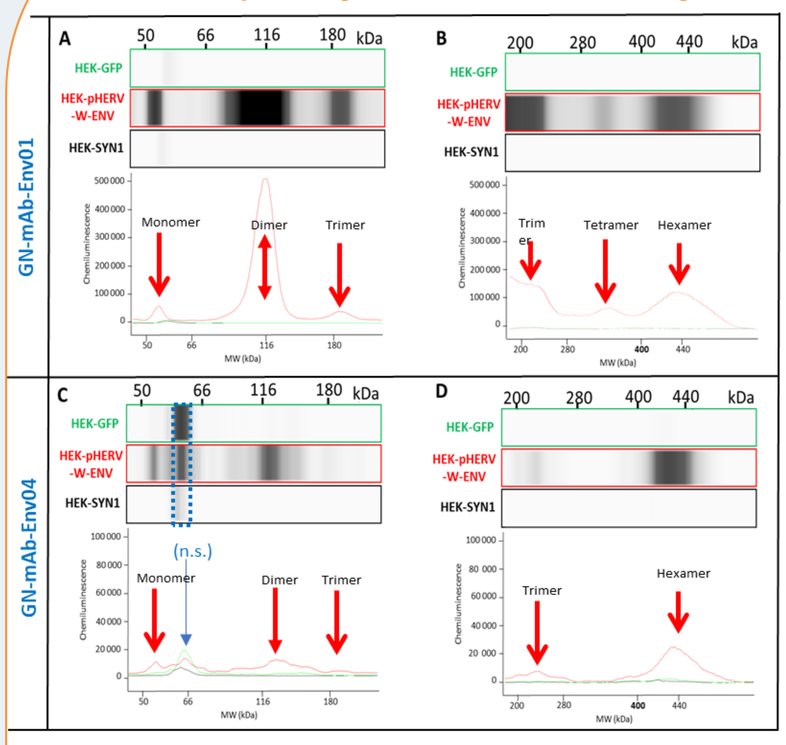


Figure 1: pHERV-W-Env forms oligomers.

The total soluble protein fraction obtained from HEK transfected cells was analyzed with simple western (WES) technology based on protein molecular weight (MW) separation. 12-230kDa (**A, C**) or 66-440kDa (**B, D**, focusing on 200-440kDa region) separation matrices were used.

A comparison was performed between GFP (used as negative control, **green label**), pHERV-W-Env (**red label**) or SYN1 (physiologic HERV-W element, **blue label**).

Two antibodies were used, GN-mAb-Env01-biotin (**A, B**) directed against pHERV-W-Env ectodomain and GN_mAb_Env04 (**C, D**) directed against the intra-cytoplasmic (or -virion) domain.

n.s. : Non specific (artefactual) band found in all conditions.

Result:

- No specific signal was obtained in cell extracts transfected with plasmids expressing GFP or SYN1
- GN_mAb_Env01 and GN_mAb_Env04 specifically detected pHERV-W-Env monomeric and oligomeric forms:
  - **Monomer: 55-60kDa**
  - Dimer: 110-120kDa
  - **Trimer: 180-220kDa**
  - Tetramer: 300-320kDa
  - **Hexamer: 400-440kDa**

NB. Dimeric and tetrameric forms are known to be generated by denaturing conditions (data not shown).

Soluble protein fraction from brain extracts: 360-400 kDa pHERV-W-Env oligomer is detected in all MS active lesions

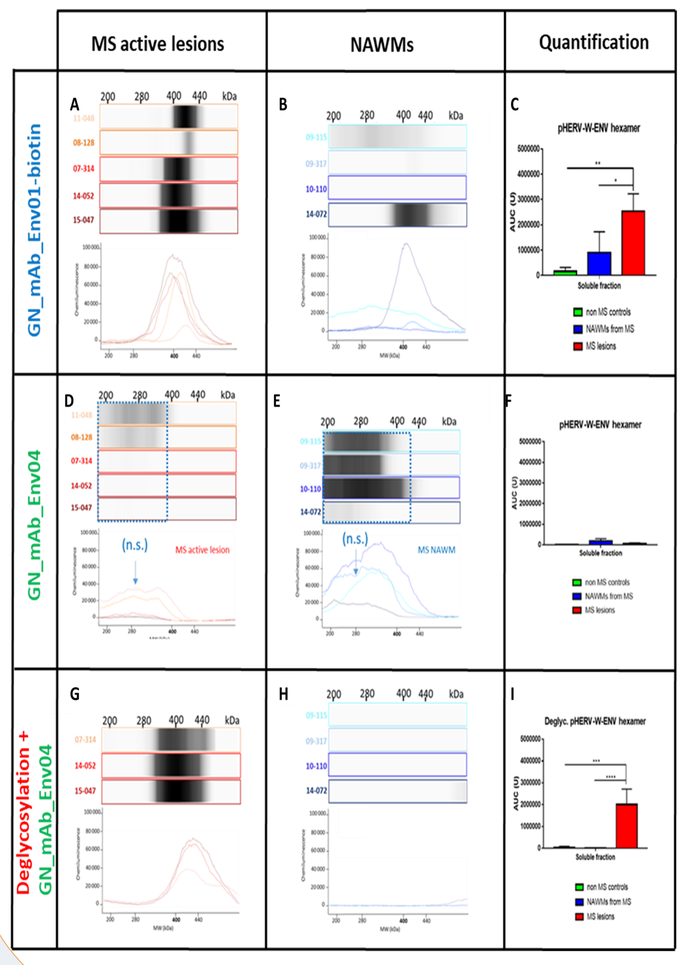


Figure 2: Detection of 360-400 kDa pHERV-W-Env oligomer in soluble fraction of MS brain lesion extracts.
Brain protein extracts (**A-F**) or deglycosylated protein extracts (G-I) were analyzed with simple western (WES) technology based on protein molecular weight (MW) separation. A 66-440kDa separation matrix was used, focusing on 200-440kDa region. Soluble protein extracts from active MS lesions (**A, D, G**), Normal appearing white matter (NAWM) from MS brain (**B, E, H**), or non-MS controls (results not shown, values reported in quantitative representation (C, F, I) were separated by capillary electrophoresis and probed with antibodies (Geneuro) directed against pHERV-W-ENV: anti-ectodomain (hydrophilic domain) GN_mAb_Env01-biotin (**A-C**),
or anti-intracytoplasmic tail (hydrophobic domain) GN_mAb_Env04 (**D-I**).
Western blotting (**A-B,D-E, G-H**) and area under curve (AUC – peak 360-400kDa) representation (**C, F, I**).
360-400kDa Peak AUC was correlated with pHERV-W-Env quantity and was compared between samples (**C, F; I**).

Results are means ± standard errors of the means (SEM) (Non-MS controls n = 5, NAWM n=4, MS lesions n=3 to 5).

Result :

- Using GN_mAb_Env01-biotin (ectodomain detection), a strong signal was detected in 360-400kDa region (**A**). pHERV-W-Env monomer MW had an apparent molecular weight about 60kDa for the monomeric form, and about 360-400kDa for the hexameric form.

- Using GN_mAb_Env04 (intracytoplasmic tail detection) No signal was detected around 360-400kDa. An important non-specific signal was present in the 200-400kDa region (**D-F**).

- Deglycosylation unmasked hidden hydrophobic domains resulting in epitope detection with GN_mAb_Env04 (**G**). No signal corresponding to an hexameric form of pHERV-W-Env was detected in any control (**H**). Column filtration concentrated pHERV-W-Env hexamer, showing statistically highly significant difference between controls and MS lesion extracts (**I**).

The transmembrane domain of pHERV-W-Env being hydrophobic, the hexameric organization of pHERV-W-Env is the "best possible" organization for a soluble form with hydrophobic domains hidden in the core of the structure, whereas hydrophilic ectodomains are exposed outwards. This soluble hexameric form of pHERV-W-ENV is significantly and strongly produced in active MS lesion. Its structure confers new solubility and diffusion properties to pHERV-W Env potentially involved in MS lesion formation and progression.

360-400kDa pHERV-W-Env oligomer can be released and detected in MS serum

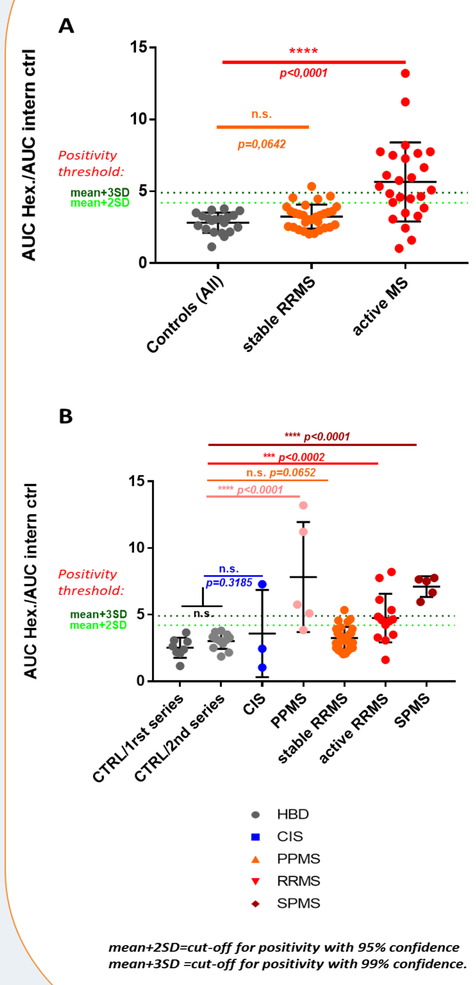


Figure 3: Detection of 360-400 kDa pHERV-W-Env oligomer in sera of MS patient.
Deglycosylated soluble protein extracts from sera of healthy controls (CTRL), of patients with active or stable relapsing-remitting MS (RRMS), with primary or secondary progressive MS (PPMS, SPMS), as well as with CIS, were analyzed with WES technology. A 66-440kDa separation matrix was used, focusing on the 200-440kDa region. Soluble protein extracts from sera were separated by capillary electrophoresis and probed with GN_mAb_Env01-biotin (Geneuro) directed against pHERV-W-ENV ectodomain.

(**A**): Global comparison between controls, stable and active MS.
(**B**): Detailed comparison between CTRL, CIS and MS forms.

CTRL n= 20; CIS n=3; MS n=53, including: PPMS n=5, RRMS n=43 and SPMS n=5. Statistics = t test for comparison of series of values.

Results :

We found significantly positive levels of pHERV-W ENV in sera of active RRMS, SPMS and PPMS compared to 20 healthy controls ($p<0.004$ to $p<0.0001$), but not in sera of stable RRMS ($p>0,5$).
10/14 RRMS subjects classified as active were positive, versus 4/30 clinically and radiologically stable RRMS patients (Chi-2 y.c. $p<0.001$).
In progressive patients 9/10 subjects were positive.
When analyzing different sub-groups with different clinical forms or phases of the disease (**B**), it appeared that detection was possible even in CIS (the positive one was later confirmed as RRMS). PPMS and RRMS showed dispersed values among which few revealed elevated, whereas SPMS patients showed rather grouped positive values.

CONCLUSION:

- pHERV-W-Env was identified as monomer and oligomers in reference transfected cell extracts. In the soluble fraction from MS lesions or MS sera, a unique large peak of hexamer was detected around 360-400 kDa. It was absent in MS NAWM and control brains, except in one MS NAWM extract and in a control with brain metastases. However, in these cases, hexameric forms disappeared after deglycosylation, like in transfected cells. In MS lesion samples, the hydrophilic N-terminus unit was readily labelled in all conditions, whereas the C-terminus hydrophobic region became detectable after deglycosylation only. A compatible structural organization is proposed here as the best possible soluble form.
- Given previously described effects of the pHERV-W-Env antigen, this soluble hexameric form now raises the question about its relationship with the soluble demyelinating and cytotoxic factor (>300kDa) described in MS (H. Lassmann Exp Neurol 2014; Lisak et al., J. Neuroimmunol. 2017).
- If antigenically related, this soluble demyelinating factor should also be targeted by anti-pHERV-W-Env neutralizing antibody (GNbAC1),
currently in clinical development in MS.