The Case FOR Human Gamma Retroviruses (XMRV/ HGRVs) in CFS/ME

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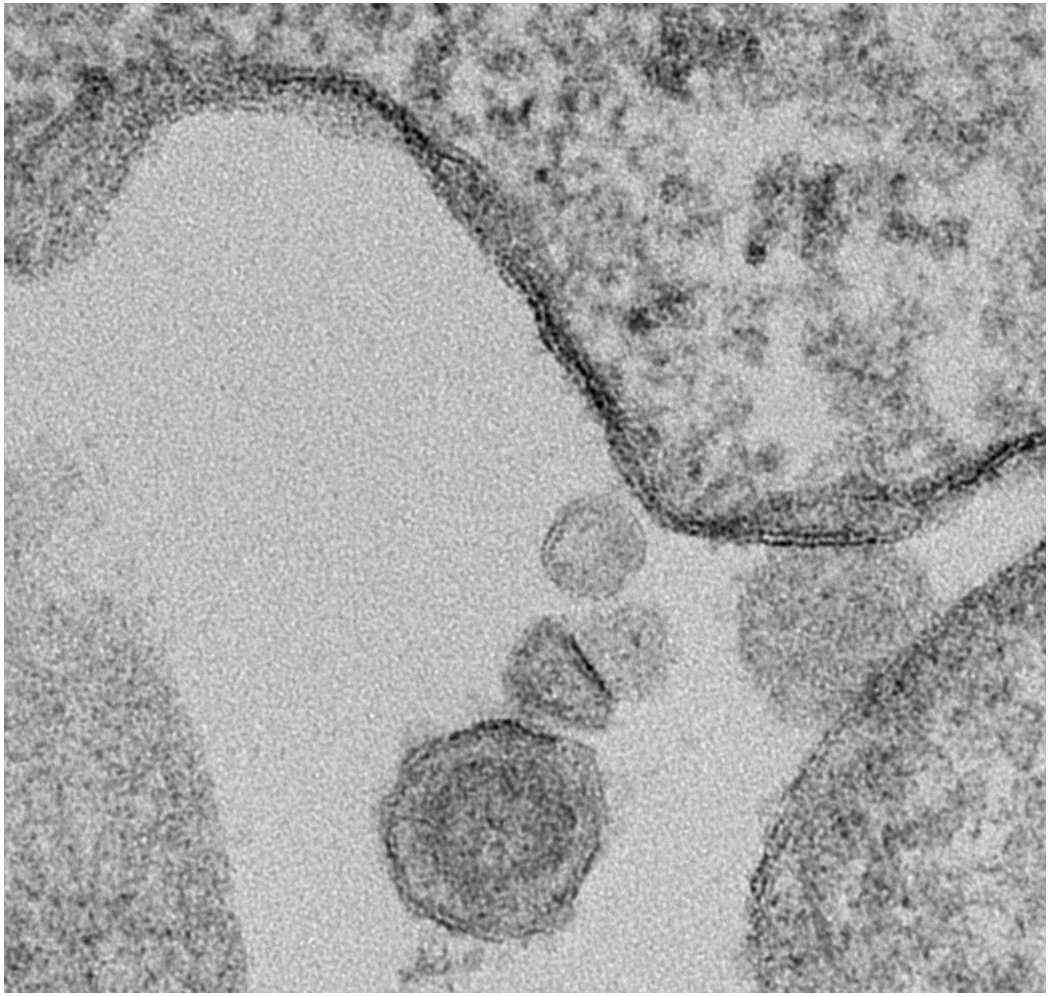
Special Thanks:

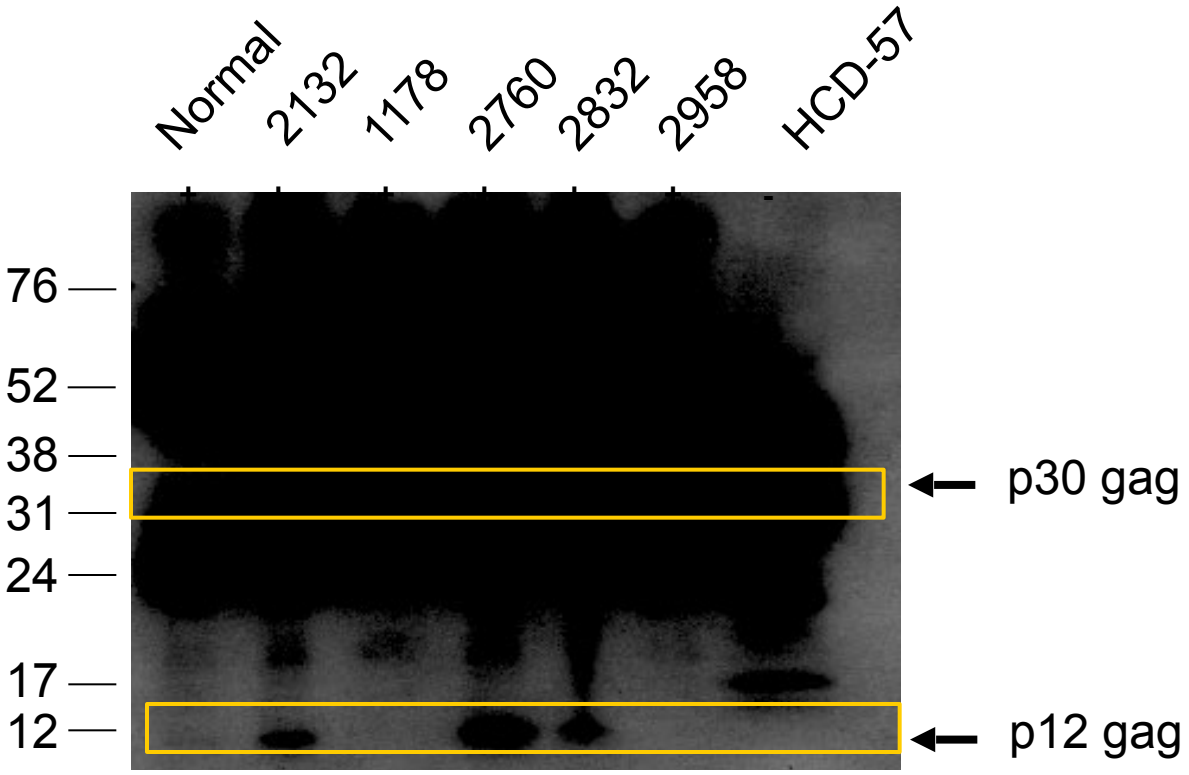
All the patients who participated in BWG, WPI, Maldarelli and Lipkin studies. Without **YOU** there is **NO** Research in ME/CFS!

Direct Isolation of a Gammaretrovirus from the PBMCs of CFS patients



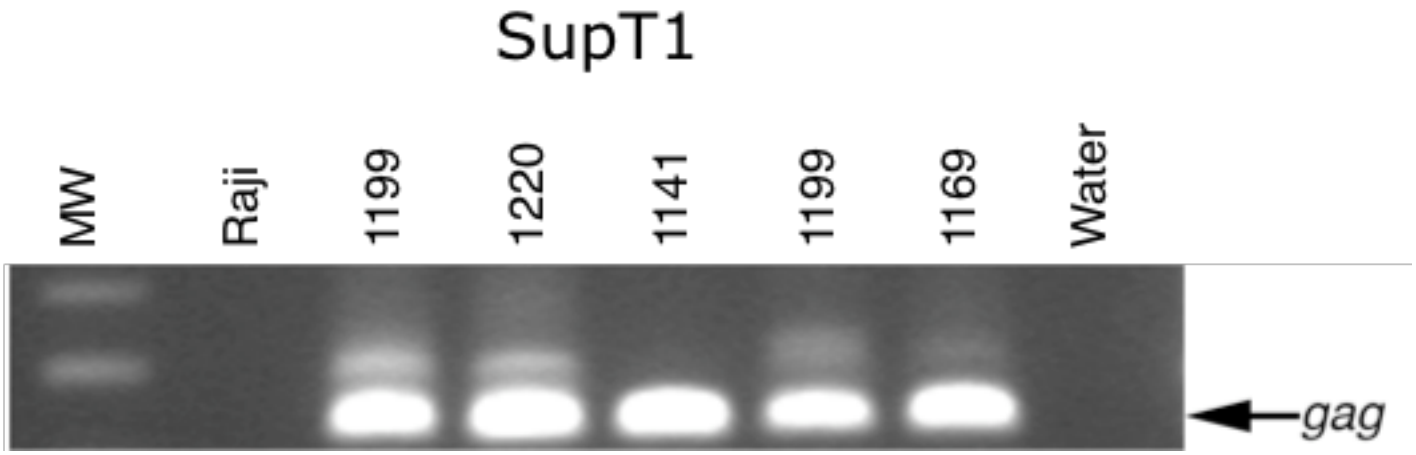
Direct Isolation of XMRV/HGRV Protein From Plasma of CFS Patients By Immunoprecipitation with Anti-X-MLV Antibodies

IP: Goat anti-X-MLV (BALB-V2)

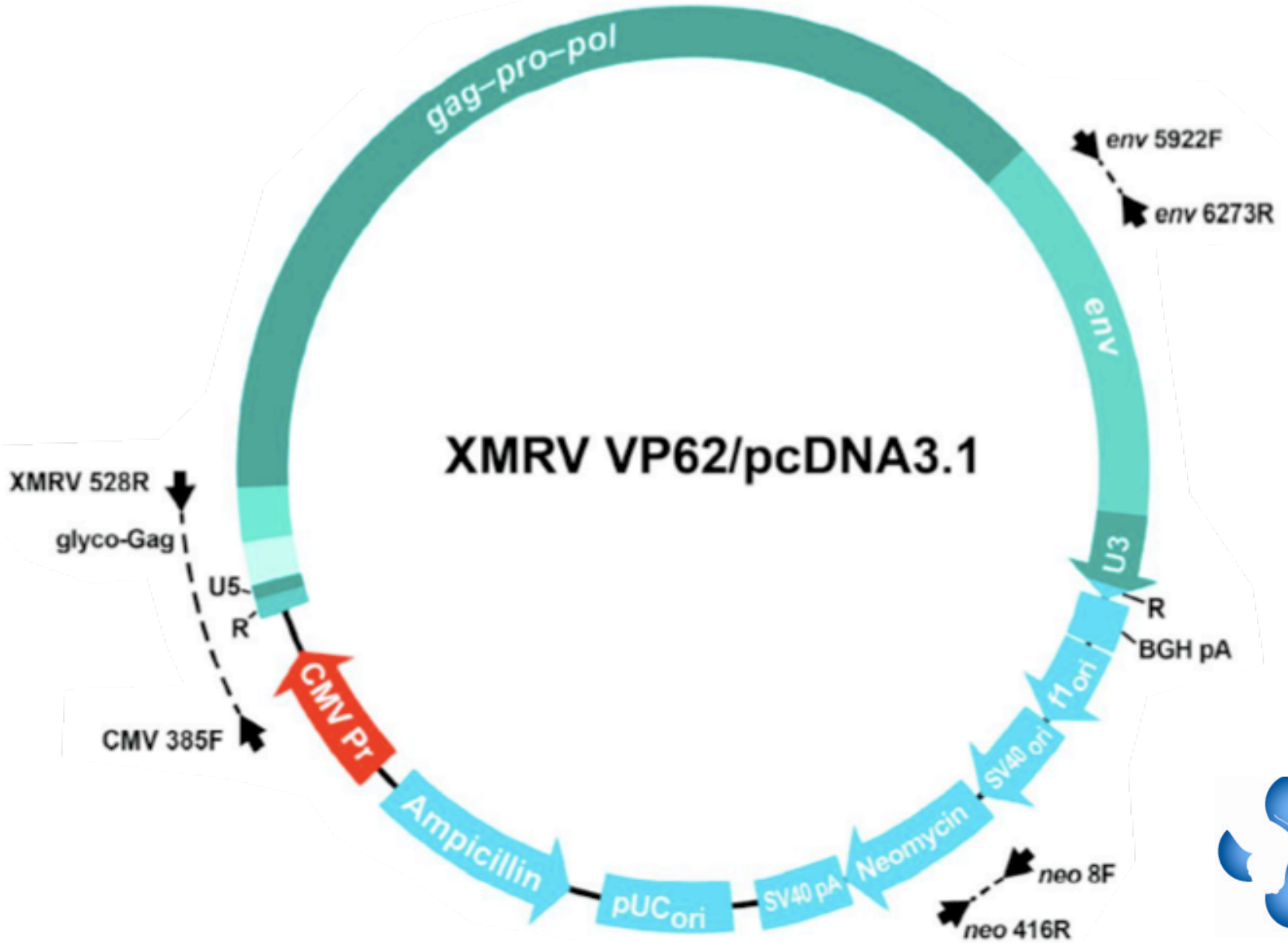


Blot: Goat anti-R-MuLV Gag

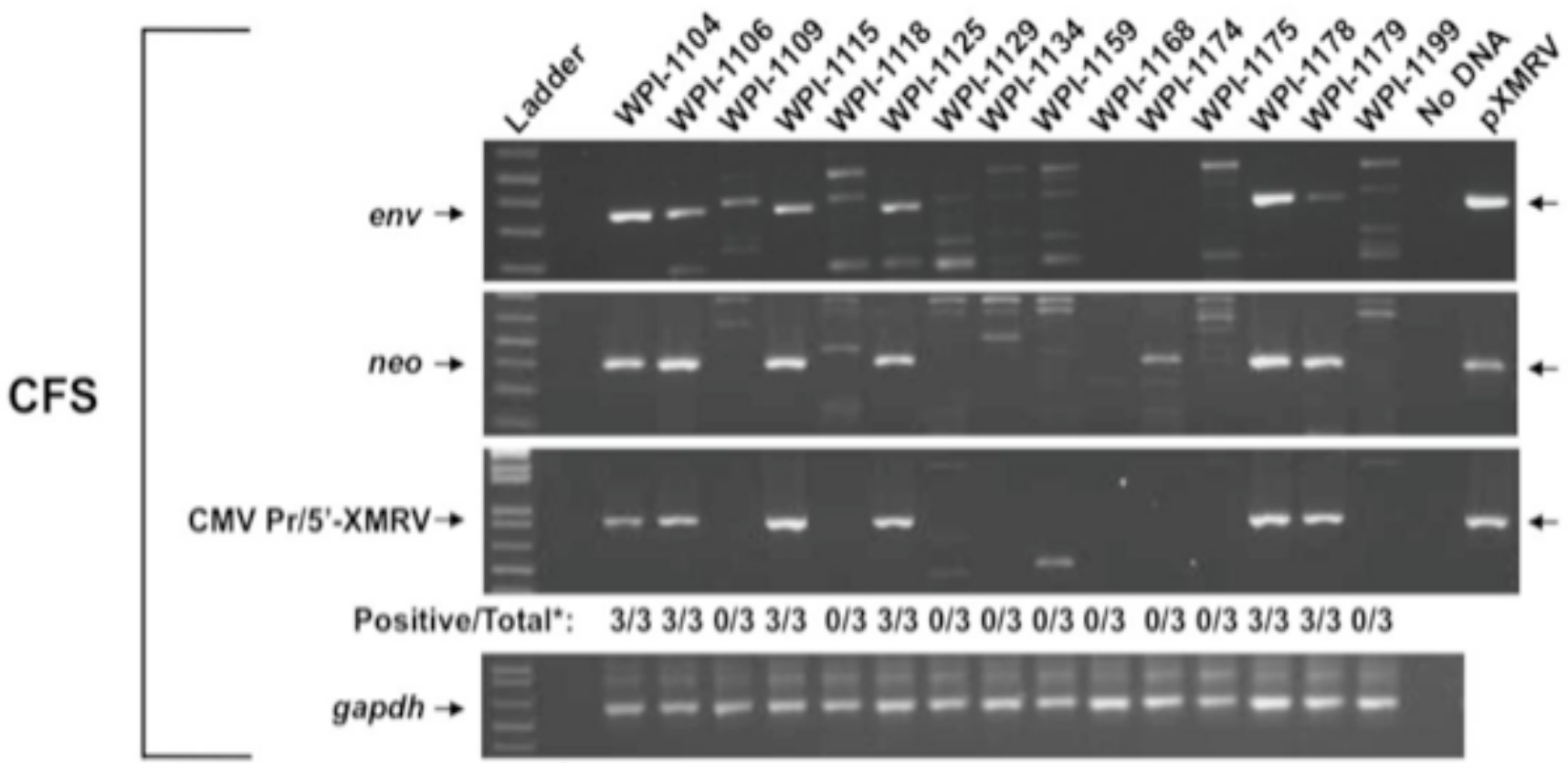
Cell-Free Transmission of XMRV from CFS Patients' PBMCs to the SupT1 Cell Line



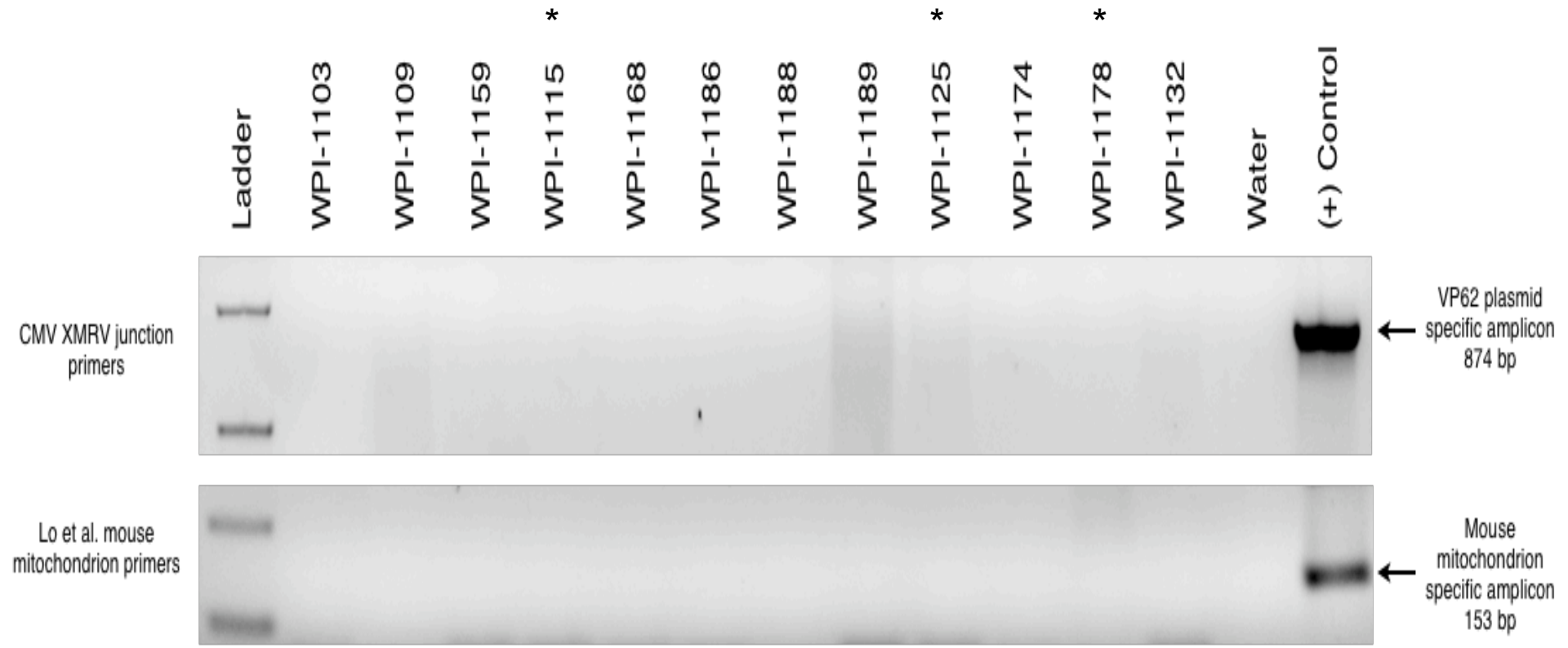
Schematic of Plasmid containing XMRV/VP62



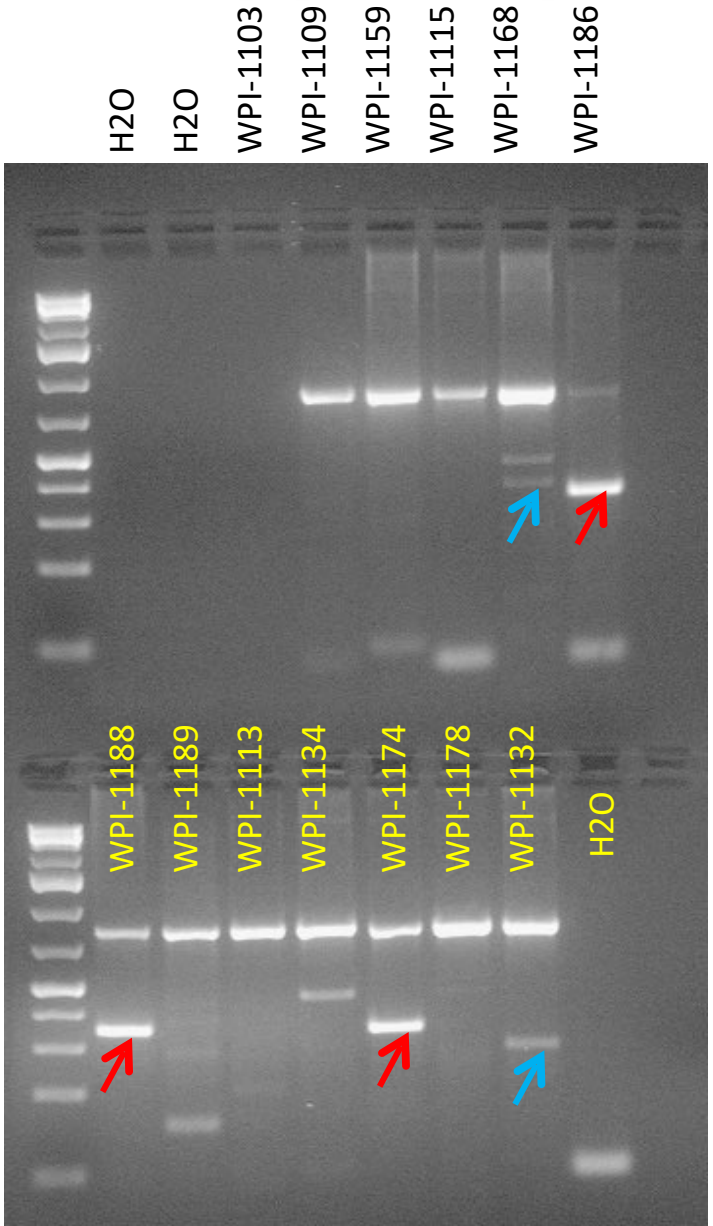
Some WPI DNA Samples shown in Fig. 1 of the original study Contain VP-62 plasmid



Original DNA Samples were negative for XMRV plasmid



Independent Reanalysis of samples used in Original Study Detected XMRV gag without plasmid or mouse contamination



PCR performed with USB HotStart-IT FideliTaq Master Mix

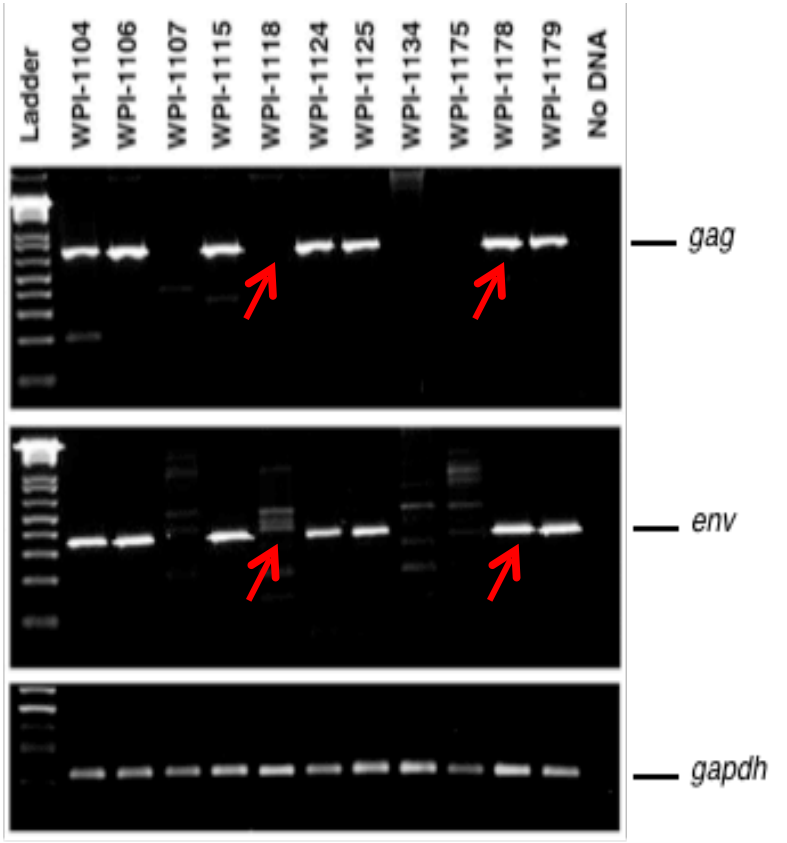
94°C 2 min
45 cycles:
94°C 30 sec, 54.8°C 30 sec, 72°C , 30 sec
72°C 3 min.

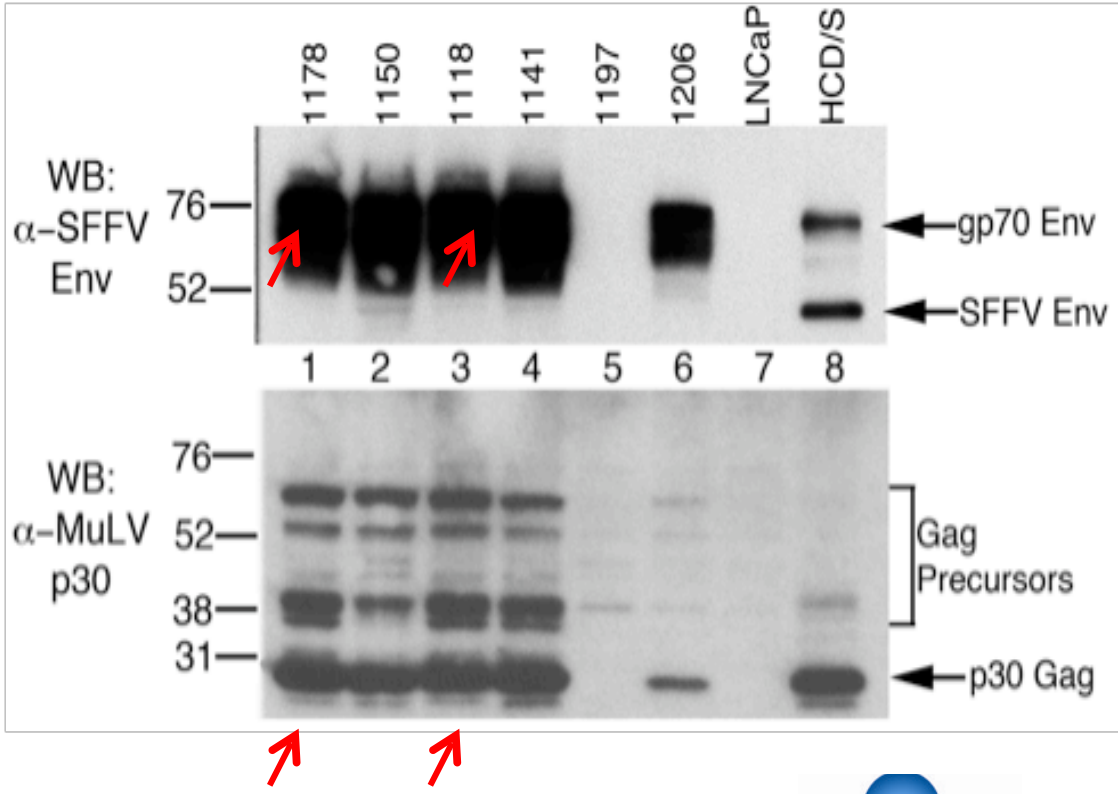
All three are negative for IAP and negative for CMV385F/XMRV528R primers for VP62 junction fragment

Sequencing of bands:

↗ Non-specific (Human DNA)
↗ XMRV Gag

Cell-Free Transmission of XMRV/HGRV from PCR-negative CFS Patients' Plasma to LNCaP cells





Horizontal Spread of Gammaretroviruses in Tissue Culture

Table 4. Characterization of murine leukemia viruses (MLV) detected in human non-xenograft cultures in xenograft culture laboratories[1]

Cell line type	MLV positive cell lines[1]	MLV sequence homology[2]	RT Enzyme (nU/μl)	Mouse DNA[3]	Other sources or passages[4]	Source: Lab PI
NSCLC	NCI-H460	ND	Negative	-	Negative	C. Rudin
NSCLC	NCI-H1155	MLV N417	ND	-	ND	A. Gazdar (NCI)[6]
SCLC	NCI-H60	MLV N417	3.6×10^6	-	Negative	A. Gazdar (NCI)[6]
SCLC	NCI-H82	MLV NZB	1.3×10^6	-	Negative	C. Rudin
SCLC	NCI-H1092	MLV N417	8.0×10^3	-	Negative	A. Gazdar (NCI)[6]
SCLC	NCI-H182	MLV N417	ND	-	ND	A. Gazdar (NCI)[6]
SCLC	NCI-H289	MLV N417	ND	-	Negative	A. Gazdar (NCI)[6]
SCLC	NCI-H1514	MLV N417	ND	-	ND	A. Gazdar (NCI)[6]
Colon	RKO	XMRV	2.9×10^3	-	Negative	A. Maitra
Prostate	PrEC2	ND	ND	-	ND	J.T. Hsieh
Prostate	LNCaP	Multiple MLV strains[5]	ND	++++	Negative	J.T. Hsieh
Prostate	PC3	ND	ND	-/+	Negative	J.T. Hsieh
SCLC	NCI-H146	MLV NZB likely	7.2×10^5	-/+	Negative	C. Rudin

Zhang et al., Cancer, Biol. Ther. 2011, 12:617

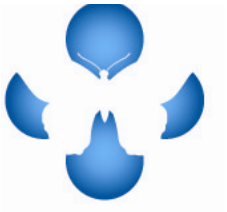
What does this mean?

1. Horizontal spread of these viruses is another potential source of viral contamination of cell lines

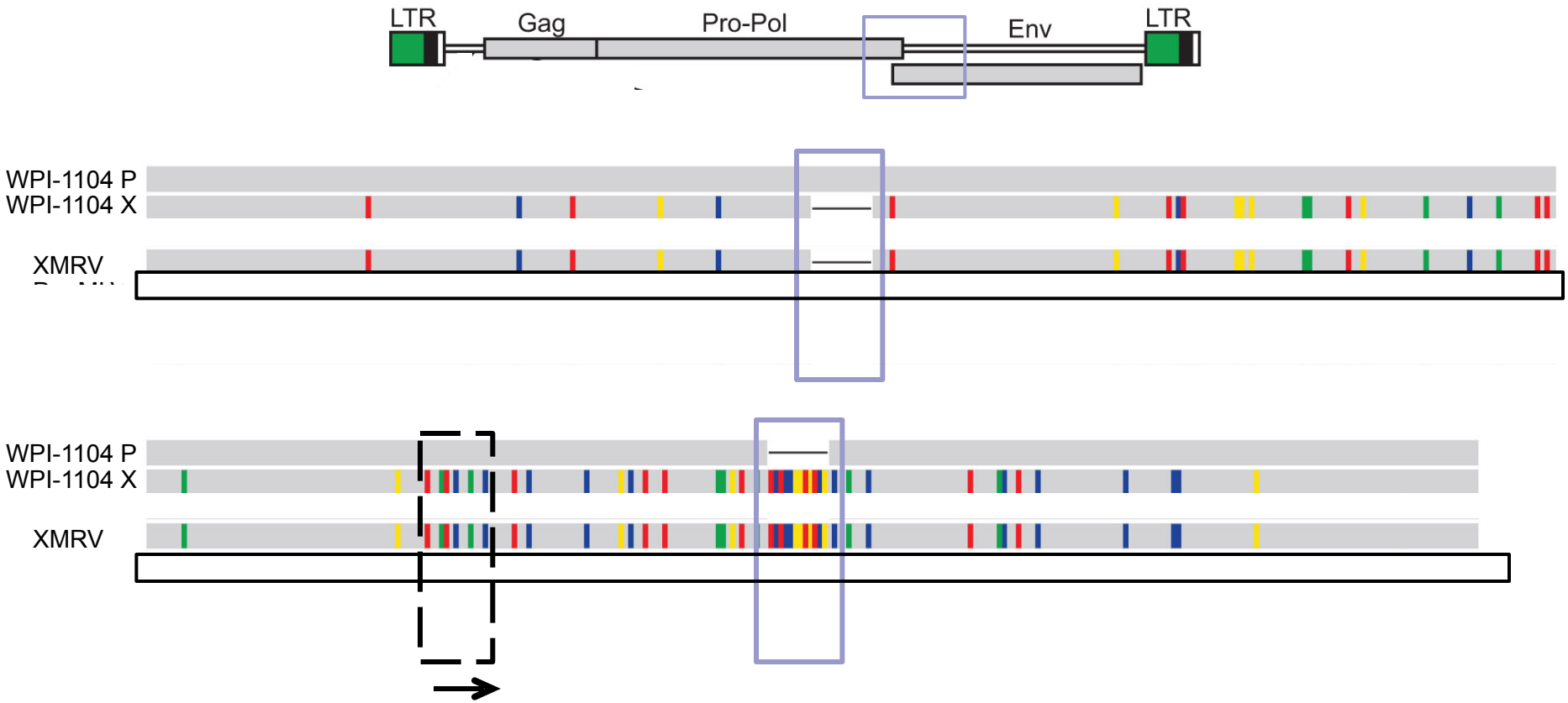
2. As presented in Ottawa, this could occur by spiking CR22V1 virus in LNCAP

3. It could occur by aerosolization, contaminated reagents, faulty technique

4. This is no evidence that such horizontal spread of gamma-retroviruses occurs between individuals

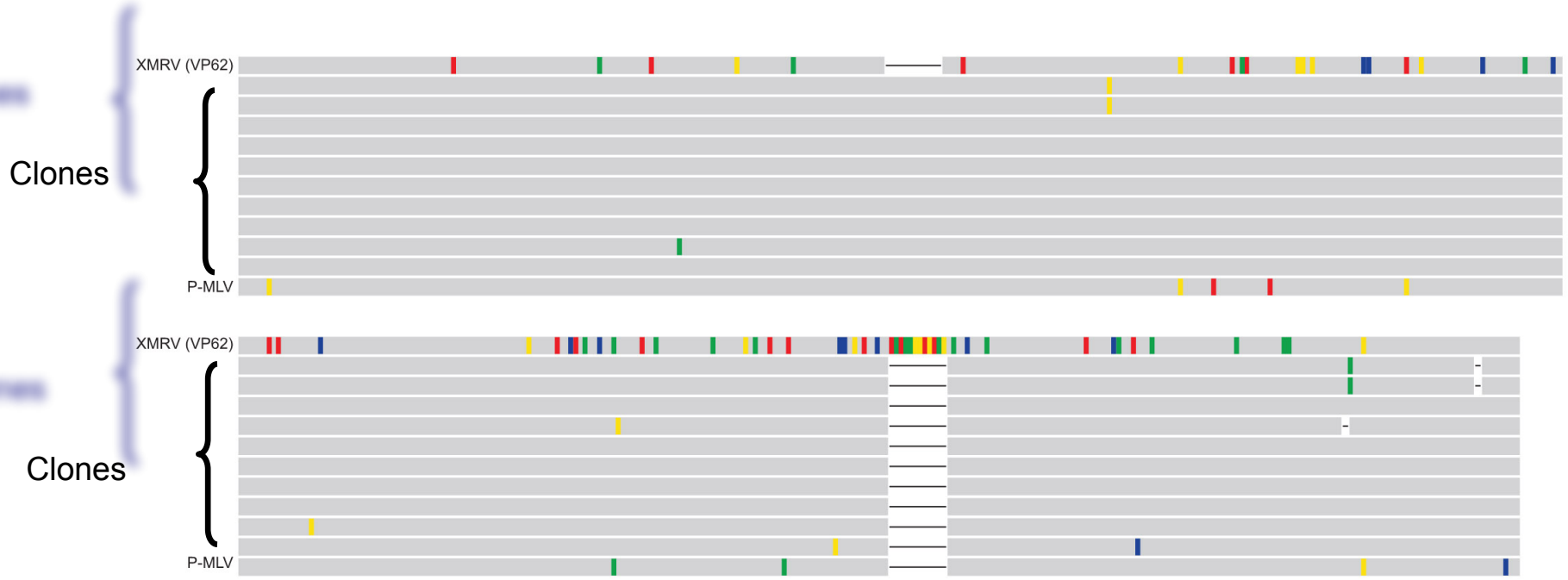


A CFS Patient has a Strain of HGRV distinct from XRMV



DNA from several individuals carry both XMRV and PMRV sequences, as confirmed by cloning and sequencing of PCR products

Clones of XMRV/HGRV Env SU Similar to Polytropic MLVs



- The main XMRV/HGRV in this patient is unlikely to be VP-62

Why No XMRV/HGRV Detection in previously Positive Patients in BWG?

- Exogenuous Infection ($3x10^6$ particles From VP62) in Rhesus Macaques revealed:
- Viral and proviral signals disappeared in the blood 1 month p.i. (acute vs chronic)
- Chronic infection present in tissues
- Latent infection and reactivation observed

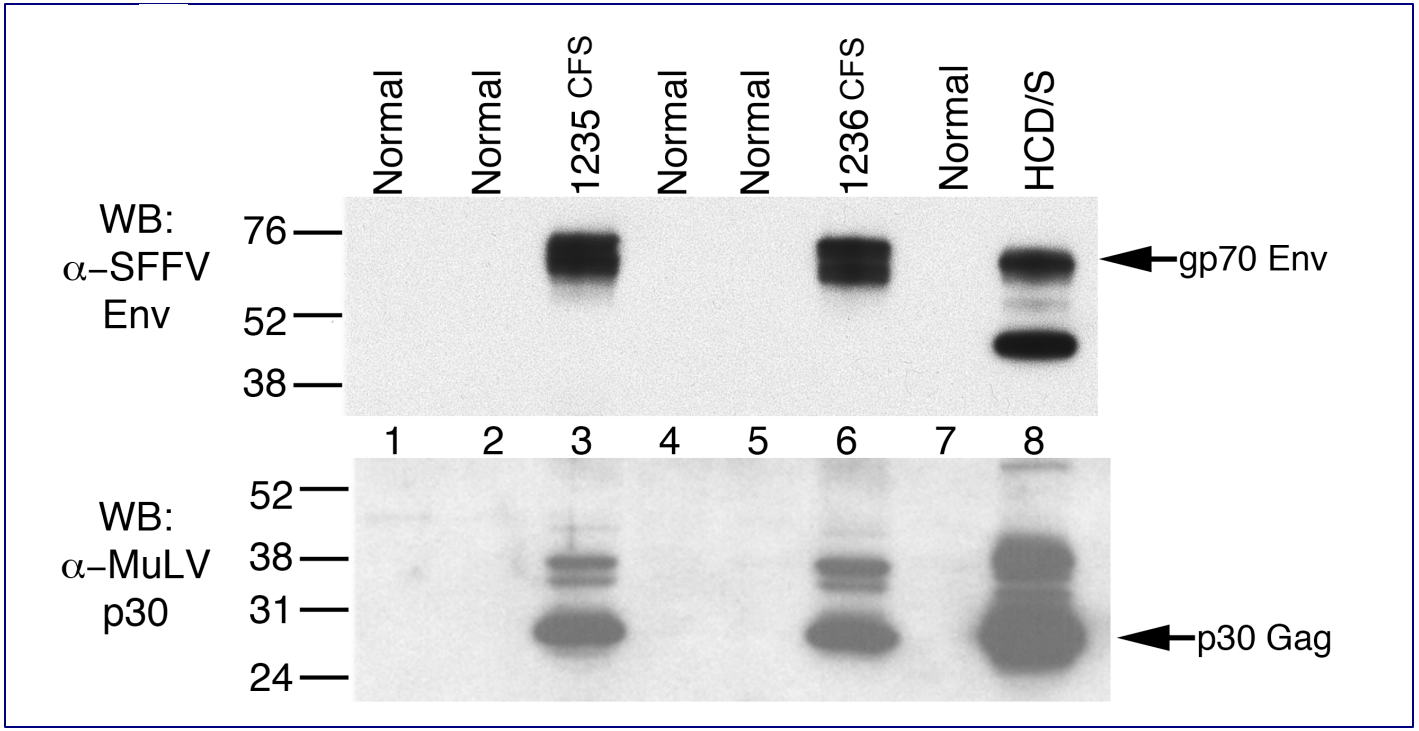
Onlamoon et al., J Virol. 85: 4547, 2011

Animate or just say an a transition could these viruses also be latent in Man

slide HGRV can be latent

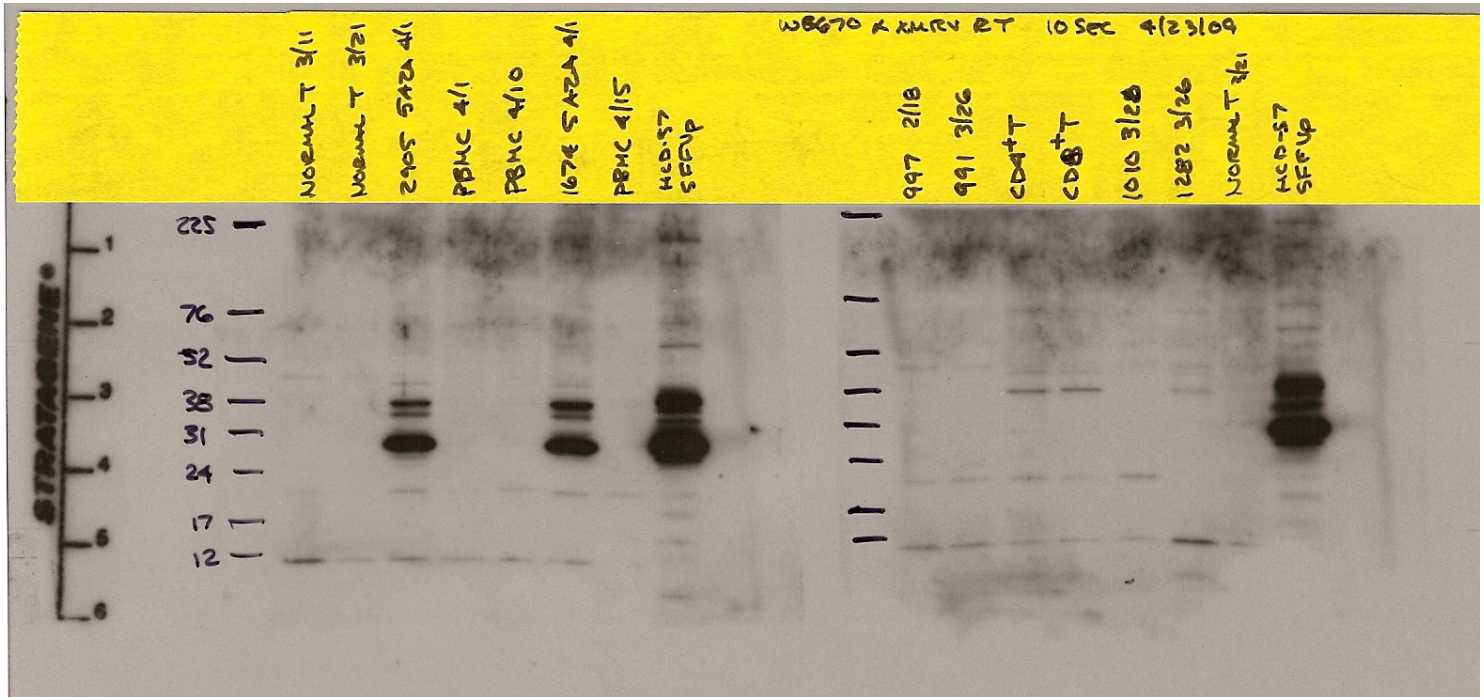
- Data here on two occasions acitvated PBMC were positive for gag PCR
- One time the PBMC for both patient were negative by gag PCR but positive after 5AZA, a demethylating agent, known to activate latent viruses

Absence of XMRV Protein Expression In Activated PBMC from Normal Donors



0/50 healthy donors were positive for XMRV proteins

Original Film for Science Fig 2C



Names changed to protect patient privacy

The 5 AZA treated samples were used as controls for negative activated normal PBMC

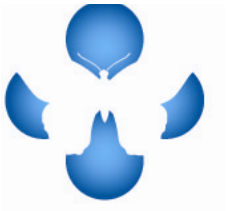
5 Aza treatment of these two samples was not identified

It was an error not to in methods..but nothing more

Why No XMRV/HGRV Antibody Detection in previously Positive Patients in BWG?

- Exogenuous expression of XMRV proteins in Rhesus Macaques and wild mice revealed:
- Antibody responses are in low in magnitude and short in duration even after boosting: no durable immune response
- Low immunogenicity– XMRV characteristic
- Reason for discrepancies in detection methods

Onlamoon et al., J Virol. 85: 4547, 2011, Makarova et al., Plos One 6: e18272 2011



N-Terminus of SFFV ENV allows recognition of most potential HGRVs

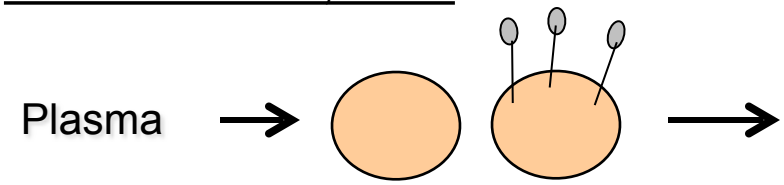
Comparison of N-terminal Env regions of SFFV and XMRV

```
VQLDSPHQVSNVTWRVTNLMTGQTANATSLLG
VQRDSPHQVFNVTWKITNLMTGQTANATSLLG

TMTEAFPKLYFDLCDLMGDDWDE      TGLGC
TMTDTFPKLYFDLCDLVGDHWDDPEPDIGDGC


RTPGGRKRARTFDFYVCPGHTVPTGCGGPREG
RSPGGRKRTRLYDFYVCPGHTVLTGCGGPREG
                              I    G

YCGKWGCETTGQAYWKPSSSWDLISLKRGN
YCGKWGCETTGQAYWKPSSSWDLISLKRGN

TPKDQGPCYDSSVSSGVL GATPGGRCNPLVL
TPKGQGPCFDSSVGSGSIQGATPGGRCNPLVL
   RN

EFTDAGRKASWDAPKVWGLRLYRSTGTDPVTR
EFTDAGKRASWDAPKTWGLRLYRSTGADPVTL

FSLTRQVLD IGPRVPIGSNPVTTD
FSLTRQVLNVGPRVPIGPNPVITE
```

--- SFFV
--- XMRV (bold shows differences from SFFV)
--- Xeno MuLV
--- Mol MCF MuLV

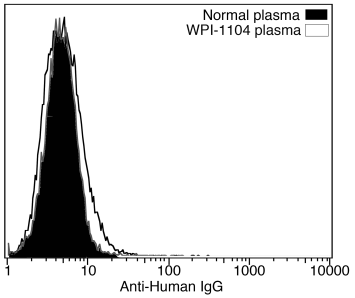
Assay used to Detect Anti-XMRV/HGRV Antibodies

Lombardi et al., 2009

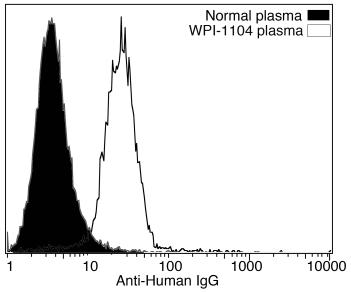


FACS analysis of Murine Cell Line
+/- SFFV Env

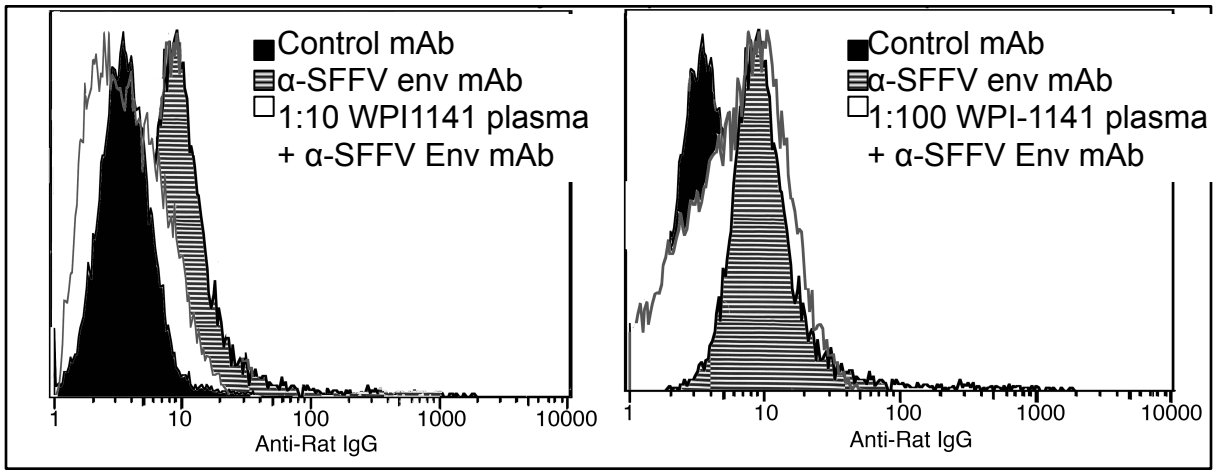
- SFFV Env



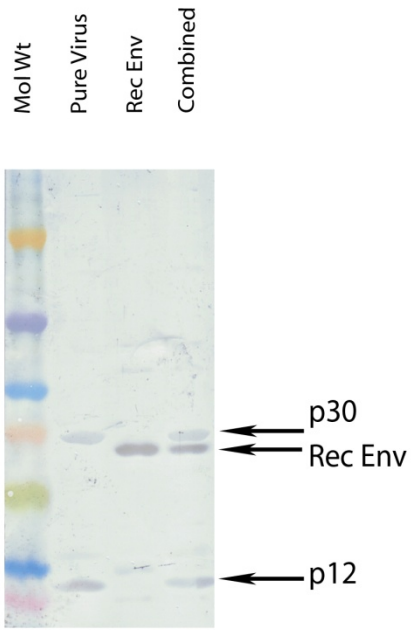
+ SFFV Env



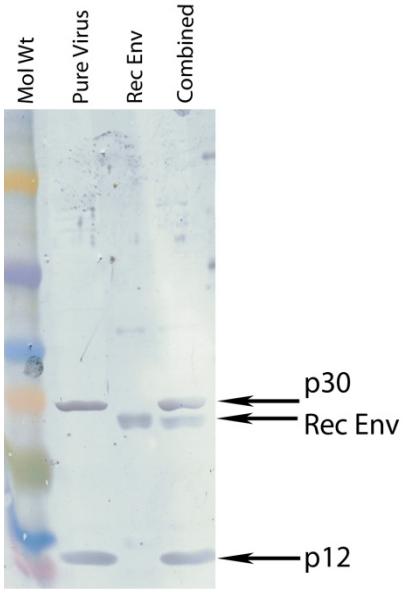
Plasma from CFS patients block binding of SFFV Env rat mAb to the B cell line expressing SFFV Env, demonstrating specificity



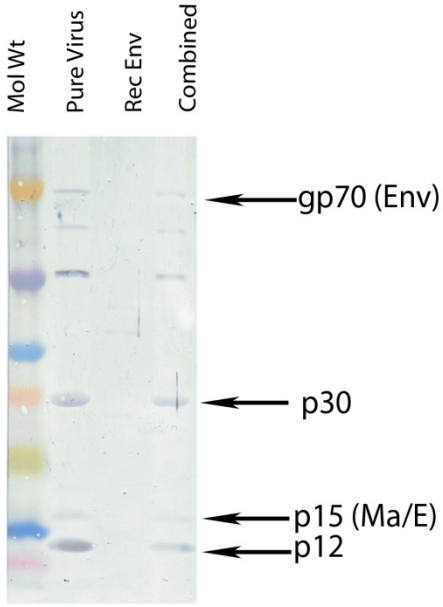
Plasma from XMRV/HGRV Infected CFS Patients are reactive to multiple XMRV proteins



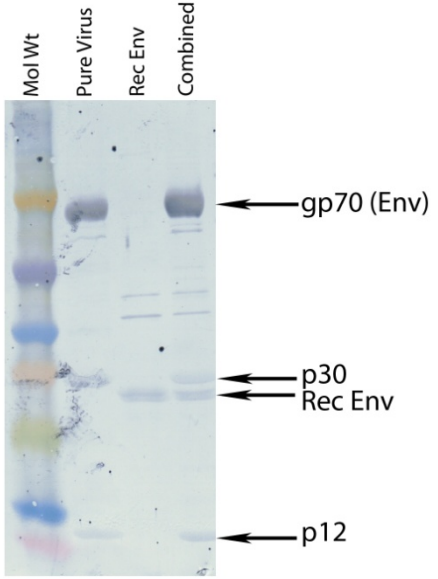
Patient 2152



Patient 2231

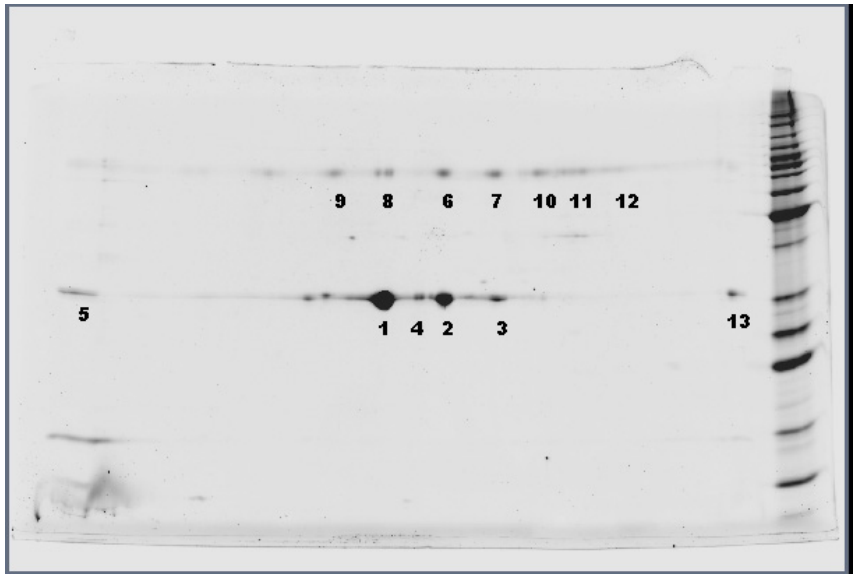


Patient 1888

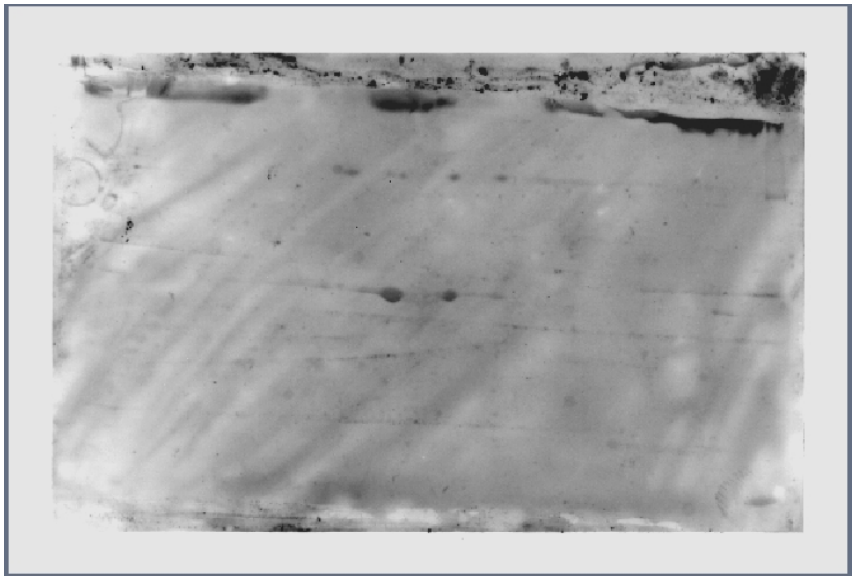


Patient 2111

Two Dimensional Western Blot Followed by Mass Spec Sequencing Confirms Human Antibody Specific Binding to XMRV/HGRV Viral Proteins



Sypro-Ruby Stained 2-D Protein Gel



Western Blot Probed with Human Serum

Both XMRV gp70 and p30 were recognized by CFS sera

❖Ability to recognize XMRV proteins does not mean that XMRV was the immunogen it could be any HGRV or a cross reactive protein

Where are we now?

Many potential kinds of contamination

1)Plasmid or Viral Nucleic Acid which to false sequencing in Silverman Lab – likely virus in paper is not VP-62

Silverman et al., Science in press

2) Horizontal passage lead to viral contamination of cell lines which put in doubt integrations into human genome

Rusmevichientong et al., J. Virol. In press

3)Mouse viral sequences in commercial reagents

All this makes it likely that VP-62 XMRV is not an human infection

Where are we now II (Lombardi et al)

The many potential kinds of contamination have not been found
In the WPI but

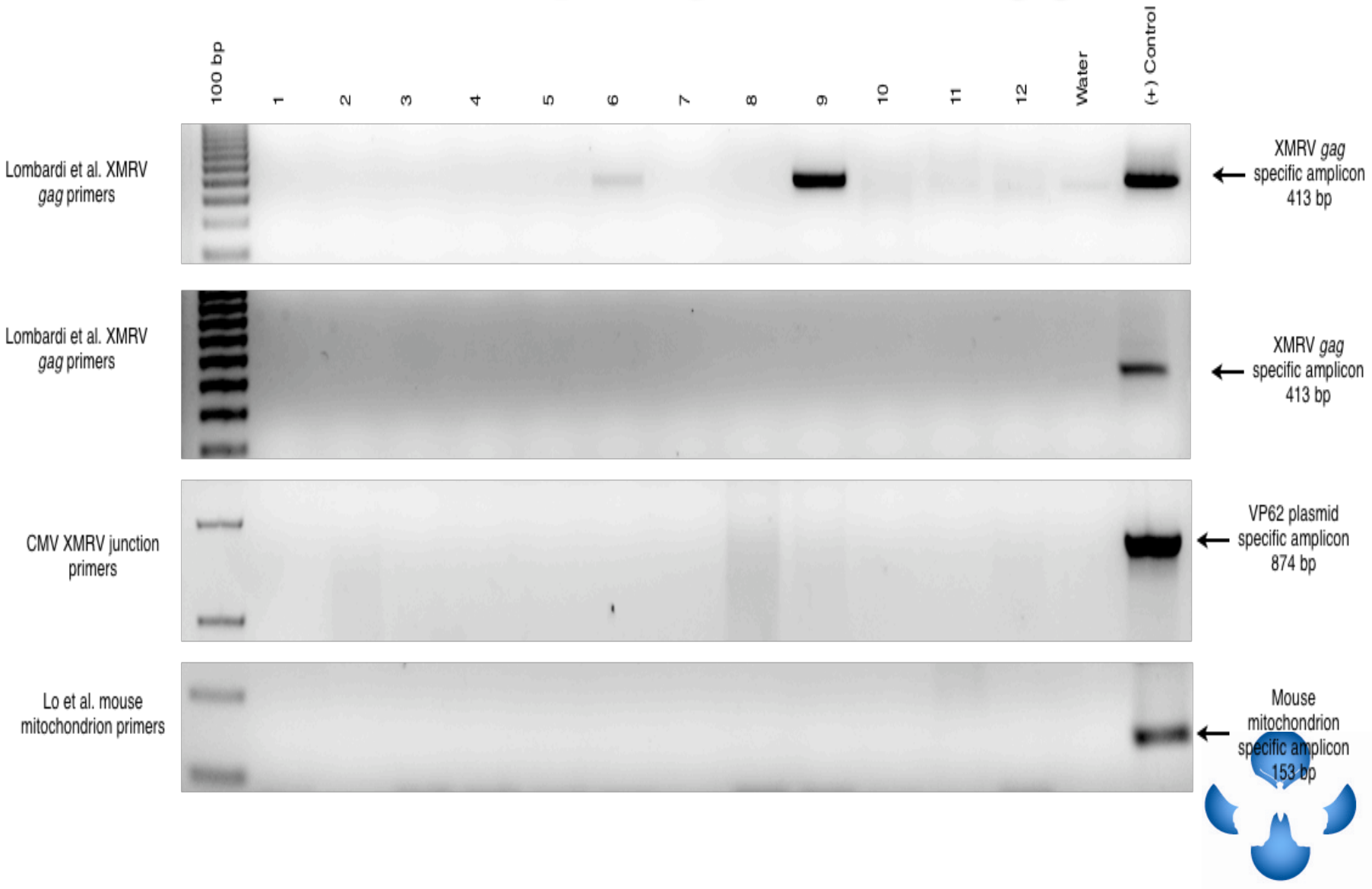
1)The results in Lombardi et al have not be reproduced

2)Science demands that results be reproduced, therefore these results remain unproven – must be considered wrong until confirmed

3) “If you can not find something, it is either not there or you can not see it”

We must persue answers in different, more difficult directions

Extended PBMC cell culture without manipulation shows XMRV *gag* infection in samples negative for XMRV *gag* RNA



Next Generation Sequencing Study

Sera from Subjects

- 8 CFS patients (Source: Hemispherx Biopharma)
- 17 Apparently Healthy Controls (Source: Vendor)
  - 15 Russian samples
  - 2 Canadian samples

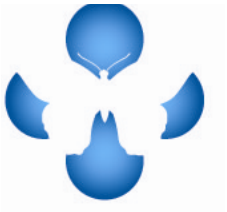
Serum samples processed for SOLiD4 sequencing:

- extraction of DNA (Std. silica method)
  - random amplification (WGA – Genomeplex)
- Emulsion PCR -> Sequencing (50bp reads)

Post-sequencing Biometry:

- First alignment to human DB (Hg18)
  - only reads that were not positive in first alignment used
- Second alignment to 6 XMRV DBs and 16 pMRV[1] DBs (partial cds)
  - Cleaned for low complexity and short hits
- Data mining for read significantly positive on Individual XMRV/pMRV DBs
  - Stringency: Similarity ≥ 95% within ≥ 95% read length

[1] pMRV= polytrophic MLV-related virus



NGS detected XMRV/HGRV in 7/8 CFS samples and 2/17 controls.

GenBank Accession #	# Samples	8	Normals	17
		+ Samples	# Reads	+ Samples
	SUM ALL XMRV	7	4	2
DQ241301.1	Xenotropic MuLV-related virus VP35	1	1	1
DQ241302.1	Xenotropic MuLV-related virus VP42	3	1	1
DQ399707.1	Xenotropic MuLV-related virus VP62	3	0	0
FN692043.2	Xenotropic MuLV-related virus 22Rv1/CWR-R1	3	0	0
GQ497343.1	Xenotropic MuLV-related virus clone WPI-1178 putative (gag-pro-pol)/(gag)/(env)	3	1	1
GQ497344.1	Xenotropic MuLV-related virus clone WPI-1106 putative (gag-pro-pol)/(gag)/(env)	2	1	1

No hits on pMRV DBs:

HQ601962.1, HQ601961.1, HQ601960.1, HQ601959..1, HQ601958.1|, HQ601957.1, HM630561.2, HM630557.2, HM630558.1, HM630562.1, HM630560.1, HM630559.1, HQ157343.1, HQ157342.1, FJ907198.1, FJ907199.1

Conclusions

- NGS technology offers analysis of the entire body pool of circulating nucleic acid DNA from apoptotic cells.
- New NGS studies indicate that CFS sera exhibit alterations in apoptotic DNA sequences. (see Strayer D, and Mitchell, B et. al. LB poster).

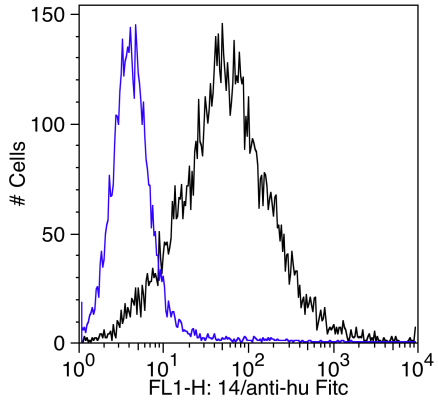
- Under conditions of high stringency and a 45bp minimum size, Low levels of XMRV reads were observed in 7 out of 8 CFS and 2 out of 17 non-matched control samples.

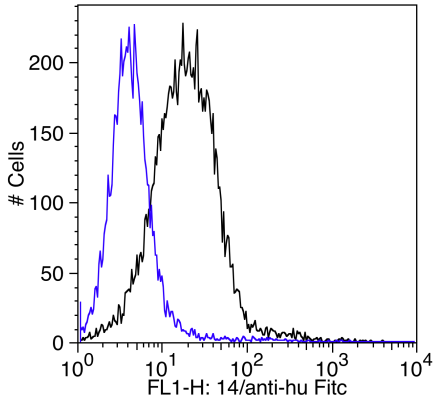
- No reads were observed with the pMRV DBs.

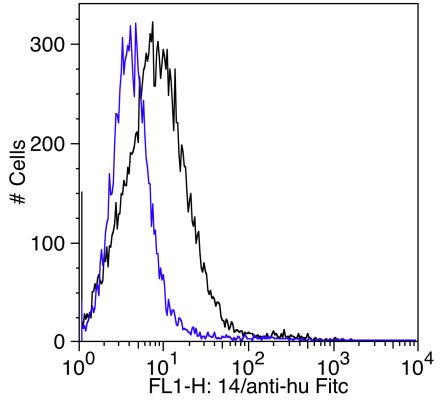
Future Plans for CFS studies Based on Initial Next Generation Sequencing Results

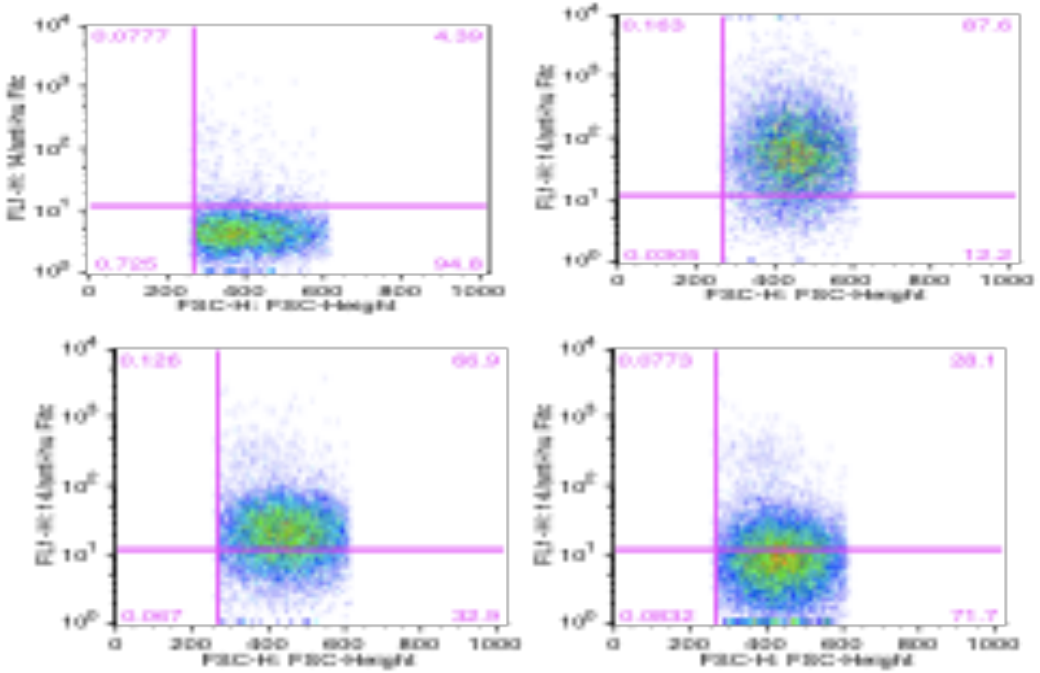
- Increase Sequencing coverage of CNA DNA per sample.
- Use age, sex, and geographical region matched non-CFS controls.
- Use >75 bp paired end reads to investigate viral-human chimeras as an indicator of viral integration.
- Compare amplified DNA with non-amplified DNA to eliminate the possibility of template switching as a potential source of method artifact.

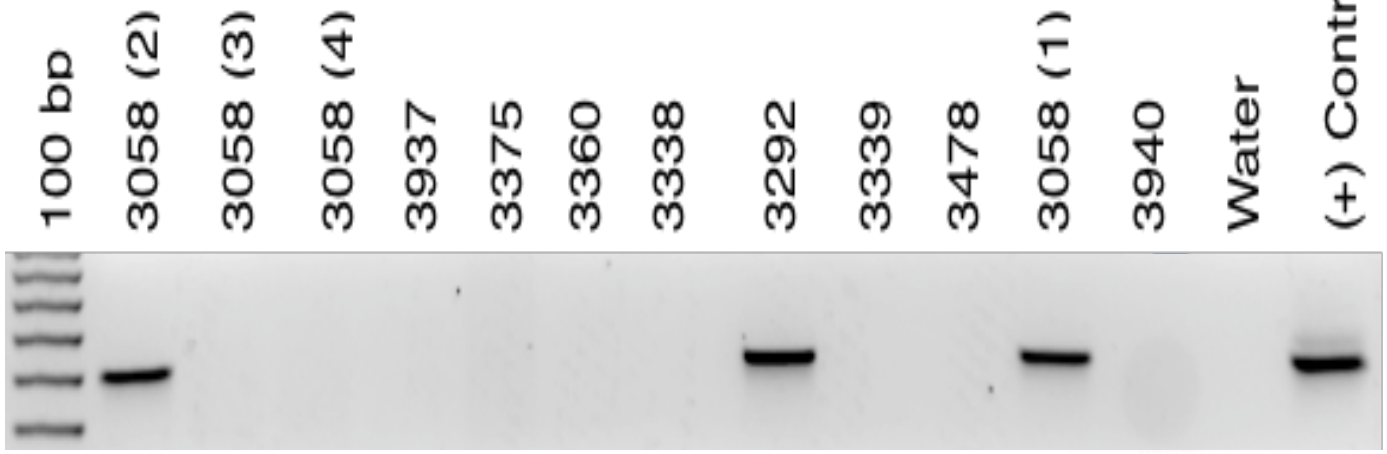
Case report 3058 : plasma Gag RNA + and seropositive CFS/CLL



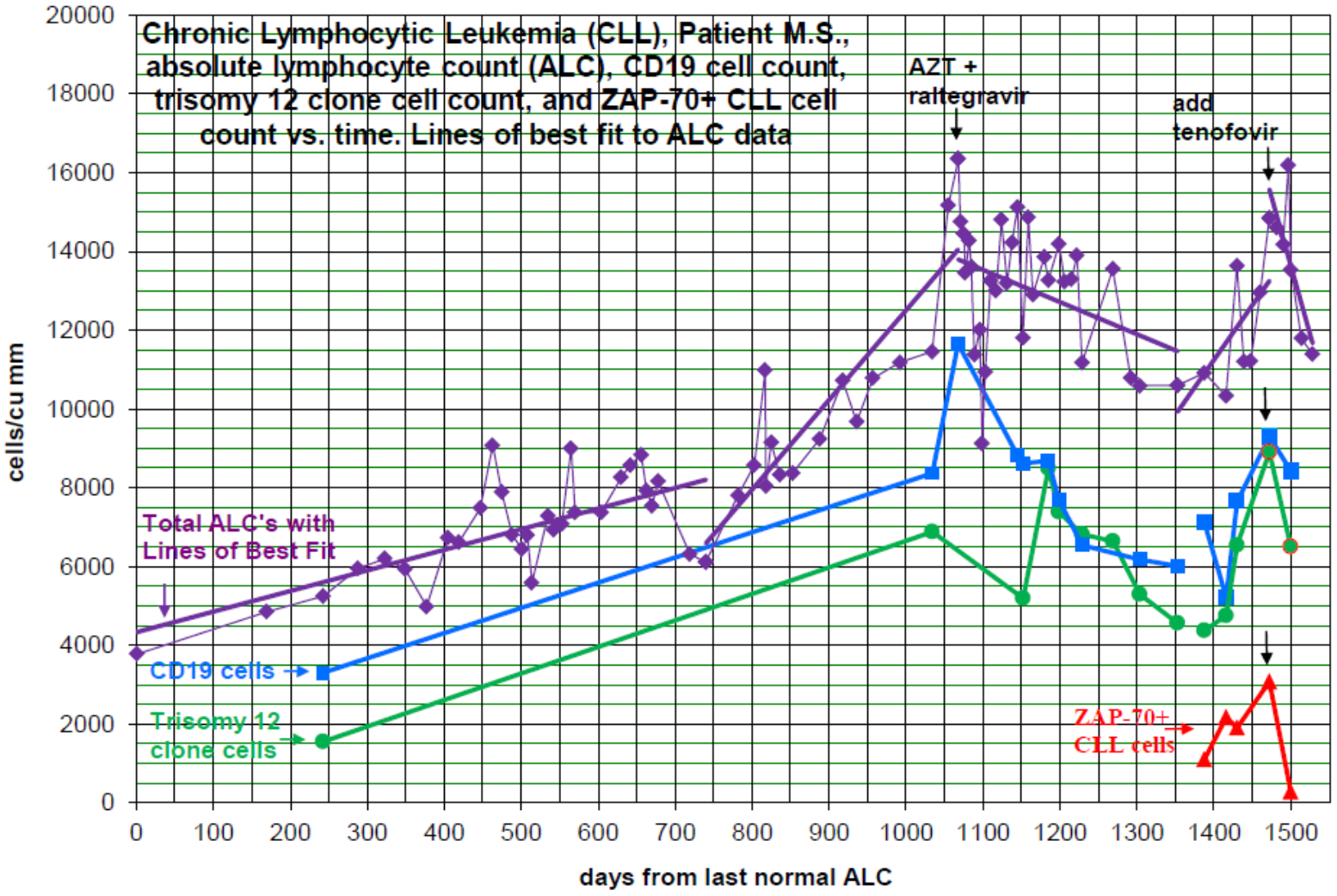






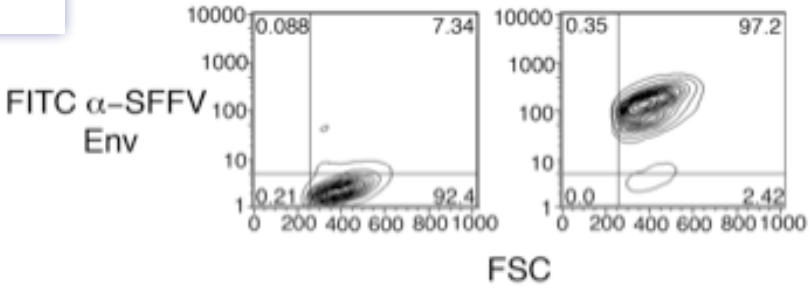


Antiretroviral Therapy of CFS and CLL



3 B-Cell Lines Derived Directly From XMRV+ CFS Patients' PBMCs

- **XMRV+ CFS patient PBMCs were cultured; 3 samples developed into immortalized cell lines**
- **All three showed high CD20+ expression and two showed high CD23+ expression.**
- **All three showed strong similarity to B cells seen in XMRV+ patients.**



Marker	MCL	WPI 1125	WPI 1186	WPI 1143
CD5	+	+	+	+
CD23	-	-	+	+
CD19	+	+	+	+
CD20	+	+	+	+
FMC7	+	+	-	-
CD3	-	-	-	-
CD4	-	-	-	-
CD7	+	-	-	-
CD8	-	-	-	-
CD10	-	-	-	-
CD38	+	+	+	+
CD45	+	+	+	+
CD56	-	-	-	-
CD122	-	-	-	-
HLA-DR	+	+	+	+
Lambda	+	+	-	-
Kappa	+	+	+	+

These Cell lines were developed from CFS patients. One, (1125) developed MCL; one (1186) was developed from a bone marrow biopsy, 3rd a CLL

HTLV-I: Sequence Conservation and Pathogenesis

As opposed to HIV, HTLV has remarkable genetic stability:
fixation of base substitutions – 1% in 1000 years
complete sequence conservation in ENV and Tax

Pathogenesis:

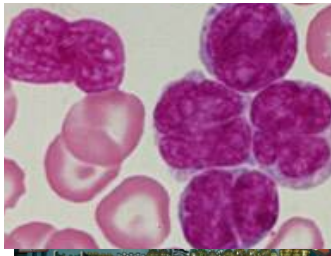
- **Asymptomatic in majority of individuals**
- **5% lifetime risk of developing either type of disease:**

- Adult T cell leukemia

- Clonal malignancy of $CD4^+$ T cells.
- Long latency; Immune deficiency
- Tax and HBZ needed for transformation

- Inflammatory syndromes

HTLV-I associated myelopathy/Tropical spastic paraparesis
- Uveitis
- Arthropathy



One reason for remarkable genetic stability is proviral load increases
By clonal expansion of infected T cells (Wattel et al 69:2863, 1995)

Summary

- We have shown that we can detect HGRV/XMRV footprints in the blood by serology and nucleic acid analysis without any evidence of contamination
- Some CFS plasma contains HGRV/XMRV proteins and antibodies that recognize XMRV/HGRV viral antigens.
- XMRV producing Hematopoietic Cell Lines (B and NK-like) were developed from CFS patients.
- XMRV/HGRV-infected individuals exhibit cytokine profiles characteristic of inflammatory processes
- Sequence data indicate there are different strains of XMRV/HGRVs that can infect humans.

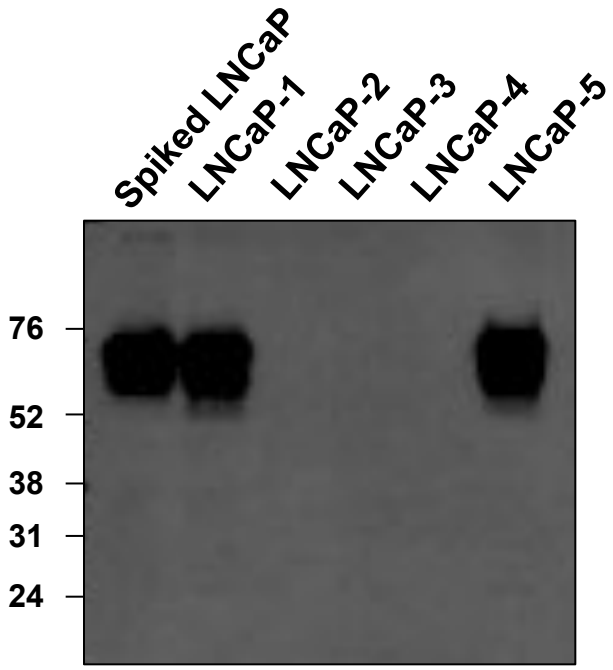
Conclusions

- The pathogenic potential of HGRVs in ME/CFS deserves further exploration

Future Plans

- Obtain full-length sequences of human HGRVs in CFS/ME
- Determine Human Integration Sites for HGRV infection in CFS/ME
- Identification of Tissue Reservoirs for human HGRV in CFS/ME
- Identify mechanisms of HGRV latency

Spiked 22Rv1 Virus in BWG Plasma Spreads to Uninfected Cells



WB: Anti-SFFV Env

In Chronic Diseases Viruses Seldom Come Alone

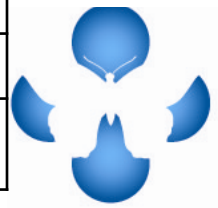
Table 1. Mechanisms of Interactions between HIV-1 and Coinfecting Viruses

Mechanisms	Viruses
Immunoactivation	HCV, HSV-2, CMV, EBV, HTLV-2[a]
HIV-1 *trans*-activation	HSV-2, HTLV-1, JCV[a]
Abnormal production of chemokines	HTLV-1, HHV-6, HTLV-2, MV, GBV-C
CD4, CCR5, or CXCR4 downregulation	HHV-7, GBV-C
Expression of virokines and viroceptors	CMV, HHV-6, HHV-7
Blockage of CD4 T cell cycle	MV
Modulation of cytokine signaling	EBV, adenovirus
Inhibition of apoptosis	CMV, EBV
Aberrant activation of autologous complement	HHV-6, HHV-7
MHC downregulation	CMV, HHV-6, HHV-7

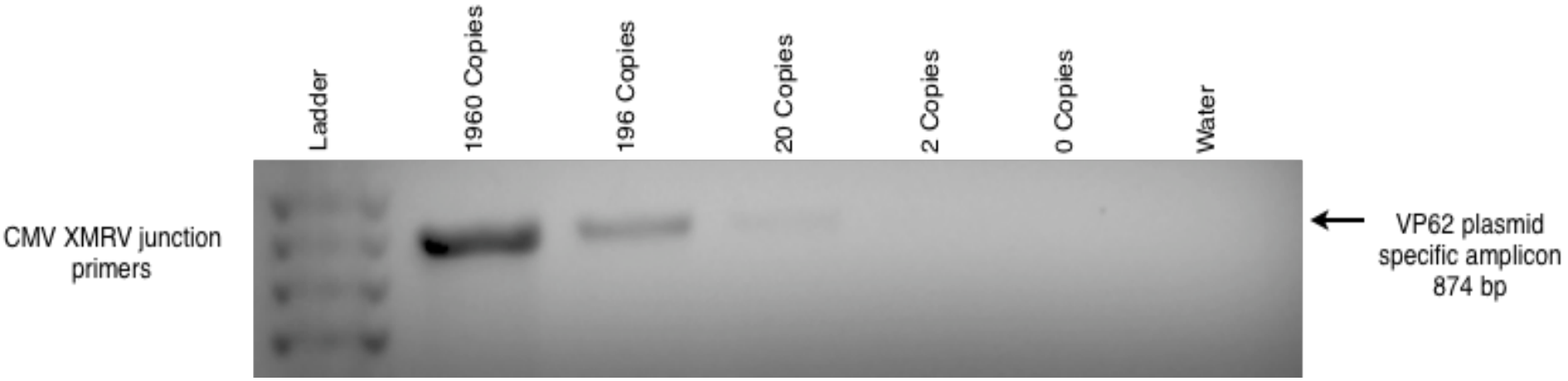
War and Peace between Microbes: HIV-1 Interactions with Coinfecting Viruses:
Cell Host & Microbe 6, November 19, 2009 A. Lisco, C Vanpouille, & L Margolis

Dysregulated Cytokine/Chemokine Production Detected in Plasma from ME/CFS patients: Inflammatory Signature of XMRV/MRV infection

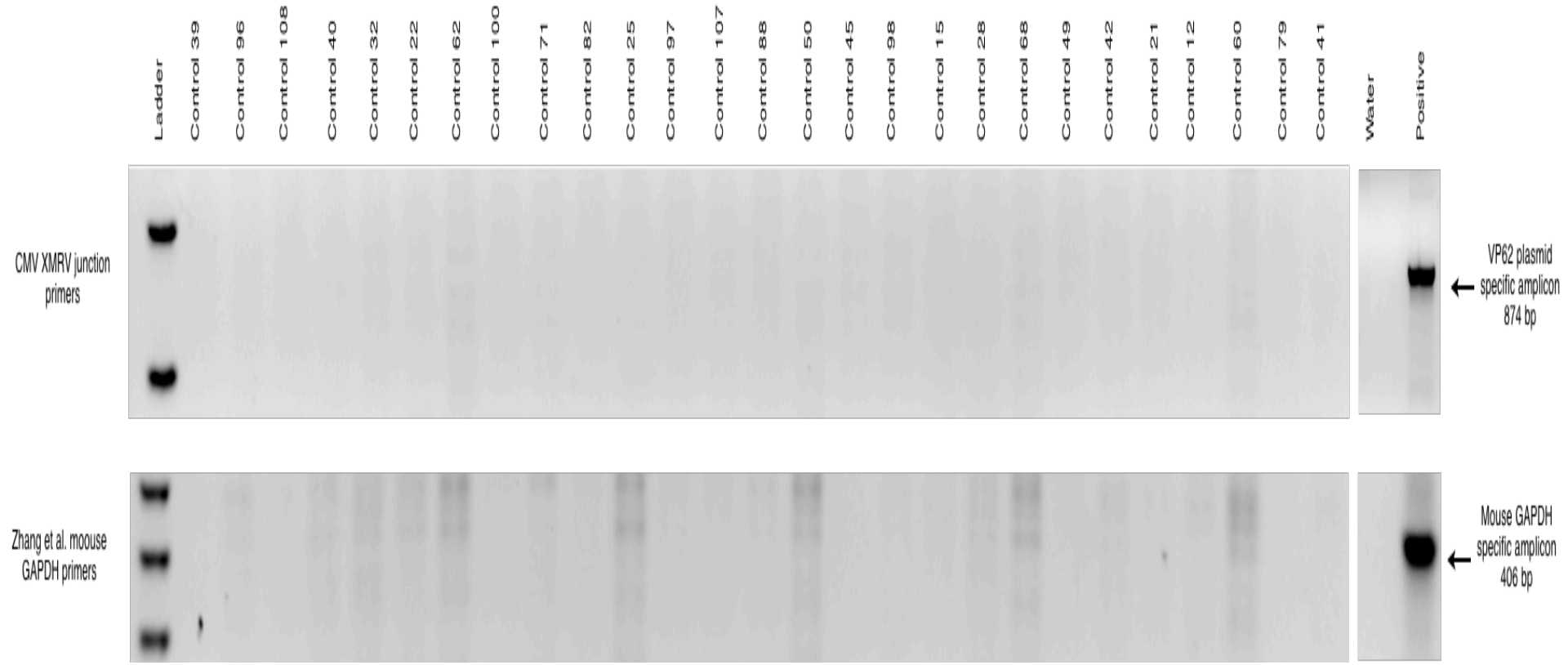
CYTOKINES/ CHEMOKINES	Patient N = 156	Control N=140	P value	FUNCTION IN INFLAMMATION
IL-8	1067	11.1	<0.0001	RNase L and CMV activated
IL-13	28	86	<0.0001	Inhibits inflammatory cytokine production
MIP-1β	1840	157	<0.0001	Elevated in Neurodegenerative disease
TNF-α	109	12.8	<0.0001	Stimulates chronic inflammation
MCP-1	468	421	0.003	Elevated in chronic inflammatory diseases
IL-7	21.1	82	<0.0001	Stimulates proliferation of B and T lymphocytes and NK cells
IFN-α	35	60	<0.0001	Stimulates macrophages and NK cells to elicit an anti-viral response
IL-6	271	29	<0.0001	Stimulates chronic inflammation
MIP-1α	673	91	0.0062	Elevated in Neurodegenerative disease
GM-CSF	108	166	<0.0001	Stimulates proliferation of B and T lymphocytes and NK cells



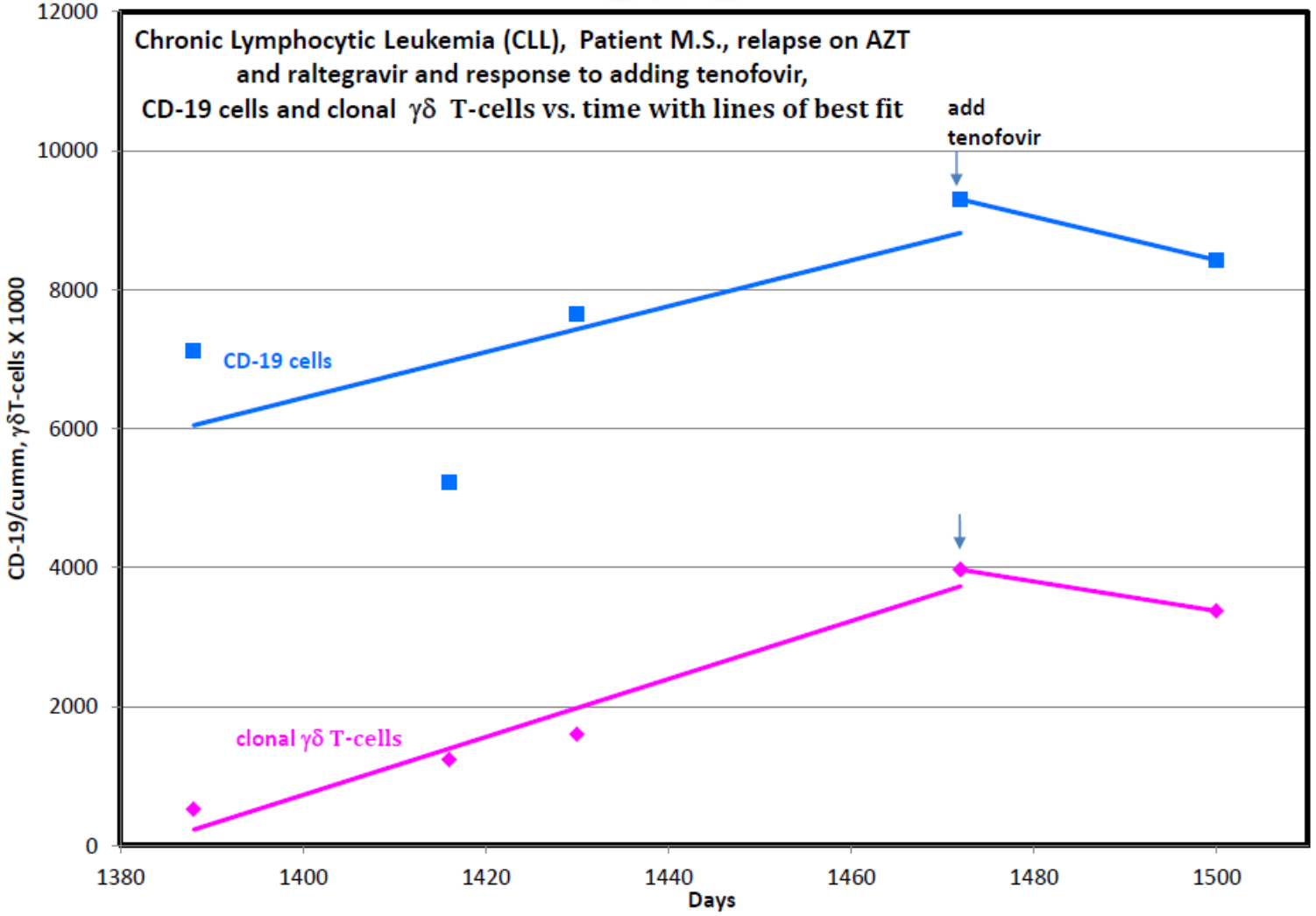
Junction Primer Titration



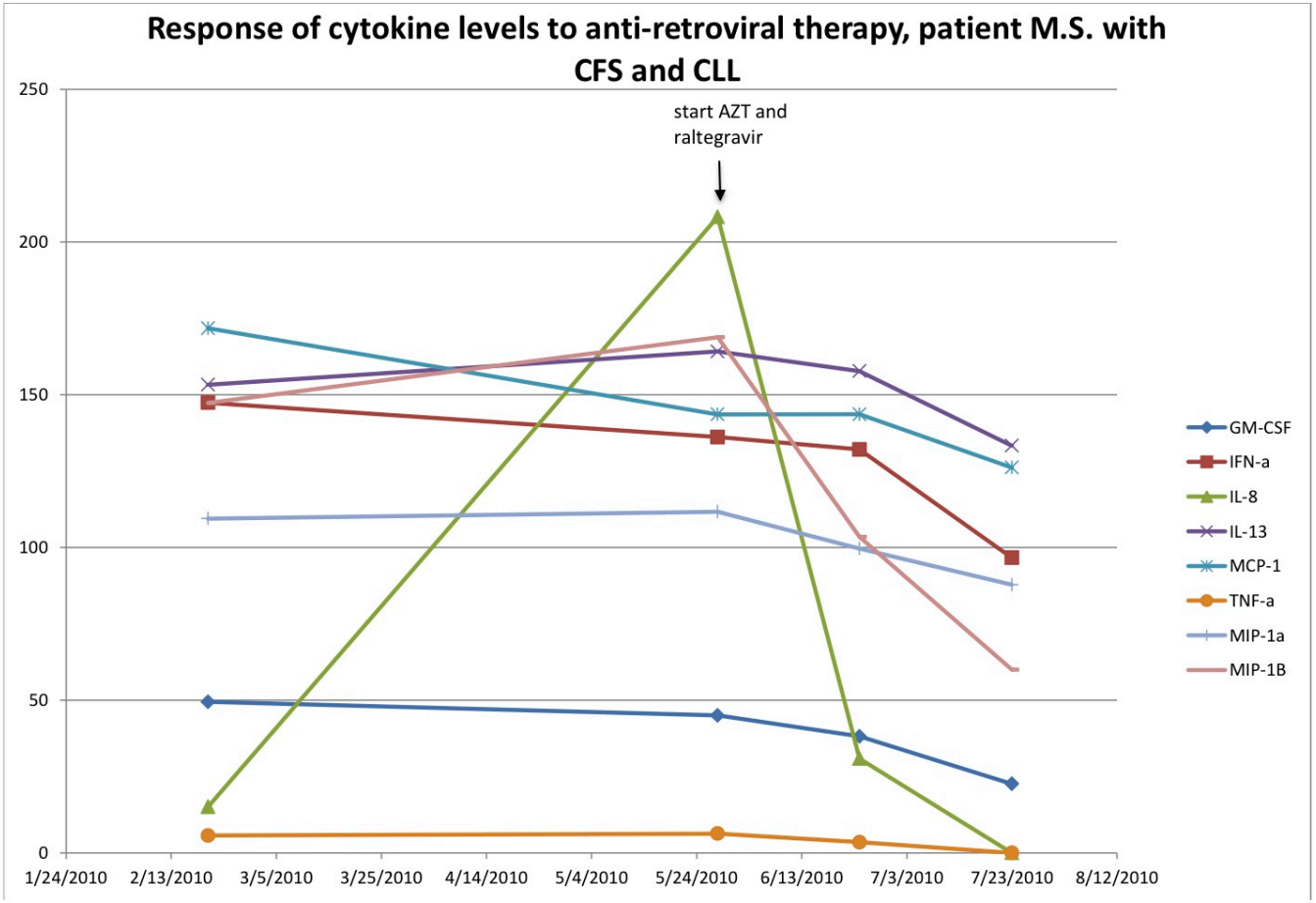
Science Healthy Controls



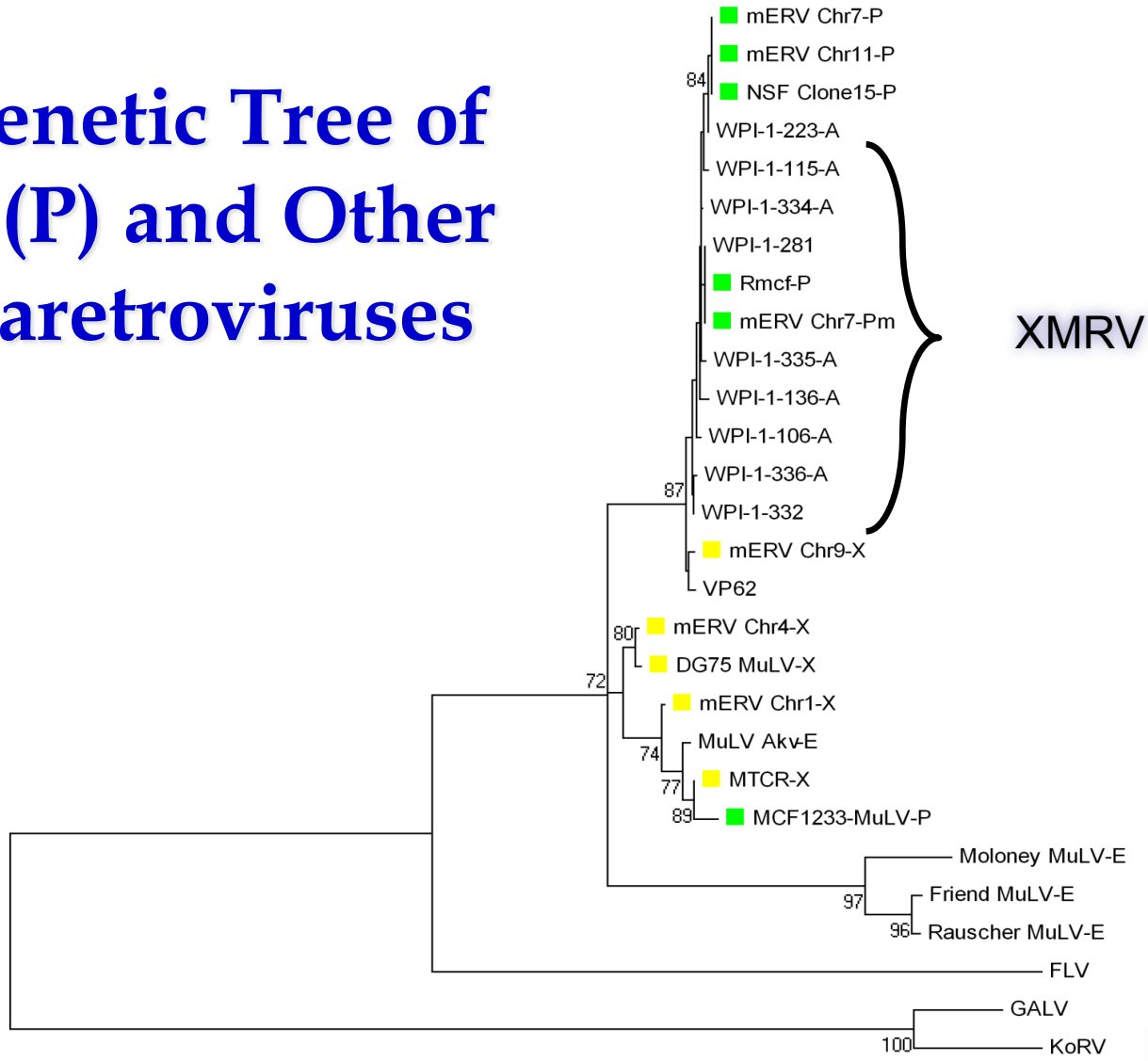
Antiretroviral Therapy of CLL and CFS



Inflammatory Signature Resolves with Antiretroviral Treatment



Phylogenetic Tree of XMRV (P) and Other Gammaretroviruses



Variation in XMRV sequences and low levels of replicating virus in the blood are reasons why some groups fail to detect; therefore we looked for footprints of XMRV infection in unmanipulated plasma