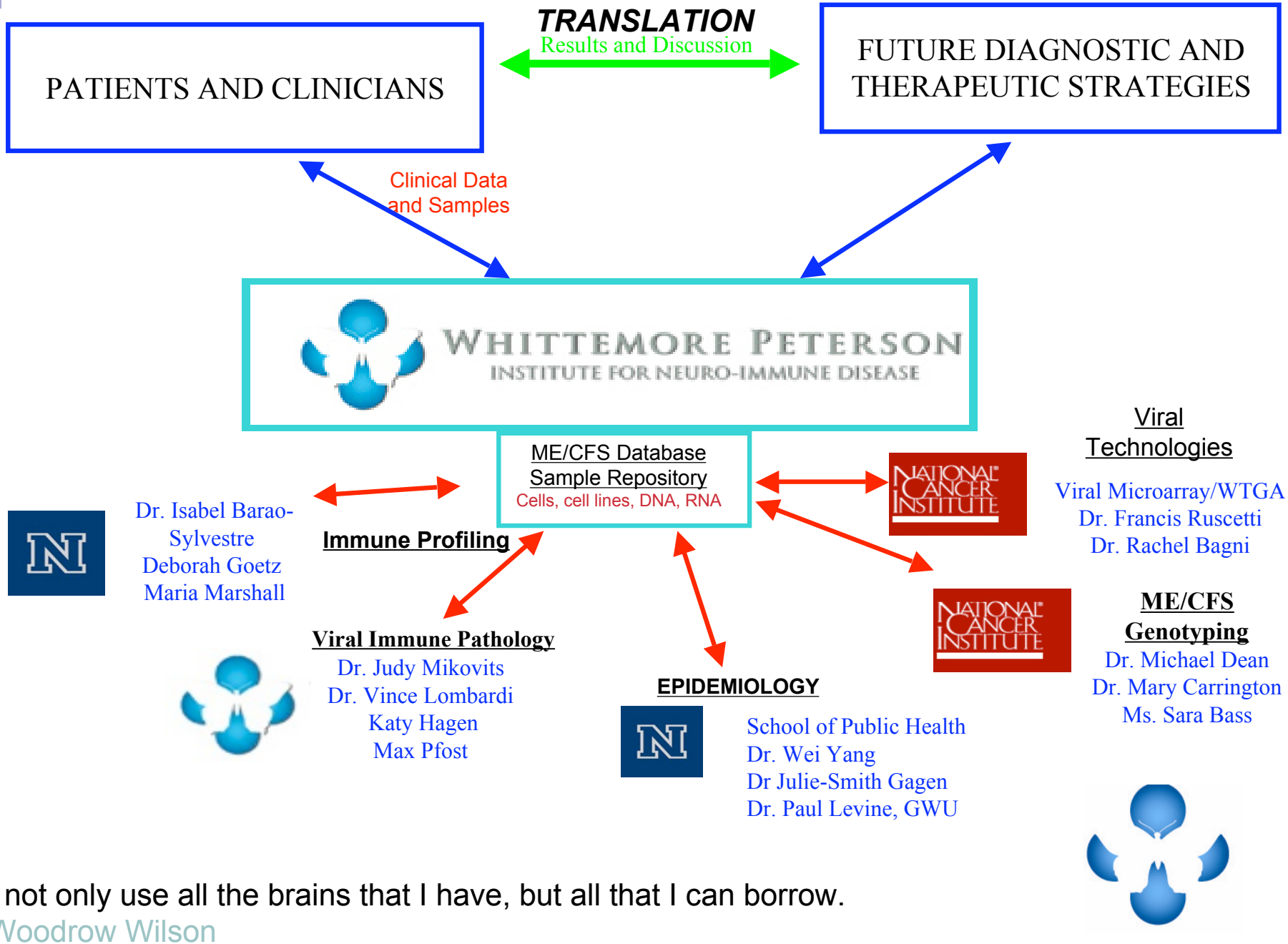
WHITTEMORE PETERSON
INSTITUTE FOR NEURO-IMMUNE DISEASE

Translational Research Towards the Diagnosis of Difficult Medical Cases of ME/CFS

Judy A. Mikovits, PhD

May 29, 2009



I not only use all the brains that I have, but all that I can borrow.
Woodrow Wilson

Nevada CFS Cohort:

- Between 1984 and 1987, a cluster of 300 cases of CFS was identified in Incline Village Nevada. Recently, a subset of the Nevada CFS cohort (77 to date) have presented with clonal T Cell receptor gamma rearrangements. This abnormality is suggestive of a persistent infection and predicative of the development of lymphoma. For these studies, we isolated RNA, DNA and plasma From ~100 of this cohort at two time points Sept 06 and July 07

Viral Microarray Profiling:

Cancer Inflammation Program

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Advanced Biomedical Computing Center

Ming Yi, PhD

Robert M. Stephens, PhD

Advanced Viral Technology Program

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Darren White

Kathryn Hagen

David Pomeranz

The Patients of Sierra Internal Medicine!

Problem: Prior to this study no study has been able to address the expression of all viruses in a single sample from a CFS population with immunological phenotypes suggesting a chronic active viral infection.

- **We addressed this problem using a custom virus oligonucleotide array-based technology that can detect all known mammalian and avian pathogenic viruses.**

- **Each virus is represented by 10 or more features (both conserved and unique regions represented) to provide redundancy and also to allow the identification of new viruses that may arise via recombination.**

- **Human influenza and avian flu viruses are represented as tiling paths to aid in the identification of viral sub-types as well as the precise definition of influenza recombination events.**

- **Both RNA and DNA viruses are represented; Highly sensitive DNA virus 5-10 virus particles; RNA virus: 20-200 virus particles.**

Some Key Points for Analysis of Viral Chips

1. Confounding issues and assumptions:

The detected virus not necessarily is the causative virus for tumor or diseases, but a consequence of impaired immunity.

- Only a portion of the disease population got the intended virus
- The control group has to be very “clean”, no intended virus at all or to large extent
- For virus chip data of each sample, the strongest probe signals are not necessarily from the intended virus. Two-class contrast analysis helpful!

2. Multiple Probes (most of them >=10) in arrays are designed to detect the same virus.

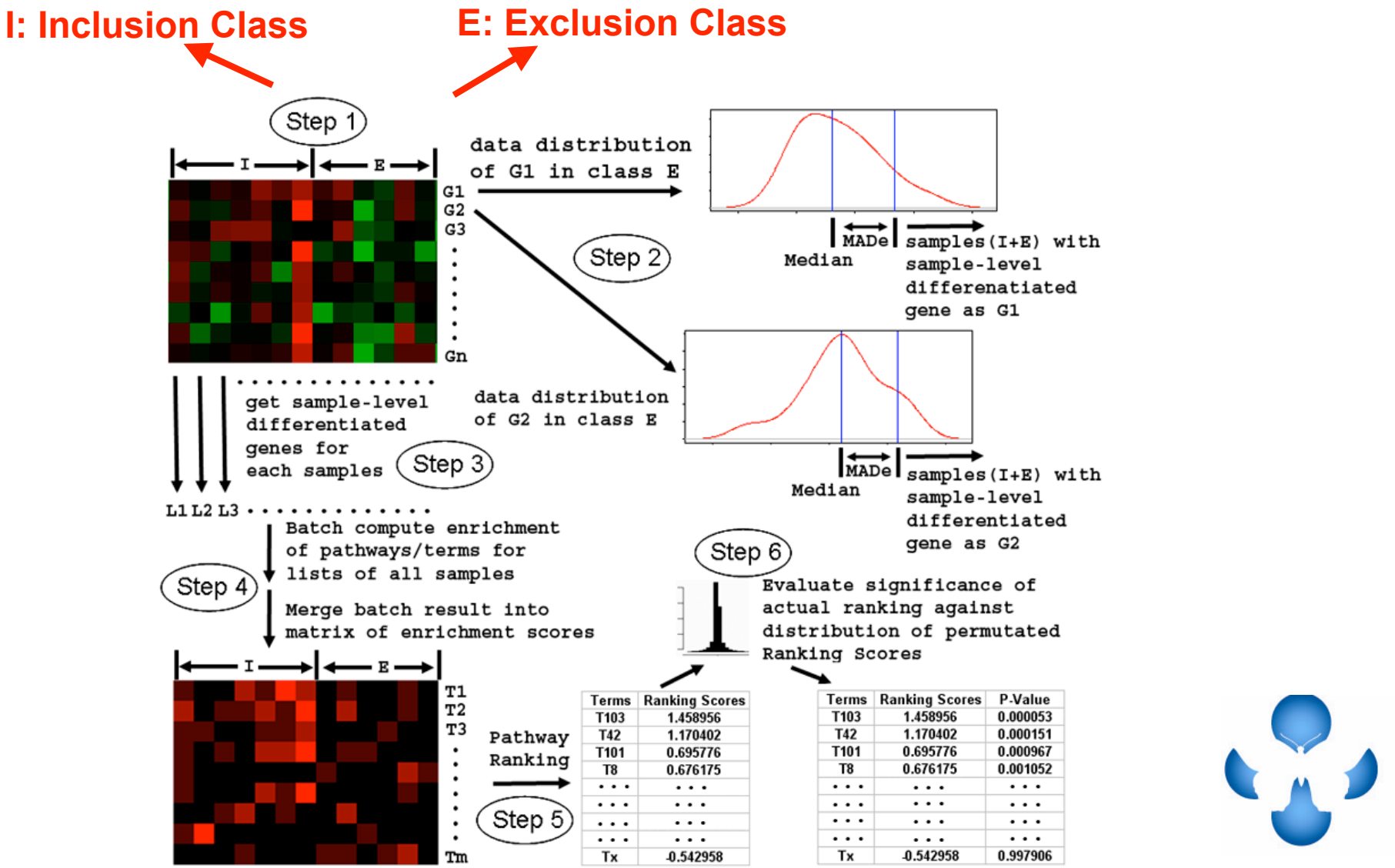
3. Two class contrast : disease vs control.
   Sample-Level-Enrichment-Based Pathway Ranking Method (SLEPR)

4. Conventional gene-level array analysis methods not suitable for such tasks

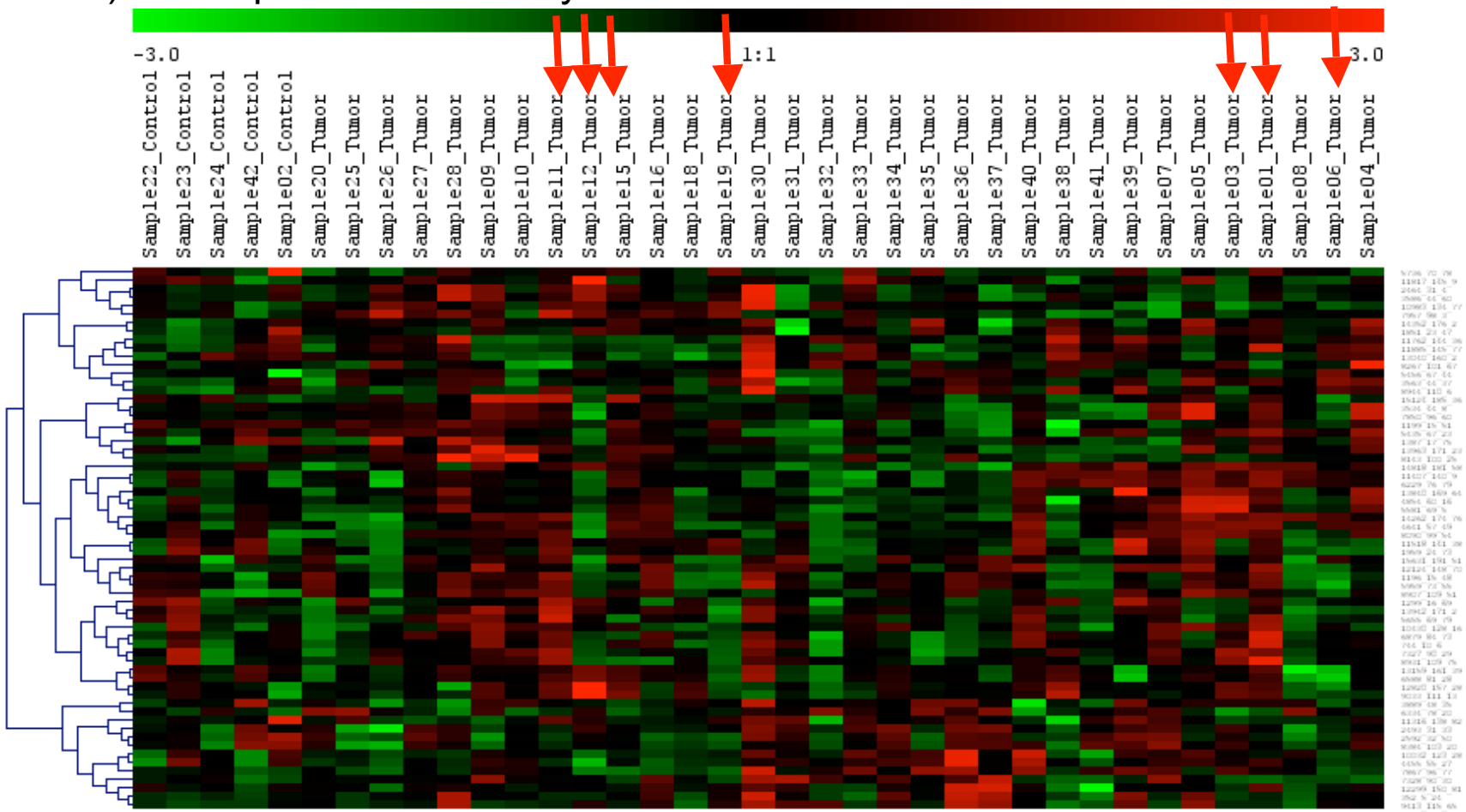
5. Rationale for the analysis: Look for the virus detected by multiple probes with “good” signals, but with biggest contrast in between the two classes.
“clean” Control samples are crucial.

Schematic Work Flow of Sample-Level Enrichment Based Pathway Ranking Method:

Yi and Stephens (2008) PLoS ONE 3(9):e3288)



All 64 probes signals of human herpesvirus 4 (in Z-scores: reflecting relative expression level) in samples of Microarray #2



Samples having more probes with higher signals for human EBV (HHV4)

Individual Sample Ranking Data

Higher Ranks

Sample26_CFS

Virus	ProbeNum
Human herpesvirus 5	241
Human endogenous retrovirus clone M3.5	10
Kaposi's sarcoma-associated herpesvirus long unique region, 80 putative ORF's and kaposin gene, complete cds	77
Influenza A virus segment 1	78
Dengue virus type 1	10
Simian-Human immunodeficiency virus	10
Influenza B virus (B/Memphis/12/97-MA) segment 5	56
Human T-lymphotropic virus 1	12
Influenza A virus segment 4 (H2)	58

Sample23_CFS

Virus	ProbeNum
HHV8 long unique region, 80 putative ORF's and kaposin gene, complete cds	77
Human herpesvirus 5	241
Human herpesvirus 4	64
Human endogenous retroviral DNA (4-1)	10
Human endogenous retrovirus clone M3.5	10
Human herpesvirus 1	38
Equine herpesvirus 2	17
Tick-borne encephalitis virus	10
Human T-lymphotropic virus 2	10

S27_Control

Virus	Probe Num
Human herpesvirus 5	241
Human endogenous retrovirus clone M3.5	10
HHV8 long unique region, 80 putative ORF's and kaposin gene, complete cds	77
Human herpesvirus 4	64
Human herpesvirus 2	39
Human herpesvirus 1	38
Ictalurid herpesvirus 1	25
Equine herpesvirus 2	17
Human herpesvirus 7	26
Human endogenous retrovirus pHE.1 (ERV9)	10

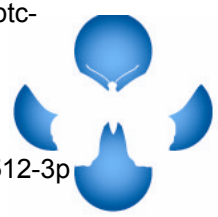
Lower Ranks

Top 7 viruses in 4 controls by individual ranking

Top	S21_Control	S27_Control	S29_Control	S30_Control
1	Ictalurid herpesvirus 1	Human herpesvirus 5	Human herpesvirus 5	Human herpesvirus 5
2	Rubella virus	Human endogenous retrovirus clone M3.5	Kaposi's sarcoma-associated herpesvirus long unique region, 80 putative ORF's and kaposin gene, complete cds	Kaposi's sarcoma-associated herpesvirus long unique region, 80 putative ORF's and kaposin gene, complete cds
3	Avian adeno-associated virus ATCC VR-865	Kaposi's sarcoma-associated herpesvirus long unique region, 80 putative ORF's and kaposin gene, complete cds	Human herpesvirus 4	Human herpesvirus 4
4	Human T-lymphotropic virus 1	Human herpesvirus 4	Human herpesvirus 1	Human endogenous retrovirus clone M3.5
5	Human endogenous retrovirus pHE.1 (ERV9)	Human herpesvirus 2	Equine herpesvirus 2	Human herpesvirus 1
6	Human endogenous retrovirus clone M3.5	Human herpesvirus 1	Influenza A virus segment 1	Human herpesvirus 2
7	Adeno-associated virus 3B	Ictalurid herpesvirus 1	Human herpesvirus 2	Ictalurid herpesvirus 1

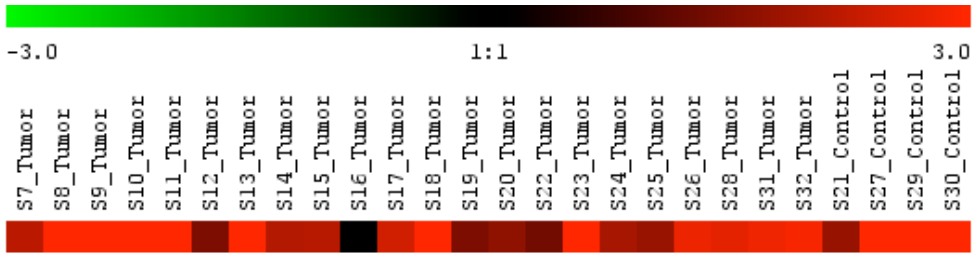
Top 6 viruses of all 5 tumors in individual ranking Microarray #1

S7_Tumor	S8_Tumor	S9_Tumor	S10_Tumor	S11_Tumor	S12_Tumor
Human endogenous retrovirus clone M3.5	Human endogenous retrovirus clone M3.5	Human endogenous retrovirus clone M3.5	Human endogenous retroviral DNA (4-1)	Human endogenous retroviral DNA (4-1)	Human endogenous retrovirus clone M3.5
Ictalurid herpesvirus 1	Human endogenous retroviral DNA (4-1)	Polyomavirus sp.	Ictalurid herpesvirus 1	Human endogenous retrovirus clone M3.5	Human endogenous retrovirus pHE.1 (ERV9)
Human endogenous retrovirus pHE.1 (ERV9)	Human endogenous retrovirus pHE.1 (ERV9)	Human endogenous retroviral DNA (4-1)	Human endogenous retrovirus clone M3.5	Ictalurid herpesvirus 1	Human endogenous retroviral DNA (4-1)
Human endogenous retroviral DNA (4-1)	Ictalurid herpesvirus 1	Ictalurid herpesvirus 1	Human endogenous retrovirus pHE.1 (ERV9)	Human herpesvirus 1	ptc-miR169o|ptc-miR169p
Adeno-associated virus 3B	Human endogenous retrovirus HERV-K-T47D	Human endogenous retrovirus pHE.1 (ERV9)	Rat cytomegalovirus Maastricht	Human herpesvirus 7	ath-miR169d|ath-miR169e|ath-miR169f|ath-miR169g|ptc-miR169n
Chimpanzee cytomegalovirus	Hepatitis D virus	Rat cytomegalovirus	Human coxsackievirus B2	Human endogenous retrovirus pHE.1 (ERV9)	hsa-miR-512-3p



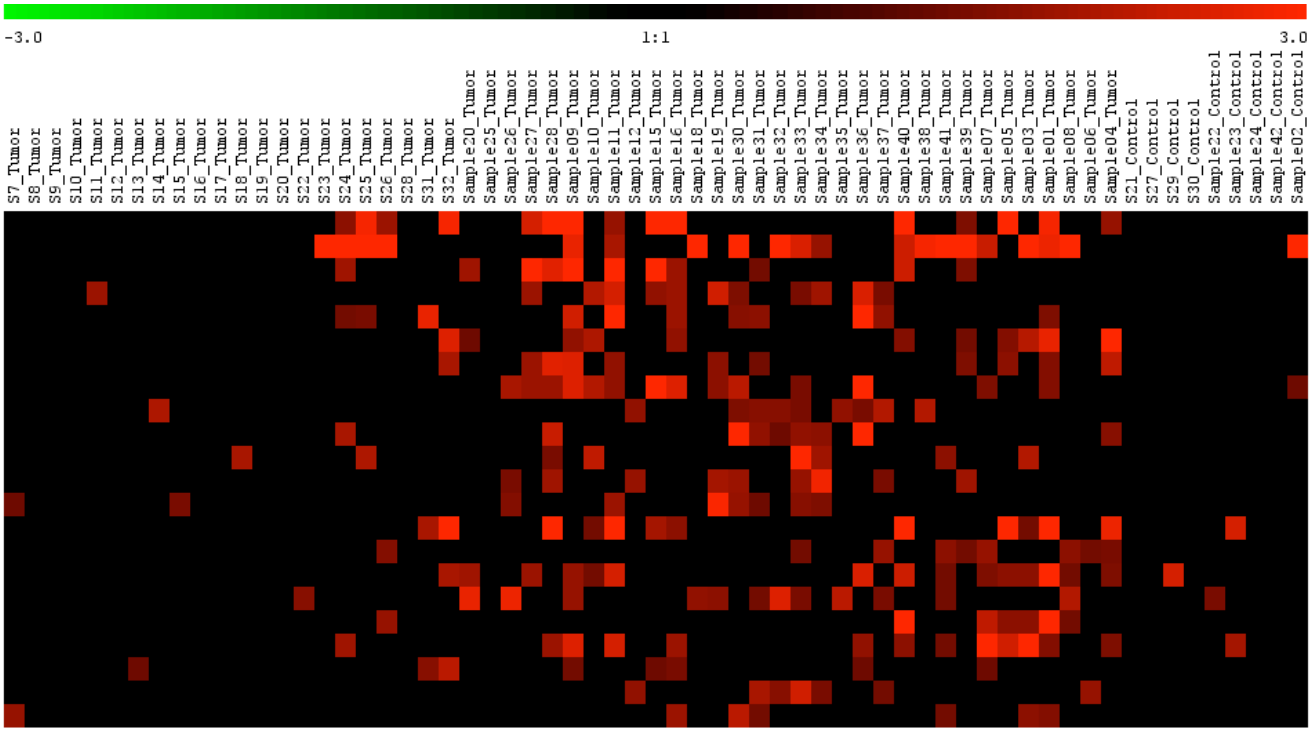
Class-Contrast-Based Ranking method lowered rank of HERV 4.1 from #1 to #1319

In Microarray #1, Human endogenous retroviral DNA (4-1) showed
Up as top ranked virus in many CFS samples , however, it is also
Highly ranked in many of the controls (ranked as top 9 in S21 and S29),
which immediately reduced its significance using class-contrast-based ranking method
Actually Put it into rank 1319



Human endogenous retroviral DNA (4-1)

Top 22 Viruses of SLEPR ranking result: Rank Result same as The result of Normalized Pooled Data



Higher Ranks

Human herpesvirus 5
Human herpesvirus 7
Feline leukemia virus
Pestivirus type 1
Equine herpesvirus 1
Human adenovirus F
Adeno-associated virus 4
Modoc virus
Influenza A virus segment 4 (H5)
Influenza A virus segment 1
Human herpesvirus 6B
Influenza A virus neuraminidase gene (N4)
Influenza A virus segment 5
Human herpesvirus 2
"La Crosse virus strain Human/78 segment M
Theilovirus
Mouse parvovirus 1
Porcine enterovirus B
Macaca mulatta rhadinovirus
Human adenovirus C
Influenza A virus segment 4 (H1)
Murine polyomavirus strain A2

Lower Ranks

In SLEPR result, any thing shown in red:
at least enrichment p<0.05 (ES score >1.3) and List Hits>=2

CMV and HHV7 : Top two ranking viruses in ME/CFS with clonal TCRg rearrangements

CONCLUSION: CFS Patients expressed significantly higher levels of all viruses. CMV>HHV6>HHV7>EBV were identified as the most actively expressed herpes viruses in this CFS cohort. These data suggest that multiple highly active herpes virus infections distinguish CFS patients from healthy and underscore the role of the innate immune deficiencies in the pathogenesis of CFS.

TRANSLATION: FUTURE PLANS:

- We (The WPI and NCI) have developed a custom herpes virus 4, 5, 6A, 6B and 7 chip to quantify expression of each virus from same sample as a diagnostic tool. This chip will be licensed by VIPDx for clinical use.
- The herpes chip and cytokine signature diagnostic tools can be licensed by physicians world-wide to stratify patients monitor clinical trials of combinations of antiviral, immune modulating and anti-inflammatory drugs

Cytokine and Chemokine Profiling

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Julie Smith-Gagen, PhD

The Patients and controls who graciously volunteered to participate!

Rationale:

- Chronic Innate Immune activation by pathogenic triggers in a genetically susceptible host mediate the pathogenesis through a cytokine/chemokine storm.

- Multiplex suspension arrays afford the opportunity to analyze the complex relationships between the cytokines and clinical disease and to determine if clinical subgroups of disease could be identified based on distinct cytokine profiles

Clonal TCRg Rearrangement Testing CFS Cohort

RATIONALE:

Clonal TCRg Rearrangements:

- Suggest chronic active infection particularly CMV
- Predictive of lymphoma development

gd T cells :

- Play active role in regulation and resolution of pathogen induced immune responses
- Accumulate at sites of inflammation
- Associated with Viral, Parasitic and bacterial Infections
- Associated with autoimmune diseases
- Upregulate MIP1a, B, TNFa, IL-10, IFNg

Clinical Criteria for Testing:

- Acute (viral) onset CFS
- Lymphadenopathy and/or splenomegaly

Inflammatory Chemokines Previously Linked to Chronic Inflammatory Diseases are Up Regulated in ME/CFS

INFLAMMATORY CHEMOKINES					
	Chemokine	Patient N = 118	Control N=138	P value	
CXC	IL-8	1045	13	<0.0001	RNAse L and CMV activated
	MIG	48	80	<0.0001	
	IP-10	98	32	<0.0001	Interferon response protein
CC	MCP-1	468	421	0.0003	
	MIP-1a	763	91	0.0062	Important in Neurodegenerative disese
	MIP-1b	1985	164	<0.0001	Important in Neurodegenerative disese
	RANTES	27107	9564	0.0018	
	EOTAXIN	271	96	<0.0001	

Mean values in pg/ml: Red denotes up regulation, Blue denotes down regulation

Shift of TH1, TH2 and TH17 Functional T-cell Subsets in ME/CFS

FUNCTIONAL T-CELLS					
	Cytokine	Patient N = 118	Control N=138	P value	
TH_1	IL-2	113	28	<0.0001	
	IFN-g	16	13	0.072	
	IL-12	289	211	0.0001	
TH_2	IL-4	40	55	0.0003	
	IL-5	7	21	<0.0001	
	IL-13	28	86	<0.0001	
	IL-10	70	49	0.56	
T_{reg}	CD-25	289	476	0.09	
TH_{17}	GM-CSF	108	166	<0.0001	
	IL1B	500	56	<0.001	
	IL-6	336	29	<0.0001	
	TNF-a	148	13	<0.0001	

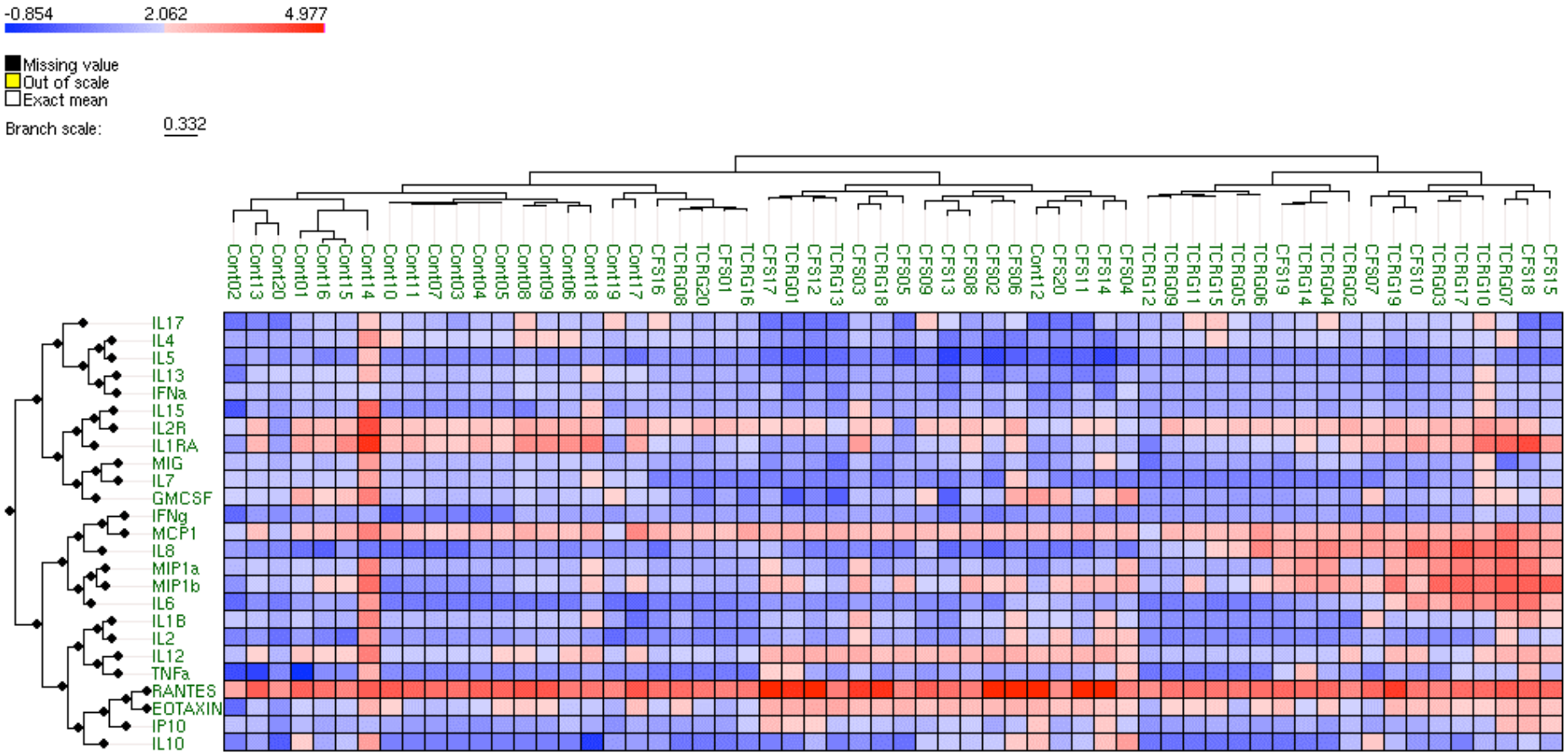
Mean values in pg/ml: Red denotes up regulation,
Blue denotes down regulation

Other Dysregulated Cytokines in ME/CFS

OTHER CYTOKINES				
Cytokine	Patient N = 118	Control N=138	P value	Function
INF-a	35	60	<0.0001	stimulate both macrophages and NK cells to elicit an anti-viral response
IL-1RA	20	14	<0.0001	modulates a variety of interleukin 1 related immune and inflammatory responses
IL-7	160	60	<0.0001	stimulates proliferation of B and T lymphocytes and NK cells

Mean values in pg/ml: Red denotes up regulation,
Blue denotes down regulation

Cluster Analysis Reveals Distinct Subgroups



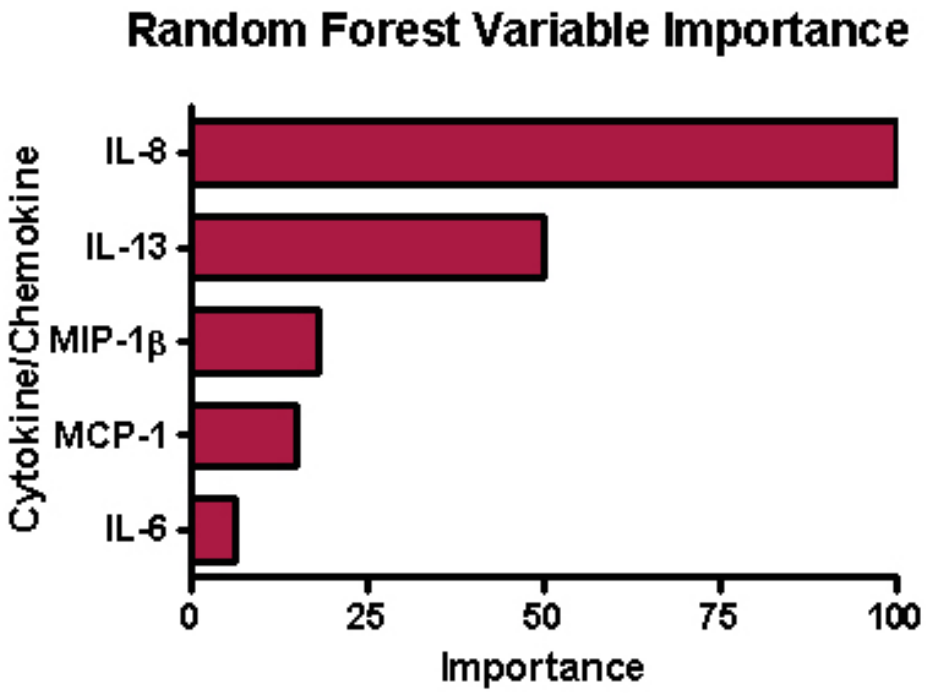
Random Forest Analysis

- The RF algorithm uses ensemble of unpruned classification or regression trees produced through bootstrap sampling of the training data and random feature selection in tree generation Cytokines and Chemokines

- Prediction is made by a majority vote of the predictions of the ensemble.

- The FR algorithm is uniquely suited for cytokine and chemokine analysis in that in can handle highly skewed cytokine and a chemokine value well and weighs the contribution of each cytokine or chemokine according to its relatedness with others.

Random Forest Predicts 5 Cytokine/Chemokine Signature of ME/CFS with 94% Specificity and Sensitivity



Random Forests Prediction Success.

Actual Class	Total Cases	Percent Correct	Control N=135	Patient N=121
Control	138	93.48	129	9
Patient	118	94.92	6	112

Genetic Profiling:

Cancer Inflammation Program

Carrington Lab

SARA BASS

Mary Carrington

Pat Martin

Xiaojiang Gao

Ying Qi

Fuh-Mei Duh

Darlene Marti

Yalun Lui

Rasmi Thomas

Smita Kulkarni

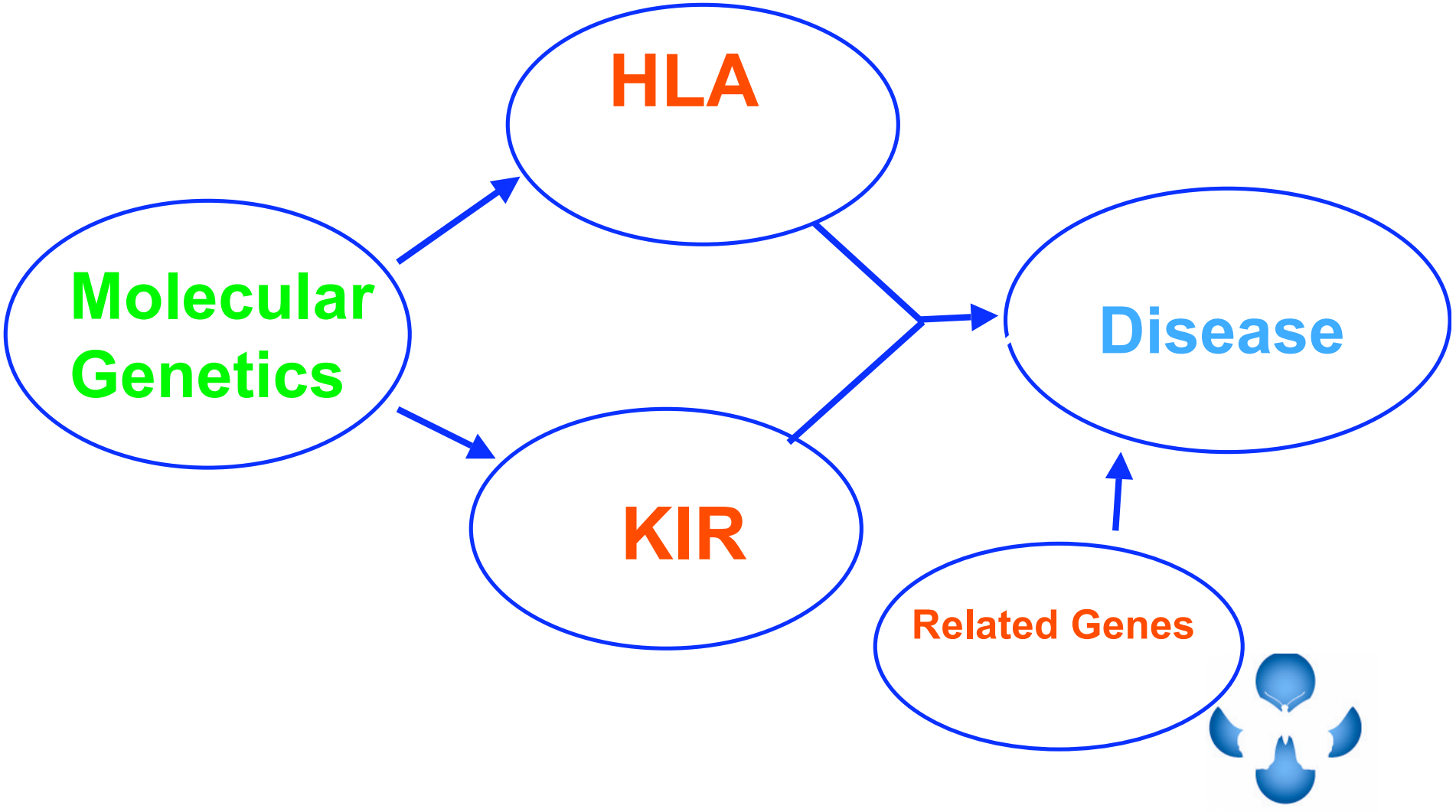
Mike Dean

Frank Ruscetti

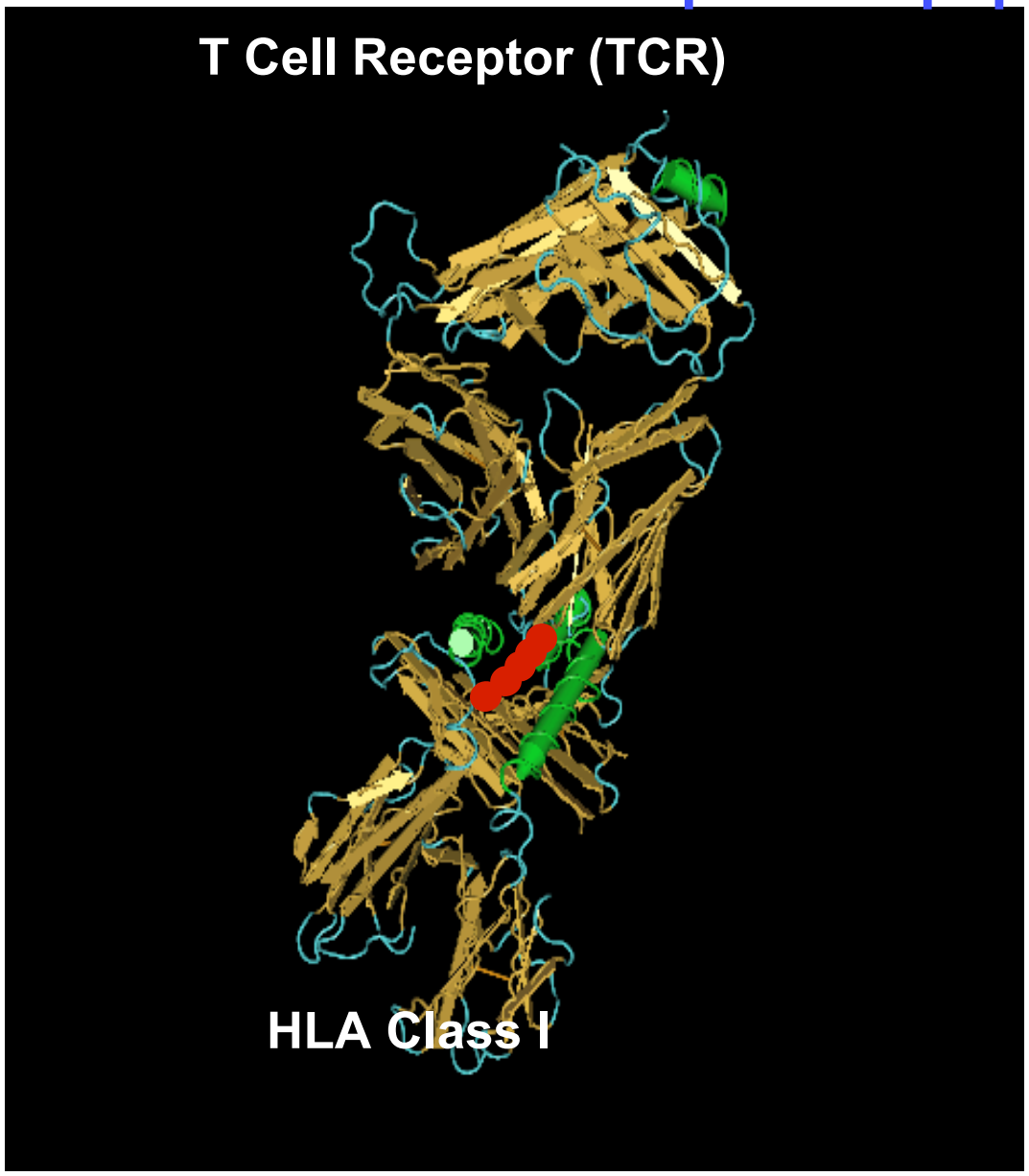
Darren White

Kathryn Hagen

Polymorphic immune response genes affect the level of host resistance against human disease. Functional and molecular genetic studies validate and clarify the nature of these associations.



The HLA class I molecules present peptides to T cells

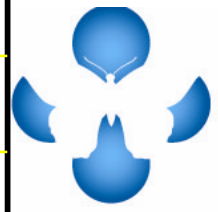


No significant associations found for HLA-A **(p < .05)**

	Cases N=67	Controls N=97		
	% (N)	% (N)	OR	p value
A*26	11 (7)	4 (4)	2.91	0.11
A*31	6 (4)	5 (5)	1.25	0.74
A*03	25 (16)	25 (24)	1.04	1.00
A*68	10 (6)	9 (9)	1.03	1.00
A*23	6 (4)	6 (6)	1.03	1.00
A*02	46 (29)	45 (44)	1.03	1.00
A*24	17 (11)	25 (24)	0.64	0.33
A*01	25 (16)	35 (34)	0.63	0.22
A*11	10 (6)	15 (15)	0.58	0.34

No significant associations found for HLA-C

	Cases N=67 % (N)	Controls N=97 % (N)	OR	p value
C*16	8 (5)	3 (3)	2.56	0.27
C*05	26 (17)	16 (15)	1.89	0.11
C*06	25 (16)	18 (17)	1.50	0.33
C*01	6 (4)	4 (4)	1.49	0.72
C*12	12 (8)	11 (10)	1.19	0.80
C*04	25 (16)	22 (21)	1.15	0.71
C*02	14 (9)	14 (13)	1.01	1.00
C*03	18 (12)	22 (21)	0.80	0.69
C*08	5 (3)	7 (7)	0.61	0.74
C*07	43 (28)	56 (53)	0.60	0.15



HLA-B*44 is associated with increased susceptibility to ME/CFS

	Cases **N=67**	Controls **N=97**		
	% (N)	% (N)	OR	p value
B*44	**37 (23)**	**19 (18)**	**2.56**	**0.02**
B*57	16 (10)	7 (7)	2.44	0.11
B*18	13 (8)	6 (6)	2.22	0.16
B*38	6 (4)	4 (4)	1.59	0.71
B*35	19 (12)	17 (16)	1.20	0.68
B*51	13 (8)	13 (12)	1.04	1.00
B*40	11 (7)	11 (11)	0.98	1.00
B*14	5 (3)	7 (7)	0.65	0.74
B*08	14 (9)	22 (21)	0.61	0.30
B*07	19 (12)	29 (28)	0.58	0.19
B*13	3 (2)	6 (6)	0.50	0.48
B*27	6 (4)	13 (12)	0.48	0.28
B*15	8 (5)	16 (15)	0.47	0.22

The association with B*44 does not appear to be subtype specific

		Cases	Controls		
		% (N)	% (N)	OR	p value
B*4402	yes	26 (16)	14 (13)	2.22	0.06
	no	74 (46)	86 (83)		
B*4403	yes	15 (9)	5 (5)	3.09	0.08
	no	85 (53)	95 (91)		

NK cells kill targets that do not express *HLA* class I in the "normal" context

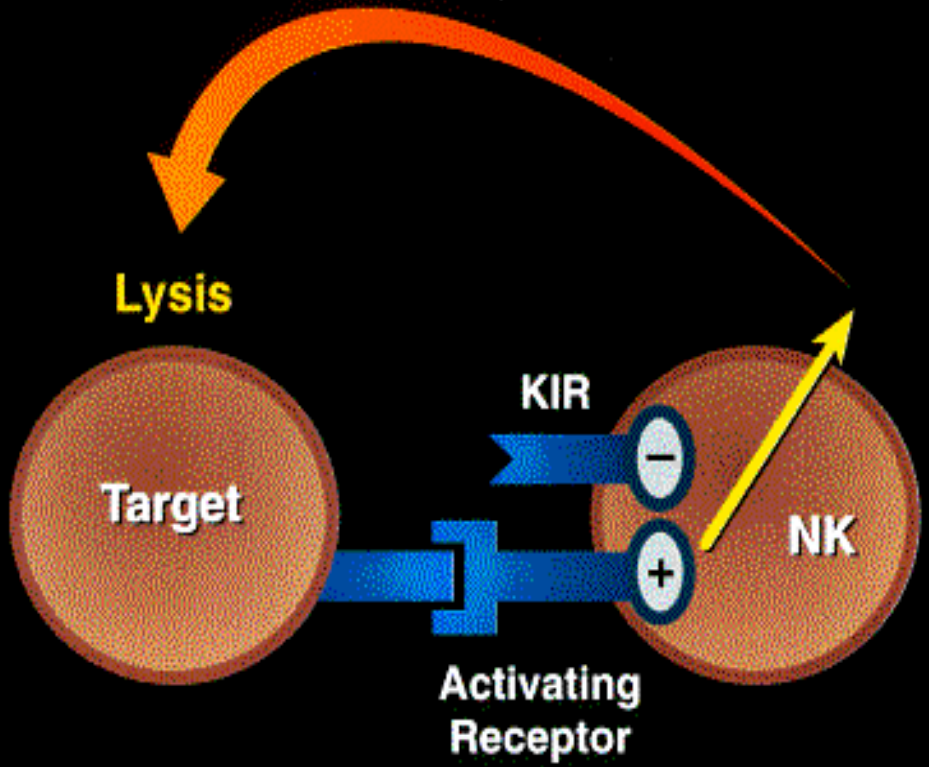


Figure 2

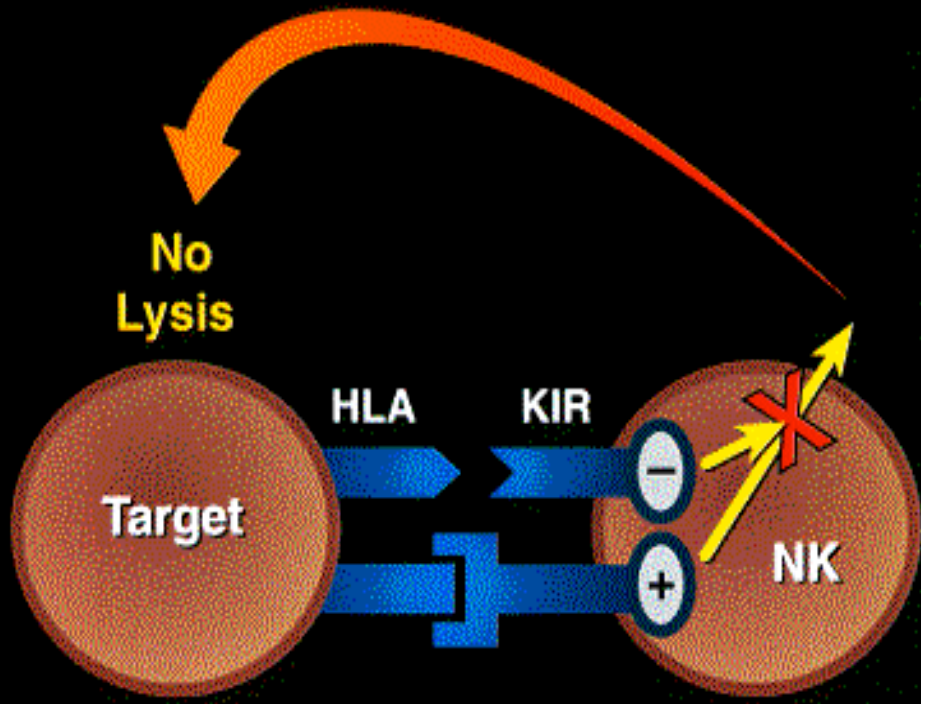
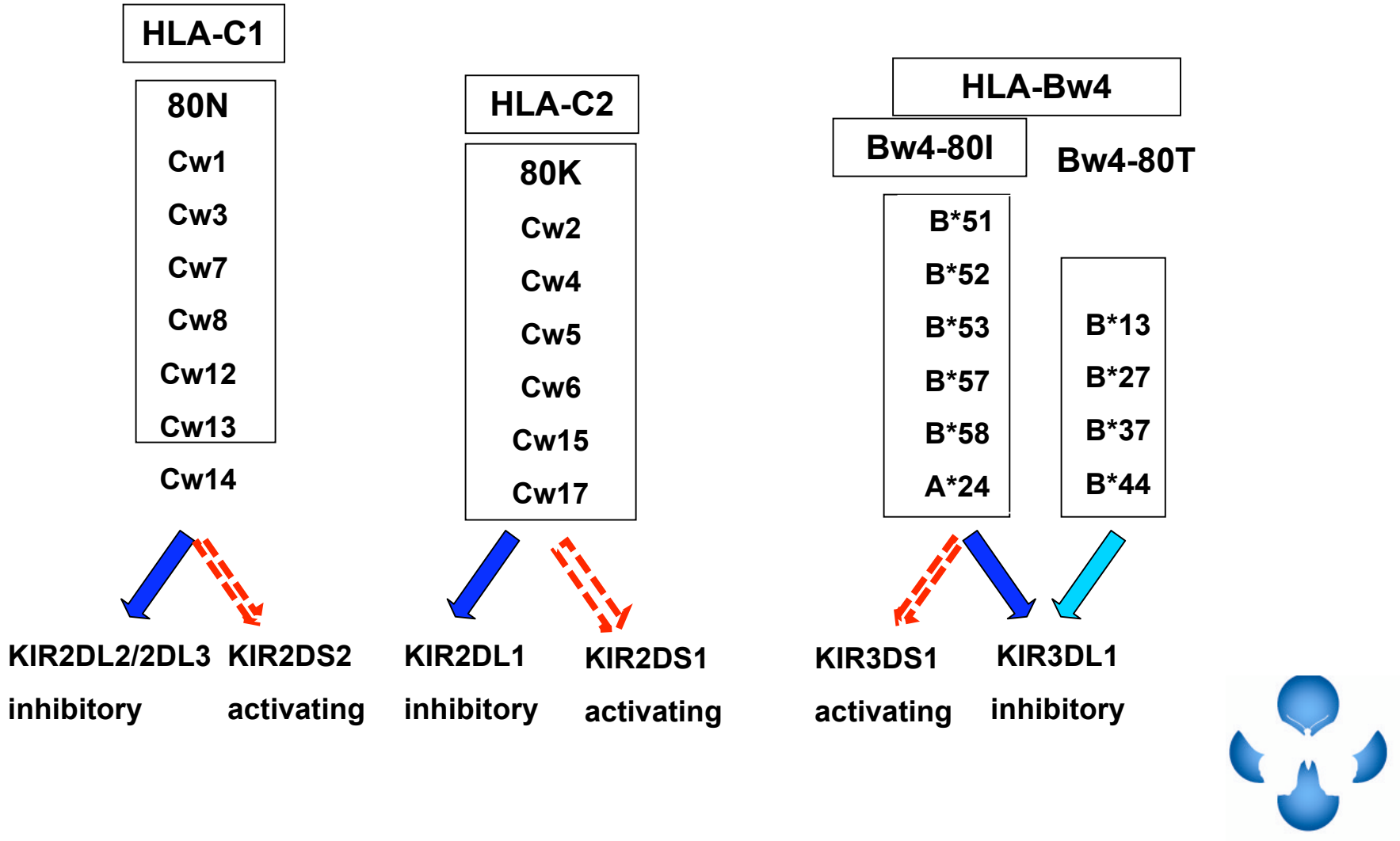


Figure 1

HLA Class I ligand binding groups for *KIR*



HLA-Cw group 1 homozygosity is protective. HLA-Cw group heterozygosity is associated with increased susceptibility.

	Cases % (N)	Controls % (N)	OR	p value
c1/c1	18 (12)	40 (37)	0.36	0.008
c1/c2	63 (41)	44 (42)	2.20	0.02
c2/c2	18 (12)	16 (15)	1.22	0.64

HLA Bw4 alleles are associated with increased disease susceptibility

	Cases % (N)	Controls % (N)	OR	p value
Bw4	79 (49)	64 (62)	2.13	0.05
Bw4 80T	48 (30)	35 (34)	1.74	0.10
Bw4 80I	39 (24)	40 (39)	0.94	0.87

Study Summary

- HLA-B*44 is significantly associated with susceptibility to ME/CFS (OR = 2.56, p = 0.02).
- There was no significant association with HLA-A or -C.
- HLA-Bw4, a ligand for *KIR* is associated with susceptibility (OR = 2.13, p = 0.05)
- HLA-Cw group 1 homozygosity was associated with protection (OR= 0.36, p = 0.008) while HLA-Cw group heterozygosity was associated with increased susceptibility to CFS (OR = 2.2, p = 0.02).
- We are currently analyzing 100 additional patients to verify these associations.

Conclusion

- Taken together these data suggest that this systems biology approach is and effective strategy for the development of serum biomarkers to stratify subgroups of ME/CFS patients for appropriate anti-inflammatory, immune modulating, antimicrobial and antiviral therapeutics