Viral Associations with Chronic Fatigue Syndrome and the Prevalence of Artifacts

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Abstract

Chronic fatigue syndrome (CFS) is a complex, heterogeneous disease affecting more than 1 million Americans. Despite extensive research into the etiology of CFS, no definitive cause of CFS has been determined; however, there is evidence supporting an infectious etiology.

In 2009, a study that reported 67% of CFS patients tested positive for the new gammaretrovirus xenotropic murine leukemia virus-related virus (XMRV), as compared to only 3.7% of healthy controls, reignited the quest for a causative viral agent for CFS. Confirming this high prevalence of XMRV in CFS patients would have been a major step forward in defining CFS. Instead, we found that murine DNA contamination in human samples or reagents can result in false-positives when using the sensitive nested PCR to detect XMRV employed by the 2009 study. Failing to detect anti-XMRV antibodies in the sera of patients who previously tested positive for XMRV using the nested PCR confirmed that the samples were, in fact, false-positives.

Two viruses under investigation as possible triggers for CFS, Epstein-Barr virus (EBV) and human herpesvirus-6 (HHV-6), can transactivate the human endogenous retrovirus-K18 (HERV- K18) *env* gene, whose product encodes a superantigen (SAg). SAgs are microbial proteins that greatly over-stimulate the immune system. Thus, these viruses could lead to induction of the HERV-K18 *env* SAg, which then could lead to overstimulation of the immune system causing the symptoms of CFS. To test this model, we first attempted to associate increased HERV-K18 *env* transcripts with CFS symptom severity; however, we found HERV-K18 *env* expression was the same in CFS patients as healthy controls and did not correlate with CFS symptoms. Next, we attempted to link

the ubiquitous herpesviruses already suspected as triggers to CFS and inducers of HERV-K18 *env*, HHV-6 and HHV-7, to HERV-K18 *env* by measuring viral copy number in CFS patients; however, viral copy number failed to correlate with HERV-K18 *env* expression. Lastly, because EBV was shown to transactivate HERV-K18 *env*, we examined the HERV-K18 *env* genotype in CFS patients with a history of EBV-infectious mononucleosis (EBV-IM) and compared it to CFS patients without a history of EBV-IM. Patients who develop CFS after having EBV-IM could be associated with a susceptible HERV-K18 *env* genotype that is not present in other CFS patients; however, we failed to find a genotypic difference between these CFS populations in our small cohort. Typical gene association studies employ hundreds, if not thousands, of samples to find significance; thus, future studies should be done on a much larger cohort.

Overall, these studies helped focus the HERV-K18 model on a more specific, stratified group of CFS patients with high viral copy numbers of HHV-6 or HHV-7. Also, these studies helped prove, without a doubt, that XMRV is not associated with CFS. Finally, these studies highlight the importance of using multiple methods to confirm results of major new findings.

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List of Abbreviations

°C – Degrees Celsius

ΔΔCT – delta delta Cycle Threshold

µl – microliter

ACTH – Adrenocorticotropic Hormone

Ag – Antigen

bp – base pairs

CBT – Cognitive Behavioral Therapy

CDC – Center for Disease Control and Prevention

cDNA – Complementary Deoxyribonucleic Acid

CFS – Chronic Fatigue Syndrome

CI – Confidence Interval

ciHHV-6 – Chromosomally Integrated Human Herpesvirus-6

CMIA – Chemiluminescence Immunoassay

CMV – Cytomegalovirus

CO – Cut Off

Ct – Cycle Threshold

DNA – Deoxyribonucleic Acid

EBV – Epstein-Barr Virus

EBV-IM – Epstein-Barr Virus Infectious Mononucleosis

FAM - 6-carboxyfluorescein

FDA – U.S. Food and Drug Administration

Gapdh – Glyceraldehyde 3-phosphate dehydrogenase

GET – Graded Exercise Therapy

GHQ – General Health Questionnaire

HBLV – Human B-Lymphotropic Virus

HCMV – Human Cytomegalovirus

HERV-K18 – Human Endogenous Retrovirus-K18

HHV-6 – Human Herpesvirus-6

HHV-6A – Human Herpesvirus-6 Variant A

HHV-6B – Human Herpesvirus-6 Variant B

HHV-7 – Human Herpesvirus-7

HIV – Human Immunodeficiency Virus

HPA – Hypothalamic-Pituitary-Adrenal Axis

hprt – Hypoxanthine Phosphoribosyltransferase

IAP – Intracisternal A-type Particle

ID – Identification

IFN-α – Interferon alpha

IgG – Immunoglobulin G

IO&NS – Inflammatory and Oxidative and Nitrosative Stress

JRA – Juvenile Rheumatoid Arthritis

K18.1 – HERV-K18.1 allele

K18.2 – HERV-K18.2 allele

K18.3 – HERV-K18.3 allele

kb – kilobase

K-S – Kolmogorov-Smirnov Test

MAOIs – Monoamine Oxidase Inhibitors

mcox, cox2 – Murine Mitochondrial Cytochrome Oxygenase

ME – Myalgic Encephalomyelitis

mg – milligram

MGB – Minor Groove Binder

MHC – Major Histocompatibility Complex

min – Minute

ml – Milliliter

MLVs – Murine Leukemia Viruses

mM – Millimolar

MPMV – Modified Polytropic Murine Leukemia Viruses

mRNA – Messenger Ribonucleic Acid

MS – Multiple Sclerosis

NADH – Nicotinamide Adenine Dinucleotide

NC – Negative Control

NFκb – Nuclear Factor-KappaB

ng – nanogram

NIH – National Institute of Health

nM – Nanomolar

NTC – No Template Control

PBMCs – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PC1 – Positive Control 1

PC2 – Positive Control 2

PCR – Polymerase Chain Reaction

PMV – Polytropic Murine Leukemia Viruses

PSS – Perceived Stress Survey

PVDF - polyvinylidene difluoride

qPCR – Quantitative Polymerase Chain Reaction

RCT – Randomized Controlled Trial

RLU – Relative Light Unit

RNA – Ribonucleic Acid

RPMI – Roswell Park Memorial Institute Culture Medium

s – Second

S/CO – Sample Relative Light Unit Value / Cut off Relative Light Unit Value

SAg – Superantigen

SDS – Sodium Dodecyl Sulfate

SLE – Systemic Lupus Erythematosus

SNP – Single Nucleotide Polymorphism

SNRIs – Serotonin-Norepinephrine Reuptake Inhibitors

SSRIs – Selective Serotonin Reuptake Inhibitors

TAMRA – 6-carboxytetramethylrhodamine

TCR – T-Cell Receptor

TH – Tufts Huber

U – Unit

UK – United Kingdom

US – United States of America

WB – Western Blot

WPI – Whittmore Peterson Institute

XMRV – Xenotropic Murine leukemia-like virus Related Virus

XMV – Xenotropic Murine Leukemia Viruses

Viral Associations with Chronic Fatigue Syndrome and the Prevalence of Artifacts

Introduction

History of Chronic Fatigue Syndrome (CFS)

Chronic fatigue syndrome (CFS) is a complex, heterogeneous disease affecting more than 1 million Americans [1]. Patients with CFS have overwhelming fatigue that is not improved with bed rest and worsens after physical activity or mental exertion. This excessive fatigue stops individuals from performing everyday tasks and limits what they can do on a day to day basis. Despite extensive research for more than two decades, CFS still remains a controversial topic among practitioners, scientists, and patients.

Although only recently defined as CFS, evidence of a diagnosis similar to CFS has been around for over a century. In 1869, New York neurologist George Beard coined the term neurasthenia, meaning weak nerves or nervous exhaustion, for patients that suffered from excessive physical and mental fatigue and muscle weakness [2]. Neurasthenia also included vague and variable symptoms including insomnia, lack of concentration, depression, head ache, dyspepsia, and a variety of non-verifiable physical complaints [3]. The fatigue was the first symptom and would come early, was extreme and lasted a long time, meaning the patients had abnormally quick fatigability and slow recuperation [2]. Careful examination failed to reveal an organic basis for these symptoms; however, the exhaustion remained and proved incapacitating to individuals who had previously led productive lives. The lack of an organic cause led Beard to classify neurasthenia as a functional condition caused by exhaustion of the nerve cells, a result of the increased mental stresses of the modern world [2].

Although initially a popular diagnosis, neurasthenia lost popularity once it became more of a psychiatric behavioral diagnosis and less of a physical neurological condition [4]. Further cases of a CFS-like disease occurred in various outbreaks worldwide throughout the 20th Century (Table 1) [5]. Two of the outbreaks given notable attention due to involving mostly the hospital staff were the 1934 Los Angeles County Hospital outbreak in Los Angeles, California and the 1955 Royal Free Hospital outbreak in London, England [5]. In the 1934 case, 198 doctors, nurses and community members at Los Angeles County Hospital suffered from an illness characterized by recurring fatigue, pain, and muscle spasms that lasted for a few months. Other symptoms were described as relapsing muscle weakness, inability to work, unusual pain syndromes, personality changes, memory loss, hysterical episodes, vertigo, major temperature fluctuations, pain in limbs, nausea, and aphasia. The onset was thought to be caused by an infective trigger, poliomyelitis, and the illness was termed atypical poliomyelitis [5]. The 1955 Royal Free Hospital case was quite similar. Between July 13th and November 25th, 292 members of the medical staff at the Royal Free Hospital in London were stricken with an illness having symptoms of overwhelming fatigue [6]. The major clinical manifestations were headache, sore throat, malaise, lassitude, vertigo, muscle pain, and depression [7]. The symptoms described were common to the early phase of most infections; however, these patients were tested for viral and bacterial infections and nothing was found. Neurological manifestations also were apparent in the more severe cases from the onset of the disease. Although the symptoms kept most of the patients hospitalized for only 1-2 months, the symptoms could persist for many months, with 4 patients still being disabled two years later. The disease became known as benign

myalgic encephalomyelitis (ME) and, although no infectious agent was found, it was thought to have an infectious etiology [6-7].

Both outbreaks seemed to be triggered by an infectious agent, had prolonged convalescence, accompanied by mental changes including depression, and were characterized by profound fatigue [8]. Although the severity of other symptoms varied from person to person, the main symptoms of muscular fatigue and generalized fatigue were present in all patients. Similar outbreaks continued throughout the 1960s and 1970s without any advances in diagnostic tools or definition until 1985 (Table 1) [5].

In 1985, another CFS-like outbreak occurred in Incline Village, Nevada, near Lake Tahoe [9]. At a small practice, Dr. Daniel Peterson and Dr. Paul Cheney saw over 150 cases of an unknown illness characterized by fatigue and muscle pain. According to Cheney, about 70% of the patients had higher than normal antibody levels for Epstein-Barr virus, and he diagnosed them as having chronic mononucleosis-like syndrome or chronic Epstein-Barr virus syndrome [9]. Fearing an outbreak, they contacted the Center for Disease Control (CDC) for help. The CDC responded by sending Gary Holmes and Jon Kaplan to Lake Tahoe to investigate the outbreak. Holmes' study could not confirm that EBV was the causative agent to the outbreak. They found similar antibody titers among the patients and a group of healthy controls. In addition, they found elevated levels of antibody against several other viruses, including cytomegalovirus, herpes simplex types I and II, and the measles virus [9-10]. The outbreak made mainstream news and was finally recognized as a serious illness, with physicians and patients contacting the CDC for help with diagnosis and treatment [11]. This led the CDC to develop a case-definition for CFS –like diseases in 1988, so patients could be

Table 1. Summary of CFS-like Outbreaks in the 20th Century

Year	Location	Who
1934	***Los Angeles, California***	***198 Hospital Staff and Community Members***
1936	Fond-du-Lac, Wisconsin	35 Convent Candidates and Novices
1937	Erstfeld, Switzerland	130 Soldiers
1937	Frohburg, Switzerland	28 Patients and Staff
1939	Harefield, England	7 Hospital Staff Members
1939	Degersheim, Switzerland	73 Soldiers
1945	Philadelphia, Pennsylvania	University Hospital
1948 -1949	Iceland	1,090 Community Members
1949-1951	Adelaide, Australia	800 Community Members
1950	Louisville, Kentucky	37 Nursing Students
1950	Upper New York State	19 Community Members
1952	Middlesex Hospital, England	14 Nursing Students
1952	Copenhagen, Denmark	70 Community Members
1952	Lakeland, Florida	27 Community Members
1953	Coventry, England	13 Hospital Staff and Community Members
1953	Rockville, Maryland	50 Nursing Students and Community Members
1954	Tallahassee, Florida	450 Community Members
1954	Seward, Alaska	175 Community Members
1954	Berlin, Germany	7 Soldiers
1954-1955	Johannesburg, South Africa	14 Community Members
1955	Dalston, Columbia, England	Community Members
1955	***Royal Free Hospital, England***	***300 Hospital Staff Members***
1955	Perth, Australia	Community Members
1955	Gilfach Goch, Wales	Community Members
1955	East Ham, London, England	Community Members
1955	Durban, South Africa	140 Hospital Staff and Community Members
1955-1956	Segbwema, Sierra Leone	Community Members
1956	Ridgefield, Connecticut	70 Community Members
1956	Punta Gorda, Florida	124 Community Members
1956	Pittsfield, Massachusetts	7 Community Members
1956-1957	Coventry, England	Community Members
1958	Athens, Greece	27 Nursing Students
1959	Newcastle-upon-Tyne, England	Community Members
1961-1962	New York State	Convent
1964-1965	Galveston County, Texas	Community Members
1969	New York State	University Medical Center
1970-1971	Great Ormond Street Hospital, London, England	Hospital Staff Members
1975	Sacramento, California	200 Hospital Staff Members
1976	South West, Ireland	Community Members
1979	Southampton, England	10 Community Members
1980-1981	Ayrshire, Scotland	Rural Practice
1980-1983	Helensburgh, Scotland	General Practioner Practice
1983-1984	West Otago, New Zealand	> 20 Community Members
1984	***Lake Tahoe, Nevada***	***> 150 Community Members***
1985	Lyndonville, New York	Community Members

The 1934 Los Angeles outbreak, the 1955 Royal Free outbreak, and the Lake Tahoe outbreak are in bold-italics and discussed in the text. Modified from Patarca-Montero [5]

more uniformly diagnosed and research studies could be more easily compared [11]. They defined CFS as a syndrome, a complex of potentially related symptoms tending to occur together that may have several causes. Because there is no diagnostic test to define CFS, it is merely a collection of symptoms and a diagnosis of exclusion [11].

Development of a Case-Definition for CFS

In 1988, a working group of public health epidemiologists, academic researchers, and clinicians was organized to develop a consensus on the clinical characteristics of CFS [11]. The 1988 definition of CFS was based on signs and symptoms and was intentionally restrictive, in order to delineate a more uniform patient population to maximize the chances that research studies will detect significant associations with causative agents [11]. The 1988 (Holmes) case definition, summarized in table 2, was the first to define the syndrome as chronic fatigue syndrome and states that a patient must present with a new onset of persistent or relapsing, debilitating fatigue or easy fatigability in a person who has no previous history of similar symptoms, that does not resolve with bed rest, and that is severe enough to reduce or impair average daily activity below 50% of the patient's premorbid activity level for a period of at least 6 months. Other clinical conditions that may produce similar symptoms must be excluded by thorough evaluation, based on history, physical examination, and appropriate laboratory findings. The patient must also fulfill 6 of the following 11 symptoms: Mild fever, sore throat, painful lymph nodes, unexplained muscle weakness, muscle discomfort, prolonged generalized fatigue after exercise, headaches, joint swelling, neuropsychologic complaints, and sleep disturbance. These minor criteria must have begun at or after the time of onset of increased fatigability and must have persisted or recurred over a period of at least 6

Table 2. Summary of 1988 Holmes Case Definition for CFS

A case of the chronic fatigue syndrome must fulfill major criteria 1 and 2, 6 or more of the 11 symptom criteria and 2 or more of the 3 physical criteria; or major criteria 1 and 2, and 8 or more of the 11 symptom criteria.

MAJOR CRITERIA

1. New onset of persistent or relapsing, debilitating fatigue or easy fatigability in a person who has no previous history of similar symptoms, that does not resolve with bed rest, and that is severe enough to reduce or impair average daily activity below 50% of the patient's premorbid activity level for a period of at least 6 months.

2. Other clinical conditions that may produce similar symptoms must be excluded by thorough evaluation, based on history, physical examination, and appropriate laboratory findings.

MINOR CRITERIA

Symptom Criteria

To fulfill a symptom criterion, a symptom must have begun at or after the time of onset of increased fatigability, and must have persisted or recurred over a period of at least 6 months.

1. Mild fever
2. Sore Throat
3. Painful cervical or axillary lymph nodes
4. Unexplained generalized muscle weakness
5. Muscle discomfort or myalgia
6. Prolonged (24 hours or greater) generalized fatigue after levels of exercise that would have been easily tolerated in the patient's premorbid state
7. Generalized headaches
8. Migratory arthralgia without joint swelling or redness
9. Neuropsychologic complaints (photophobia, transient visual scotomata, forgetfulness, excessive irritability, confusion, difficulty thinking, inability to concentrate, depression)
10. Sleep disturbance
11. Symptom complex initially developing over a few hours or a few days

Physical Criteria

Physical criteria must be documented by a physician on two occasions, at least a month apart.

1. Low-grade fever
2. Nonexudative pharyngitis
3. Palpable or tender cervical or axillary lymph nodes

From Holmes *et al.* [11]

months [11].

Now that there was a case definition, multiple groups could diagnose a patient population and do comparative studies to possibly find a cause for CFS. Although it was a step forward to have a definition for comparative analysis, some researchers and patients thought that the definition was too vague and open to interpretation which lead to differing patient populations between studies [12]. Some patients felt that the name was misleading, pointing to mainly psychiatric problems [13]. A study by Wayne Katon and Joan Russo looked at the number of unexplained physical complaints a CFS patient had and looked at the prevalence of current and lifetime psychiatric disorders. They found that patients with the highest numbers of medically unexplained physical symptoms had high rates of current and lifetime psychiatric disorders [14]. They suggested modifying the case definition to include few physical symptoms to lower the prevalence of psychiatric patients diagnosed with CFS. Another study set out to test whether the case definition for CFS could successfully discriminate CFS patients from healthy controls as well as discriminate CFS patients from two diseases that have fatigue as a major symptom, multiple sclerosis and major depression [15]. They compared symptom surveys from a CFS group, a healthy control group, an MS group, and a major depression group. They found that the CDC minor criteria symptoms that were the most successful discriminators were myalgias, post-exertional malaise, headaches, and an infectious-type group of symptoms. The group also agreed with the Katon and Russo study and suggested that the physical examination signs were mostly not important in making the CFS diagnosis [16].

In 1991, a workshop was held at the National Institute of Health (NIH) to discuss the 1988 case-definition along with confounding diagnoses and exclusion criteria. It was recommended that the CDC case-definition be revised [17]. In 1994, the CDC and the International Chronic Fatigue Syndrome Study Group published its revised case-definition (summarized in Table 3) [12]. The point of the revisions was to address some of the criticism that the 1988 definition had received and to facilitate a more systematic collection of data internationally. The new definition removed all physical signs from the inclusion criteria, because their presence had been unreliably documented in past studies. The new definition also lowered the required number of symptoms from 8 to 4 and decreased the list of symptoms from 11 to 8, by removing mild fever, unexplained muscle weakness, and initially happening over a few hours/days from the list [12]. The requirement for an "average daily activity below 50%" was also eliminated, because the level of impairment was difficult to verify. Although this new definition was still met with some complaints, it is the current generally accepted definition for CFS research [1].

Table 3. Summary of 1994 Fukuda Case Definition for CFS

A case of chronic fatigue syndrome must fulfill the first two major criteria and 4 or more of the 8 minor symptoms.

MAJOR CRITERIA

1. The individual has severe chronic fatigue for 6 or more consecutive months that is not due to ongoing exertion or other medical conditions associated with fatigue. All other medical conditions associated with fatigue must be ruled out by a doctor.

2. The fatigue significantly interferes with and reduces daily activities and work.

MINOR CRITERIA

CFS patients must have 4 or more of the following symptoms persisting or recurring for 6 or more months, first appearing after the initial fatigue.

1. Post-exertional malaise lasting more than 24 hours (extreme, prolonged exhaustion and exacerbation of symptoms following physical or mental exertion)
2. Unrefreshing sleep
3. Significant impairment of short-term memory or concentration
4. Muscle pain
5. Pain in the joints without swelling or redness
6. Headaches of a new type, pattern, or severity
7. Tender cervical or axillary lymph nodes
8. Sore throat that is frequent or recurring

From Fukuda *et al.* [12]

Other Case-Definitions for CFS

Neither the 1988 case-definition nor the 1994 revision were intended to be used by physicians for the clinical diagnosis of CFS [18]. In 2003, an expert subcommittee of Health Canada selected an expert medical consensus panel to develop a clinical case-definition to aid the family physician and other treating clinicians in recognizing CFS. This clinical definition placed more emphasis on the symptoms other than fatigue. The definition makes it compulsory that in order to be diagnosed with CFS, a patient must become symptomatically ill after exercise and must also have neurological, neurocognitive, neuroendocrine, dysautonomic, circulatory, and immune manifestations [18]. There are two other accepted case-definitions, the Oxford criteria [19], and the pediatric case-definition [20]; however, almost all studies use the 1994 CDC case-definition or the Canadian case-definition [21-22].

Demographics/Prevalence

Since the first case-definition of CFS, many studies have been carried out to estimate the overall prevalence of CFS in the general population. Early studies relied on referrals from physicians, however, these studies underestimate prevalence because low-income families lack access to medical care and some CFS patients are too sick to even go to the doctor [23]. These studies also led to population bias and the overall thought that CFS was a disease of white middle- and upper-class people, which is not true. CFS affects people of all races and socioeconomic classes [1]. According to a 1999 community-based study in Chicago, IL, in which researchers telephoned over 28,000 adults, representing a stratified random sample of the population, including Caucasian, Latino, Asian, and African Americans, CFS affects about 422 adults per 100,000 in the

population [23]. A similar 2003 study in Wichita, Kansas by Reyes *et al*. suggested that CFS affects about 235 persons per 100,000 in the population [24]. The difference in prevalence between the two studies could reflect a difference between metropolitan and urban populations; however, there were other differences in the designs as well. Although the Wichita study did attempt to recruit people from all races, the study turned out to be of mostly Caucasians [24]. Both studies used random dialing; however, the questions asked on the questionnaire were different, which could lead to differing interpretations. The authors describing these studies suggested that a standardized questionnaire should be developed, so future studies could be more accurately compared [24]. Both studies did show a higher prevalence of CFS in females than males. Other studies outside the U.S. also suggest a different prevalence, which could be a result of different genetic populations, cultural differences, or diagnostic criteria. In a 1997 study at primary care hospitals in Southern England, Wessley *et al*. reported a prevalence of 2600 per 100,000 people; however, they did not have as rigorous an exclusionary policy for other medical or psychological causes of fatigue [25]. After exclusion of patients with comorbid psychological disorders, the prevalence was more similar to the other population studies at 500 per 100,000 people [25].

Overall, the prevalence of CFS appears to be between 0.23 % and 0.5% with females being affected more often than males [26]. Previous reports showed a 1.3:1 female to male ratio; however, other reports showed a 4:1 ratio [27]. It affects all ethnic groups and is seen in all socioeconomic groups [23-24, 28]. The estimated prevalence of CFS is much lower among children and adolescents than among adults [29-31].

Symptoms/Clinical Manifestations

The major complaint of patients suffering with CFS is persistent severe fatigue lasting longer than six months that is not alleviated with rest, but most patients also complain of muscle pain and cognitive dysfunction [32]. Other reported symptoms include myalgia, impaired memory or concentration, gastrointestinal problems, headaches, pain in muscles or several joints, dizziness, nausea, anorexia, and night sweats [1]. Postexertional malaise after performing simple tasks such as walking short distances is also a common complaint [33]. In almost all cases, symptoms substantially reduce a person's everyday activities. Although these are the common symptoms among CFS patients, not all patients have every symptom. Many patients report an acute onset of symptoms after an infectious flu-like illness [32]. The CDC lists the following symptoms lasting at least 6 months: increased malaise following physical activity or mental exertion, problems with sleep, difficulties with memory and concentration, persistent muscle pain, joint pain without swelling, headache, tender lymph nodes in the neck or armpit and sore throat [1]. Patients may also experience brain fog, difficulty maintaining an upright position, dizziness or fainting, allergies or sensitivities to foods, irritable bowel, chills and night sweats, visual disturbances and depression or mood swings [1]. The severity of CFS varies from patient to patient. CDC studies show that CFS can be as debilitating as multiple sclerosis, lupus, and similar chronic conditions. CFS symptoms tend to affect patients in cycles with periods of illness, followed by periods of remission [1].

Treatment / Management of Fatigue

Although there is no cure for CFS, treatments exist to manage the symptoms of CFS and improve a patient's quality of life. While one particular treatment does not always work for every CFS patient, multiple treatment options are available. Most treatment regimens start with a well-balanced diet and a discussion with the patient about their nutritional habits [1]. Eating right can make a person feel better, give a person more energy, and boost their immune system [34]. Treatment strategies for CFS include psychological, physical, and pharmacological intervention [35]. Because CFS is such a heterogeneous disease, it is recommended that treatments be somewhat personalized by starting with the most problematic symptoms as prioritized by the patient. Patients are encouraged to exercise, but they must know their limits. The main goal of treatment is not to return to a pre-disease state, but to get relief from symptoms [1].

Pharmacological Treatments

Currently there are no approved drugs for the treatment of CFS [36]. However, physicians have prescribed drugs to treat various symptoms, such as sleep-aids and pain-relievers. Patients often complain of difficulty falling asleep, extreme sleepiness, frequent awakening, and nocturnal myoclonus [32]. They also report feeling less refreshed after sleep [37]. One way to manage sleep problems is to help patients adopt good sleep habits. Patients should establish a regular bed-time routine, avoid napping during the day, and avoid caffeine, alcohol, and tobacco. When patients use these techniques and are still unable to get restful sleep, sleep-aids can be prescribed, such as antihistamines or over the counter sleep products [1]. Another main complaint by CFS patients is pain in the muscles and joints along with headaches [27]. Regular pain-

relievers like aspirin, acetaminophen or ibuprofen can help. Stretching and movement therapies along with gentle massage, heat, and relaxation techniques can also aid in pain management [38].

Antidepressants

Antidepressants are the most commonly prescribed medications to CFS patients [38]. They can help improve sleep, energy levels, cognitive impairment, and alleviate pain [39-41]. Adequately powered placebo-controlled randomized clinical trials of most antidepressants are lacking; however, there is evidence of some benefits. A small double-blind crossover study of the tricyclic antidepressant nortriptyline demonstrated benefit for depressive and fatigue symptoms [42]. Studies involving selective serotonin reuptake inhibitors (SSRIs) have shown less benefit. Two randomly controlled trials (RCTs) involving fluoxetine failed to demonstrate any improvement in CFS symptoms [43-44]. Some evidence exists that serotonin-norepinephrine reuptake inhibitors (SNRIs) could be beneficial to CFS patients, but a random controlled trial involving CFS patients has not been done. Dhir and Kulkarni demonstrated a positive effect of the SNRI venlafaxine in a rodent model of CFS [45]. The venlafaxine treatment produced a significant reduction in immobility time and reversed behavioral, biochemical, and neurotransmitter alterations induced by chronic forced swim [45]. An earlier report describes two CFS patients that had clinical reduction in global fatigue symptoms and immunological aberration after 6 weeks of treatment with venlafaxine [40]. Still, a large random controlled trial has not been done using CFS patients with the SNRI drug class. Monoamine oxidase inhibitors (MAOIs) have shown some modest improvements independent of depressive illness [46]. An RCT using moclobimide showed an overall

response in 51% of patients as compared to 23% in the placebo group [46]. However, moclobimide is not approved by the FDA for use in the United States. Studies involving the MAOIs phenelzine [39] and selegiline [47] also demonstrated a modest therapeutic effect in CFS patients; however, these drugs have well-known side effects such as hypertensive crisis. Therefore, MAOIs are rarely prescribed for CFS patients [27, 36].

Stimulants

Several stimulants have been studied in clinical trials of CFS patients. Dexamphetamine, modefinil, and pyridostigmine improved fatigue symptoms in one small RCT [48] and three case studies [49-50]. Methylphenidate was also found to be superior to placebo in reducing fatigue and concentration disturbance [51]. Although stimulants have shown some therapeutic benefit in CFS, the risks of misuse, abuse, and withdrawal have limited their use [38].

Anti-viral, Antibiotic, and Immunological Treatments

In general, studies of antiviral therapies in CFS patients have had no effect on symptoms. Studies of both acyclovir [52] and ganciclovir [53] showed no beneficial effect in CFS patients. However, a study of a subset of CFS patients having elevated EBV serum IgM antibodies treated with valacyclovir did demonstrate positive effects with decreases in anti-EBV antibodies, improved tachycardia, and increased physical functioning [54]. This suggests that antivirals may work in subsets of CFS patients with proven viral infections, although the viral infection should exclude the patient from a CFS diagnosis. Another trial treating a CFS group with the immunostimulant inosine pranobex found significant improvements in natural killer cell-mediated cytotoxicity, but no differences in symptoms, activity, or cognitive function [55]. Potential serious side

effects limit the use of antiviral medications in CFS patients, and therefore, are only prescribed when a proven infection is present.

Like antivirals, antibiotics have also shown limited success in alleviating the symptoms of CFS. Treatment with minocycline or doxycycline of a small group of CFS patients with *Coxiella burnetii* infections did kill off the infection; however, the CFS signs and symptoms were unchanged [56]. Antibiotics also have side effects, such as nausea, vomiting and diarrhea, that limit their use to only proven infections [57].

Evidence that CFS is associated with immune system dysregulation is abound [58]. Consequently, trials of immune-modulating agents have been carried out. The benefit of immunoglobulin treatment for CFS patients is questionable. Two studies reported significant improvement in functional outcome [59-60]; however, two other studies reported no functional improvements [61-62]. Treatment with interferon-alpha is just as controversial. One small trial led to an increase in physical activity [63] while a second trial showed no physical improvements based on quality of life measures [64].

New Drug Candidate

Ampligen® is a mismatched double-stranded RNA with immunomodulatory and antiviral properties that acts as a toll-like receptor-3 agonist [65]. A phase III prospective, double-blind, randomized, placebo-controlled trial compared twice-weekly Ampligen® to a placebo in 234 subjects with long-standing, debilitating CFS. The 40 week study showed improvement in exercise tolerance, as well as reduction in other medication usage [66]. In 2009, the FDA rejected the application for approval of Ampligen® as a CFS treatment and recommended another trial be conducted in more

than 300 CFS patients. The initial study was released in early 2012, and Hemispherx is currently recruiting for a clinical trial of Ampligen® in CFS patients [67].

Supplements/Alternative medicines

As stated earlier, a well-balanced diet is one of the first recommendations a physician makes to a CFS patient. Vitamins and other supplements can also be helpful in alleviating symptoms [34]. One trial that investigated the effect of essential fatty acid supplements on CFS symptoms failed to show significant improvements in depression and general symptoms [68], but a second trial looking at patients suffering from post-viral fatigue syndrome did show moderate improvement in symptoms[69]. Magnesium supplements did lead to mild improvements in measures of energy and pain, but no improvement in sleep or physical mobility [70]. On the other hand, acetyl-L-carnitine and propionyl-L-carnitine did show significant improvements in fatigue and cognitive function; however no control treatment was used as comparison [71]. Two trials showed statistically significant effects of NADH on symptom scores when compared with placebo after 1 month [72] and psychological therapy after 3 months [73]. Pollen extract [74], medicinal mushrooms [75], and acclydine [76] treatments failed to show significant improvements in general CFS symptoms. There are a few trials assessing the effectiveness of alternative medicine; however, most of these studies are poorly designed and their overall effectiveness cannot be ascertained. Evidence does exist that qigong, massage, and tuina have demonstrated positive symptom relief in CFS patients, but larger studies with better control groups must be done [77].

Failed Pharmacotherapies/ Other Pharmacotherapies

Although these compounds have been investigated, no benefit was found in CFS patients from treatment with the antihypertensive agent clonidine [78], immune-modulating agent (histamine receptor antagonist) terfenadine [79], hydrocortisone and fludrocortisones treatments [80-86], anticholinergics (sulbutiamine or galantamine hydrobromide) [87-89], or growth hormone [90]. Treatment with melatonin did significantly improve sleep, vitality and mental health, but bodily pain was found to increase [91-92].

Exercise/Graded Exercise Therapy (GET)

Graded exercise therapy (GET) involves a structured activity management program that gradually increases aerobics over time [1]. CFS patients are very sensitive to any type of exertion, so therapy usually starts slow and advances slowly [27]. Patients typically receive personal treatment goals dependent on their own capacity for activity. The objective is to do just enough activity without over-doing it to avoid crashes [93]. Studies show an overall beneficial effect on fatigue and functional work capacity compared to control groups [44, 94-96]. Exercise decreased psychological stress [97] and improved fatigue, functional capacity, and fitness [27, 98]. GET is usually combined with cognitive behavioral therapy (CBT).

Cognitive Behavioral Therapy (CBT)

One of the most successful treatments for CFS is cognitive behavioral therapy (CBT) [32]. CBT is a psychological therapy model that is used to treat a range of chronic pain and psychological conditions [35]. CBT identifies unhelpful, anxiety-provoking thoughts and challenges these negative thoughts and dysfunctional assumptions to

encourage a better attitude toward managing the disease [35]. CBT focuses mainly on the factors that may be maintaining fatigue, rather than the initial trigger [1]. CBT for CFS involves planned activity and rest, graded increases in activity, a sleep routine and cognitive restructuring of unhelpful beliefs and assumptions about CFS to develop a more positive attitude [27]. In a randomized controlled trial comparing CBT to relaxation therapy, 70% of patients completing CBT therapy showed substantial improvement in physical functioning, whereas only 19% completing relaxation therapy showed the same improvement [99]. An earlier study by Sharpe *et al.* [100] showed similar effectiveness of CBT over control treatments.

However, some researchers have found that CBT and GET are not beneficial and in some cases even counterproductive to CFS patients. One randomized controlled study [101] and one controlled study [102] showed no benefit to CBT, but these trials were much shorter than the studies showing improvement. Other researchers, patients, and physicians have brought up concerns involving the safety and the long term efficacy of CBT and GET [103]. Some physicians believe that increased exercise leads to a crash, leaving patients more fatigued than they were previously [104]. A follow-up study showed a statistically significant decline in physical function compared to baseline 12 months after treatments involving CBT and GET [105]. Light *et al.* demonstrated in CFS patients that exercise led to a significant increase in the expression of receptors detecting muscle metabolites, as well as genes in the sympathetic nervous system and immune system, compared to healthy subjects [106]. These increases in expression highly correlated with the level of physical fatigue, mental fatigue, and pain [106].

Although the opinions of physicians, patients, and researchers are mixed about the effectiveness and safety of CBT and GET, both CBT and GET are the only treatments from randomized controlled trials that seem to yield a positive outcome [32]. Again, it must be remembered that the CFS population is a very heterogeneous group, and a treatment that might work for one individual will not necessarily work for another individual and vice versa. All treatment options must be discussed on an individual patient basis instead of using one treatment for all CFS patients.

Causes/ Possible Etiologies

Although extensive research has attempted to find a universal cause for CFS, none has been found. Multiple theories exist; however, no individual theory seems to cover all cases of CFS. In reality, CFS may have multiple causes resulting in the same symptoms [21, 27, 32]. Most experts agree that there is a triggering physical or psychological event for CFS [32, 58].

Infectious Etiology

Most cases of CFS report an infectious flu-like illness that triggers the fatigue, which has led to a multitude of studies attempting to link viral infections to CFS [32, 107]. Early studies found high antibody titers to Epstein-Barr virus (EBV) in patients with CFS [9], but subsequent studies failed to discern a difference in antibody titers between CFS patients and healthy controls [108-109]. High rates of CFS have also been reported after Q fever and Lyme disease [110], but no causal evidence exists. Other infectious agents linked to CFS include Borna disease virus [111-112], Enterovrius [113-114], parvovirus B19 [115-116], glandular fever [117], Nipah virus encephalitis [118],

and EBV and CMV leading to infectious mononucleosis [119]. The human herpesviruses 6 and 7 have also been implicated in CFS [10, 120-122] and this topic will be discussed in more detail later. Human herpesviruses have the ability to stay dormant and then reactivate later in life. Even though there is some evidence for human herpesvirus 6 and 7 to be associated with CFS [120-122], our studies found no association between human herpesvirus 6 and 7 viral load and disease state (Oakes et al. 2012 in preparation). Retroviruses, like xenotropic murine leukemia virus-related virus (XMRV), have also been associated with CFS [123]; however, our studies found no association of XMRV with CFS [124-125]. Furthermore, we proved that the PCR test for XMRV would come up positive if mouse DNA was contaminating the human sample [124-125]. This was confirmed by other studies [126-128] and will be discussed in more detail later. Although studies have found associations between some of these pathogens and CFS, there is no evidence that any one of these viruses causes CFS.

Genetic Etiology

Another possible theory is that CFS patients are somehow genetically predisposed to develop CFS. CFS is sometimes seen in members of the same family [129] and has a higher concordance rate in monozygotic female twins than dizygotic female twins [130]; however, no specific genes have been linked to CFS. Some studies have isolated different mRNA expression patterns in CFS patients compared to healthy controls [131-132], but these findings were not confirmed in other studies [133]. Further work is necessary to determine if there is a true genetic link.

Endocrinology/Metabolism Etiologies

Hypothalamic-pituitary-adrenal (HPA) axis abnormalities have been linked to CFS [27]. Studies have shown both HPA hypoactivity and higher chronic adrenocorticotropic hormone (ACTH) autoantibody levels in CFS [134-135]. However, a subsequent study showed no role for deficiency in central opioids or the HPA axis in the symptoms of CFS [136]. Nevertheless, HPA dysfunction does occur in some CFS patients and cannot be overlooked as possessing a possible etiological role [137].

Mental/Neurologic Etiology

As stated above, most experts agree that there is a triggering physical or psychological event to CFS [32]. Psychosocial factors are frequently thought to contribute to fatigue. Serious life events, such as the loss of a loved one or other stressful situations have been found to precipitate CFS [138-139]. Although stress by itself cannot cause CFS, it can be a contributing factor to the development and prolonging of CFS [140].

Inflammation and Oxidative and Nitrosative Stress Pathways (IO&NS)

A newer theory on the cause of CFS is disruption in the inflammation and oxidative and nitrosative stress pathways [141]. The theory is that a nonspecific trigger activates a systemic inflammatory response marked by increases in proinflammatory cytokines. The proinflammatory stimuli increase oxygen radicals such as peroxides and superoxides, which lead to oxidative damage of the cell membranes causing them to be immunogenic. Inflammatory cytokines also activate neutrophils and monocytes, which produce nitrogen monoxide and peroxynitrite (ONOO-). Nitration causes chemical modifications of proteins, which render them immunogenic [141]. Systemic

inflammation can lead to a central neuroinflammation with increased levels of proinflammatory cytokines, which remain for several months [142]. There is a strong correlation between inflammation and vegetative symptoms, such as symptoms of depression, sleep disorders and psychomotor retardation [143]. Two studies show increased oxidative stress and decreased antioxidant levels in CFS patients as compared to healthy controls [144-145]. Maes *et al.* measured the immune response to neoepitopes of modified lipids and proteins, indicating damage caused by oxidative and nitrosative stress, and found an increase in CFS patients compared to controls [144]. Richards *et al.* presented evidence of oxidative damage in the red blood cells of CFS patients by showing statistically significant increases in methemoglobin and malondialdehyde [145]. The free radicals that cause oxidative stress are also linked to muscle fatigue and muscle pain [146]. Nuclear factor kappa beta (NF-κB) is the main upstream regulator of the IO&NS pathway [147]. A study looking at the production of NF-κB p50 subunit in peripheral blood lymphocytes of 18 unmedicated patients with CFS and 18 matched controls showed significantly higher levels in CFS patients than controls. The level of NF-κB also highly correlated with the severity of illness as measured with the FibroFatigue scale [147].

These findings suggest that future studies should look at inhibiting NF-κB activation and translocation, as well as antioxidant treatment as strategies for alleviating the symptoms of CFS; however, these are relatively new findings and more independent studies must be done to confirm these findings.

Viruses Associated with CFS

As stated above, most experts agree that there is a triggering physical or psychological event for CFS [32], as well as immune dysfunction [58]. Viral triggers are continually being investigated and associated with CFS, although the evidence of their association is controversial. Four potential viruses associated with CFS that we studied are xenotropic murine leukemia virus-related virus (XMRV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and human endogenous retrovirus K18 (HERV-K18). In the following sections, I will review these viruses.

Xenotropic Murine Leukemia Virus-Related Virus (XMRV)

Discovery of a new virus

Xenotropic murine leukemia virus-related virus (XMRV) was first described in a 2006 study looking for infectious agents in prostate cancer associated with a mutation in the RNASEL gene, which functions as part of the innate immune response to virus infection [148]. A ViroChip bearing conserved virus sequences was used for these studies, testing RNA isolated from prostate tissue [148]. Full length viral genomes were constructed and were found to have homology to genomes of endogenous murine leukemia virus, giving XMRV its name. This was the first pathogenic gammaretrovirus found to infect humans [148].

In 2009, noting that CFS patients suffer immunological abnormalities including RNASEL deficiencies [149], Lombardi *et al.* demonstrated a link between CFS and XMRV [123]. The study conducted at the Whitmore Peterson Institute in Nevada, detected XMRV in the DNA from peripheral blood mononuclear cells (PBMCs) of 67%

of CFS patients, compared to only 3.7% of healthy controls, using a nested PCR [123]. A nested PCR uses two sets of primers in two successive runs of PCR, the second set intended to amplify a target within the product of the first run. These data were received with enthusiasm from CFS patients and researchers, because they pointed to a possible infectious etiology of CFS. To patients, the finding of XMRV helped validate CFS as a real disease and to doctors, the discovery of XMRV could have been used as a diagnostic tool. Some patients jumped to the conclusion that XMRV was the cause of CFS and started taking anti-retrovirals to lessen their symptoms [150]. The study instigated a worldwide hunt to confirm that XMRV was associated with CFS.

Initial failures to detect XMRV in other CFS patients

Despite initial excitement, early studies failed to find an association of XMRV to CFS. Two studies in the UK used the same nested PCR as Lomardi *et al*. to detect XMRV; however, not one CFS sample nor healthy control in either study tested positive for XMRV [151-152]. Studies in the Netherlands [153] and China [154] that both utilized a real time PCR specific for XMRV, as well as the Lombardi nested PCR, also failed to find XMRV in any patients. Initial attempts to explain why XMRV was not detected in these studies involved the patient populations. Some thought that the U.S. population merely had a higher prevalence of XMRV than the international community, but this conclusion was quickly disproven by an American study. A group at the CDC also failed to detect XMRV in archived blood specimens of CFS patients and healthy controls from Wichita, Kansas and metropolitan, urban, and rural Georgia [155]. They also used a serologic test to look for XMRV specific antibodies and failed to detect any XMRV reactions [155]. Another explanation for the failure to detect XMRV was the

diagnosis of CFS and the severity of symptoms at the time of the blood draw. Most groups used the 1994 CFS definition to define their cohort; however, the initial group linking CFS to XMRV said they used the 1994 CDC definition and the more stringent Canadian definition and chose patients presenting with severe disability [156]. Although patient selection could be an important issue in CFS studies, the fact that no other group detected XMRV at all was still disconcerting.

To determine if an altered immune system may play a role in XMRV detection, Henrich *et al.* not only tested a group of CFS patients, but also tested a group with human immunodeficiency virus (HIV) infection, a rheumatoid arthritis group, an organ transplant group, and a general group of patients presenting for medical care [157]. Using the established nested PCR techniques from both Erlwein *et al.* [151] and Urisman *et al.* [148], Henrich *et al.* failed to detect XMRV in any sample. They made it clear that their CFS patients were diagnosed using the revised 1994 CFS case-definition and the majority of them (69%) had stopped work as a direct result of their CFS symptoms, showing that they were severely disabled. The authors concluded that XMRV was not associated with CFS or altered immune function and again cited geographical differences in the prevalence of XMRV, further weakening the link between XMRV and CFS [157].

New association of MLVs and CFS

Later in 2010, a second group ignited excitement by publishing that they detected the presence of murine leukemia virus-like (MLV) sequences in PBMCs of 86% of CFS patients, compared to only 7% of healthy controls [158]. These sequences were more similar to those of polytropic MLVs than those of xenotropic viruses and further confounded the initial XMRV finding. Instead of reinforcing and confirming the initial

finding that XMRV was associated with CFS, the study by Lo *et al.* weakened the evidence for XMRV.

Contamination of Human Samples

Soon after that, four independent studies, including our own, published in *Retrovirology,* examined contamination of human samples with mouse DNA. XMRV is closely related to abundant endogenous retroviruses in mice [148]. Using the very sensitive PCR methods employed by most groups, a very small amount of contaminating murine DNA could yield a false-positive result [124]. We blindly tested 112 CFS patients and 36 healthy controls for XMRV using a TaqMan qPCR assay specific for XMRV *pol,* as well as the nested PCR used by Urisman *et al.* [148]. All samples tested were negative using the XMRV specific qPCR; however, we did start to see some positive results using the nested PCR. Due to the close relationship between XMRV and endogenous MLVs, we developed a novel PCR with our collaborators in the Coffin Lab to detect contaminating murine DNA by using the Intracisternal A-type Particle which is abundant in the mouse genome [124]. We also utilized a qPCR that tested for the murine mitochondrial cytochrome oxidase gene, *cox2,* developed by Switzer *et al.* [159]. Every sample that had tested positive with the XMRV nested PCR, also tested positive for mouse DNA, meaning our XMRV samples were probably false positives. We noted the importance of testing for mouse DNA contamination when working with viruses related to the mouse genome [124]. Other papers in the same issue of *Retrovirology* also noted false XMRV positives from commercial reverse-transcription kits [127] and from mouse DNA contamination in human tissues [128].

Although our paper did show mouse DNA contamination in all of our XMRV-positive samples [124], we could not rule out that our samples had XMRV as well as mouse contamination. To prove without a doubt that our XMRV-positive samples were only due to murine DNA contamination, we tested plasma samples for antibodies against XMRV proteins [125]. None of our CFS samples or our healthy control samples tested positive for an antibody response to XMRV proteins; thus, we concluded without a doubt that XMRV-positive PCR results were due to mouse contamination [125].

Further evidence that XMRV may just be a contaminate came with the realization that a widely used cell line, 22Rv1, is a chronically XMRV-infected cell line that generates high titers of XMRV in culture [160]. To understand the origins of XMRV, Hue *et al.* compared proviral DNA sequences derived from the 22Rv1 cells with MLV and XMRV sequences reportedly isolated from patients. In their analysis, they concluded that sequences from the cell line and the patients were equally similar and that some cell line-derived sequences were ancestors to the patient isolates. This suggested that the 22Rv1 cells were the probable source of the patient isolates [160].

XMRV Cannot Circumvent Intracellular Defenses

Evidence that XMRV could not be an infective virus in humans came from two studies demonstrating that XMRV lacked the ability to circumvent intracellular defense mechanisms that restrict viral replication. APOBEC3 proteins, TRIM5α, and BST2/tetherin are host restriction factors that potently inhibit HIV-1 and other retroviruses and are present in PBMCs [161]. Both Groom *et al.* and Paprotka *et al.* showed *in vitro* that XMRV replication was potently inhibited by human APOBEC3 proteins, as well as BST2/tetherin, but not human TRIM5α [161-162]. These findings

were quickly confirmed by other laboratories [163-164]. An *in vivo* study infecting pigtail macaques with $>10^{10}$ RNA copy equivalents of XMRV showed that virus replication peaked at low levels at around 2000 RNA copies/ml and became undetectable four weeks after infection. These data indicate that the primate's intracellular defenses fought off the infection [165]. This was also independently confirmed by infecting rhesus macaques with XMRV [166]. These studies show that if XMRV does infect humans, its replication would be severely limited in people with normal intracellular defenses.

The Origin of XMRV

The chronically XMRV-infected human prostate cancer cell line 22Rv1 was derived by serial passage of human prostate tumor cells in nude mice [150]. Observing the lack of genetic diversity between 22Rv1-derived and patient-derived XMRVs [160] and similarities between XMRV and murine endogenous viruses [124], and previous reports of infection of human xenografts with murine retroviruses [150], the Coffin and Pathak groups hypothesized that XMRV could have been generated during xenograft passage in nude mice. Both groups analyzed DNA and RNA from early and late passages of the tumor used to generate 22Rv1 and found that early passages did not have XMRV, while the later passages did. This suggested that the original tumor did not have XMRV. Further analysis identified two unknown mouse endogenous proviruses with complementary stretches identical to XMRV. Sequence analysis suggested that six crossover events between these two viruses generated a recombinant that differed from XMRV by only four nucleotides, only one of which led to an amino acid change. This would have occurred between 1993 and 1996, since the late xenograft samples after 1996

were all positive for XMRV, but the early samples before 1993 were negative. The probability of these exact crossover events occurring independently is exceedingly low; thus, it can be safely assumed that all XMRV sequences were derived from this one crossover event that occurred in a single mouse [167].

The Death of XMRV's Association with CFS

Although proving that XMRV could not actively replicate in primates and showing the origin of this virus should have brought an end to the claim that XMRV was a novel human infectious retrovirus, some researchers and patients awaited the results of a large multi-laboratory study by the Blood XMRV Working Group [168]. Replicate CFS blood samples that previously tested positive for XMRV by the original group linking XMRV and CFS, along with known XMRV-negative samples were blinded and sent out to nine laboratories. Each laboratory chose their own testing methods which included PCR assays, serological, and co-culture tests. Only the group led by Judy Mikovits, the lab that originally linked XMRV to CFS [123], and her collaborators in the Ruscetti laboratory at the National Cancer Institute, reported positive XMRV samples. The rest of the laboratories, who all used more sensitive methods than Mikovits and Ruscetti, showed that no XMRV was present in any sample [168]. Soon after this, a partial retraction of the original Lombardi paper was issued by *Science* after two of the authors had found that an XMRV plasmid had contaminated their original nucleic acid analysis [169]. A full editorial retraction of the Lombardi paper was made a few months later after *Science* investigated allegations of image manipulation with regard to the paper [170]. This was shortly followed by the retraction of the Lo study [171], which brought a definitive end to XMRV being associated with CFS.

Human Herpesviruses 6 (HHV-6)

Human herpesvirus-6 (HHV-6) was first isolated in 1986 from the blood of patients with lymphoproliferative disorders [172]. Originally it was called human B-lymphotropic virus (HBLV), but later it was found to have greater cell tropism for T lymphocytes [173]. HHV-6 is characterized as a β-herpesvirus and is closely related to human cytomegalovirus (HCMV) and human herpesvirus-7 (HHV-7). Two genetically distinct variants, designated HHV-6A and HHV-6B, have been distinguished among HHV-6 strains based on restriction enzyme maps, monoclonal antibody reactions, and cell culture properties [174-175]. HHV-6 is an enveloped double-stranded DNA virus with an icosahedral capsid [176].

HHV-6 Cellular Tropism

HHV-6 uses human CD46 as a cellular receptor [177]. CD46 is a ubiquitous type-I glycoprotein expressed on the surface of all nucleated human cells [177]. Although CD46 is expressed on all cells, HHV-6 demonstrates predominantly CD4+ T cell tropism [173]. There is limited HHV-6 replication in CD8+ T cells, natural killer cells, and monocytes [178-179]. Neural, epithelial and fibroblastic cell lines have shown some levels of permissiveness for HHV-6 growth *in vitro* [180]. HHV-6A and HHV-6B have shown different neurological tropisms. Although HHV-6B is frequently detected in cerebrospinal fluid, only HHV-6A persists in cerebrospinal fluid, suggesting that 6A has greater neurotropism than 6B [181]. HHV-6A can establish a productive infection in human astrocytes, but HHV-6B lacks this ability [182]. Both variants of HHV-6 DNA are readily detected in saliva and salivary glands [183].

Chromosomally integrated HHV-6 (ciHHV-6)

HHV-6 has also demonstrated the ability to integrate itself into a host's genome, known as chromosomally integrated HHV-6 (ciHHV-6), and is present in about 1% of the population [184]. In ciHHV-6, the entire HHV-6 genome inserts itself into the telomere of a host cell chromosome [185]. If this occurs in a germline cell, ciHHV-6 can be inherited in a Mendelian manner, with a 50% chance of being passed to a child. Because it is inserted in the germline, all nucleated cells will have a copy of the HHV-6 genome. These individuals will always test high for HHV-6 DNA because it is present in every cell in their body. Typically, they will have greater than $1x10^6$ HHV-6 genomes/ml of whole blood and they will also have detectable levels of HHV-6 in serum, plasma, and cerebrospinal fluid which could lead to a misdiagnosis of active HHV-6 infection leading to unnecessary anti-viral treatments that have severe side effects [184]. No disease has been linked to ciHHV-6 [186].

Prevalence of HHV-6

HHV-6 has a high prevalence all over the world, usually infecting children within the first 2 years of life and develops lifelong latency [187]. The peak age of acquisition is 6-9 months [188]. HHV-6 DNA was detected in 90% of immunocompetent adults [189], and more than 90% of adults are seropositive for infection [190]. A study comparing viral sequences between mothers and their infants suggests mother-to-infant transmission, which most likely occurs through saliva [191].

HHV-6 Associated Diseases

HHV-6B is the main causative agent of exanthem subitum (also called roseola or sixth disease), which occurs in children and is characterized by a high fever and the

development of a rash after resolution of the fever [192]. Seizures and neurological symptoms are also common [188]. However, most cases of primary infection are benign. In adults, primary infection, although rare, can cause a mononucleosis-like disease, involving fatigue, headache, fever, sore throat, and swollen lymph glands [193]. HHV-6A has not been proven to be the causative agent of any disease and is currently considered an orphan virus [194].

HHV-6 and Multiple Sclerosis

Several studies suggest an association between HHV-6 infection and multiple sclerosis. Sera from patients with multiple sclerosis show significantly higher anti-HHV-6 antibody titers than healthy controls [195]. Furthermore, HHV-6 virion proteins have been detected at high frequency in oligodendrocytes near plaque formations in the brain of patients with multiple sclerosis [196]. However, HHV-6 has also been detected in the brain tissues of healthy people [197]. Due to the ubiquitous nature of HHV-6, it is difficult to prove an etiological role of HHV-6 in diseases.

HHV-6 in Transplant Patients

Due to HHV-6's ability to establish lifelong latency following primary infection, reactivation in immunocompromised hosts does occur [198]. Evidence suggests that reactivation of HHV-6 occurs in kidney, liver, and bone marrow transplant patients and most likely occurs due to immunosuppressive treatment to avoid organ rejection [198]. Clinical symptoms include fever, skin rash, and malaise, but most cases do not cause any clinical symptoms [199]. A recent study suggests that HHV-6 reactivation during transplant is associated with acute graft rejection (odds ratio = 2.94) [200]. Although

infection may be transmitted through an organ transplant, reactivation is generally believed to result from reactivation of the organ recipient's endogenous virus [198].

HHV-6 and CFS

HHV-6 has been linked to CFS as a possible triggering agent since the first case definition of CFS was established in 1988. Analysis of the 1985 Lake Tahoe outbreak patient population found active replicating HHV-6 in 79 of 113 (70%) patients compared to only 8 of 40 (20%) controls by testing primary cell culture of lymphocytes [10]. This was also confirmed in the same study using monoclonal antibodies specific for HHV-6 proteins and by PCR assays [10]. Another study confirmed the association of HHV-6 and CFS using serological testing, finding increased HHV-6 infection in CFS patients, but few in healthy adults [201]. Studies report higher HHV-6 DNA levels in PBMCs of CFS patients compared to healthy controls [202-205] and increased anti-HHV-6 antibodies present in the sera of CFS patients compared to controls [202, 206-208]. However, researchers have also found the same or similar levels of HHV-6 DNA in PBMCs of patients and controls [209-213], as well as the same levels of anti-HHV-6 antibodies in the sera of CFS patients and matched controls [211-214]. In fact, our study of HHV-6 failed to note any difference in HHV-6 viral load in PBMCs of CFS patients and healthy controls (Oakes 2012, in preparation). These contradictory results are difficult to interpret because, as stated earlier, HHV-6 is a ubiquitous virus being prevalent in over 90% of the population [215]. Although HHV-6 reactivation is probably not associated with all cases of CFS, it still could be a triggering agent for some cases of CFS [107].

Human Herpesvirus-7 (HHV-7)

Human Herpesvirus-7 (HHV-7) was isolated in 1990 from the peripheral blood of a healthy adult [216]. It is characterized as a β-herpesvirus and is closely related to human cytomegalovirus (HCMV) and human herpesvirus-6 (HHV-6). Together with HHV-6, they are the sole members in the β-herpesvirus subclass *Roseoloviruses* [194]. The HHV-7 genome is about 145 kb and codes for more than 70 proteins [179]. Like HHV-6, HHV-7 is an enveloped double-stranded DNA virus with an icosahedral capsid [176].

HHV-7 Cell Tropism

HHV-7 displays a tropism for CD4+ T lymphocytes. Lusso *et al.* demonstrated that HHV-7 uses CD4 as one of its receptors [217], but other receptors must exist due to the viruses' ability to infect non-CD4 cells [218].

HHV-7 Prevalence

Like HHV-6, HHV-7 is ubiquitous in the adult population and has worldwide distribution with primary infection usually occurring early in childhood [219]. Seroprevalence in the adult population is more than 90% [215]. Studies suggest that the peak for HHV-7 infection is observed between 1 and 2 years of age [220]. Infectious HHV-7 virus is continually shed in saliva of healthy adults [221] and is the probable route of transmission to children [215]. HHV-7 develops chronic infection, taking place mostly in salivary glands [222], with lifelong latent infection occurring in macrophages and CD4+ T cells [223].

Diseases Associated with HHV-7

Evidence shows that HHV-7 infection can reactivate HHV-6 [224]. Therefore, the two viruses are usually studied together and appear to be associated with the same diseases. Like HHV-6, HHV-7 can cause exanthem subitum [225], but usually primary infection is asymptomatic. HHV-7 has been associated with pityriasis rosea, a relapsing skin disease associated with rash, which occurs during states of altered immunity [226-227]; however, studies have failed to confirm this association [228]. Due to HHV-7's ability to establish lifelong latency following primary infection, reactivation in immunocompromised hosts does occur. Like HHV-6, evidence suggests that reactivation of HHV-7 occurs in transplant patients, most likely due to immunosuppressive treatment to avoid organ rejection [229]. There is growing concern showing an association of HHV-6 reactivation with acute graft rejection; however, a recent study showed no association of HHV-7 reactivation with graft rejection [200].

HHV-7 and CFS

HHV-7 has controversially been associated with CFS. Early studies showed higher HHV-7 antibody titers in CFS patients [207, 230], but Di Luca *et al.* found no difference in HHV-7 DNA in PBMCs from patients with CFS and controls [203]. Reeves *et al.* showed no difference in seroactivity to HHV-7 in 26 CFS patients compared to 52 non-fatigued matched controls [211], while Chapenko *et al.* demonstrated an increase in dual infection of HHV-6 and HHV-7 in PBMCs of 17 CFS patients compared to 20 healthy controls [205]. Studies in our own lab failed to show a difference in HHV-7 viral load in both PBMCs and saliva in 40 CFS patients and 10 healthy controls (Oakes 2012 in preparation). However, a recent study by Chapenko *et*

al. indicates active viral infections of HHV-6, HHV-7 or Parvovirus B19 occur more often in CFS patients (70/108) than healthy controls (12/90) [231]. They also showed that CFS patients with active infections display the typical symptoms of CFS (malaise after exertion, muscle pain, and headaches) [231]. Although evidence exists for HHV-7 to play a role in CFS, the collection of studies as a whole is inconclusive.

Treatment of both HHV-6 and HHV-7

As of now, there have not been any clinical trials of anti-viral therapy against either HHV-6 or HHV-7 [198], and most cases of infection resolve without treatment [176]. *In vitro* studies have found that ganciclovir, foscarnet, and cidofovir have antiviral activity against HHV-6 [232-233], but they all have serious side effects and should only be used when absolutely needed [194]. In a large randomized trial comparing ganciclovir and valganciclovir in the treatment of CMV in liver transplant patients, the response to anti-viral treatment of concomitant HHV-6 and HHV-7 infections showed no clear effect on HHV-6 and HHV-7 viremia [234]. Despite this recent study, intravenous ganciclovir and foscarnet are both considered to be first-line agents for established infections [235].

Human Endogenous Retrovirus K18

As stated earlier, most CFS patients cite a 'flu-like' illness or infection prior to the symptoms of CFS [58]. Uncontrolled persistent viral infections may trigger chronic activation of the immune system leading to abnormal regulation of cytokine production resulting in the symptoms of CFS [149]. It is believed that CFS could have multiple causes resulting in the same symptoms. Therefore, numerous viruses such as EBV [9, 117], Q Fever [110], Borna disease virus [111-112], enterovirus [113-114], parvovirus

B19 [115-116, 231], Nipah virus [118], HHV-6, and HHV-7 [10, 201-214, 231] have all been investigated for their association with CFS; however, expression of a human endogenous retroviral gene may link all of these potentially triggering viruses together.

What is an Endogenous Retrovirus?

A retrovirus is a type of RNA virus that contains a protein, reverse transcriptase, which converts the viral RNA into DNA once inside a host cell [236]. The retroviral DNA then uses another viral protein, integrase, to insert itself into the host-cell DNA where it can be actively transcribed, resulting in virus production [237]. Viral particles can then go on to infect other host cells and can also be transmitted to other hosts. Retroviruses usually infect somatic cells, although sometimes, a retrovirus will infect a germline cell. If this cell survives, and goes on to produce offspring, then every nucleated cell in the offspring produced from that germ cell will contain a copy of the retrovirus and it will be part of the host's genome [236]. These retroviruses are then passed on to future generations via classical Mendelian inheritance and are known as endogenous retroviruses [237].

History of Human Endogenous Retrovirus-K18

In 1996, Sutkowski *et al.* studied the early events that occur in the immune system during Epstein-Barr viral (EBV) infection [238]. EBV is the causative agent of infectious mononucleosis (glandular fever), a self-limiting lymphoproliferative disease characterized by extensive T cell activation [239]. Sutkowski *et al.* measured the appearance of the early activation marker CD69 on individual T cell Vβ subsets and demonstrated the selective activation of human Vβ13+ T cells [238]. They concluded

that this was the tell tale sign of a superantigen and that EBV infection is associated with the expression of a superantigen in B cells [238].

Superantigens

Superantigens (SAg) are a class of pathogen-derived proteins that elicit a powerful T cell response, activating whole families of T cells with identical T-cell receptor (TCR) Vβ chains [240]. TCRs are composed of five variable elements, Vα, Jα, Vβ, Dβ, and Jβ. All of these variable elements contribute to the specific interaction of T-cells with conventional peptide antigens presented in the context of major histocompatibility complex (MHC) molecules [240]. There are potentially millions of possible combinations of these variable elements, so the frequency of responding to a given antigen is very low, but SAg bypass the normal T-cell activation pathway [241]. SAgs form a bridge between MHC class II molecules on antigen presenting cells and a region on the TCR Vβ chain outside of the unique antigen-binding domain (Figure 1). This results in activation of every T-cell containing the same Vβ variable element [242].

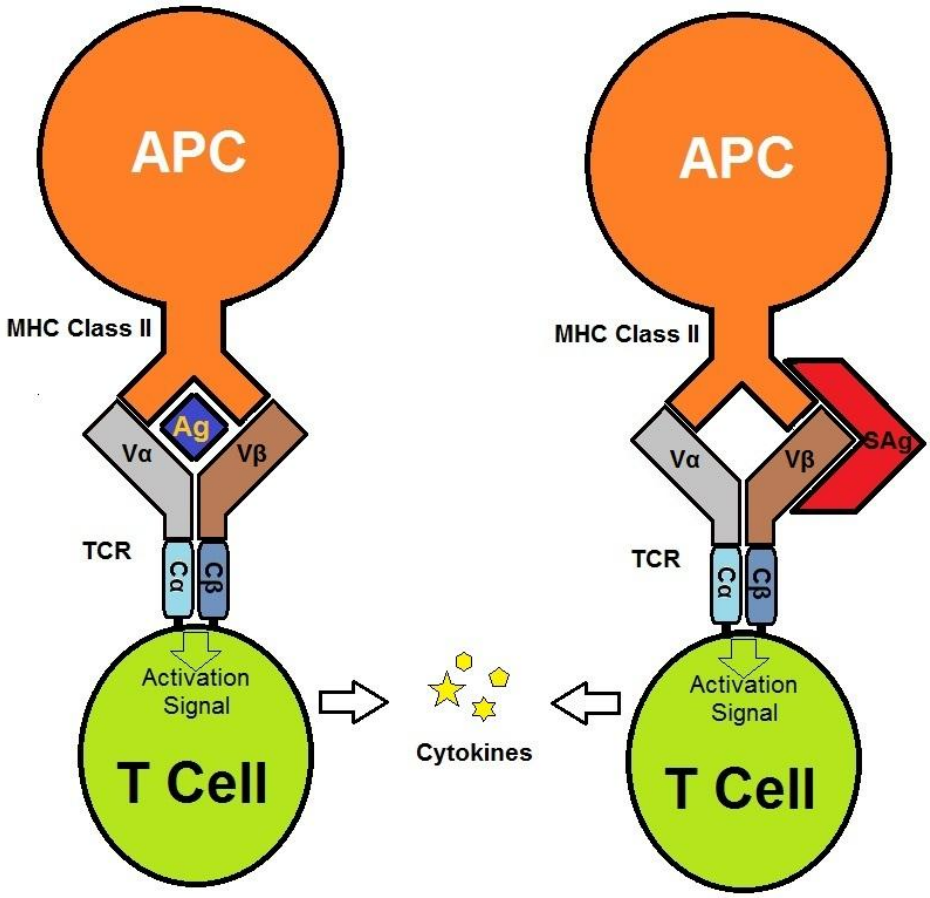

Figure 1. Conventional T-Cell Activation and SAg T-Cell Activation. On the left, an antigen presenting cell (APC) is activating a T cell through the conventional pathway. APCs process peptide antigens (Ag) and present them on their cell surface in the context of the major histocompatibility complex (MHC) class II. Only one or a very small number of T-Cells can recognize a given Ag. The T-Cell receptor's (TCR) variable region recognizes the Ag in the context of the MHC II and is then activated and releases cytokines to activate the immune system. On the right, a superantigen (SAg) is bypassing the specific TCR binding groove and activating all T-Cells that have the same Vβ subunit, resulting in massive immune system activation.

EBV Transactivates a Superantigen

Despite an extensive search, an EBV gene encoding a superantigen could not be identified, which led Sutkowski *et al.* to look for an endogenous gene with superantigen activity that is transactivated by EBV [243]. Previously, human endogenous retrovirus-K18 (HERV-K18) was mapped to the first intron of CD48 [244], an EBV-transactivated gene [245]. Also, Conrad *et al.* previously showed that the HERV-K18 *env* gene encodes superantigen activity [246]. Sutkowski *et al.* then showed that EBV infection led to transcriptional activation of HERV-K18 *env* and that HERV-K18 *env* specifically stimulates Vβ13+ T cells, confirming HERV-K18 *env* as the EBV-inducible gene encoding a superantigen [243]. Simultaneously, Stauffer *et al.* demonstrated that interferon-α (IFN-α) also transactivates HERV-K18 *env* [247]. IFN-α is an antiviral cytokine released by infected cells to activate the immune system meaning any infection inducing an IFN-α response could activate the HERV-K18 superantigen.

HERV-K18 Location and Structure

The HERV-K18 *env* gene is localized to chromosome 1q21.2-q22 in the first intron of CD48 [244]. There are three alleles of the HERV-K18 *env* in the human population, and all of them show superantigen activity [247]. However, due to differences in amino acid sequence, biochemical differences are predicted between alleles (See Figure 10). Allele K18.1 has a stop codon after the first 152 amino acids, eliminating the transmembrane portion seen in the other two alleles. The three alleles are not evenly distributed within the Caucasian population. The allelic frequencies are 46.6%, 42.5%, and 10.8% for K18.1, K18.2, and K18.3, respectively [247].

Association of HERV-K18 to Diseases with Immune Dysfunction

Multiple studies have attempted to link HERV-K18 *env* expression to different diseases involving immune dysfunction. Sicat *et al.* showed significantly elevated HERV-K18 expression in peripheral blood from patients with juvenile rheumatoid arthritis (JRA), but not in patients with pediatric systemic lupus erythematosus (SLE) suggesting a role for autoimmunity by SAg stimulation of autoreactive T cells in JRA, but not in SLE [248]. An early study linked the HERV-K18.3 haplotype to type 1 diabetes [249], however a larger subsequent study failed to see any association between HERV-K18 polymorphisms and type 1 diabetes [250]. A large study by Tai *et al.* showed an increased relative risk of multiple sclerosis for individuals with the rare K18.3/K18.3 genotype compared to the K18.2/K18.2 genotype (relative risk = 2.7) [251], however, no study has been done to confirm these findings at this time.

XMRV, HHV-6 & -7, HERV-K18 and CFS: Thesis Premise

CFS is a complex, heterogeneous disease affecting more than 1 million Americans [1, 23-24]. Patients with CFS have overwhelming fatigue that is not improved with bed rest and worsens after physical activity or mental exertion. CFS is a debilitating disease that stops people from performing even everyday tasks. Other symptoms of CFS include impaired memory, sore throat, tender cervical or axillary lymph nodes, muscle pain, pain in joints, and headaches [32]. Despite extensive research into a cause of CFS for over the past three decades, no definitive cause of CFS has been determined; however, there is evidence supporting an infectious etiology.

A large percentage of CFS patients note an acute infectious event that triggers their fatigue [252]. The exciting discovery of the new gammaretrovirus, xenotropic murine leukemia virus-related virus (XMRV), present in 67% of CFS patients PBMCs compared to only 4% of healthy controls reignited the search for a viral cause to CFS [123]. Confirming this exciting discovery in an independent cohort of CFS patients would provide a major step forward in defining CFS. Previous studies have also attempted to link numerous other viruses [9, 110-118, 231], including the ubiquitous β-herpesviruses, human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7) to CFS [10, 201-214, 231]; however most of these studies are controversial and one virus has never been linked to all cases of CFS. One explanation to this quandary could be the involvement of human endogenous retrovirus-K18 (HERV-K18).

HERVs are ancient retroviruses that infected germ line cells and became permanently integrated into the genome [237]. About 8% of the entire human genome is

believed to be HERVs, however most of these proviral genes are silenced or only expressed in response to an environmental trigger, such as an infecting virus that can activate them in susceptible cells [248]. Recently, it was shown that the HERV-K18 *env* gene can be activated in B cells by EBV [243, 253], IFN-α [247], HHV-6A [254], and HHV-6B [255]. The HERV-K18 envelope protein encodes a superantigen (SAg) which is recognized by the human Vβ13 T-cell receptor (TCR) variable unit [238]. SAgs are microbial proteins that greatly over-stimulate the immune system by directly interacting with the Vβ segment of the TCR and the MHC II complex of antigen presenting cells. This is unlike conventional peptide antigens that are recognized by a specific hypervariable region of the TCR, which is different in every T cell clone [241].

Working Hypothesis

One of the major hypotheses for the pathogenesis of CFS is that persistent viral infections may trigger and lead to chronic activation of the immune system with abnormal regulation of cytokine production [149]. Past viruses associated with CFS and under investigation as possible triggers to CFS include EBV, HHV-6A, and HHV-6B; all of which have been shown to transactivate the HERV-K18 SAg [253-255]. IFN-α, an antiviral cytokine produced in response to infection, can also induce HERV-K18 *env* [247]. These viruses, or any infection inducing IFN-α, could lead to induction of the HERV-K18 *env* SAg, which then could lead to overstimulation of the immune system and the symptoms of CFS.

To further understand the role of viruses in CFS, I have essentially undertaken two projects. In the first project, I attempted to confirm that XMRV was present in a large cohort of CFS patients. This led to the development of a novel PCR to test for

murine DNA contamination in human samples and the debunking of the association between XMRV and CFS. In the second project, I attempted to develop the theory that chronic infecting viruses lead to HERV-K18 SAg expression and immune dysfunction resulting in the symptoms seen in CFS (Figure 2). First, I attempted to show that the HERV-K18 *env* genotype was associated with CFS. Second, I attempted to show that the HERV-K18 *env* expression level in CFS patients was increased and varied over time in correlation with the patient's symptom severity. Finally, I looked at HHV-6 and HHV-7 viral load in an attempt to link it to HERV-K18 superantigen expression and CFS symptom severity.

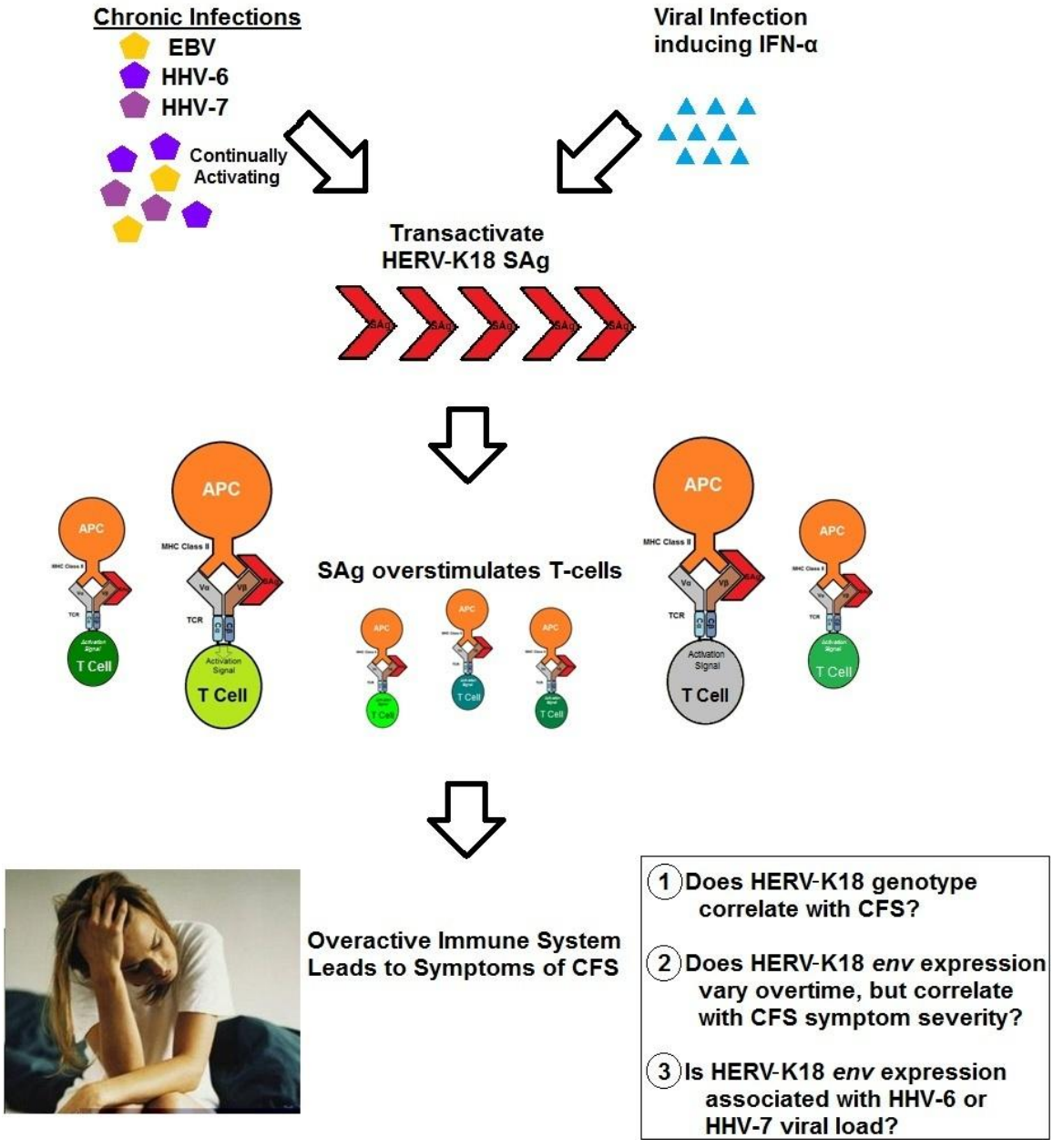

Figure 2. Working Hypothesis. Chronic infections, or viral infections inducing IFN-α, transactivate the HERV-K18 *env* SAg. The SAg then activates Vβ13+ T-Cells and over-stimulates the immune system resulting in the symptoms of CFS. The three major questions investigated are listed in the bottom right box.

Materials and Methods

Patient Cohorts

Levine Cohort

All samples were collected according to the institutional guidelines of Tufts University, after receiving informed consent. The 36 healthy individuals (15 females and 21 males) were recruited on a voluntary basis by the Huber lab and were between 18 and 65 years of age. The 112 CFS patients (89 females, 20 males and 3 unknown), recruited by Dr. Susan Levine, were between 18 and 65 years of age and resided in the northeastern United States. All patients were diagnosed for CFS according to CDC criteria [12], and the majority were completely disabled. The cohort comprised a combination of those with an abrupt and others with a gradual onset of symptoms. Blood was collected from each patient once and shipped overnight to the Huber lab at Tufts University.

Taylor Cohort

Participants in our study were recruited by an expert in CFS, Dr. Renee Taylor at the University of Illinois, Chicago and were diagnosed using the 1994 CDC criteria [12]. Participants were separated into two groups. The 53 patients in Group A (44 females and 9 males) developed CFS after having a history of EBV-infectious mononucleosis (EBV-IM). EBV-IM was previously diagnosed by physicians and part of a patient's history. EBV-IM diagnosis was done on the basis of the symptoms of fever, sore throat, and swollen lymph glands and was confirmed with serological testing showing elevated white blood cell counts, an increased percentage of atypical white blood cells, and a positive

reaction to a "mono spot" test. The 48 patients in Group B (40 females and 8 males) developed CFS with no history of EBV-IM. The patients in Group A were between 16 and 70 years of age with an average age of 49. The patients in Group B were between 15 and 64 years of age with an average age of 49. Blood was collected from each patient approximately every 6 months for two years and shipped overnight to the Huber lab at Tufts University. At the time of blood draw, patients were interviewed to determine the severity of their symptoms using 5 different scales: The Fatigue Scale, The Chronic Fatigue Syndrome Rating Form, The Perceived Stress Scale, The General Health Questionnaire, and The SPHERE and SOMA Questionnaire. All samples were collected according to the institutional guidelines of Tufts University, after receiving informed consent.

Komaroff Cohort

Participants in our study were recruited by an expert in CFS, Dr. Anthony Komaroff at Harvard University. Blood and saliva samples were collected from 40 CFS patients (29 females and 11 males) as well as 10 healthy controls (6 females and 4 males) at Harvard University. The CFS patients were between 20 and 77 years of age and resided in the northeastern United States. The healthy controls were between 24 and 58 years of age and also resided in the northeastern United States. All patients were diagnosed for CFS according to CDC criteria [12], and the majority was completely disabled. The cohort comprised a combination of those with an abrupt and others with a gradual onset of symptoms. Saliva samples were collected using a previously used protocol involving mouth rinse and gargling with 5 ml of PBS [256]. Samples were then blinded and immediately transported to Tufts University. Blood samples were processed

immediately. Saliva samples were immediately frozen at -80 °C until processing. At the time of sample collection, patients were interviewed to determine the severity of their fatigue. All samples were collected according to the institutional guidelines of Tufts University, after receiving informed consent.

Sample Processing

Blood

Approximately 30 ml of blood were drawn into three heparinized tubes (Becton Dickinson) and shipped overnight (CFS patients Levine Cohort and Taylor Cohort) or processed immediately (healthy controls and Komaroff Cohort). The blood collection tubes from each individual were consolidated into one 50 ml tube and diluted with PBS, containing $CaCl_2$ and $MgCl_2$ (Sigma) at a 1:1 ratio. 15 ml of Ficoll (GE Healthcare) was added to two new 50 ml tubes, and 25 ml of the diluted blood was gently layered on top of the Ficoll, followed by a 30 min centrifugation in a Sorvall RT7plus rotor at 2000 rpm at room temperature. The PBMCs were collected from the interface following the spin and were used for DNA/RNA isolation. Ten ml of plasma were also collected from each sample and stored at -80° C. One ml of plasma from the XMRV-Levine Cohort was sent to Abbott Labs on dry ice overnight for further testing. The collected PBMCs were diluted with PBS (Levine Cohort) or RPMI-1640 Medium (Sigma), supplemented with 10% FCS (Gemini BioProducts), 100 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), 2 mM L-glutamine (Sigma), and 1 mM sodium pyruvate (Sigma) (Taylor Cohort) (complete RPMI) at a 1:1 ratio and then pelleted at 2000 rpm for 5 min. The supernatant was aspirated, and the pellet of PBMCs was resuspended in 20 ml of PBS

(Levine Cohort) or complete RPMI (Taylor and Komaroff Cohorts). Cells were counted using a light microscope and a hemocytometer, aliquoted to $5x10^6$ cells per tube, spun down and resuspended in 350 µl of Buffer RLT Plus (Qiagen) (1% β-mercaptoethanol). Samples were stored in this lysis buffer at -80°C.

Saliva (Komaroff Cohort)

Saliva collection was performed using established methods [256]. Patients and healthy controls were asked to do a mouth rinse and gargling with 5 ml of phosphate buffered saline (PBS), which was kept in a 15 ml tube. Saliva samples were transported to Tufts University from Harvard University the day of collection and immediately frozen at -80°C until all samples had been collected. In prior experiments, there was no detectable change in recovery of viral DNA after storage periods of up to 1.5 years [256].

DNA/RNA Isolation

PBMCs

DNA and RNA were isolated using the procedures provided by the AllPrep DNA/RNA Mini Kit (Qiagen). Briefly, 350 µl of PBMCs lysate ($5x10^6$ cells in RLT buffer) were placed on the DNA spin column. Samples were spun down at 10,000 rpm for 30 s in an Eppendorf centrifuge model 5417C. The column was then transferred to a new collection tube and put aside until after RNA was isolated. The flow-through was used to isolate RNA. 350 µl 70% ethanol was added to the flow-through, mixed well and placed on an RNA spin column followed by a 15 s spin at 10,000 rpm. The flow-through was discarded, and the column was transferred to a new collection tube. 700 µl Buffer

RW1 was added to the column, followed by a 15 s spin at 10,000 rpm. The flow-through was discarded, and the column was transferred to a new collection tube. 500 µl of Buffer RPE was added to the column, followed by a 15 s spin at 10,000 rpm. The flow-through was discarded, and the column was transferred to a new collection tube. 500 µl Buffer RPE was added to the column, followed by a 1 min spin at 10,000 rpm. The flow-through was discarded, and the column was transferred to a new collection tube. The column was then spun at full speed for 1 min to remove any residual ethanol. The flow through was discarded, and the column was transferred to a new 1.5 ml collection tube. 35 µl of RNase-Free water was added directly to the column and incubated at room temperature for one minute followed by a 1 min spin at 10,000 rpm. Columns were discarded and isolated RNA was stored on ice until concentration was determined using 1 µl of sample on a Thermo Scientific Nanodrop 2000 Spectrophotometer. DNA isolation continued with 500 µl Buffer AW1 being added to the column, followed by a 15 s spin at 10,000 rpm. The flow-through was discarded, and the column was transferred to a new collection tube. 500 µl of Buffer AW2 was added to the column, followed by a 2 min spin at full speed. The flow-through was discarded, and the column was transferred to a new 1.5 ml collection tube. 100 µl of Buffer EB was added directly to the column, followed by 1 min incubation at room temperature. Finally, the column was spun down at 10,000 rpm for 1 min to elute DNA. DNA concentration was determined using 1 µl of sample on a Thermo Scientific Nanodrop 2000 Spectrophotometer. RNA was stored at -80°C and DNA was stored at -20°C until PCR testing.

DNA Isolation from Saliva Wash

DNA was isolated from the saliva wash using the QIAGEN supplementary Protocol using the QIAamp DNA Blood Mini Kit spin procedure (Qiagen). Briefly, the 5 ml saliva wash was spun down at 1800g for 5 min. The supernatant was discarded and the pellet was resuspended in 180 µl PBS. 20 µl QIAGEN Protease and 200 µl Buffer AL were added and mixed immediately by vortexing for 15 s. Samples were then incubated at 56°C for 10 min. After the 10 min incubation, 200 µl 100% ethanol was added to the sample and mixed by vortexing. The entire sample was then added to a QIAamp Spin column and then spun down at 8000 rpm for 1 min. The flow-through was discarded and the column was placed in a clean collection tube. 500 µl Buffer AW1 was added to the column followed by a spin at 8000 rpm for 1 min. The flow-through was discarded and the column placed in a clean collection tube. 500 µl of Buffer AW2 were added to the column followed by a spin at full speed for 3 min. The flow-through was discarded and the column was placed in a 1.5 ml collection tube. 150 µl of Buffer AE was added to the column followed by a 5 min incubation at room temperature and then a spin at 8000 rpm for 1 min. Isolated DNA was kept at -20°C until PCR testing.

Generation of cDNA

RNA was converted into cDNA using iScript reverse transcriptase (Bio-Rad) after utilizing the TURBO-DNase kit (Invitrogen). Briefly, 500 ng of total RNA from PBMCs was incubated at 37°C with DNase for 30 min. DNase was removed using the provided DNase Inactivation reagent, incubating for 5 min, and spinning down the tube, leaving only RNA in the supernatant. The iScript reverse transcriptase kit uses random primers to amplify all RNA resulting in cDNA.

Polymerase Chain Reactions (PCRs)

TaqMan qPCR for XMRV pol

Primers and probe, designed by Schlaberg *et al.*[257], were ordered from Applied Biosystems (see Table 4 for sequences). The reaction mix for the TaqMan qPCRs contained 1X Gene Expression Master Mix (Applied Biosystems), 900 nM forward and reverse primers, 250 nM probe, and 200 ng of DNA in a reaction volume of 20 µl. The assay was validated with DNA from the WPI-1282 cell line containing VP62 XMRV (kindly supplied by J. Mikovits, WPI). The same DNA served as positive control in each assay, which also included a no-template negative control. Thermocycler conditions were 95° C for 10 min, followed by 60 cycles of 95° C for 15 s and then 60° C for 1 min, using 96-well Optical Reaction Plates (Applied Biosystems) on a 7300 Real Time PCR System by Applied Biosystems. All reactions were performed in triplicate. Quality of DNA was assessed using a TaqMan qPCR for the ribosomal 18S gene in the same reaction (Applied Biosystems).

Nested PCR for XMRV gag

Identical primers as originally described by Urisman *et al.* [148] and also employed by the Mikovits group [123] were used. The reaction mix for all PCRs consisted of 1X HotStart-IT^{TM} FideliTaq^{TM} Master Mix, 200 nM forward and reverse primers, and 200 ng of sample DNA in a 50 µl reaction volume. The WPI-1282 lymphoblastoid cell line was used as a positive control [123]. Thermocycler conditions for the first PCR were 2 min at 94° C, followed by 30 cycles of 94° C for 30 s, 58° C for 30 s, and 72° C for 45 s and then finished off with 72° C for 7 min. Once the first PCR was complete, 2 µl of DNA from the first PCR was used for the second PCR. The

second PCR consisted of 1X HotStart-IT™ FideliTaq™ Master Mix, 200 nM forward and reverse primers, and 200 ng of sample DNA in a 50 µl reaction volume. Thermocycler conditions for the second PCR were 2 min at 94° C, followed by 30 cycles of 94° C for 30 s, 60° C for 30 s, and 72° C for 30 s and then finished off with 72° C for 7 min. Once the second PCR was complete, 15 µl of the samples were run on a 1.5% agarose gel for 1 h at 100 volts. Images of gels were taken using a VersaDoc Imaging System (Biorad). The expected fragment size of the second PCR is 413 bp [148].

All positive samples from the second XMRV nested PCR were isolated using a Qiaquick PCR Purification Kit (Qiagen). DNA sequencing was performed by the Tufts University Core Facility. Once sequenced, the traces were monitored for double peaks, and sequences with double peaks were discarded. Samples that had mixed sequences were diluted, and the nested PCR was repeated. Only clean sequences with the forward sequence matching the reverse sequence were used for phylogenetic analysis.

TaqMan qPCR for mouse mitochondrial cox2

Sequences for primers and probes were kindly supplied by Dr. Switzer, CDC [159] (see Table 4). Primers and probes were ordered from Applied Biosystems. The reaction mix contained 1X Gene Expression Master Mix (Applied Biosystems), 900 nM forward and reverse primers, 250 nM probe, and 200 ng of DNA in a reaction volume of 20 µl. DNA isolated from the murine EL4 cell line, diluted in 200 ng of human LNCaP DNA, was used as a positive control. Thermocycler conditions were 95° C for 9 min, followed by 60 cycles of 95° C for 30 s and 62° C for 30 s. 96-well plates were used on a 7300 Real Time PCR System by Applied Biosystems. All reactions were performed in

duplicate or triplicate. Quality of DNA was assessed using a TaqMan qPCR for the ribosomal 18S gene in the same reaction (Applied Biosystems).

PCR for Mouse Intracesternal A-type Particle (IAP) sequences

Primers were designed by the Coffin Lab and ordered from Invitrogen (see table 4 for sequences) [124]. The reaction mix for all PCRs consisted of 1X HotStart-IT™ FideliTaq™ Master Mix, 1 µM forward and reverse primers, and 200 ng of sample DNA in a 50 µl reaction volume. DNA isolated from the murine EL4 cell line was diluted into 200 ng of human DNA (LNCAP) and used as a positive control. Thermocycler conditions were 94° C for 2 min, followed by 45 cycles of 94° C for 30 s, 58° C for 30 s, and 72° C for 20 s and then finished off with 72° C for 7 min. Samples were then run on a 1.5% agarose gel with sequence lengths varying between 200 and 300 bp. Images of gels were taken using a VersaDoc Imaging System (Biorad).

TaqMan-based SNP genotyping for HERV-K18

To characterize the potential association between the HERV-K18 *env* and CFS, we used a highly specific, sensitive and efficient screening method, using TaqMan MGB probe based single nucleotide polymorphism (SNP) genotyping for the determination of the allelic and genomic distribution of the three alleles of HERV-K18 *env* which was previously developed in our lab [251]. A primer pair was selected for sequences that are unique to HERV-K18, but common between the three alleles. One TaqMan MGB probe was designed for each allele around a SNP that is unique to that particular allele (all sequences listed in table 4). SNP PCR was carried out on an ABI 7300 Sequence Detection System. The reaction was performed in a volume of 10 µl containing ABI TaqMan PCR MasterMix, DNA template, 450 nM of each primer and 125 nM of each

TaqMan MGB probe. The PCR was carried out with 10 min initial denaturation at 95°C, followed by 60 cycles of 95°C for 15 s and 62°C for 1 min. All samples were tested in triplicate. The data were then analyzed with the system software to determine genotype.

TaqMan-based HERV-K18 env expression

cDNA was generated from 500 ng of total RNA using iScript reverse transcriptase (Bio-Rad) per the manufacturer's instructions. The Taqman probe and primers specific for the read-through transcript of HERV-K18 were designed to recognize HERV-K18 *env* in human cells by Hsiao *et al.* (sequences shown in table 4) [258]. Thermocycler conditions were 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and then 60°C for 1 minute using 96-well Optical Reaction Plates (Applied Biosystems) on a 7300 Real Time PCR System by Applied Biosystems. The housekeeping gene hypoxanthine phosphoribosyltransferase (hprt) was used to measure the quality of DNA (Applied Biosystems). The ΔΔCt method was used to compare relative expression of HERV-K18 *env*. HERV-K18 *env* transcript levels were measured relative to a standard that was always present on every plate. All samples were tested in triplicate.

TaqMan-based quantitative real-time HHV-6 qPCR

Primers and probe, designed by Karlsson *et al.* [259], were ordered from Applied Biosystems (See table 4 for sequences). The target sequence for the HHV-6 qPCR was chosen from a conserved region of the HHV-6 U67 gene, which was a perfect match for both HHV-6A and HHV-6B. The analytical sensitivity and linear detection range of the assay corresponds to 0.5-5 x 10^5 HHV-6 genome copies/PCR reaction. This was

determined using a quantitated viral DNA control given to us by D. Ablashi of the HHV-6 Foundation. The reaction mix for the Taqman qPCRs contained 1X Gene Expression Master Mix (Applied Biosystems), 900 nM forward and reverse primers, 250 nM probe, and 200 ng of DNA in a reaction volume of 20 µl. A standard curve using the quantitated HHV-6 viral DNA was run on each plate as a positive control and samples containing no DNA were used as negative controls. Thermocycler conditions were 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and then 60°C for 1 min using 96-well Optical Reaction Plates (Applied Biosystems) on a 7300 Real Time PCR System by Applied Biosystems. All reactions were performed in triplicates. Quality of DNA was assessed, using a Taqman qPCR for the *hprt* gene or ribosomal 18s gene in the same reaction (Applied Biosystems).

TaqMan-based HHV-6A and 6B variant discrimination

A subtype-specific, probe based, real time PCR for detection and typing of HHV-6 was developed by Lou *et al* [260]. Briefly, the DNA polymerase genes (U38) were selected as target genes. Three nucleotides differ between the strains in the region selected (see table 4). The 5' end of the probes for HHV-6A and HHV-6B was labeled with the fluorescent reporter dye VIC and 6-carboxyfluorescein (FAM), respectively; while the 3' end was quenched with 6-carboxytetramethylrhodamine (TAMRA). Primers and probes were synthesized by Applied Biosystems. The reaction mix for the Taqman qPCRs contained 1X Gene Expression Master Mix (Applied Biosystems), 900 nM forward and reverse primers, 250 nM probe, and approximately 200 ng of DNA in a reaction volume of 20 µl. All samples that first tested positive for HHV-6 in the quantitative real time PCR assay were subsequently tested for subtype variant. All

samples were tested in triplicate. Quality of DNA was assessed, using a Taqman qPCR for the 18s gene in the same reaction (Applied Biosystems). Thermocycler conditions were 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and then 60°C for 1 min using 96-well Optical Reaction Plates (Applied Biosystems) on a 7300 Real Time PCR System by Applied Biosystems. HHV-6A and HHV-6B standard DNA was used as positive controls. Water was used as a no template negative control.

Taqman-based quantitative real-time HHV-7 qPCR

A quantitative real time PCR for HHV-7 was developed by Fernandez *et al.* [261]. Briefly, the primers and probe were chosen in the conserved U100 gene (sequences shown in Table 4). The 5' end of the probe was labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM). Primers and probe were synthesized by Applied Biosystems. The reaction mix for the qPCR contained 1X Gene Expression Master Mix (Applied Biosystems), 900 nM forward and reverse primers, 250 nM probe, and approximately 200 ng of DNA in a reaction volume of 20 µl. All samples were tested in triplicate. Quality of DNA was assessed using a Taqman qPCR for the 18s gene in the same reaction (Applied Biosystems). Thermocycler conditions were 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and then 60°C for 1 minute using 96-well Optical Reaction Plates (Applied Biosystems) on a 7300 Real Time PCR System by Applied Biosystems. Quantitated HHV-7 standard DNA (Advanced Biotechnologies, Inc.) was used to make a standard curve for each plate. The analytical sensitivity and linear detection range of the assay corresponds to 0.5-5 x 10^5 HHV-7 genome copies/PCR reaction. A water blank sample was used as a non-template control on each plate.

Table 4. Primers and Probes for PCR

Assay	Primer	Sequence
Taqman qPCR for XMRV *pol*	XMRV4552F	5'-CGA GAG GCA GCC ATG AAG G-3'
	XMRV4673R	5'-CCC AGT TCC CGT AGT CTT TTG AG-3'
	XMRV4572MGB	5'-6FAM-AGT TCT AGA AAC CTC TAC ACT C-MGBNFQ-3'
Nested PCR for XMRV *gag*	GAG-O-F	5'-CGC GTC TGA TTT GTT TTG TT-3'
	GAG-O-R	5'-CCG CCT CTT CTT CAT TGT TC-3'
	GAG-I-F	5'-TCT CGA GAT CAT GGG ACA GA-3'
	GAG-I-R	5'-AGA GGG TAA GGG CAG GGT AA-3'
TaqMan qPCR for mouse mitochondrial *cox2*	MCox2-F2	5'-TTC TAC CAG CTG TAA TCC TTA-3'
	MCox2-R1	5'-GTT TTA GGT CGT TTG TTG GGA T-3'
	MCox2-PR1	5'-FAM-CGT AGC TTC AGT ATC ATT GGT GCC CTA TGG T-MGBNFQ-3'
	MCox2-P1	5'-FAM-TTG CTC TCC CCT CTC TAC GCA TTC TA-MGBNFQ-3'
PCR for Mouse IAP	IAP-Forward	5'-ATA ATC TGC GCA TGA GCC AAG G-3'
	IAP-Reverse	5'-AGG AAG AAC ACC ACA GAC CAG A-3'
TaqMan SNP Genotyping for HERV-K18 *env*	SNP-Forward	5′-CTA AAT TCC ATT CTA ACG GTT CCT TT-3′
	SNP-Reverse	5′-GTT TGG GAG GCT GGT TTA ATA ACT AT-3′
	K18.1 Probe	5′-FAM-AAA GTT GC**C** TAA AGC-MGBNFQ-3′
	K18.2 Probe	5′-VIC-AAA GTT GC**G** TAA AGC-MGBNFQ-3′
	K18.3 Probe	5′-NED-AAA GTT GC**A** TAA AGC-MGBNFQ-3′
TaqMan HHV-6 qPCR	HHV-6 Forward	5'-CCC GAG CGC TAG GTT GAG-3'
	HHV-6 Reverse	5'-AAC GCT CGT CAC AAA CAT AAA ATT C-3'
	HHV-6 Probe	5'-FAM-ATG ATC GAA ACG CCT ACA C-MGB-3'
TaqMan HHV-6A and 6B Variant Discrimination	HHV-6AB Forward	5'-CTA AGG TGA GCC AGA TTC G-3'
	HHV-6AB Reverse	5'-GAA TAC CCA CAG GCA CTC C-3'
	HHV-6A Probe	5'-VIC-TGC AGC CA**T** **T**TC **T**TT GGA AAG C-TAMARA-3'
	HHV-6B Probe	5'-FAM-TGC AGC CA**C** **C**TC **C**TT GGA AAG-TAMARA-3'
TaqMan HHV-7 qPCR	HHV-7 Forward	5'-AGA GCT TGC GTT GTG CAT GTT-3'
	HHV-7 Reverse	5'-ATG TAC CAA TAC GGT CCC ACT TG-3'
	HHV-7 Probe	5'-FAM-CACGGCAATAACTCTAG-MGB-3'
TaqMan HERV-K18 *env* Expression	HERV-K18 Forward	5′-CCG CCT TTT GAG CAG AAG TAT AAG A-3′
	HERV-K18 Reverse	5′-CAG TAA TGG CAA TGC TGG CTA TG-3′
	HERV-K18 Probe	5′-FAM-TAA GTC CTA CAG ACA AAC TT-MGB-3′

XMRV Chemiluminescent Immunoassays (CMIAs)

A detailed procedure can be seen here [262]. Briefly, 100 µL of neat plasma were screened for antibodies to XMRV envelope proteins, gp70 and p15E, using two prototype ARCHITECT® chemiluminescent immunoassays (CMIAs; Abbott Diagnostics, Abbott Park, IL). The CMIAs utilize a direct assay format in which *E. coli*-expressed XMRV p15E or mammalian-expressed XMRV gp70 were used as both capture and detection antigens. Assay positive controls were derived from XMRV-infected macaque plasmas at 1:1000 (PC1) or 1:4000 (PC2). A pool of normal human plasma was used as negative control (NC) and as sample diluents. Cutoff (CO) values of the ARCHITECT® CMIAs were calculated based on the following formulas: CO = 0.45 X (Calibrator 1 Mean Relative Light Units (RLU)) for p15E CMIA and CO=0.078 X (Calibrator 2 Mean RLU) for gp70 CMIA. Assay results were reported as the ratio of the sample RLU to the cutoff RLU (S/CO) for each specimen. Specimens with S/CO values <1.00 were considered non-reactive; specimens with S/CO values $\geq$1.00 were considered initially reactive. The S/CO values of the NC, PC1 and PC2 were 0.16, 12.8 and 3.5 for the gp70 CMIA and 0.13, 7.4 and 2.2 for the p15E CMIA. Initially reactive specimens were retested in duplicate by either ARCHITECT® p15E or gp70 CMIAs. Repeatedly reactive specimens were analyzed at 1:100 dilution by investigational western blot assays using purified XMRV viral lysate as well as recombinant gp70 protein.

Western blot analysis for XMRV viral proteins

Western blot (WB) analysis using purified XMRV viral lysate as well as recombinant gp70 protein was performed as described [262]. Briefly, viral lysate (80

µg/gel) or recombinant gp70 protein (20 µg/gel) were separated by electrophoresis on a 4-12% NuPAGE Bis-Tris 2-dimension gel (Invitrogen, Carlsbad, CA) in the presence of sodium dodecylsulfate (SDS). The protein bands on the gel were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). After blocking, the PVDF membrane was cut into 2 mm strips. Strips were incubated with human samples diluted 1:100 or XMRV infected macaque plasma diluted 1:200 overnight at 2-8 °C. After removal of unbound antibodies, strips were incubated with alkaline phosphatase conjugated goat anti-human IgG (Southern Biotech, Birmingham, AL) for 30 min at room temperature. The strips were washed and chromogenic substrate solution was added.

Symptom Severity Evaluations

The Fatigue Scale

The Fatigue Scale, developed by Chalder *et al.* [263], is an 11-item scale intended to measure the severity of fatigue-related symptoms experienced by individuals with ME/CFS. Responses to items are measured using a Likert-style format with four possible response choices related to symptom frequency (0 = less than usual, 1 = no more than usual, 2 = worse than usual, 3 = much worse than usual). The scores are then summed and a higher score indicates more severe fatigue-related symptomatology. The 'Physical Fatigue' items include questions such as "Do you have problems with tiredness?" or "Do you lack energy?" The remaining items constitute a 'Mental Fatigue' factor with questions such as "Do you have difficulty concentrating?" or "Do you make slips of the tongue when speaking?" The Total scale demonstrated sufficient internal consistency with alpha coefficients of .89 [263].

The Chronic Fatigue Syndrome Rating Form

Using this form, participants rate the severity of their fatigue and the severity of the eight chronic fatigue syndrome definitional symptoms [12]. Responses to items are measured using a Likert-style format with four possible response choices related to symptom frequency (0 = never, 1 = seldom, 2 = often or usually, 3 = always). In a previous study [264], a modified version of this form was demonstrated to have high test-retest reliability over a 2-week period (test-retest agreement: 76%- 92%) [265].

The Perceived Stress Scale Scale

The Perceived Stress Scale (PSS) is the most widely used psychological instrument for measuring the perception of stress. It is a measure of the degree to which situations in one's life are appraised as stressful. Items were designed to tap how unpredictable, uncontrollable, and overloaded respondents find their lives. The scale also includes a number of direct queries about current levels of experienced stress. The questions in the PSS ask about feelings and thoughts during the last month [266].

The General Health Questionnaire

The GHQ-28 was developed by Goldberg *et al.* [267] and has since been translated into 38 languages. Developed as a screening tool to detect those likely to have or to be at risk of developing psychiatric disorders, the GHQ-28 is a 28-item measure of emotional distress in medical setting. Through factor analysis, the GHQ-28 has been divided into

four subscales. These are somatic symptoms, anxiety/insomnia, social dysfunction, and severe depression.

The SPHERE and SOMA Questionnaire

The SPHERE-34 is a screening tool for common mental disorders most commonly used in medical settings. Although composed of 34 items, the scoring is based on a subset of 12 items in order to create two subscales, PSYCH-6 (comprised of six items assessing psychological symptoms of depression and anxiety) and SOMA-6 (comprised of six items assessing somatic symptoms such as fatigue and pain). SPHERE questions are scored on a Likert scale with a score of 0 for "never or some of the time", 1 for "a good part of the time", and 2 for "most of the time". The timeframe applied to all questions is "the last few weeks."

Komaroff Patient Interviews

At the time of the sample collection, CFS patients were asked 5 questions to determine the severity of their fatigue. These are: 1. On a scale of 1 to 10 (10 indicating the most fatigue), how would you rate your fatigue over the last 24 hours? 2. At what % of your pre-illness level have you been functioning? 3. Which of the following statements best describes the severity of your fatigue at its worst over the past several months (1 indicating the most fatigue)? 4. Which of the following statements best describes the severity of your fatigue on an average day over the past months (1 indicating the most fatigue)? 5. Have you been so fatigued that you had to reduce your

average activity level below half of what was your normal level before you became ill (1 indicating the most fatigue)?

Statistics

Statistical analysis was performed using Microsoft Excel, GraphPad and SAS. Comparison of viral loads and HERV-K18 *env* expression levels between CFS group and healthy controls was done using the Kolmogorov-Smirnov (K-S) test. Correlations between viral loads and symptom severity scales were tested using the Spearman Rank Correlation test.

Results

Chapter 1. XMRV is a contaminant and is not associated with CFS

XMRV Study Population

We analyzed a library of 111 stored DNA samples that had been collected from the PBMCs of CFS patients in 2005. The patients had been diagnosed with CFS using the 1994 CDC criteria [12]. In addition, we collected 37 blood samples (one CFS and 36 healthy controls) from 2009-2010. Some of the blood samples collected in 2009-2010 were processed immediately after draw and some had been shipped overnight to the Huber lab and were processed the next day.

TaqMan qPCR specific for XMRV did not reveal positive individuals

The original XMRV results from patients with prostate cancer and CFS were obtained using a sensitive nested PCR assay for XMRV [123, 148] that also detects endogenous MLV sequences in murine genomic DNA. A new qPCR assay specific for the IN region in the XMRV *pol* gene not cross-reactive with any sequence known to be present in mouse DNA was later developed [257]. To test our cohort for the presence of XMRV sequences, we analyzed PBMC DNA using the qPCR specific for XMRV. Titration of DNA from an XMRV positive lymphoblastoid cell line, WPI-1282 (kindly provided to us by the Whittemore Peterson Institute (WPI)), resulted in detection of XMRV down to 10-12 pg, equivalent to two cells, in the presence or absence of 5 µg control DNA isolated from the human LNCaP cell line (Figure 3). However, no positive response (Ct < 60) was obtained with DNA from 112 CFS patients and 36 healthy controls, when tested at 600 ng to 5 µg per reaction. These data indicated that our

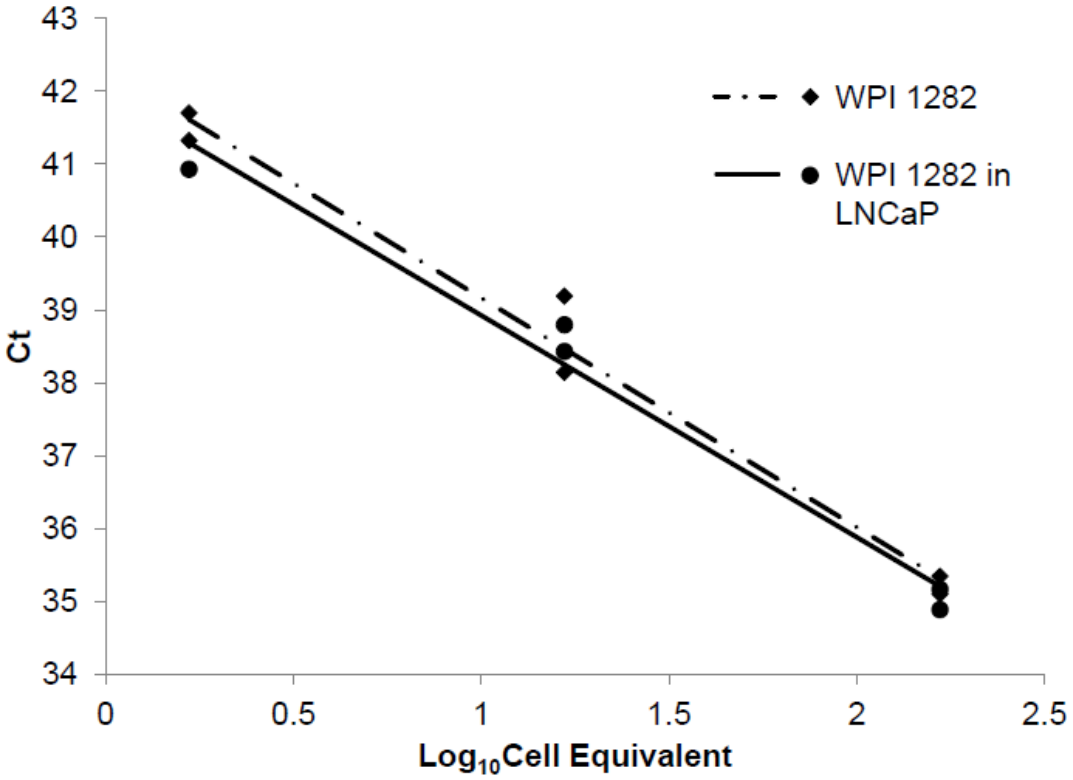

Figure 3. Sensitivity of TaqMan qPCR for IN region in XMRV *pol.* Titration of DNA from WPI-1282 (1.7, 16.7, and 166.7 cell equivalents) in the absence (Diamond, solid line, slope = -3.14) or in the presence of 8.3 x 10^5 cell equivalents of background DNA. Samples were run in duplicates. All qPCR reactions were run for 60 cycles. Samples that did not produce a signal after 60 cycles were assumed negative for XMRV. Ct = Cycle Threshold

samples were either XMRV-negative or had more divergent MLV sequences than originally described [123, 148]. In the latter case, the qPCR assay used, which is sensitive to small sequence differences, would not have allowed detection.

Nested PCR for XMRV gag yielded a high frequency of positive samples

To explore the possibility that XMRV sequences in humans are more divergent than previously reported, we used the nested PCR assay for XMRV *gag* sequences used by earlier studies [123, 148] that also detects many endogenous MLV proviruses. A preliminary titration experiment revealed that MLV-like sequences could be detected in 2-3 pg of WPI-1282 DNA, equivalent to <1 cell, when mixed with 200 ng control background DNA (Figure 4). This assay was used to test DNA in triplicates of 200 ng each from my CFS and control cohorts. Surprisingly, a high proportion of DNA samples from the healthy volunteers (19/36), but only 2/112 of the CFS patients, yielded PCR products of the correct size, as tested on an agarose gel. None of the "no template" control samples, included in each assay in triplicate, gave positive results. These data suggested that XMRV-related viruses may be highly prevalent in the human population, but no special link of these viruses to CFS patients was indicated. While all the blood samples were processed in the Huber laboratory, it should be noted that the CFS cohort mainly consisted of banked samples collected and processed in 2005, whereas the healthy volunteers were recruited more recently, between November of 2009 and May of 2010, and were processed using a slightly modified protocol.

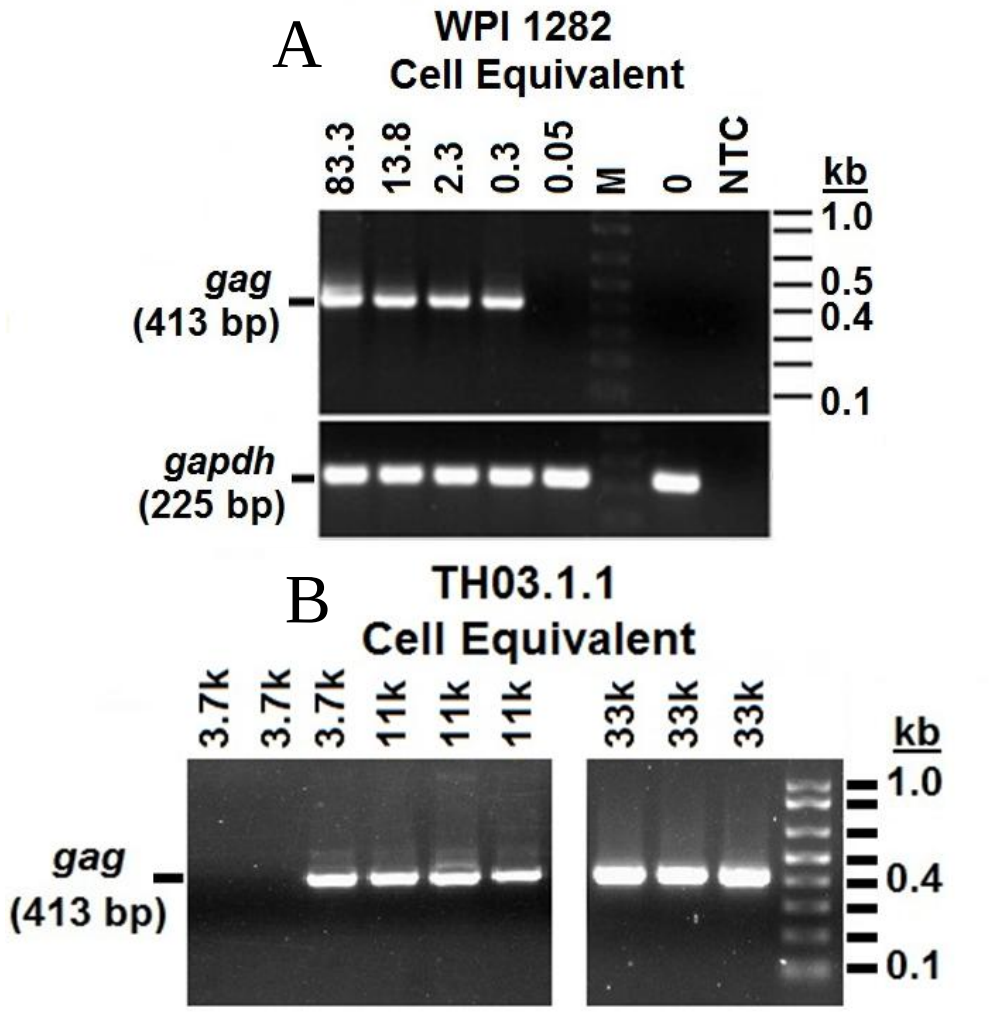

Figure 4. Sensitivity of nested PCR for XMRV *gag*. (A) Titration of genomic DNA from WPI-1282. PCR amplicons from 83.3, 13.8, 2.3, 0.3, 0.05 and 0 cell equivalents of genomic DNA from the WPI-1282 cell line in the presence of 3.3×10^4 cell equivalents of LNCaP genomic DNA were run on a 1.5% agarose gel to show the sensitivity of the assay. *Gapdh* was used as the loading control. XMRV *gag* yields an expected product of 413 bp. NTC = No Template Control. (B) Representative example of nested PCR for XMRV *gag*. Sample TH03.1.1 was first tested as 3.3×10^4 cell equivalents of genomic DNA, followed by limiting dilutions of 1.1×10^4 and 3.7×10^3 cell equivalents. Once a dilution had 1 out of 3 samples positive for *gag*, the positive band was purified and sequenced.

Sequence analysis of the gag PCR products revealed high polymorphism

To determine the relationship among the various PCR products, we obtained their DNA sequences. We observed that most amplicons contained mixtures of sequences, thus, necessitating limiting dilutions of the original DNA samples to obtain pure sequences for analysis (Figures 4B and 5). A total of 37 clean sequences of single PCR products (designated TH for "Tufts Huber") were obtained in this way from 21 samples (19 healthy controls and 2 CFS). Surprisingly, a high degree of diversity was seen in these viral sequences (Figure 5), revealing both XMRV-like and endogenous MLV sequences and implying 15 different virus strains. While 3 healthy controls had sequences that were identical to the corresponding segment of XMRV strain VP42, a viral isolate that was originally found in prostate cancer [148] and later in CFS patients [123], the remaining samples were either identical or closely related to known endogenous MLVs [268-270].

The sequences obtained were also analyzed by constructing neighbor-joining trees (Figure 6). Again, the data indicate a high degree of polymorphism in the MLV-like sequences found in our cohort. In contrast to the published VP [148] and WPI [123] XMRV sequences, which are tightly clustered, the *gag* sequences found in our study are dispersed, similar to the sequences reported in Lo *et al.* [271]. The 15 unique XMRV-related partial *gag* sequences found from the 37 single PCR products are distributed over a minimum of 3 clusters, each of which contains endogenous MLV sequences of a different subtype (XMV, PMV, and MPMV (xenotropic, polytropic, modified polytropic MLV)).

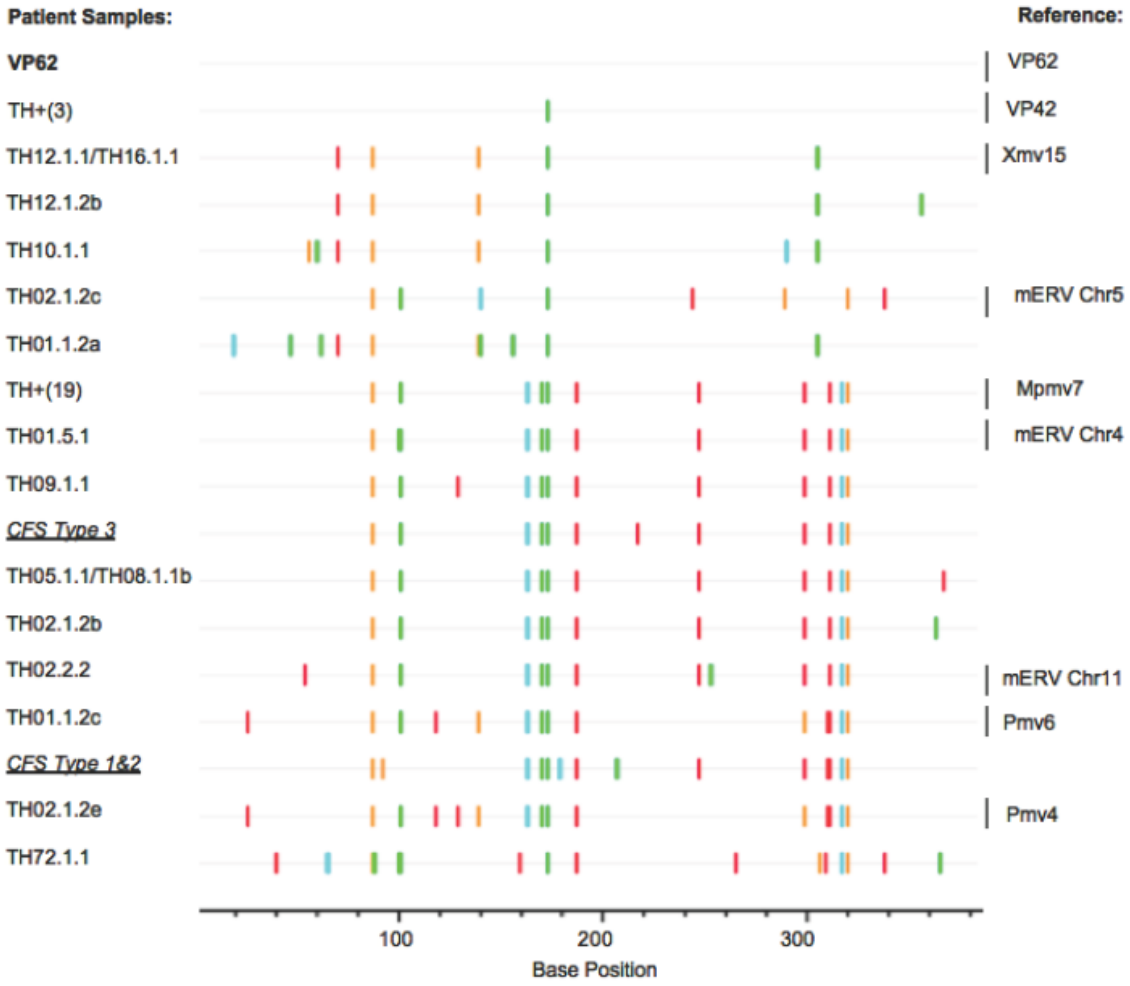

Figure 5. *Gag* sequences from patient samples. Individual 382 bp sequences free of double peaks and confirmed through forward and reverse sequencing, are compared in a Highlighter plot to the control WPI-1282 cell line sequence, VP62. The samples were coded to remain anonymous, with the first number being the patient number, the second number being the bleed number, the third number being the tube of DNA, and the letter showing that we have multiple sequences in the same tube of DNA. Identical sequences were collapsed into individual clusters, those with more than two sequences are labeled TH+(N), where N is the total number of sequences that cluster. CFS Type1, 2 & 3 are from Lo *et al.* [271]. Each vertical line shows a single nucleotide difference between the labeled sequence and the control VP62 sequence.

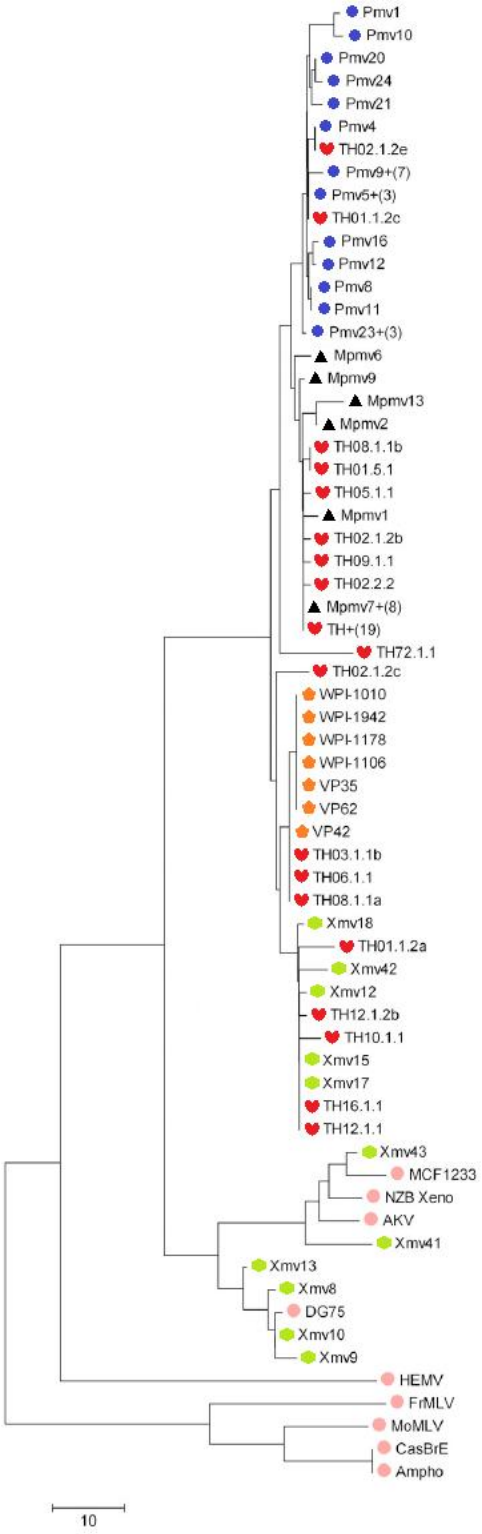

Figure 6. Neighbor Joining Tree of *gag* fragments.
A neighbor-joining tree was constructed using the 382 bp *gag* fragments detected from the PBMC DNA of 2 CFS patients and 17 healthy controls, along with various exogenous and endogenous MLV sequences. Identical sequences are collapsed into individual clusters, where a representative member is shown followed by "+(N)", where N is the total number of sequences in that cluster. Distances were calculated based on the absolute number of base substitutions; all sites containing gaps were ignored. Note the extensive variation of sequences detected in our samples (TH, shown in red), which cluster with known Xmv ( ), Pmv ( ), Mpmv ( ), and XMRV ( ) sequences.

Tests for Mouse DNA Contamination Revealed Correlation with Viral Sequences

Endogenous MLVs are present in high copy number in all inbred and wild mice species, making mouse DNA a possible source of the XMRV-related sequences observed. To test whether contamination with mouse DNA might account for the observed results, all human DNA samples were screened using two different assays, a TaqMan qPCR for murine mitochondrial cytochrome oxidase, *cox2* [155] and a single PCR assay for the highly abundant intracisternal A-type particle (IAP) long terminal repeat sequences, developed in collaboration with the Coffin lab. Both assays had similar sensitivity, detecting the target sequences in 0.6 pg of mouse DNA, equivalent to 1/10 of a cell in a background of 200 ng LNCaP DNA (Figure 7). Using these two test systems, we observed that many samples, both CFS and control, were positive for murine sequences, while all "no template" controls were negative. A direct comparison of the *gag* PCR results with those obtained in the two assays for mouse DNA revealed a 100% correlation between samples positive for *gag* and mouse DNA. All human DNA samples that were positive in the *gag* PCR assay were also positive for IAP sequences, and all but 2 were positive for mouse *cox2* sequences (Table 5). In addition, nearly half (62/127) of the samples were positive for mouse DNA by either IAP or both assays, but did not have a detectable MLV signal. These findings are in agreement with our observation that the two PCR assays for mouse DNA are at least 10-fold more sensitive than the XMRV *gag* PCR assay, when tested on genomic mouse DNA. Overall, the data are consistent with the conclusion that the positive results obtained with the XMRV *gag* PCR assay are due to variable contamination of the human samples with mouse DNA.

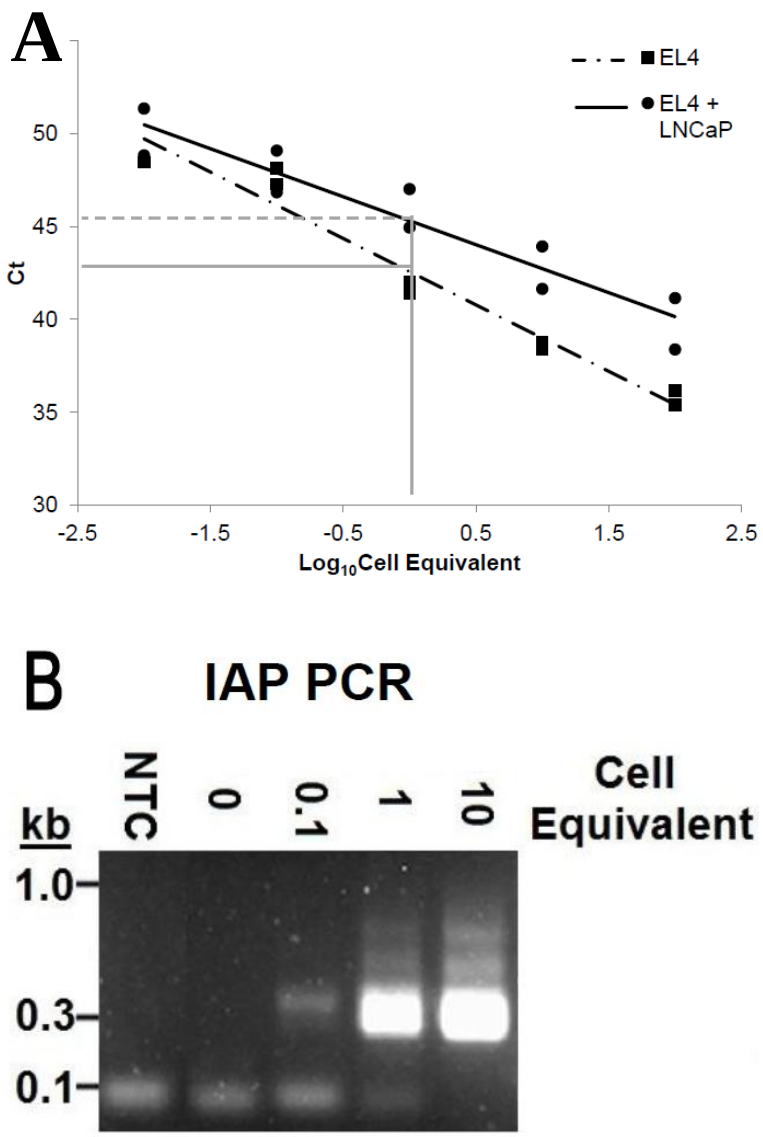

Figure 7. Tests for mouse DNA. A) TaqMan qPCR for murine mitochondrial cytochrome oxidase (*mcox2*). Titration of DNA from the murine EL4 cell line (100, 10, 1, 0.1, and 0.01 cell equivalents) in the absence (square, dotted line, slope = -3.58) or in the presence of 3.3 x 10^4 cell equivalents of background genomic LNCaP DNA (circle, solid line, slope = -2.58). 0.1 cell equivalents of murine DNA was detectable in 3.3 x 10^4 cell equivalents of background DNA. Samples were run in duplicates. All qPCR reactions were run for 60 cycles. Samples that did not produce a signal after 60 cycles were considered negative for murine DNA. B) IAP PCR. Titration of DNA from murine EL4 cell line (10, 1, 0.1, and 0 cell equivalents) in the presence of 3.3 x 10^4 cell equivalents of genomic LNCaP DNA. The limit of detection was 0.1 cell equivalents of murine DNA in 3.3 x 10^4 cell equivalents of background DNA. NTC = No Template Control.

Table 5

Correlation of MLV DNA sequence detection with mouse DNA contamination

			CFS Patients		Healthy Controls	
XMRV GAG	*cox*	IAP	# of Samples (n=112)	Percent	# of Samples (n=36)	Percent
+	+	+	2*	1.79	17	47.22
-	-	-	53	47.32	12	33.33
+	-	-	0	0.00	0	0.00
+	-	+	0	0.00	2	5.56
+	+	-	0	0.00	0	0.00
-	+	+	10	8.93	1	2.78
-	-	+	47	41.97	4	11.11
-	+	-	0	0.00	0	0.00

*One CFS sample from 2005 collection, and one CFS sample from 2010 collection.
All other CFS samples were collected in 2005.

**All collected in 2009-2010.

Lack of XMRV-Specific Antibodies in CFS and Healthy Control Sera

The presence of anti-XMRV antibodies in the sera of patients and healthy controls cannot be due to mouse DNA contamination. 148 blinded CFS and healthy control plasma samples from our XMRV-Levine cohort were analyzed for the presence of XMRV-specific antibodies using the direct format ARCHITECT p15E and gp70 CMIAs. None of the 148 plasma samples were reactive in the p15E CMIA (Figure 8A). Two of the 148 samples (ID = 137, 138) were positive in the gp70 CMIA (Figure 8B). Both specimens were weakly reactive in the gp70 CMIA with sample/cut-off (S/CO) values of 7.77 (log N of S/CO = 2.05) and 9.02 (log N of S/CO = 2.20), respectively. Although the samples were repeat-reactive in the gp70 CMIA, they were not reactive by western blot. As shown in Figure 9, both samples showed no visible western blot bands using either XMRV viral lysate proteins (Figure 9B) or recombinant gp70 protein (Figure 9A). Unblinding of the samples revealed that the two gp70 reactive samples came from two sequential blood collections of a single healthy control (Table 6).

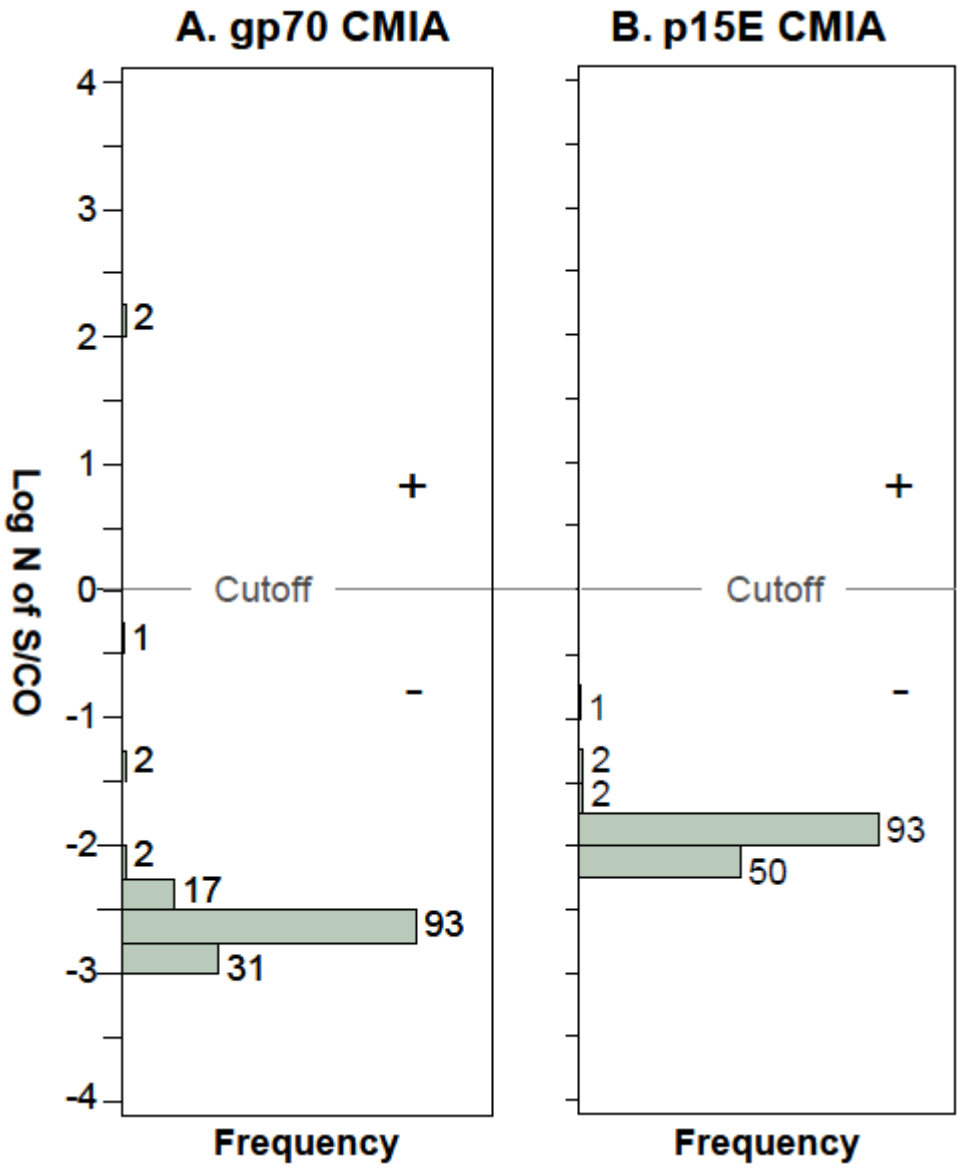

Figure 8. CMIA detection of anti-XMRV antibodies. Distribution of gp70 CMIA (A) and p15E CMIA (B) log N of S/CO on 148 samples collected from 112 CFS patients and 36 healthy controls. Numbers of specimens within each log N of S/CO value are shown above the solid bars. Assay cutoffs were equivalent to mean 12 SD and 16 SD for gp70 and p15E CMIAs, respectively, based on blood donor populations [262]. Log N of S/CO, natural log transformation of S/CO.

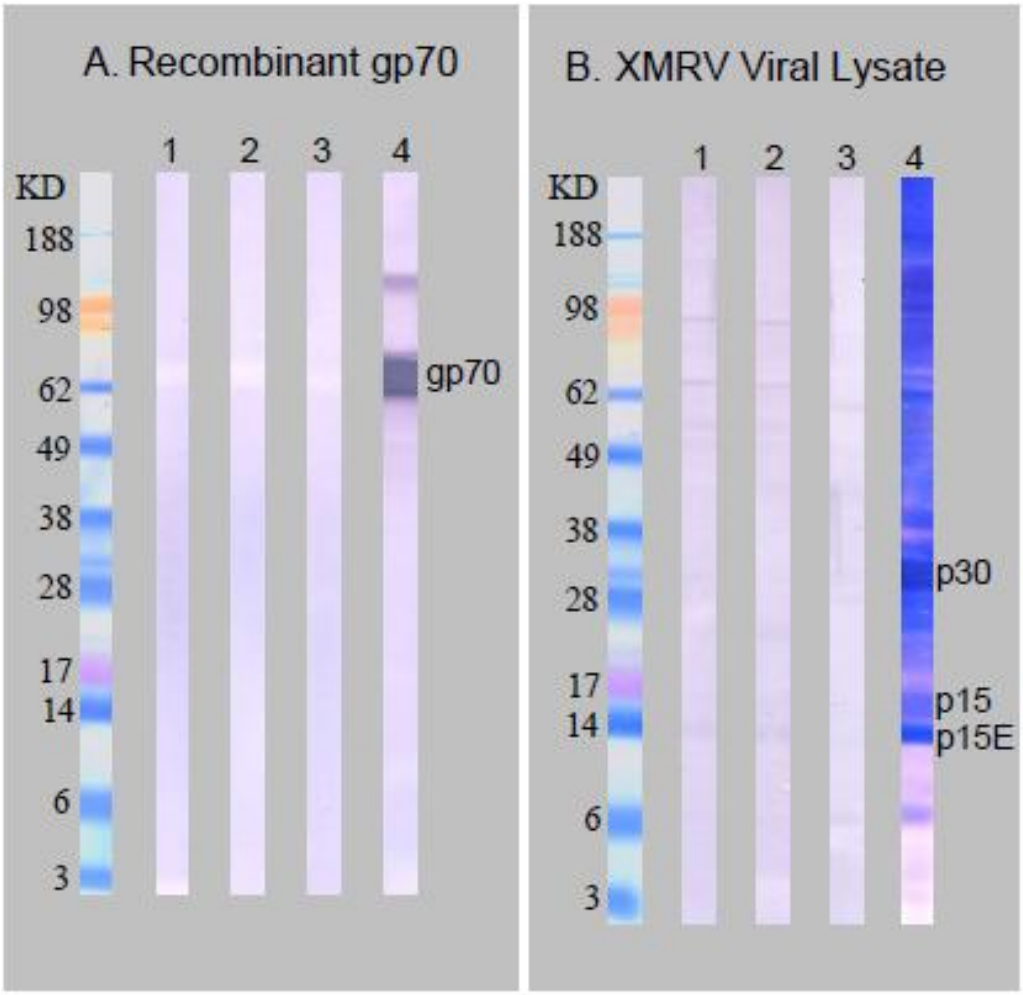

Figure 9. Western blot analysis of gp70 CMIA reactive samples. (A) Recombinant gp70 western blot. (B) Native XMRV viral proteins western blot. Key: 1 & 2 - gp70 reactive samples 137 and 138; 3- normal blood donor plasma as negative control; 4 – XMRV-infected macaque plasma as positive control. The faint white band in the 65-70 kd region in (A), strips 1-3, indicates a lack of specific anti-gp70 antibody.

Table 6

Results Summary for XMRV Positive PCR Samples

All samples that tested positive for XMRV *gag* sequence, as well as the two samples that reacted with the gp70 CMIA are displayed. Boxed and **bolded** samples showed the VP42 *gag* sequence, but did not react with the CMIAs. The red shows the two samples that were reactive in the gp70 CMIA. CMIA values less than 1 are considered nonreactive. XMRV GAG = Nested *gag* PCR. Mcox = murine mitochondrial cytochrome oxidase qPCR. IAP = Intracisternal A-type particle PCR

		PCR Results			Initial Test	Initial Test	Repeat Test
ID	Unblinded ID	XMRV GAG	Mcox	IAP	p15E S/CO	gp70 S/CO	gp70 S/CO
72	TH72.1	+	+	+	0.38	0.06	
128	TH04.1	+	+	+	0.16	0.07	
129	TH01.7	+	+	+	0.15	0.06	
131	TH01.8	+	-	+	0.12	0.06	
132	TH01.3	+	+	+	0.15	0.06	
134	**TH06.1**	**+**	**+**	**+**	**0.15**	**0.07**	
135	TH01.1	+	+	+	0.14	0.09	
136	TH05.1	+	+	+	0.16	0.06	
137	TH07.1	+	+	+	0.16	7.77	7.17, 7.21
138	TH07.2	-	-	-	0.14	9.02	8.65, 8.77
143	TH10.1	+	+	+	0.14	0.07	
144	TH11.1	+	-	+	0.14	0.06	
147	TH02.1	+	+	+	0.14	0.07	
152	TH01.5	+	+	+	0.13	0.07	
153	TH21.1	+	+	+	0.15	0.07	
155	TH20.1	+	+	+	0.16	0.06	
156	TH02.2	+	+	+	0.17	0.07	
158	**TH08.1**	**+**	**+**	**+**	**0.13**	**0.07**	
160	**TH03.1**	**+**	**+**	**+**	**0.13**	**0.07**	
161	TH12.1	+	+	+	0.11	0.06	
163	TH19.1	+	+	+	0.16	0.72	0.75, 0.72
164	TH16.1	+	+	+	0.15	0.07	

Significance

Through this study, we demonstrated, without a doubt, that the established ultra-sensitive nested PCR method for the detection of XMRV was prone to detecting false positives due to mouse DNA contaminating human samples. Laboratory mouse strains, as well as wild mouse strains, carry numerous endogenous MLVs that could potentially skew results when testing for viruses related to the murine genome. We helped develop the murine DNA detection IAP PCR and urged other scientists studying XMRV to test all of their samples for mouse DNA contaminants. We provided some of the first evidence that XMRV was merely a contaminant and not associated with CFS or present at high frequency in the general population.

Chapter 2. The HERV-K18 *env* superantigen and CFS

Study Population

It was established by our lab that the HERV-K18 *env* SAg is induced by EBV [243] and IFN-α. [247]. A previous pilot study done in our lab on a group of patients who developed CFS after prolonged iatrogenic IFN-α treatment suggested that the HERV-K18 *env* genotype could be a risk factor for CFS [272]. A follow-up pilot study testing a group of non-IFN-associated CFS patients failed to see this risk factor [272]. Therefore, we hypothesized that a patient's HERV-K18 *env* genotype may be a risk factor in patients who develop CFS after having EBV-IM. We collected blood samples from 53 CFS patients who had a previous medical history of EBV-IM and 48 CFS patients who had no previous history of EBV-IM, groups A and B, respectively. Both groups were diagnosed with CFS using the 1994 CDC criteria [12] and were mostly Caucasian. Both groups had equal percentages of females and males, same average age, and same average symptom severity scores at the time of blood draw (Table 7).

HERV-K18 env Genotype

There are three distinct HERV-K18 *env* alleles in the Caucasian population known as HERV-K18.1 (K18.1), HERV-K18.2 (K18.2), and HERV-K18.3 (K18.3). All three of the alleles encode a SAg; however, due to differences in amino acid sequence, biochemical differences are predicted between alleles (Figure 10). These alleles are unevenly distributed in the population with allelic frequencies of 46.6%, 42.5%, and 10.8% for K18.1, K18.2, and K18.3, respectively [247].

Table 7

Taylor Cohort Group Demographics

	Group A			Group B	
Males (% of Group)	9	(17 %)		8	(17 %)
Females (% of Group)	44	(83 %)		40	(83 %)
Total # of Patients	53			48	
Age Range (years)	16-70			15-64	
Avg. Age (years)	49			49	
Avg. Fatigue Score (95% CI)	25.5	(24.0-26.9)		28.0	(26.6-29.4)
Avg. CFS Score (95% CI)	23.2	(22.1-24.4)		24.2	(22.9-25.5)
Avg. PSS Score (95% CI)	32.6	(31.6-33.5)		32.6	(31.6-33.7)
Avg. GHQ Score (95% CI)	56.0	(53.2-58.8)		55.3	(52.0-58.6)
Avg. SPHERE Score (95% CI)	58.9	(56.1-61.7)		61.8	(58.4-65.2)
Avg. SOMA Score (95% CI)	7.6	(6.8-8.4)		9.0	(8.1-9.8)

(95% CI = 95% Confidence Interval)

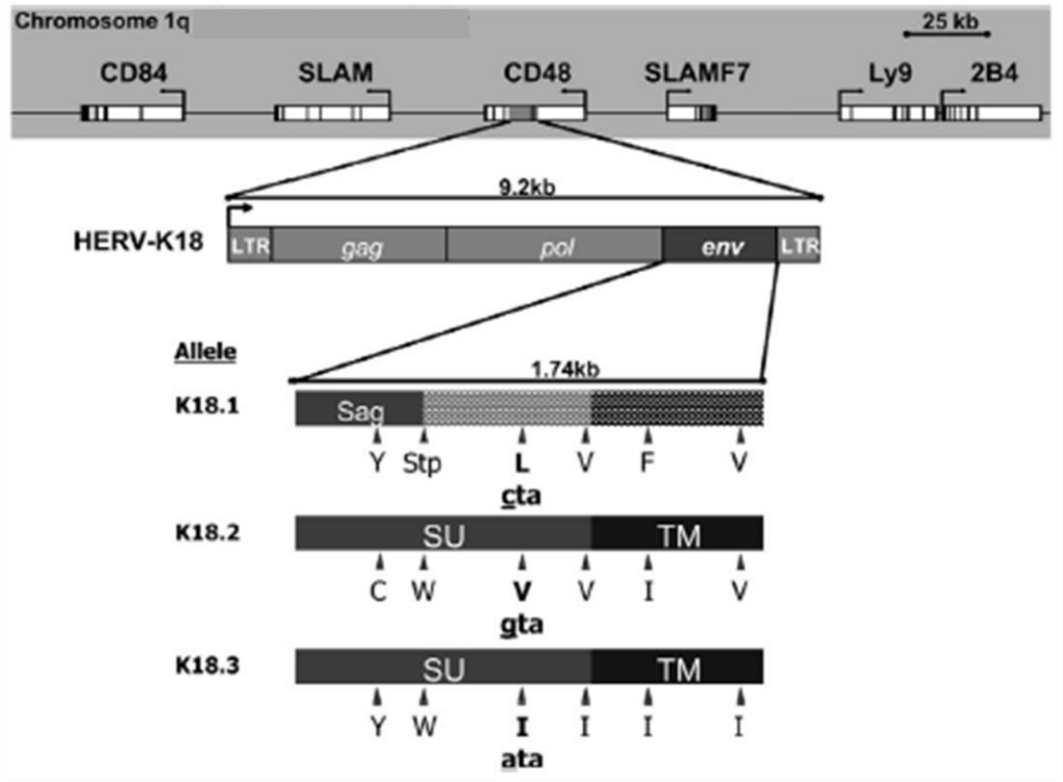

Figure 10. HERV-K18 Alleles. HERV-K18 is located in the first intron of CD48, is approximately 9.2kb long, and is transcribed in the opposite direction as CD48. The three HERV-K18 *env* alleles are 1.74kb long, but K18.1 has a premature stop codon. The amino acid differences among alleles are labeled below each allele. The bolded amino acids (**L, V, and I**) with their given codon were used in a TaqMan based real-time PCR to distinguish alleles. Sag=Superantigen, TM = Transmembrane domain, SU = Surface domain. Modified from [251]

Using a TaqMan MGB probe based real-time PCR, we assessed the HERV-K18 *env* genotype of patients in Group A and Group B. There was no statistically significant difference in genotype between the CFS patients in each group (χ^2 p-value of 0.54) (Table 8). This suggests that there is no difference in HERV-K18 *env* genotype between individuals who develop CFS with a history of EBV-IM and individuals who develop CFS without a history of EBV-IM.

HERV-K18 env Expression Fluctuates over Time

It is well known that CFS symptoms fluctuate with patients having remission and relapse [1]. To determine if HERV-K18 *env* expression levels fluctuate at the same time as symptom severity, we followed the same CFS patients from Group A and B for two years, drawing blood once approximately every six months. At the time of the blood draws, each patient was asked to fill out symptom severity forms using the Fatigue Scale, the Chronic Fatigue Syndrome Rating Form, The Perceived Stress Scale, The General Health Questionnaire, and SPHERE AND SOMA Questionnaire. Each questionnaire works independently of the others. All of the surveys use a Likert-style scoring system which ultimately rates the severity of a patient's symptoms with a number; the higher the number, the more severe the symptoms of CFS. The blood samples were collected by our collaborator, Renee Taylor, and shipped overnight to us for processing. Once all of the samples had been collected, we measured each patient's HERV-K18 *env* expression level at each time point. Following the same individuals over time, we were able to monitor changes in relative HERV-K18 *env* expression within an individual patient over time. Figure 11 illustrates examples of changes in relative HERV-K18 *env* expression within an individual at each time point.

Table 8. HERV-K18 *env* genotype frequency in CFS groups

Group A = CFS with history of EBV infectious mononucleosis

Group B = CFS without history of EBV infectious mononucleosis

Genotype	Group A Cases n (%)	Group B Cases n (%)
1/1	9 (17.0)	5 (10.6)
1/2	27 (50.9)	22 (46.8)
1/3	1 (1.9)	3 (6.4)
2/2	10 (18.9)	9 (19.1)
2/3	5 (9.4)	8 (17.0)
3/3	1 (1.9)	0 (0.0)

χ^2 p-value = 0.54

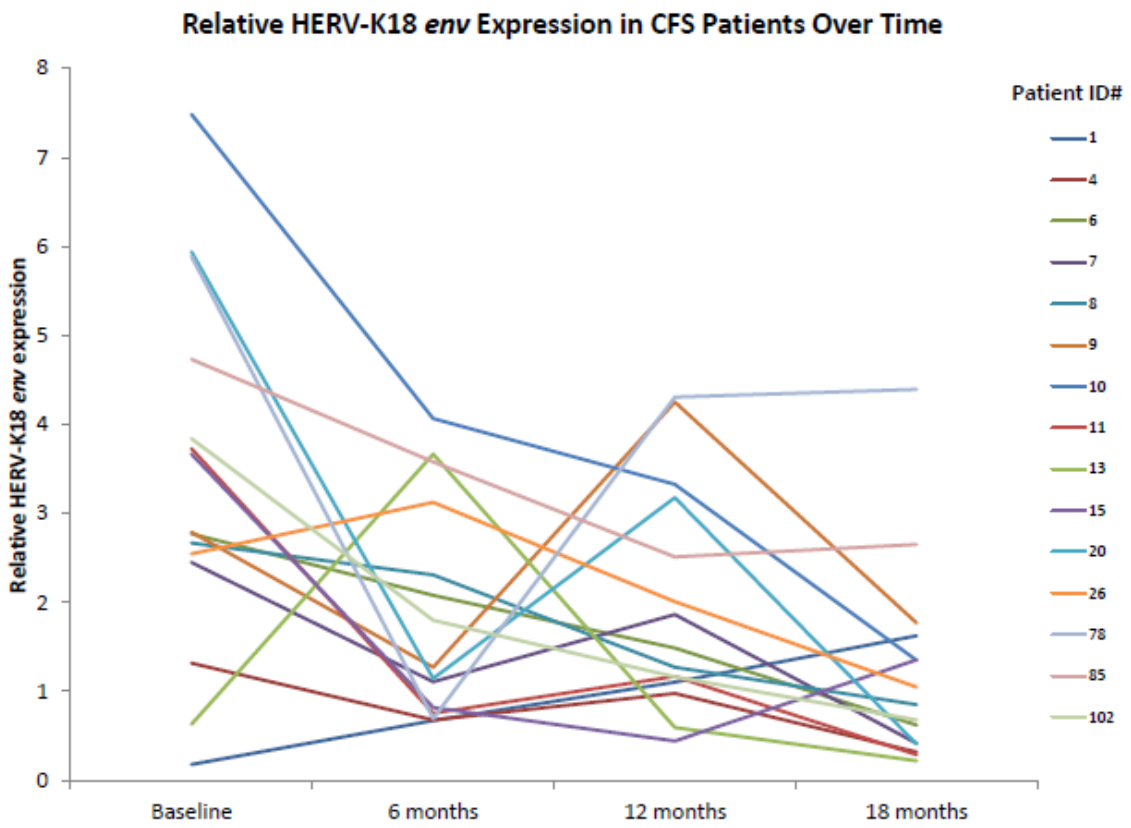

Figure 11. Relative HERV-K18 *env* expression in CFS patients over time. Example of 15 CFS patients from our HERV-K18-Taylor Cohort showing relative HERV-K18 *env* expression fluctuates at different time points. Each line represents an individual patient who was bled at 4 separate time points six months apart.

No Correlation between HERV-K18 env Expression and Symptom Severity

Once it was established that HERV-K18 *env* expression fluctuates, we attempted to determine if the changes in HERV-K18 *env* expression correlated with symptom severity using Spearman's rank order correlations. In other words, if a patient's HERV-K18 *env* expression level is high, do they also have more severe CFS symptoms? Each group was split into bleed number categories and tested against results from the symptom severity questionnaires. Surprisingly, no correlation was found between the HERV-K18 *env* expression level and any of the symptom severity surveys (Table 9).

HERV-K18 env Expression Levels Appear Higher in CFS Patients

We recruited a small healthy control cohort (n=10) consisting mostly of Caucasians from the northeastern United States. Healthy control blood samples were drawn in our lab and processed immediately. Once all the CFS and healthy control samples had been processed, we measured HERV-K18 *env* transcript levels relative to the *hprt* housekeeping gene using a real-time PCR probe that recognizes all three of the HERV-K18 alleles. Initial analysis comparing the initial bleeds of Group A and Group B to the healthy controls revealed a statistically significant increase in HERV-K18 *env* expression in both CFS groups compared to controls ($p = 0.0034$ and 0.0305, respectively) (Figure 12). Both the CFS groups appeared to have an approximate 3 fold increase in expression of HERV-K18 *env* compared to our small cohort of controls. This suggests that CFS patients, independent of whether they have had EBV-IM, have higher expression levels of HERV-K18 *env* than healthy controls.

Table 9. Lack of correlation between HERV-K18 *env* expression and symptom severity scores

Spearman Correlation Coefficients (ρ)

Group A	Fatigue	CFS	PSS	GHQ	SPHERE	SOMA
Bleed 1	0.020	-0.067	0.026	-0.010	-0.190	-0.100
Bleed 2	-0.076	-0.157	0.076	-0.121	-0.076	-0.084
Bleed 3	-0.137	-0.090	0.201	-0.116	0.002	0.043
Bleed 4	0.372	0.013	0.171	-0.074	0.251	0.233

Group B	Fatigue	CFS	PSS	GHQ	SPHERE	SOMA
Bleed 1	0.044	-0.029	-0.193	-0.180	-0.037	-0.002
Bleed 2	-0.124	-0.082	0.058	-0.003	0.032	0.160
Bleed 3	-0.355	-0.211	0.133	-0.415	-0.239	-0.088

All *p*-values > 0.05

Fatigue = The Fatigue Scale; CFS = The Chronic Fatigue Syndrome Scale; PSS = Perceived Stress Scale; GHQ = General Health Questionnaire; SPHERE = SPHERE Questionnaire; SOMA = SOMA Questionaire

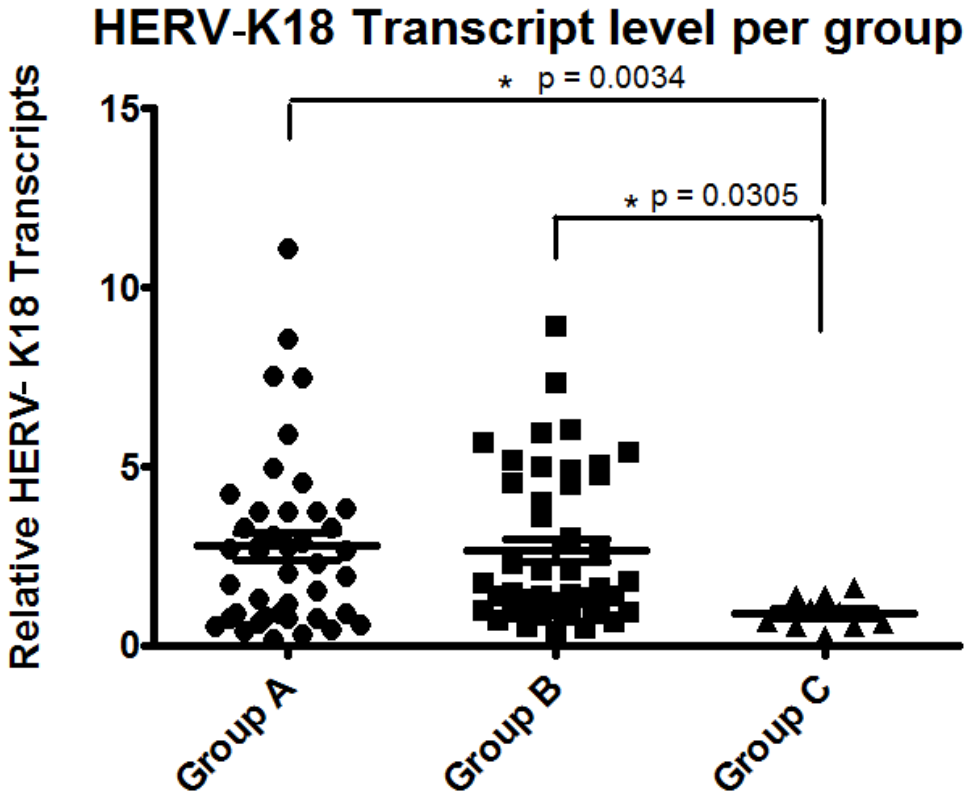

Figure 12. Relative HERV-K18 transcript level per group. Relative HERV-K18 *env* transcript levels were measured using a real-time PCR. Group A = CFS group with history of EBV-IM; Group B = CFS group with no history of EBV-IM; Group C = Healthy controls. Bars represent means +/- SEM. *p*-values were calculated using the Kolmogorov-Smirnov test.

Failure to confirm increased HERV-K18 env expression levels in independent CFS cohort

To confirm that CFS patients express higher levels of HERV-K18 *env*, we analyzed a second independent cohort of CFS patients. 50 blinded samples (40 CFS patients and 10 healthy controls) were obtained at Harvard University. Samples were transported immediately after blood draw to the Huber lab where they were processed the same day as the blood draw. These CFS patients were diagnosed using the same 1994 case-definition as the previous CFS cohort [12]. Using the same real-time PCR as above, we measured HERV-K18 *env* transcripts. Once measurements were complete, the samples were unblinded for analysis. No statistically significant difference was found in HERV-K18 expression between this cohort of CFS patients and healthy controls (Figure 13). In fact, the HERV-K18 *env* transcript levels in this cohort of CFS patients were much lower than the previous CFS cohort (Figure 14).

Determination of differences in analysis

After noting the difference in HERV-K18 *env* expression between our two CFS cohorts, we examined the differences in how the samples were processed and analyzed. The major difference between the two cohorts was the time between blood draw and processing. The Taylor cohort was shipped overnight to our lab, whereas the Komaroff cohort was immediately brought to our lab the day of the draw. To determine if an overnight incubation changes the expression of HERV-K18 *env*, twelve healthy controls were bled in our lab. Half of their blood was processed immediately and half was incubated overnight at room temperature and processed the next day. HERV-K18 *env* expression levels were then measured. The average HERV-K18 *env* expression levels

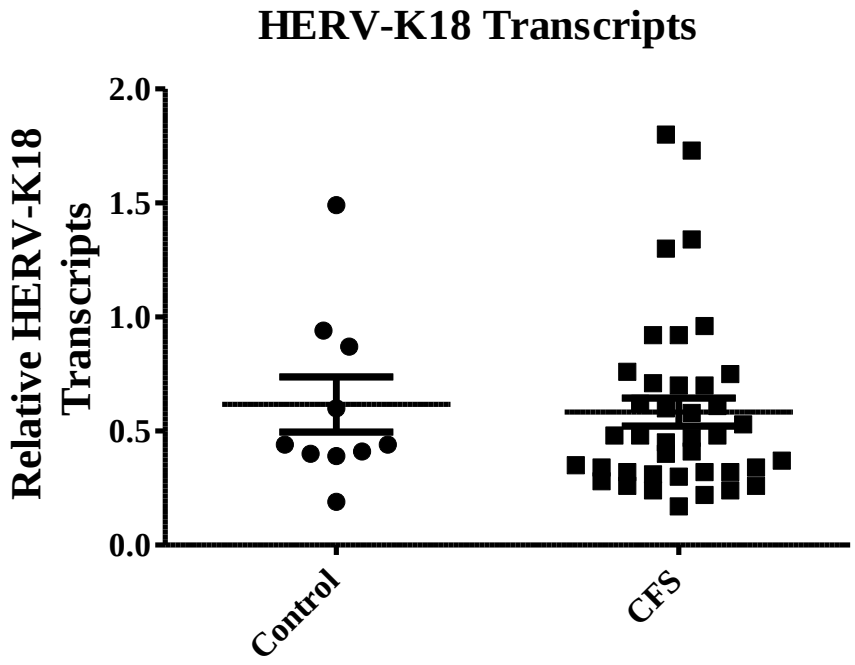

Figure 13. HERV-K18 *env* Transcripts in the Komaroff Cohort. HERV-K18 transcripts were measured in PBMCs of 10 healthy controls and 40 CFS patients. Transcript levels are relative to a standard sample. Bar = mean +/- SEM.

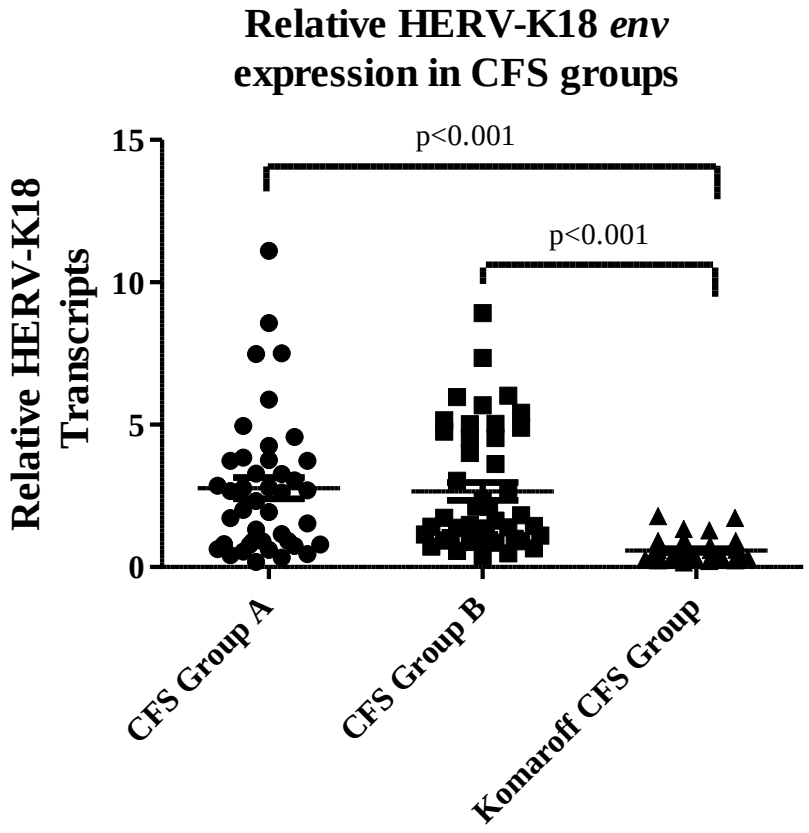

Figure 14. Relative HERV-K18 *env* expression in all three CFS groups. The HERV-K18 *env* expression level in the Komaroff CFS Group is significantly lower than the other CFS groups ($p < 0.001$). CFS Group A = CFS with history of EBV-IM; CFS Group B = CFS with no history of EBV-IM. Bars represent means +/- SEM. *p*-values were calculated using the Kolmogorov-Smirnov test.

were significantly lower in the freshly processed samples when compared to the samples processed after the overnight incubation (Figure 15).

New HERV-K18 env Analysis Reveals No Increased Expression

After determining that an overnight incubation can alter relative HERV-K18 *env* expression, the Taylor cohort CFS samples that were shipped overnight to our lab were compared to controls that also sat overnight (Figure 16). None of the CFS bleeds from Group A or Group B had HERV-K18 *env* expression levels that were significantly higher than the healthy controls. In fact, the healthy controls average HERV-K18 *env* expression were significantly higher than Group A bleed 4 and Group B bleed 2 (*p*-values = 0.007 and 0.041, respectively).

Significance

Through this study, we established that, although HERV-K18 *env* transcripts vary over time within an individual, they do not correlate with any of the symptom severity scales commonly employed by CFS researchers. Although we recognize our genotyping cohort is small, we failed to show a link between HERV-K18 *env* genotype and CFS. Despite failing to show a link between HERV-K18 *env* expression and CFS in our cohorts, we did make the important note that simple assumptions can bias results and lead to misinterpretations. Many people assume overnight blood incubations do not alter expression levels of genes; however, we discovered that this is an unwise assumption to make. Controls must be processed in the exact same manner as experimental samples without exception.

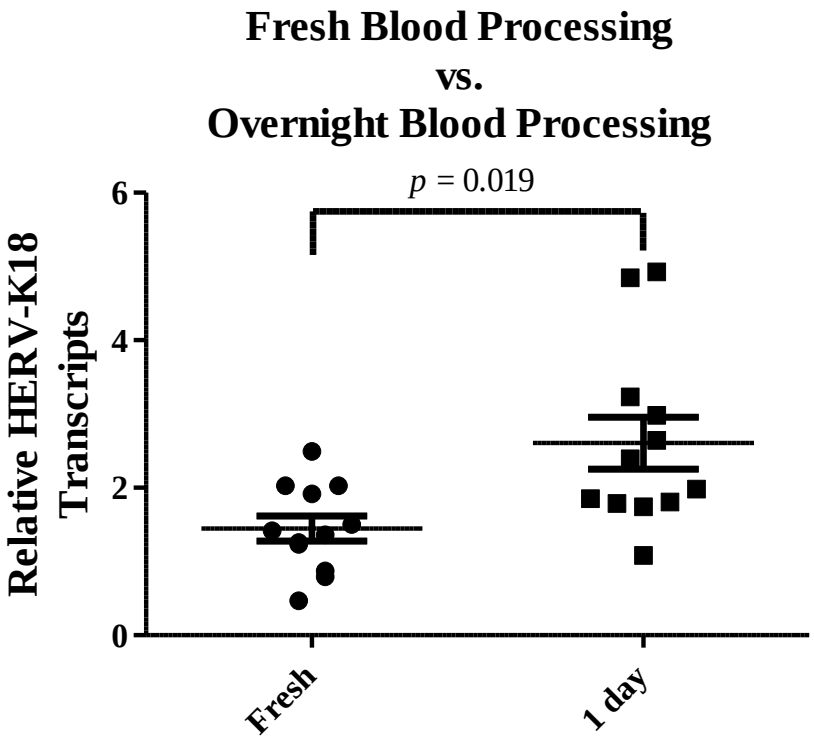

Figure 15. Fresh blood processing vs. processing after overnight incubation. Blood samples were collected from 12 healthy volunteers. Half of each person's blood was processed immediately (Fresh) while the other half was incubated at room temperature overnight and processed the next day (1 day). Relative HERV-K18 *env* transcripts were measured after both samples had been processed. Bars represent means +/- SEM. *p*-values were calculated using the Kolmogorov-Smirnov test.

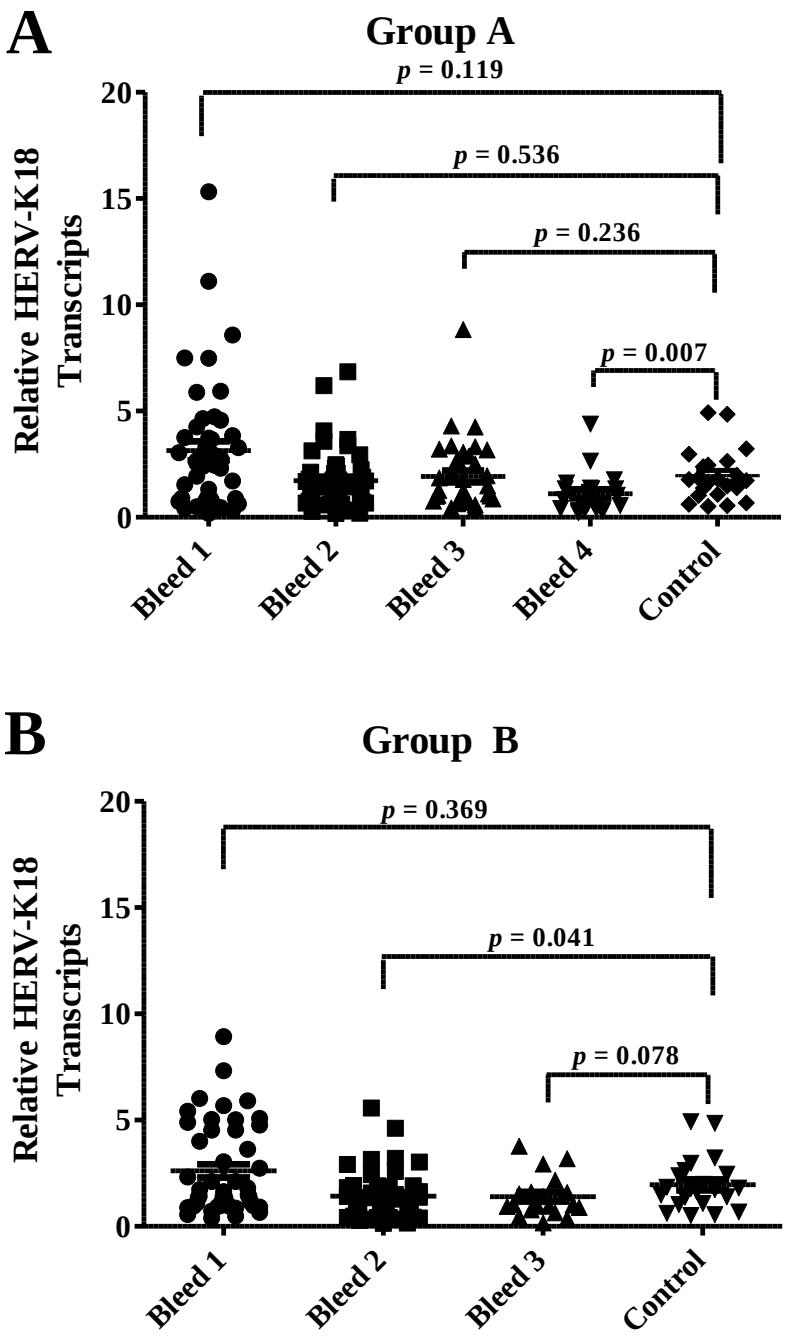

Figure 16. Relative HERV-K18 *env* transcripts compared to overnight control. Taylor Cohort Group A (A) and Taylor Cohort Group B (B) relative HERV-K18 *env* expression compared to overnight control. None of the CFS bleeds are significantly higher than the control. In fact, the control is significantly higher than Group A bleed 4 and Group B bleed 2. Bars represent means +/- SEM. *p*-values were calculated using the Kolmogorov-Smirnov test.

Chapter 3. Human herpesvirus-6 and -7 and HERV-K18 *env* in CFS patients

Study Population

It was previously shown that HHV-6A and HHV-6B both can induce the HERV-K18 *env* SAg [254-255]. Both HHV-6A and 6B, along with the closely related HHV-7, are continually being investigated as potential triggering viruses to CFS [107, 231]. To investigate the potential relationship of HHV-6 and HERV-K18 in CFS patients, we collected blood and saliva samples from 40 established CFS patients and 9 healthy controls (description of cohort in Table 10). Originally, we had 10 healthy controls; however, one of the controls had unusually high HHV-6 viral copy number in saliva, PBMCs, and plasma. It was assumed that this person had ciHHV-6 and was removed from the study[184]. Samples were collected at Harvard University under the supervision of Dr. Komaroff and transported immediately to the Huber lab for processing. Patients were diagnosed according to the 1994 CDC criteria [12] and were interviewed by a physician to determine their severity of symptoms at the time of sample collection.

HERV-K18 env Transcripts and HHV-6 Viral Loads

As described in the previous chapter, relative HERV-K18 *env* transcripts were measured using a real-time PCR. HERV-K18 *env* transcripts were not significantly different in the CFS patients compared to the healthy controls (Figure 13). HHV-6 viral loads were measured in PBMCs and saliva from these same patients using a quantitative real-time PCR. 4 of the 9 healthy controls (44%) and 17 of the 40 CFS patients (42.5%) had detectable levels of HHV-6 in their saliva. No difference was seen in HHV-6 viral load in saliva between the controls and the CFS patients (Figure 17A). Six of the 9

Table 10. Komaroff Cohort Demographics

	Controls	**CFS**
Males (% of Group)	4 (40.0 %)	11 (27.5 %)
Females (% of Group)	6 (60.0 %)	29 (72.5 %)
Total # in Group	10	40
Age Range (years)	24-58	20-77
Avg. Age (years)	35.9	50.4

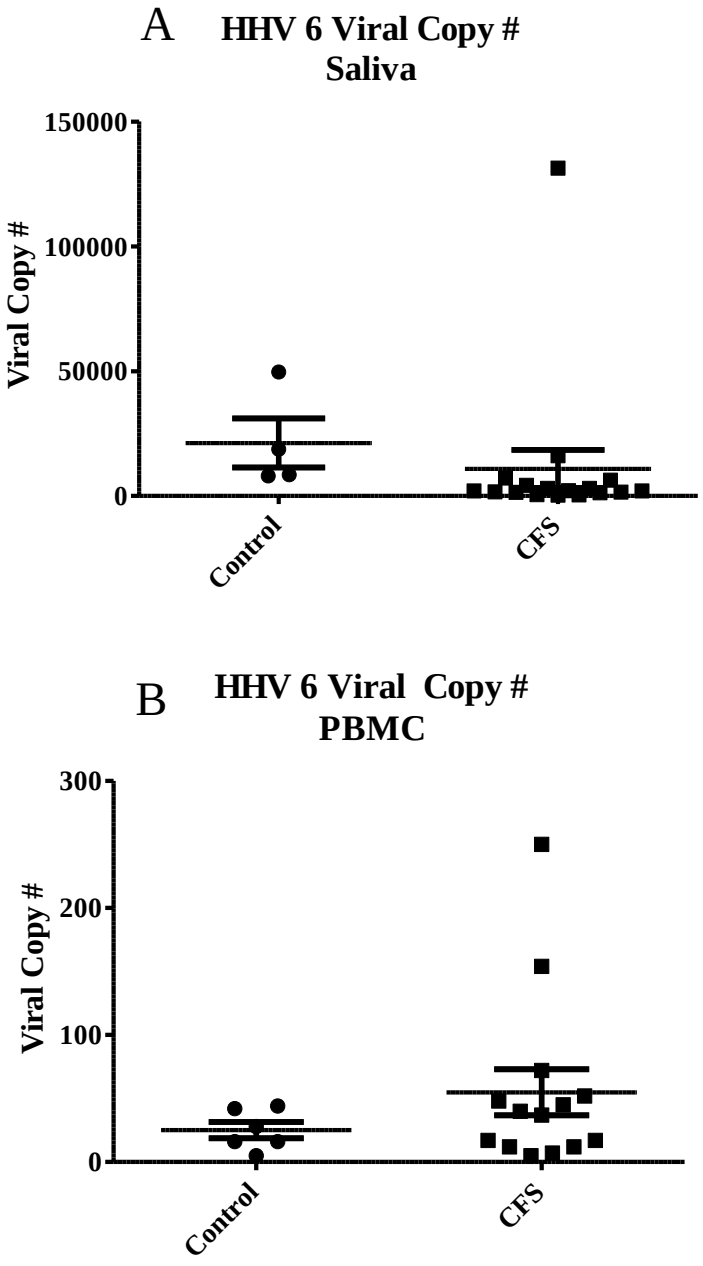

Figure 17. HHV-6 Viral Copy Number. (A) HHV-6 viral copy number was measured using real time PCR in the saliva of 9 healthy controls and 40 CFS patients. 4/9 healthy controls and 17/40 CFS patients had detectable levels of HHV-6. Viral copy number represents viral copies present in an entire 5 ml wash. (B) HHV-6 viral copy number was measured using real time PCR in the PBMCs of 9 healthy controls and 40 CFS patients. 6/9 healthy controls and 14/40 CFS patients had detectable levels. Viral copy number represents viral copies present in 500 ng PBMC DNA. Bars = Mean +/- SEM

healthy controls (67%) and 14 of the 40 CFS patients (35%) had detectable levels of HHV-6 in DNA isolated from PBMCs. No difference was seen between CFS patients and healthy controls in HHV-6 viral load in the DNA of PBMCs (Figure 16B). A Spearman correlation calculation failed to detect any correlation between the samples that had a measurable HHV-6 viral load in PBMCs and HERV-K18 *env* transcripts (ρ=-0.4073, $p > 0.05$). A second Spearman correlation calculation also failed to detect any correlation between the samples that had a measurable HHV-6 viral load in saliva and HERV-K18 *env* transcripts ($\rho = 0.0643$, $p > 0.05$). As expected, HHV-6 viral load was higher in saliva than in PBMCs. All HHV-6 viral sequences were of the HHV-6B variant. HHV-6 viral copy number did not correlate with any of the interview questions assessing CFS symptom severity.

HERV-K18 env Transcripts and HHV-7 Viral Load

HHV-7 viral copy number was measured in PBMCs and saliva using a quantitative real-time PCR. All 9 of the healthy controls and 35 of the 40 (87.5%) CFS patients had detectable levels of HHV-7 in the DNA of their PBMCs. All saliva samples also had detectable levels of HHV-7. No difference in viral copy number was seen between controls and CFS patients in saliva or PBMCs (Figure 18). There was no correlation between HHV-7 viral copy number in saliva or PBMCs and HERV-K18 *env* expression ($\rho = 0.003$, $p > 0.05$ for saliva; $\rho = -0.1242$, $p > 0.05$ for PBMC). As expected, HHV-7 viral copy number was higher in saliva than in PBMCs. Like HHV-6 viral copy number, HHV-7 viral copy number did not correlate with any of the interview questions assessing CFS symptom severity.

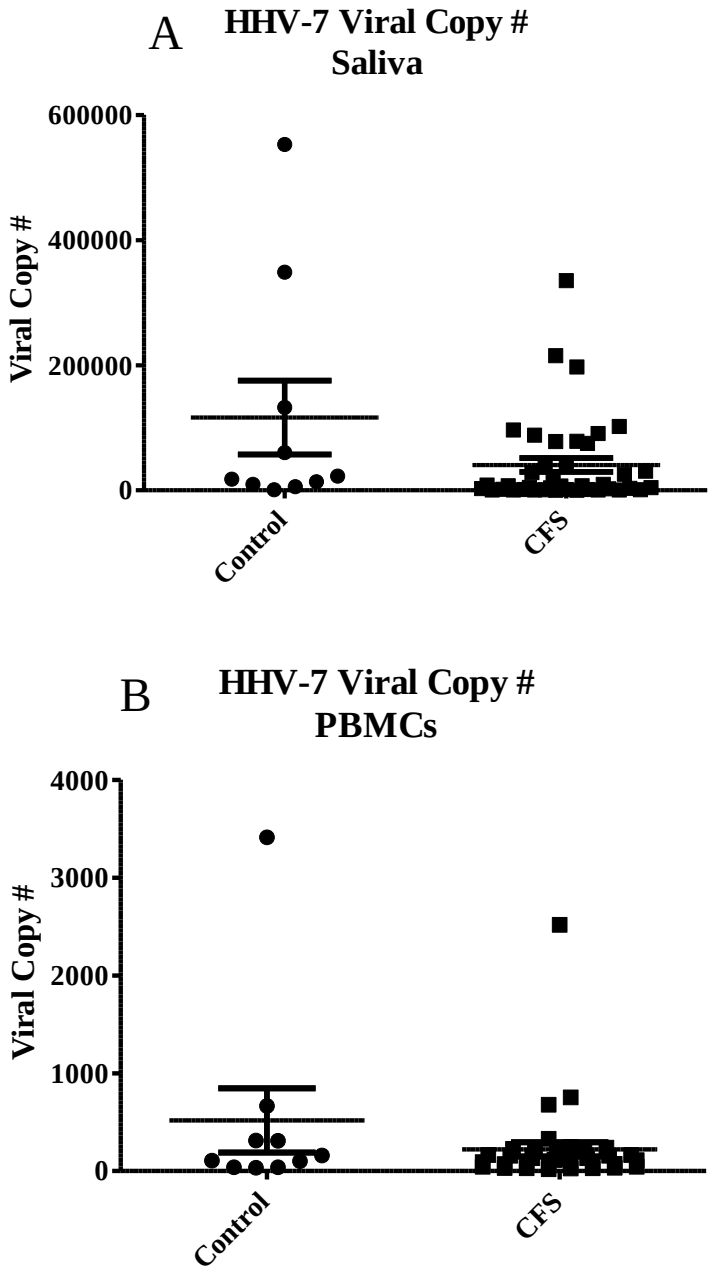

Figure 18. HHV-7 viral copy number. (A) HHV-7 viral copy number was measured using real time PCR in the saliva of 9 healthy controls and 40 CFS patients. 9/9 healthy controls and 40/40 CFS patients had detectable levels of HHV-7. Viral copy number represents viral copies present in an entire 5 ml wash. (B) HHV-7 viral copy number was measured using real time PCR in the PBMCs of 10 healthy controls and 40 CFS patients. 9/9 healthy controls and 35/40 CFS patients had detectable levels. Viral copy number represents viral copies present in 500 ng PBMC DNA. Bars = Mean +/- SEM

Significance

Although HHV-6 has been proven to induce HERV-K18 *env* expression *in vitro* [254-255], we failed to see any HERV-K18 *env* induction in the presence of HHV-6. As expected, HHV-6 and HHV-7 were readily detectable in both saliva and PBMCs of both healthy controls and CFS patients; however, HERV-K18 *env* transcripts failed to correlate with HHV-6 viral load. Both HHV-6 and HHV-7 are highly prevalent in the worldwide population and, again, we must be cautious in how we interpret data. Although a large viral genome copy number is indicative of reactivation, there are other explanations that must be considered before jumping to a conclusion. Our study does not support the hypothesis of reactivation of HHV-6 or HHV-7 in CFS and encourages the use of proper controls treated in the exact same manner as the experimental samples.

Discussion

XMRV

In 2009, Lombardi *et al.* demonstrated a link between CFS and the new gammaretrovirus XMRV [123]. The study detected XMRV *gag* sequences in the DNA from PBMCs of 67% of CFS patients compared to only 3.7% of healthy controls using a nested PCR. This exciting finding was met with enthusiasm and seen by some as a major breakthrough in CFS research. Having previously banked a large library of PBMC DNA samples from CFS patients for an unrelated study, we were poised to confirm this game-changing finding.

Method of Detecting XMRV

The original findings by Lombardi *et al.* used a non-XMRV-specific nested PCR that also detects endogenous MLV sequences within the mouse genome [123]. To avoid potential contamination issues, we chose to initially analyze our cohort of CFS patients and healthy controls using a qPCR assay specific for the IN region in the XMRV *pol* gene that is not cross-reactive with any sequence known to be present in closely related MLVs. Using DNA from an XMRV-positive cell line, WPI-1282, we demonstrated that this specific qPCR could detect down to 10-12 pg of XMRV DNA, the equivalent of two cells, in the presence or absence of 5 µg control DNA isolated from the XMRV-negative human LNCaP cell line (Figure 3). Despite this low limit of detection, we failed to detect XMRV sequences in any of our 112 CFS samples or 36 healthy controls. This meant that all samples were XMRV-negative, XMRV had a more divergent sequence than previously described [123, 148], or XMRV levels were below the limit of detection for

the qPCR. Due to the large percentage of CFS samples reported to be positive for XMRV by Lombardi *et al.*, it was difficult to reconcile that all of our samples were either XMRV-negative or had XMRV levels below the detection limit; thus, we surmised that XMRV must have more divergent sequences that could not be detected by our qPCR assay.

For this reason, we employed the nested PCR used in the original Lombardi *et al.* study that could detect more divergent XMRV-sequences, but this assay also came with the pitfall of being able to detect endogenous MLV proviruses. Using DNA from the XMRV-positive cell line, WPI-1282, we demonstrated that MLV-like sequences could be detected in 2-3 pg of WPI-1282 DNA, equivalent to < 1 cell, when mixed with 200 ng of background DNA (Figure 4). This means that the nested PCR is approximately 10 times more sensitive than the XMRV-specific qPCR. Once the cohort was tested using the nested PCR, a surprisingly high proportion of DNA samples from the healthy controls (19/36) tested positive, whereas only 2/112 of the CFS patients yielded PCR products of the correct size. This contradicted the original finding and suggested that XMRV was more highly prevalent in the population and was not associated with CFS.

The failure to detect any XMRV sequences with the qPCR and the subsequent detection of PCR products using the nested PCR could have meant one of three things; First, the samples could contain extremely low levels of XMRV that were not detected with the qPCR assay. As stated earlier, the qPCR was 10 fold less sensitive than the nested PCR. If sequencing the PCR product revealed an XMRV sequence, this would suggest that the difference between the two assays was merely a sensitivity issue. Second, the XMRV sequence could have been more divergent than previously described

and the specific qPCR could not detect the variation. Again determining the sequence of the PCR product would determine any variations in XMRV sequence. Third, the nested PCR could be presenting false positives by amplifying contaminating mouse or XMRV DNA. We used DNA from the WPI-1282 cell line as our positive control every time we ran a PCR. If the WPI-1282 DNA were to contaminate some nested PCR runs, then sequencing the PCR product would reveal the WPI-1282 sequence; thus, sequencing the PCR product could also determine this. Our lab routinely works with mice; thus contaminating murine DNA could not be ruled out. Contaminating mouse DNA could be introduced to the samples while they were initially processed or during the set up of the PCR. None of our NTC samples, included in triplicate in every run, ever tested positive in the nested PCR, suggesting that mouse DNA was not being introduced to the samples at the time of PCR set up. While all the blood samples were processed in our lab, it should be noted that the CFS cohort mainly consisted of banked samples collected and processed in 2005, whereas the healthy controls were recruited between November of 2009 and May of 2010; thus, the healthy control samples were processed using different lots of reagents and were handled by a different person. The rather large difference in PCR positive samples between the CFS group and the healthy controls suggests that different processing may be a contributing factor. To determine what the reason was for the discrepancy between the two series of PCRs, we sequenced all nested PCR products and tested all samples for mouse DNA.

Sequencing Reveals both Evidence of Mouse Genomic Contamination and the presence of XMRV

In order to determine if our PCR products were XMRV, MLV, or contaminating WPI-1282, we sequenced the PCR products. The observation that most of our amplicons contained mixtures of sequences and needed limiting dilutions to obtain a pure sequence for analysis suggests multiple viruses were present or, more likely, there was contamination. Nevertheless, a total of 37 clean sequences were found to have a high degree of diversity, revealing both XMRV-like and endogenous MLV sequences (Figure 5). The majority of sequences were either identical or closely related to known endogenous MLVs; however, 3 healthy controls had sequences that were identical to the corresponding segment of XMRV strain VP42. The WPI-1282 sequence (VP62) was not found in any samples, so it can be ruled out as a possible contaminate. Our lab never had a VP42 plasmid, or a VP42-containing cell line, suggesting that the three samples with the VP42 sequence, which is not present in the sequenced C57Bl/6 genome, may actually be XMRV; however, the 3 samples containing the VP42 sequence also contained other MLV sequences. All of the sequences were distributed over a minimum of 3 clusters, each of which contains endogenous MLV sequences of a different subtype (XMV, PMV, and MPMV). Given the fact that these sequences were mostly mixed in the same samples, that MLVs have not been observed to infect human hosts, and the large distribution of endogenous MLV sequences, these data seem to support the idea of murine genomic DNA contamination; however, the presence of the VP42 sequence may suggest that XMRV is also present.

Massive Murine DNA Contamination

To rule out contaminating mouse DNA, we employed two PCR assays specific for minute amounts of murine DNA; a qPCR for the mouse mitochondrial *cox2* gene and a single PCR assay for the highly abundant IAP long terminal repeat sequences developed by us. Both assays are extremely sensitive, detecting the target sequences in 0.6 pg of mouse DNA, equivalent to 1/10 of a cell in a background of 200 ng LNCaP. Importantly, these murine DNA detection assays are more sensitive than the MLV nested PCR; therefore, if genomic murine DNA was the cause of the MLV sequences, the IAP or *cox2* assays should detect murine DNA. As expected, all samples that tested positive for MLV sequences using the nested PCR also tested positive for mouse DNA (Table 5). Surprisingly, some samples that did not have a PCR product in the MLV nested PCR also tested positive for mouse DNA. This shows the higher sensitivity of both the mouse DNA detection PCRs over the MLV nested PCR and also suggests that mouse DNA is a universal contaminate, most like contaminating during collection of blood, during isolation of PBMCs, or during the preparation of DNA from PBMCs. Despite a thorough search, we could not pinpoint a specific reagent or laboratory vessel that consistently tested positive for mouse DNA. We suspect it to be a common laboratory reagent such as fetal calf serum or PBS which are both used during PBMC isolation and have inconsistently tested positive for mouse DNA.

Lack of XMRV-specific Antibodies Confirms False Positives

Although we demonstrated that the MLV sequences detected in the DNA of PBMCs of both our healthy controls and our CFS patients correlated with detection of mouse DNA, we still detected 3 sequences identical to the XMRV sequence, VP42. We

cannot rule out the possibility that XMRV is present in these samples along with mouse DNA contamination. The presence of anti-XMRV antibodies in the sera of patients and healthy controls cannot be due to mouse DNA contamination. To this end, we employed two novel direct format ARCHITECT p15E and gp70 CMIAs. Recent animal studies showed that XMRV infection elicited a potent humoral immune response in rhesus macaques [262]. The infected macaques developed XMRV-specific antibodies within two weeks of infection and persisted for more than 158 days. The predominant responses were to all three structural proteins of XMRV: the envelope protein gp70, the transmembrane protein p15E, and the capsid protein p30. The gp70 and p15E CMIA are the most sensitive and can detect antibodies as early as 9 days post infection [262]. We were unable to detect XMRV p15E and gp70 specific antibodies in any of our 112 CFS patients, proving that XMRV is not present in our CFS cohort. 34 of our healthy controls, including the samples that previously were found to contain the VP42 XMRV sequence, also tested negative for XMRV p15E and gp70, suggesting the samples that contained the XMRV *gag* sequence, were actually XMRV negative (Table 6). Two of the healthy controls had weak reactivity in the gp70 CMIA (Figure 8), but were negative for reactivity in the WB to recombinant gp70. Both samples were also non-reactive in the p15E CMIA and had no detectable p15E and p30 antibodies by viral lysate WB. The weak reactivity in the gp70 CMIA most likely represents nonspecific reactivity since specificity of the gp70 CMIA was reported as 99.5% [262]. The lack of anti-XMRV antibodies present in the sera of CFS patients and healthy controls is indicative of XMRV not being present in any of our samples. Taken with the mouse DNA contamination data,

we can unequivocally conclude that XMRV is not present in our CFS patient or healthy control cohort.

XMRV: Final Conclusions

Although the initial discovery of a new retrovirus associated with CFS was exciting, invigorated researchers, and brought new interest to CFS, extreme caution should have been exercised in the interpretation of the initial results. The identity of the virus being closely related to endogenous MLVs and the use of the nested PCR that could admittedly amplify other MLVs should have been a tell tale signal to scientists everywhere to proceed with caution. In our initial analysis utilizing the XMRV-specific qPCR for the IN region in the XMRV *pol* gene that is not cross-reactive with any sequence known to be present in closely related MLVs, we made the naive assumption that what Lombari *et al.* [123] reported as the prevalence of XMRV in CFS patients (67%) was fact. Since Lombardi *et al.* detected XMRV in such a large proportion of CFS patients, we assumed that XMRV must be present in our cohort and that the XMRV-specific qPCR assay was too restricted, even though both Lombardi and Urisman reported the lack of sequence divergence in XMRV sequences [123, 148]. Making assumptions is dangerous and can lead researchers astray. We must let the data speak for themselves. Given that all of our samples that tested positive for an MLV or XMRV sequence also tested positive for mouse DNA contamination and none of our samples tested positive for XMRV specific antibodies, we can conclude without a doubt that XMRV is not present in our cohort and is not associated with CFS.

HERV-K18 *env* Model for CFS

One of the major hypotheses for the pathogenesis of CFS is that persistent viral infections may trigger and lead to chronic activation of the immune system with abnormal regulation of cytokine production [149]. Past viruses associated with CFS and under investigation as possible triggers to CFS include EBV, HHV-6A, and HHV-6B; all of which have been shown to transactivate the HERV-K18 SAg [253-255]. SAgs are microbial proteins that greatly over-stimulate the immune system by directly interacting with the Vβ segment of the TCR and the MHC II complex of antigen presenting cells. This is unlike conventional peptide antigens that are recognized by a specific hypervariable region of the TCR, which is different in every T cell clone [241]. IFN-α, an antiviral cytokine produced in response to infection, can also induce HERV-K18 *env* [246]. This led me to the model that chronic virus infection with EBV or HHV6, or any infection inducing IFN-α, could lead to induction of the HERV-K18 *env* SAg, which then could lead to overstimulation of the immune system, resulting in the symptoms of CFS (Figure 2). A previous small pilot study done in our lab on a group of patients who developed CFS after prolonged iatrogenic IFN-α treatment suggested that the HERV-K18 *env* genotype could be a risk factor for CFS. A follow-up pilot study testing a group of non-IFN-associated CFS patients failed to see this risk factor [272]. Because of these data, we hypothesized that subsets of CFS patients associated with known inducers of the HERV-K18 *env* SAg could have increased risk of CFS due to their HERV-K18 *env* genotype.

A CFS population associated with a HERV-K18 env-inducing agent

In order to test my hypothesis, we needed two groups of CFS patients; a CFS patient population that was associated with a known HERV-K18 inducer and a CFS patient population that was not associated with a HERV-K18 *env* inducer. To this end, we recruited 53 CFS patients who had a previous medical history of EBV-IM (Group A, the inducer group) and 48 CFS patients who had no previous history of EBV-IM (Group B, non-inducers). Importantly, the only apparent difference between the two groups was the EBV-IM association. Both groups were diagnosed with CFS using the 1994 CDC criteria [1], had equal percentage of females and males, same average age, and same average symptom severity scores at the time of the blood draw (Table 7).

The three distinct K18 alleles are unevenly distributed in the Caucasian population with K18.1 (K18.1 (46.6%) and K18.2 (42.5%) being the most common and K18.3 (10.8%) being the most rare. All three alleles encode a SAg; however, due to differences in amino acid sequence, biochemical differences are predicted between alleles. In the pilot study, the group of patients who developed CFS after prolonged iatrogenic IFN-a treatment suggested that the HERV-K18 *env* genotype, K18.1/K18.3, had a significantly increased odds ratio for developing CFS [272]. Although we were aware that a typical gene association study has hundreds, if not thousands, of different samples, the initial pilot study only contained 42 samples, and if HERV-K18 *env* genotype was a strong indicator of genetic risk, than our small cohort should detect a difference in genotype. Surprisingly, there was no statistically significant difference in genotype between Group A, the EBV-IM associated CFS group, and Group B, the non-EBV-IM associated CFS group (Table 8). Although the K18.1/K18.3 genotype was the

genotype with the increased risk in the pilot study [272], the K18.1/K18.3 genotype was one of the rarest genotypes in both groups in this study. This suggests that there is no difference in HERV-K18 *env* genotype between individuals who develop CFS with a history of EBV-IM and individuals who develop CFS without a history of EBV-IM. Another possible explanation is that our sample size was too small and the relationship between HERV-K18 *env* genotype and CFS is not as strong as originally thought. Lastly, although group B had no history of EBV-IM, those patients could still be associated with other HERV-K18 *env* inducers that we have not accounted for such as HHV-6 or other IFN-α inducing infections, which could alter our comparison. However, the fact that both Group A and Group B expressed similar levels of HERV-K18 *env as* healthy controls (Figure 16), suggests neither patient group induces HERV-K18 env expression.

HERV-K18 env expression and symptom severity

One of our main research questions was to determine if HERV-K18 *env* expression varies over time in the same individual. To this end, we followed patients for two years, taking blood sample every six months and measuring their HERV-K18*env* expression. Figure 11 illustrates that HERV-K18 *env* transcripts do fluctuate within an individual and inter-individuals variation also occurs. Since it is well established that CFS patients' symptoms have cycles of remission and relapse, we wanted to determine if the fluctuations in HERV-K18 *env* transcripts correlated with symptom severity. We expected to see a positive correlation, with increased HERV-K18 *env* transcripts seen with increased symptom severity; however, we found no correlation between HERV-K18 *env* transcripts and any of the symptom severity scales.

Assessment of Symptom Severity Scales

CFS patients are traditionally a heterogeneous group of patients with varying symptoms [1, 12]. Not all patients are expected to have the exact same symptoms, and the severity of each symptom, such as fatigue and pain, can be interpreted differently by each individual. There is no physical reading to determine the level of a somatic symptom to compare one individual to another. The best method for determining the level of somatic symptoms is interview questionnaires. The six surveys employed in this study employ a Likert-style scoring system that assigns a number to the level of a somatic symptom; the higher the overall number, the more severe the symptom. The Fatigue Scale measures both physical and mental fatigue, the most common symptom in CFS [263]. The Chronic Fatigue Syndrome Rating Form rates the severity of a patients fatigue, as well as the 8 CFS definitional symptoms from the 1994 CDC criteria (Table 3) [12, 264-265]. The SOMA Questionnaire measures somatic symptoms, such as fatigue and pain. We expected that if HERV-K18 *env* transcripts were influencing symptoms, that the Fatigue Scale, the Chronic Fatigue Syndrome Rating Form, and SOMA scales would all have a positive correlation using the Spearman test. Surprisingly, none of these scales correlated with HERV-K18 *env* transcripts at all, suggesting that the HERV-K18 *env* transcript level is not associated with CFS symptom severity.

The other 3 surveys used in this analysis help measure psychiatric or mental disorders. The Perceived Stress Scale measures how stressful an individual sees his own life. The more stressed a person feels, the higher the perceived stress score. The General Health Questionnaire was developed as a screening tool to detect those likely to have or to be at risk for developing psychiatric disorders. Questions can be broken into the four

subclasses: somatic symptoms, anxiety/insomnia, social dysfunction, and severe depression. The SPHERE also screens for common mental disorders with a focus on depression and anxiety. One of the main exclusionary criteria for a CFS diagnosis is mental disorder so we expected none of these scales to correlate with HERV-K18 *env* if HERV-K18 *env* is only influencing CFS symptom severity. Not surprisingly, HERV-K18 *env* transcript levels did not correlate with any of these scales.

Performing a group analysis of symptom severity based on scales measuring somatic symptoms is difficult to do. Although these scales have reliably been used to evaluate symptom severity [263-264], they still are just measures of an individual's personal assessment of his own symptoms. Unfortunately, no other reliable method to measure symptom severity in CFS patients exists. The Spearman rank order correlation test attempts to measure a monotonic relationship between two variables. Basically, it tests if one variable increases, does the second variable also increase. The Spearman test is less restrictive than the Pearson test; the latter assumes a linear relationship. The failure to see any correlation between HERV-K18 *env* transcripts and any of the symptom severity scales, suggests that HERV-K18 is not associated with any symptoms in our cohorts.

Assessment of HERV-K18 env in CFS vs. healthy controls

Our initial analysis of HERV-K18 *env* expression showed increased levels of HERV-K18 transcripts in CFS patients compared to healthy controls; however, when we tried to confirm this in an independently diagnosed CFS group, we found much lower levels of HERV-K18 *env* expression. In the initial analysis, we compared CFS samples that had been shipped overnight before processing to healthy controls that were processed

the same day as collection. In the second study, both healthy controls and CFS patient samples were processed the same day as the blood draw. One of the major assumptions in testing patient populations is that blood samples that take a day to reach a lab before being processed are the same as blood samples that are processed immediately. This assumption allows labs to study populations not near where they are located.

To determine if this assumption is correct, that blood samples do not change after an overnight shipment, we collected two blood samples from 12 healthy volunteers. One sample was processed immediately, while the second sample was allowed to sit overnight at room temperature and processed the next day. The samples that sat overnight had significantly higher levels of HERV-K18 *env* transcripts than the freshly processed samples (Figure 15). Our assumption that samples do not change with an overnight incubation before processing was wrong and had skewed our results. Analysis of the initial CFS population with proper controls that had also sat overnight revealed that the CFS samples did not express higher levels of HERV-K18 *env* transcripts than controls.

HHV-6 and HHV-7

Although both HHV-6A and 6B, along with the closely related HHV-7, are continually being investigated as potential triggering viruses for CFS [107, 232], we found that CFS patients and healthy controls have similar viral copy numbers of these viruses in both their saliva and PBMCs, respectively (Figures 17, 18). Noting that HHV-6 is known to induce HERV-K18 *env*, we attempted to correlate viral load to HERV-K18 *env* expression using the Spearman rank-order correlation test. No correlation was found between HHV-6 viral load and HERV-K18 *env* expression. Not surprisingly, since our

CFS patients expressed similar levels of HERV-K18 with controls, no correlation was seen between symptom severity and HERV K18 *env* either. This suggests that HERV-K18 *env* is not associated with CFS in this patient population.

Both HHV-6 and HHV-7 viral copy number did not correlate with patient symptom severity. HHV-6 and HHV-7 are ubiquitous in our population, with greater than 90% of the population becoming infected asymptomatically as young children. Active infections of HHV-6 and HHV-7 in adults are usually only found in immunocompromised individuals such as transplant recipients or HIV patients showing that these are "opportunistic" infectious agents [179]. Although some past studies have shown an association of these viruses with CFS [202-204, 206-208], this does not mean that these viruses are causing CFS. HHV-6 and HHV-7 could be bystanders that are taking advantage of the immune dysfunction seen in CFS patients; however, our study could not support any association of HHV-6 or HHV-7 with CFS.

Our HERV-K18 CFS Model and Future Studies

In our working hypothesis (Figure 2), we proposed that chronic infection, or infection that induced IFN-α, induces the HERV-K18 *env* SAg leading to overstimulation of the immune system, resulting in the symptoms of CFS. The first step in this model is the induction of HERV-K18 *env.* For this model to be relevant, we must find a patient population with increased HERV-K18 *env* expression. Initially, we looked at a population of CFS patients who had been associated with prior EBV-IM. Although, EBV has previously been shown to induce the HERV-18 SAg, this CFS group expressed similar levels of HERV-K18 *env* as healthy controls. Two other CFS patient populations also showed similar HERV-K18 *env* levels as healthy controls. Tests to reveal if any of

these CFS patients had high viral copy number of HHV-6, a known inducer of HERV-K18 *env*, revealed that our CFS patients had similar viral copy numbers to healthy controls. None of the CFS patient populations investigated had increased HERV-K18 *env*. This suggests that our model is either completely wrong or we are not studying the correct patient populations. Numerous studies have started to look at post-viral fatigue syndrome [213, 273-275]. Future studies to investigate if HERV-K18 expression is associated with CFS should start in these patient populations to confirm our model.

A limit to our studies of HERV-K18 is the fact that we only analyzed transcript levels of HERV-K18 *env*. We attempted to resurrect a reporter assay that measured HERV-K18 SAg stimulation of a T-cell Hybridoma expressing Vβ13 using IL-2 as readout, but we could not get these cells to grow. Future studies should reestablish this SAg activity assay, attempt to measure HERV-K18 SAg protein levels or look for other signs of SAg presence. It is known that the HERV-K18 SAg selectively activates human Vβ13+ T cells [243]. If SAg activity is present in CFS patients, we should see increased activation of Vβ13+ T cell subsets, along with increased cytokine levels.

Although our data do not support our proposed model, a different population of CFS patients could yield more promising results. CFS is a heterogeneous disease with more than one likely cause. Further stratification of CFS patients into subclasses may be necessary to prove individual causes. Our model is dependent on an infectious trigger leading to CFS; therefore, future studies should focus on a CFS population classified as having post viral fatigue.

Bibliography

1. Prevention, C.f.D.C.a., *Chronic Fatigue Syndrome*. 2012.
2. Wessely, S., *History of postviral fatigue syndrome.* British Medical Bulletin, 1991. **47**(4): p. 919-941.
3. Gosling, F.G., *Before Freud: Neurasthenia and the American Medical Community, 1870-1910.* 1987, Urbana, IL: University of Illinois Press.
4. Taylor, R.E., *Death of neurasthenia and its psychological reincarnation.* The British Journal of Psychiatry, 2001. **179**(6): p. 550-557.
5. Patarca-Montero, R., *Medical Etiology, Assessment, and Treatment of Chronic Fatigue and Malaise: Clinical Differentiation and Intervention*. 2004, Binghamtom, NY: The Hawthorne Medical Press.
6. Hospital, T.M.S.o.t.R.F., *An Outbreak of Encephalomyelitis in the Royal Free Hospital Group, London, in 1955.* British Medical Journal, 1957. **2**(5050): p. 895-904.
7. Compston, N.D., *An outbreak of encephalomyelitis in the Royal Free Hospital Group, London, in 1955.* Postgraduate Medical Journal, 1978. **54**: p. 722-724.
8. Parish, J.G., *Early outbreaks of 'epidemic neuromyasthenia'.* Postgraduate Medical Journal, 1978. **54**(637): p. 711-717.
9. Barnes, D., *Mystery diseasee at Lake Tahoe challenges virologists and clinicians.* Science, 1986. **234**(4776): p. 541-542.
10. Buchwald, D., et al., *A Chronic Illness Characterized by Fatigue, Neurologic and Immunologic Disorders, and Active Human Herpesvirus Type 6 Infection.* Annals of Internal Medicine, 1992. **116**(2): p. 103-113.
11. Holmes, G.P., et al., *Chronic Fatigue Syndrome: A Working Case Definition.* Annals of Internal Medicine, 1988. **108**(3): p. 387-389.
12. Fukuda, K., et al., *The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group.* Ann Intern Med, 1994. **121**: p. 953 - 959.
13. Radford, C., *The Chronic Fatigue Syndrome.* Annals of Internal Medicine, 1988. **109**(2): p. 166.
14. Katon, W. and J. Russo, *Chronic fatigue syndrome criteria: A critique of the requirement for multiple physical complaints.* Archives of Internal Medicine, 1992. **152**(8): p. 1604-1609.
15. Komaroff, A., et al., *An examination of the working case definition of chronic fatigue syndrome.* American Journal of Medicine, 1996. **100**(1): p. 56-64.
16. Straus, S.E., *DEfining the chronic fatigue syndrome.* Archives of Internal Medicine, 1992. **152**(8): p. 1569-1570.
17. Schluederberg, A., et al., *NIH conference. Chronic fatigue syndrome research. Definition and medical outcome assessment.* Annals of Internal Medicine, 1992. **117**(4): p. 325-331.
18. Carruthers, B.M., et al., *Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: Clinical Working Case Definition, Diagnostic and Treatment Protocols.* Journal of Chronic Fatigue Syndrome, 2003. **11**(1): p. 7-36.
19. Sharpe, M.C., et al., *A report--chronic fatigue syndrome: guidelines for research.* Journal of the Royal Society of Medicine, 1991. **84**(2): p. 118-121.
20. Jason, L.A., et al., *A Pediatric Case Definition for Myalgic Encephalomyelitis and Chronic Fatigue Syndrome.* Journal of Chronic Fatigue Syndrome, 2006. **13**(2/3): p. 1-44.
21. Holgate, S.T., et al., *Chronic fatigue syndrome: understanding a complex illness.* Nat Rev Neurosci, 2011. **12**(9): p. 539-544.

22. Christley, Y., T. Duffy, and C.R. Martin, *A review of the definitional criteria for chronic fatigue syndrome.* Journal of Evaluation in Clinical Practice, 2012. **18**(1): p. 25-31.
23. Jason, L., et al., *A community-based study of chronic fatigue syndrome.* Archives of Internal Medicine, 1999. **159**(18): p. 2129-2137.
24. Reyes, M., et al., *Prevalence and incidence of chronic fatigue syndrome in wichita, kansas.* Archives of Internal Medicine, 2003. **163**(13): p. 1530-1536.
25. Wessely, S., et al., *The prevalence and morbidity of chronic fatigue and chronic fatigue syndrome: a prospective primary care study.* Am J Public Health, 1997. **87**(9): p. 1449-55.
26. Bassi, N., et al., *Chronic fatigue syndrome: characteristics and possible causes for its pathogenesis.* The Israeli Medical Association Journal, 2008. **10**(1): p. 79-82.
27. Griffith, J.P. and F.A. Zarrouf, *A Systematic Review of Chronic Fatigue Syndrome: Don't Assume It's Depression.* The Primary Care Companion to the Journal of Clinical Psychiatry, 2008. **10**(2): p. 120-128.
28. Steele, L., et al., *The epidemiology of chronic fatigue in San Francisco.* American Journal of Medicine, 1998. **105**(3A): p. 83S-90S.
29. Jordan, K.M., et al., *Chronic fatigue syndrome in children and adolescents: A review.* Journal of Adolescent Health, 1998. **22**(1): p. 4-18.
30. Chalder, T., et al., *Epidemiology of chronic fatigue syndrome and self reported myalgic encephalomyelitis in 5-15 year olds: cross sectional study.* BMJ, 2003. **327**(7416): p. 654-655.
31. Jones, J.F., et al., *Chronic fatigue syndrome and other fatiguing illnesses in adolescents: a population-based study.* Journal of Adolescent Health, 2004. **35**(1): p. 34-40.
32. Prins, J.B., J.W.M. van der Meer, and G. Bleijenberg, *Chronic fatigue syndrome.* The Lancet, 2006. **367**(9507): p. 346-355.
33. Nijs, J., et al., *Can exercise limits prevent post-exertional malaise in chronic fatigue syndrome? An uncontrolled clinical trial.* Clinical Rehabilitation, 2008. **22**(5): p. 426-435.
34. Werbach, M., *Nutritional strategies for treating chronic fatigue syndrome.* Alternative Medicine Review, 2000. **5**(2): p. 93-108.
35. Price, J., et al., *Cognitive behaviour therapy for chronic fatigue syndrome in adults (Review).* The Cochrane Library, 2008(3): p. 1-102.
36. Pae, C.-U., et al., *Pharmacological treatment of chronic fatigue syndrome: focusing on the role of antidepressants.* Expert Opinion on Pharmacotherapy, 2009. **10**(10): p. 1561-1570.
37. Mariman, A., et al., *Subjective sleep quality and daytime sleepiness in a large sample of patients with chronic fatigue syndrome (CFS).* Acta Clinica Belgica, 2012. **67**(1): p. 19-24.
38. Spotila, L., *Pharmacotherapy for CFS.* CFIDS Chronicle, 2006: p. 1-10.
39. Natelson, B., et al., *Randomized, double blind, controlled placebo-phase in trial of low dose phenelzine in the chronic fatigue syndrome.* Psychopharmacology, 1996. **124**(3): p. 226-230.
40. Goodnick, P., *Treatment of chronic fatigue syndrome with venlafaxine.* American Journal of Psychiatry, 1996. **153**(2): p. 294.
41. White, P. and K. Cleary, *An open study of the efficacy and adverse effects of moclobemide in patients with the chronic fatigue syndrome.* International Clinical Psychopharmacology, 1997. **12**(1): p. 47-52.
42. Gracious, B. and K.L. Wisner, *Nortriptyline in chronic fatigue syndrome: A double blind, placebo-controlled single case study.* Biological Psychiatry, 1991. **30**(4): p. 405-408.
43. Vercoulen, J., et al., *Randomised, double-blind, placebo-controlled study of fluoxetine in chronic fatigue syndrome.* Lancet, 1996. **347**(9005): p. 858-61.

44. Wearden, A.J.M., R. K.; Mullis, R.; Strickland, P.L.; Pearson, D.J.; Appleby, L.; Campbell, I. T.; Morris, J.A., *Randomised, double-blind, placebo-controlled treatment trial of lfuoxetine and graded exercise for chronic fatigue syndrome.* The British Journal of Psychiatry, 1998. **172**(6): p. 485-490.
45. Dhir, A. and S.K. Kulkarni, *Venlafaxine reverses chronic fatigue-induced behavioral, biochemical and neurochemical alterations in mice.* Pharmacology Biochemistry and Behavior, 2008. **89**(4): p. 563-571.
46. Hickie, I., et al., *A randomized, double-blind placebo-controlled trial of moclobemide in patients with chronic fatigue syndrome.* Journal of Clinical Psychiatry, 2000. **61**(9): p. 643-8.
47. Natelson, B., et al., *Single-blind, placebo phase-in trial of two escalating doses of selegiline in the chronic fatigue syndrome.* Neuropsychobiology, 1998. **37**(150-4).
48. Olson, L.G., A. Ambrogetti, and D.C. Sutherland, *A Pilot Randomized Controlled Trial of Dexamphetamine in Patients With Chronic Fatigue Syndrome.* Psychosomatics, 2003. **44**(1): p. 38-43.
49. Turkington, D., et al., *Recovery from chronic fatigue syndrome with modafinil.* Human Psychopharmacology: Clinical and Experimental, 2004. **19**(1): p. 63-64.
50. Kawamura, Y., et al., *Efficacy of a half dose of oral pyridostigmine in the treatment of chronic fatigue syndrome: three case reports.* Pathophysiology, 2003. **9**(3): p. 189-194.
51. Blockmans, D., et al., *Does Methylphenidate Reduce the Symptoms of Chronic Fatigue Syndrome?* The American journal of medicine, 2006. **119**(2): p. 167.e23-167.e30.
52. Straus, S.E., et al., *Acyclovir Treatment of the Chronic Fatigue Syndrome.* New England Journal of Medicine, 1988. **319**(26): p. 1692-1698.
53. Lerner, A.M., et al., *A Small, Randomized, Placebo-Controlled Trial of the Use of Antiviral Therapy for Patients with Chronic Fatigue Syndrome.* Clinical Infectious Diseases, 2001. **32**(11): p. 1657-1658.
54. LERNER, A.M., et al., *Valacyclovir Treatment in Epstein-Barr Virus Subset Chronic Fatigue Syndrome: Thirty-six Months Follow-up.* In Vivo, 2007. **21**(5): p. 707-713.
55. Diaz-Mitoma, F., et al., *Clinical improvement in chronic fatigue syndrome is associated with enhanced natural killer cell-mediated cytotoxicity: the results of a pilot study with Isoprinosine.* Journal of Chronic Fatigue Syndrome, 2003. **11**(2): p. 71-95.
56. Iwakami, E., et al., *Treatment of Chronic Fatigue Syndrome with Antibiotics: Pilot Study Assessing the Involvement of Coxiella burnetii Infection.* Internal Medicine, 2005. **44**(12): p. 1258-1263.
57. Rimes, K.A. and T. Chalder, *Treatments for chronic fatigue syndrome.* Occupational Medicine, 2005. **55**(1): p. 32-39.
58. Lorusso, L., et al., *Immunological aspects of chronic fatigue syndrome.* Autoimmunity Reviews, 2009. **8**(4): p. 287-291.
59. Rowe, K.S., *Double-blind randomized controlled trial to assess the efficacy of intravenous gammaglobulin for the management of chronic fatigue syndrome in adolescents.* Journal of Psychiatric Research, 1997. **31**(1): p. 133-147.
60. Lloyd, A., et al., *A double-blind, placebo-controlled trial of intravenous immunoglobulin therapy in patients with chronic fatigue syndrome.* The American journal of medicine, 1990. **89**(5): p. 561-568.
61. Peterson, P.K., et al., *A controlled trial of intravenous immunoglobulin G in chronic fatigue syndrome.* The American journal of medicine, 1990. **89**(5): p. 554-560.
62. Vollmer-Conna, P.U., et al., *Intravenous Immunoglobulin is Ineffective in the Treatment of Patients with Chronic Fatigue Syndrome.* The American journal of medicine, 1997. **103**(1): p. 38-43.
63. Brook, M., B. Bannister, and W. Weir, *Interferon-alpha therapy for patients with chronic fatigue syndrome.* The Journal of Infectious Diseases, 1993. **168**(3): p. 791-792.

64. See, D. and J. Tilles, *alpha-Interferon treatment of patients with chronic fatigue syndrome.* Immunological Investigations, 1996. **25**(1-2): p. 153-64.
65. *Mismatched Double-Stranded RNA: PolyI:PolyC12U.* Drugs in R&D, 2004. **5**(5): p. 297-304.
66. Strayer, D.R., et al., *A Double-Blind, Placebo-Controlled, Randomized, Clinical Trial of the TLR-3 Agonist Rintatolimod in Severe Cases of Chronic Fatigue Syndrome.* PLoS ONE, 2012. **7**(3): p. e31334.
67. Biopharma, H., *Study of Ampligen in Chronic Fatigue Syndrome*, in *ClinicalTrials.gov [Internet]*. 2011, Bethesda (MD): National Library of Medicine (US).
68. Warren, G., M. McKendrick, and M. Peet, *The role of essential fatty acids in chronic fatigue syndrome. A case-controlled study of red-cell membrane essential fatty acids (EFA) and a placebo-controlled treatment study with high dose of EFA.* Acta Neurologica Scandinavica, 1999. **99**(2): p. 112-116.
69. Behan, P., W. Behan, and D. Horrobin, *Effect of high doses of essential fatty acids on the postviral fatigue syndrome.* Acta Neurologica Scandinavica, 1990. **82**(3): p. 209-216.
70. Cox, I., M. Campbell, and D. Dowson, *Red blood cell magnesium and chronic fatigue syndrome.* Lancet, 1991. **337**(8744): p. 757-60.
71. Vermeulen, R.C.W. and H.R. Scholte, *Exploratory Open Label, Randomized Study of Acetyl- and Propionylcarnitine in Chronic Fatigue Syndrome.* Psychosomatic Medicine, 2004. **66**(2): p. 276-282.
72. Forsyth, L.M., et al., *Therapeutic effects of oral NADH on the symptoms of patients with chronic fatigue syndrome.* Annals of Allergy, Asthma & Immunology, 1999. **82**(2): p. 185-191.
73. Santaella, M., I. Font, and O. Disdier, *Comparison of oral nicotinamide adenine dinucleotide (NADH) versus conventional therapy for chronic fatigue syndrome.* Puerto Rico Health Sciences Journal, 2004. **23**(2): p. 89-93.
74. Ockerman, P.-A., *Antioxidant Treatment of Chronic Fatigue Syndrome.* Clinical Practice of Alternative Medicine, 2000. **1**(2): p. 88-91.
75. Walker, M., *Clinically Proven Treatment for Chronic Fatigue Syndrome.* Townsend Letter for Doctors & Patients, 2002(230): p. 80-5.
76. De Becker, P., et al., *A double-blind, placebo-controlled study of acclydine in combination with amino acids in patients with chronic fatigue syndrome.* AHMF Proceedings, "Myalgic Encephalopathy/Chronic Fatigue Syndrome 'The Medical Practitioners' Challenge in 2001", 2001.
77. Alraek, T., et al., *Complementary and alternative medicine for patients with chronic fatigue syndrome: A systematic review.* Complementary and Alternative Medicine, 2011. **11**(87).
78. Morriss, R., M. Robson, and W. Deakin, *Neuropsychological performance and noradrenaline function in chronic fatigue syndrome under conditions of high arousal.* Psychopharmacology, 2002. **163**(2): p. 166-173.
79. Steinberg, P., et al., *Double-blind placebo-controlled study of the efficacy of oral terfenadine in the treatment of chronic fatigue syndrome.* Journal of Allergy and Clinical Immunology, 1996. **97**(1): p. 119-126.
80. McKenzie, R., et al., *Low-dose hydrocortisone for treatment of chronic fatigue syndrome: a randomized controlled trial.* Journal of the American Medical Association, 1998. **280**(12): p. 1061-6.
81. Cleare, A., V. O'Keane, and J. Miell, *Plasma leptin in chronic fatigue syndrome and a placebo-controlled study of the effects of low-dose hydrocortisone.* Clinical Endocrinology, 2001. **55**(1): p. 113-119.
82. Cleare, A., et al., *Low-dose hydrocortisone for chronic fatigue syndrome: a randomised crossover trial.* Lancet, 1999. **353**(9151): p. 455-458.

83. Peterson, P., et al., *A preliminary placebo-controlled crossover trial of fludrocortisone for chronic fatigue syndrome.* Archives of Internal Medicine, 1998. **158**(8): p. 908-914.
84. Rowe, P., et al., *Fludrocortisone acetate to treat neurally mediated hypotension in chronic fatigue syndrome: A randomized controlled trial.* JAMA: The Journal of the American Medical Association, 2001. **285**(1): p. 52-59.
85. Blockmans, D., et al., *Combination therapy with hydrocortisone and fludrocortisone does not improve symptoms in chronic fatigue syndrome: a randomized, placebo-controlled, double-blind, crossover study.* The American journal of medicine, 2003. **114**(9): p. 736-741.
86. Kakumanu, S., et al., *Effect of topical nasal corticosteroids on patients with chronic fatigue syndrome and rhinitis.* JAOA: Journal of the American Osteopathic Association, 2003. **103**(9): p. 423-427.
87. Snorrason, E., A. Geirsson, and K. Stefansson, *Trial of a Selective Acetylcholinesterase Inhibitor, Galanthamine Hydrobromide, in the Treatment of Chronic Fatigue Syndrome.* Journal of Chronic Fatigue Syndrome, 1996. **2**(2-3): p. 35-54.
88. Blacker, C., et al., *Effect of galantamine hydrobromide in chronic fatigue syndrome: A randomized controlled trial.* JAMA: The Journal of the American Medical Association, 2004. **292**(10): p. 1195-1204.
89. Tiev, K.P., J. Cabane, and J.C. Imbert, *Traitement de l'asthénie en période postinfectieuse: étude randomisée en double aveugle de deux doses de sulbutiamine (400–600 mg/j) versus placebo.* La Revue de Médecine Interne, 1999. **20**(10): p. 912-918.
90. Moorkens, G., H. Wynants, and R. Abs, *Effect of growth hormone treatment in patients with chronic fatigue syndrome: a preliminary study.* Growth Hormone and IGF Research, 1998. **8**(Suppl B): p. 131-3.
91. Williams, G., et al., *Therapy of circadian rhythm disorders in chronic fatigue syndrome: no symptomatic improvement with melatonin or phototherapy.* European Journal of Clinical Investigation, 2002. **32**(11): p. 831-837.
92. Bagnall, A.-M., et al., *The treatment and management of chronic fatigue syndrome (CFS) / myalgic encephalomyelitis (ME) in adults and children*, C.f.R.a. Dissemination, Editor. 2007, University of York: York.
93. Bavinton, J., L. Darbishire, and P. White, *Graded Exercise Therapy for CFS/ME*, P.t.m. group, Editor. 2004.
94. Fulcher, K.Y. and P.D. White, *Randomised controlled trial of graded exercise in patients with the chronic fatigue syndrome.* BMJ, 1997. **314**(7095): p. 1647.
95. Powell, P., et al., *Patient education to encourage graded exercise in chronic fatigue syndrome.* The British Journal of Psychiatry, 2004. **184**(2): p. 142-146.
96. Powell, P., et al., *Randomised controlled trial of patient education to encourage graded exercise in chronic fatigue syndrome.* BMJ, 2001. **322**(7283): p. 387.
97. Coutts, R., R. Weatherby, and A. Davie, *The use of a symptom "self-report" inventory to evaluate the acceptability and efficacy of a walking program for patients suffering with chronic fatigue syndrome.* Journal of Psychosomatic Research, 2001. **51**(2): p. 425-429.
98. Edmonds, M., H. McGuire, and P. J, *Exercise therapy for chronic fatigue syndrome.* The Cochrane Library, 2004(3): p. 1-22.
99. Deale, A.C., Trudie; Marks, Isaac; Wessely, Simon, *Cognitive Behavior Therapy for Chronic Fatigue Syndrome: A Randomized Controlled Trial.* American Journal of Psychiatry, 1997. **154**(3): p. 408-414.
100. Sharpe, M., et al., *Cognitive behaviour therapy for the chronic fatigue syndrome: a randomised controlled trial.* BMJ, 1996. **312**(7022): p. 22-26.

101. Lloyd, A., et al., *Immunologic and psychologic therapy for patients with chronic fatigue syndrome: a double-blind, placebo-controlled trial.* The American Journal of Medicine, 1993. **94**(2): p. 197-203.
102. Friedberg, F. and L. Krupp, *A comparison of cognitive behavioral treatment for chronic fatigue syndrome and primary depression.* Clinical Infectious Diseases, 1994. **18**(Suppl 1): p. S105-10.
103. Santhouse, A.M., *Review: CBT reduces fatigue in adults with chronic fatigue syndrome but effects at follow-up unclear.* Evidence Based Mental Health, 2009. **12**(1): p. 16.
104. Maes, M. and F. Twisk, *Chronic fatigue syndrome: Harvey and Wessely's (bio)psychosocial model versus a bio(psychosocial) model based on inflammatory and oxidative and nitrosative stress pathways.* BMC Medicine, 2010. **8**(1): p. 35.
105. Núñez, M., et al., *Health-related quality of life in patients with chronic fatigue syndrome: group cognitive behavioural therapy and graded exercise versus usual treatment. A randomised controlled trial with 1 year of follow-up.* Clinical Rheumatology, 2011. **30**(3): p. 381-389.
106. Light, A.R., et al., *Moderate Exercise Increases Expression for Sensory, Adrenergic, and Immune Genes in Chronic Fatigue Syndrome Patients But Not in Normal Subjects.* The Journal of Pain, 2009. **10**(10): p. 1099-1112.
107. Komaroff, A.L., *Is human herpesvirus-6 a trigger for chronic fatigue syndrome?* Journal of Clinical Virology, 2006. **37, Supplement 1**(0): p. S39-S46.
108. Swanink, C., et al., *Epstein-Barr virus (EBV) and the chronic fatigue syndrome: normal virus load in blood and normal immunologic reactivity in the EBV regression assay.* Clinical Infectious Diseases, 1995. **20**(5): p. 1390-1392.
109. Swanink, C., et al., *Viral antibodies in chronic fatigue syndrome.* Clinical Infectious Diseases, 1995. **21**(3): p. 708-709.
110. Lloyd, A., *Postinfective fatigue*, in *Handbook of chronic fatigue syndrome*, F.P. Jason LA, Taylor RR, Editor. 2003, John Wiley & Sons: Hoboken, NJ. p. 108-123.
111. Nakaya, T., et al., *Demonstration on Borna disease virus in patients with chronic fatigue syndrome.* Nihon Rinsho, 1997. **55**(11): p. 3064-71.
112. Li, Y., et al., *Clinical characteristics of patients with chronic fatigue syndrome: analysis of 82 cases.* Zhonghua YI Xue Za Zhi, 2005. **85**(10): p. 701-4.
113. Chia, J., *The role of enterovirus in chronic fatigue syndrome.* Journal of Clinical Pathology, 2005. **58**: p. 1126-1132.
114. Chia, J.K.S. and A.Y. Chia, *Chronic fatigue syndrome is associated with chronic enterovirus infection of the stomach.* Journal of Clinical Pathology, 2008. **61**(1): p. 43-48.
115. Matano, S., et al., *Acute Parvovirus B19 Infection Mimicking Chronic fatigue Syndrome.* Internal Medicine, 2003. **42**(9): p. 903-905.
116. Kerr, J.R., *Pathogenesis of Parvovirus B19 Infection: Host Gene Variability, and Possible Means and Effects of Virus Persistence.* Journal of Veterinary Medicine, Series B, 2005. **52**(7-8): p. 335-339.
117. White, P.T., JM; Amess, J; Crawford, DH; Grover, SA; Kangro, HO; Clare, AW, *Incidence, risk and prognosis of acute and chronic fatigue syndromes and psychiatric disorders after gladular fever.* The British Journal of Psychiatry, 1998. **173**(12): p. 475-481.
118. Ng, B., et al., *Neuropsychiatric sequelae of Nipah virus encephalitis.* The Journal of Neuropsychiatry and Clinical Neurosciences, 2004. **16**(4): p. 500-504.
119. White, P., et al., *The nosology of sub-acute and chronic fatigue syndromes that follow infectious mononucleosis.* Psychological Medicine, 2004. **34**(3): p. 499-507.
120. Kondo, K., *Human herpesvirus latency and fatigue.* Uirusu, 2005. **55**(1): p. 9-17.
121. Komaroff, A., *Chronic fatigue syndromes: a preliminary overview.* Canada Diseases Weekly Report, 1991. **17**(Suppl 1E): p. 23-28.

122. Ablashi, D., *Viral studies of chronic fatigue syndrome.* Clinical Infectious Diseases, 1994. **18**(Suppl 1): p. S130-3.
123. Lombardi, V., et al., *Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome.* Science, 2009. **326**: p. 585 - 589.
124. Oakes, B., et al., *Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences.* Retrovirology, 2010. **7**(1): p. 109.
125. Oakes, B., et al., *Failure to Detect XMRV-Specific Antibodies in the Plasma of CFS Patients Using Highly Sensitive Chemiluminescence Immunoassays.* Advances in Virology, 2011. **2011**: p. 5.
126. Switzer, W., et al., *Absence of evidence of xenotropic murine leukemia virus-related virus infection in persons with chronic fatigue syndrome and healthy controls in the United States.* Retrovirology, 2010. **7**: p. 57.
127. Sato, E., R. Furuta, and T. Miyazawa, *An Endogenous Murine Leukemia Viral Genome Contaminant in a Commercial RT-PCR Kit is Amplified Using Standard Primers for XMRV.* Retrovirology, 2010. **7**(1): p. 110.
128. Robinson, M., et al., *Mouse DNA contamination in human tissue tested for XMRV.* Retrovirology, 2010. **7**(1): p. 108.
129. Rangel, L., et al., *Family health and characteristics in chronic fatigue syndrome, juvenile rheumatoid arthritis, and emotional disorders of childhood.* Journal of the American Academy of Child and Adolescent Psychiatry, 2005. **44**(2): p. 150-158.
130. Buchwald, D., et al., *A Twin Study of Chronic Fatigue.* Psychosomatic Medicine, 2001. **63**(6): p. 936-943.
131. Kaushik, N., et al., *Gene expression in peripheral blood mononuclear cells from patients with chronic fatigue syndrome.* Journal of Clinical Pathology, 2005. **58**(8): p. 826-832.
132. Kerr, J.R., et al., *Gene Expression Subtypes in Patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis.* Journal of Infectious Diseases, 2008. **197**(8): p. 1171-1184.
133. Frampton, D., et al., *Assessment of a 44 Gene Classifier for the Evaluation of Chronic Fatigue Syndrome from Peripheral Blood Mononuclear Cell Gene Expression.* PLoS ONE, 2011. **6**(3): p. e16872.
134. Wheatland, R., *Chronic ACTH autoantibodies are a significant pathological factor in the disruption of the hypothalamic–pituitary–adrenal axis in chronic fatigue syndrome, anorexia nervosa and major depression.* Medical Hypotheses, 2005. **65**(2): p. 287-295.
135. Wessely, S., *The neuropsychiatry of chronic fatigue syndrome.* Ciba Foundation Symposium, 1993. **173**: p. 212-229.
136. Inder, W., T. Prickett, and R. Mulder, *Normal opiod tone and hypothalamic-pituitary-adrenal axis function in chronic fatigue syndrome despite marked functional impairment.* Clinical Endocrinology, 2005. **62**(3): p. 343-348.
137. Cleare, A.J., *The HPA axis and the genesis of chronic fatigue syndrome.* Trends in Endocrinology & Metabolism, 2004. **15**(2): p. 55-59.
138. Hatcher, S. and A. House, *Life events, difficulties and dilemmas in the onset of chronic fatigue syndrome: a case-control study.* Psychological Medicine, 2003. **33**(7): p. 1185-92.
139. Theorell, T., et al., *Critical Life Events, Infections, and Symptoms During the Year Preceding Chronic Fatigue Syndrome (CFS): An Examination of CFS Patients and Subjects With a Nonspecific Life Crisis.* Psychosomatic Medicine, 1999. **61**(3): p. 304-310.
140. Prins, J.B., et al., *Social Support and the Persistence of Complaints in Chronic Fatigue Syndrome.* Psychotherapy and Psychosomatics, 2004. **73**(3): p. 174-182.

141. Maes, M., *Inflammatory and oxidative and nitrosative stress pathways underpinning chronic fatigue, somatization and psychosomatic symptoms.* Current Opinion in Psychiatry, 2008. **21**: p. 1-9.
142. Qin, L., et al., *Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration.* Glia, 2007. **55**(5): p. 453-462.
143. Maes, M., et al., *The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression.* Metabolic Brain Disease, 2009. **24**(1): p. 27-53.
144. Maes, M., Mihaylova I, Leunis JC, *Chronic fatigue syndrome is accompanied by an IgM-related immune response directed against neopitopes formed by oxidative nitrosative damage to lipids and proteins.* Neuro Endocrinology Letters, 2006. **27**(5): p. 615-21.
145. Richards, R.S., L. Wang, and H. Jelinek, *Erythrocyte Oxidative Damage in Chronic Fatigue Syndrome.* Archives of Medical Research, 2007. **38**(1): p. 94-98.
146. Fulle, S., et al., *Specific correlations between muscle oxidative stress and chronic fatigue syndrome: a working hypothesis.* Journal of Muscle Research and Cell Motility, 2007. **28**(6): p. 355-362.
147. Maes, M., I. Mihaylova, and E. Bosmans, *Not in the mind of neurasthenic lazybones but in the cell nucleus: patients with chronic fatigue syndrome have increased production of nuclear factor kappa beta.* Neuro Endocrinology Letters, 2007. **28**(4): p. 456-62.
148. Urisman, A., et al., *Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant.* PLoS Pathog, 2006. **2**(3): p. e25.
149. Bansal, A.S., et al., *Chronic fatigue syndrome, the immune system and viral infection.* Brain, Behavior, and Immunity, 2012. **26**(1): p. 24-31.
150. Groom, H.C.T. and K.N. Bishop, *The tale of xenotropic murine leukemia virus-related virus.* Journal of General Virology, 2012. **93**(Pt 5): p. 915-924.
151. Erlwein, O., et al., *Failure to Detect the Novel Retrovirus XMRV in Chronic Fatigue Syndrome.* PLoS ONE, 2010. **5**(1): p. e8519.
152. Groom, H.C.B., Virginie C; Makinson, Kerry; Randal, Edward; Baptista, Sarah; Hagan, Suzanne; Gow, John W; Mattes, Frank M; Breuer, Judith; Kerr, Jonathan R; Stoye, Jonathan P; Bishop, Kate N, *Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome.* Retrovirology, 2010. **7**: p. 10.
153. Kuppeveld, F.J.M.v., et al., *Prevalence of xenotropic murine leukaemia virus-related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort.* BMJ, 2010. **340**.
154. Hong, P.L., Jinming; Li, Yongzhe, *Failure to detect Xenotropic murine leukaemia virus-related virus in Chinese patients with chronic fatigue syndrome.* Virology Journal, 2010. **7**: p. 224.
155. Switzer, W., et al., *Absence of evidence of xenotropic murine leukemia virus-related virus infection in persons with chronic fatigue syndrome and healthy controls in the United States.* Retrovirology, 2010. **7**: p. 57.
156. Lloyd, A., et al., *Comment on "Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome".* Science, 2010. **328**(5980): p. 825.
157. Henrich, T., et al., *Xenotropic Murine Leukemia Virus-Related Virus Prevalence in Patients with Chronic Fatigue Syndrome or Chronic Immunomodulatory Conditions.* J Infect Dis, 2010. **202**(10): p. 1478 - 81.
158. Lo, S.-C., et al., *Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors.* Proceedings of the National Academy of Sciences, 2010. **107**(36): p. 15874-15879.
159. Satterfield, B., et al., *Serologic and PCR testing of persons with chronic fatigue syndrome in the United States shows no association with xenotropic or polytropic murine leukemia virus-related viruses.* Retrovirology, 2011. **8**(1): p. 12.

160. Hue, S., et al., *Disease-associated XMRV sequences are consistent with laboratory contamination.* Retrovirology, 2010. **7**(1): p. 111.
161. Groom, H.C.T., et al., *Susceptibility of xenotropic murine leukemia virus-related virus (XMRV) to retroviral restriction factors.* Proceedings of the National Academy of Sciences, 2010. **107**(11): p. 5166-5171.
162. Paprotka, T., et al., *Inhibition of Xenotropic Murine Leukemia Virus-Related Virus by APOBEC3 Proteins and Antiviral Drugs.* Journal of Virology, 2010. **84**(11): p. 5719-5729.
163. Bogerd, H.P., et al., *Human APOBEC3 proteins can inhibit xenotropic murine leukemia virus-related virus infectivity.* Virology, 2011. **410**(1): p. 234-239.
164. Stieler, K. and N. Fischer, *Apobec 3G Efficiently Reduces Infectivity of the Human Exogenous Gammaretrovirus XMRV.* PLoS ONE, 2010. **5**(7): p. e11738.
165. Del Prete, G.Q., et al., *Restricted Replication of Xenotropic Murine Leukemia Virus-Related Virus in Pigtailed Macaques.* Journal of Virology, 2012. **86**(6): p. 3152-3166.
166. Zhang, A., et al., *In vivo hypermutation of xenotropic murine leukemia virus-related virus DNA in peripheral blood mononuclear cells of rhesus macaque by APOBEC3 proteins.* Virology, 2011. **421**(1): p. 28-33.
167. Paprotka, T., et al., *Recombinant Origin of the Retrovirus XMRV.* Science, 2011. **333**(6038): p. 97-101.
168. Simmons, G., et al., *Failure to Confirm XMRV/MLVs in the Blood of Patients with Chronic Fatigue Syndrome: A Multi-Laboratory Study.* Science, 2011. **334**(6057): p. 814-817.
169. Silverman, R.H., et al., *Partial Retraction.* Science, 2011. **334**(6053): p. 176.
170. Alberts, B., *Retraction.* Science, 2011. **334**(6063): p. 1636.
171. Lo, S.-C., et al., *Retraction for Lo et al., Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors.* Proceedings of the National Academy of Sciences, 2012. **109**(1): p. 346.
172. Salahuddin, S., et al., *Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders.* Science, 1986. **234**(4776): p. 596-601.
173. Lusso, P., et al., *In vitro cellular tropism of human B-lymphotropic virus (human herpesvirus-6).* The Journal of Experimental Medicine, 1988. **167**(5): p. 1659-1670.
174. Schirmer, E.C., et al., *Differentiation between two distinct classes of viruses now classified as human herpesvirus 6.* Proceedings of the National Academy of Sciences, 1991. **88**(13): p. 5922-5926.
175. Ablashi, D.V., et al., *Genomic polymorphism, growth properties, and immunologic variations in human herpesvirus-6 isolates.* Virology, 1991. **184**(2): p. 545-552.
176. Dockrell, D.H., *Human herpesvirus 6: molecular biology and clinical features.* Journal of Medical Microbiology, 2003. **52**(1): p. 5-18.
177. Santoro, F., et al., *CD46 Is a Cellular Receptor for Human Herpesvirus 6.* Cell, 1999. **99**(7): p. 817-827.
178. Takahashi, K., et al., *Predominant CD4 T-lymphocyte tropism of human herpesvirus 6-related virus.* Journal of Virology, 1989. **63**(7): p. 3161-3163.
179. Kosuge, H., *HHV-6, 7 and their related diseases.* Journal of Dermatological Science, 2000. **22**(3): p. 205-212.
180. Mori, Y., *Recent topics related to human herpesvirus 6 cell tropism.* Cellular Microbiology, 2009. **11**(7): p. 1001-1006.
181. Hall, C.B., et al., *Persistence of Human Herpesvirus 6 According to Site and Variant: Possible Greater Neurotropism of Variant A.* Clinical Infectious Diseases, 1998. **26**(1): p. 132-137.
182. Donati , D., et al., *Variant-Specific Tropism of Human Herpesvirus 6 in Human Astrocytes.* Journal of Virology, 2005. **79**(15): p. 9439-9448.

183. Harnett, G., et al., *Frequent shedding of human herpesvirus 6 in saliva.* Journal of Medical Virology, 1990. **30**(2): p. 128-130.
184. Pellett, P.E., et al., *Chromosomally integrated human herpesvirus 6: questions and answers.* Reviews in Medical Virology, 2012. **22**(3): p. 144-155.
185. Luppi, M., et al., *Human Herpesvirus 6 Latency Characterized by High Viral Load: Chromosomal Integration in Many, but Not All, Cells.* Journal of Infectious Diseases, 2006. **194**(7): p. 1020-1021.
186. Arbuckle, J.H. and P.G. Medveczky, *The molecular biology of human herpesvirus-6 latency and telomere integration.* Microbes and Infection, 2011. **13**: p. 731-741.
187. Caserta, M.T., D.J. Mock, and S. Dewhurst, *Human Herpesvirus 6.* Clinical Infectious Diseases, 2001. **33**(6): p. 829-833.
188. Hall, C.B., et al., *Human Herpesvirus-6 Infection in Children -- A Prospective Study of Complications and Reactivation.* New England Journal of Medicine, 1994. **331**(7): p. 432-438.
189. Cone, R.W., et al., *Human herpesvirus 6 DNA in peripheral blood cells and saliva from immunocompetent individuals.* Journal of Clinical Microbiology, 1993. **31**(5): p. 1262-1267.
190. Levy, J.A., A. Landay, and E.T. Lennette, *Human herpesvirus 6 inhibits human immunodeficiency virus type 1 replication in cell culture.* Journal of Clinical Microbiology, 1990. **28**(10): p. 2362-2364.
191. Mukai, T., et al., *Molecular epidemiological studies of human herpesvirus 6 in families.* Journal of Medical Virology, 1994. **42**(3): p. 224-227.
192. Yamanishi, K., et al., *IDENTIFICATION OF HUMAN HERPESVIRUS-6 AS A CAUSAL AGENT FOR EXANTHEM SUBITUM.* The Lancet, 1988. **331**(8594): p. 1065-1067.
193. Akashi, K., et al., *Severe Infectious Mononucleosis-like Syndrome and Primary Human Herpesvirus 6 Infection in an Adult.* New England Journal of Medicine, 1993. **329**(3): p. 168-171.
194. Ward, K.N., *The natural history and laboratory diagnosis of human herpesviruses-6 and -7 infections in the immunocompetent.* Journal of Clinical Virology, 2005. **32**(3): p. 183-193.
195. Sola, P., et al., *Human herpesvirus 6 and multiple sclerosis: survey of anti-HHV-6 antibodies by immunofluorescence analysis and of viral sequences by polymerase chain reaction.* Journal of Neurology, Neurosurgery, and Psychiatry, 1993. **56**: p. 917-919.
196. Challoner, P.B., et al., *Plaque-associated expression of human herpesvirus 6 in multiple sclerosis.* Proceedings of the National Academy of Sciences, 1995. **92**(16): p. 7440-7444.
197. Luppi, M., et al., *Human herpesvirus-6: A survey of presence and distribution of genomic sequences in normal brain and neuroglial tumors.* Journal of Medical Virology, 1995. **47**(1): p. 105-111.
198. Lautenschlager, I. and R.R. Razonable, *Human herpesvirus-6 infections in kidney, liver, lung, and heart transplantation: review.* Transplant International, 2012. **25**(5): p. 493-502.
199. Morris, D., et al., *Human herpesvirus 6 infection in renal-transplant recipients.* New England Journal of Medicine, 1989. **320**(23): p. 1560-1561.
200. Caïola, D., et al., *Confirmation of the low clinical effect of human herpesvirus-6 and -7 infections after renal transplantation.* Journal of Medical Virology, 2012. **84**(3): p. 450-456.
201. Daugherty, S., et al., *Chronic fatigue syndrome in northern Nevada.* Reviews of Infectious Diseases, 1991. **13**(Suppl 1): p. S39-44.
202. Yalcin, S., et al., *Prevalence of human herpesvirus 6 variants A and B in patients with chronic fatigue syndrome.* Microbiology and Immunology, 1994. **38**(7): p. 587-590.

203. Di Luca, D., et al., *Human herpesvirus 6 and human herpesvirus 7 in chronic fatigue syndrome.* Journal of Clinical Microbiology, 1995. **33**(6): p. 1660-61.
204. Krueger, G., et al., *Dynamics of chronic active herpesvirus-6 infection in patients with chronic fatigue syndrome: data acquisition for computer modeling.* In Vivo, 2001. **15**(6): p. 461-465.
205. Chapenko, S., et al., *Activation of human herpesviruses 6 and 7 in patients with chronic fatigue syndrome.* Journal of Clinical Virology, 2006. **37, Supplement 1**(0): p. S47-S51.
206. Patnaik, M., et al., *Prevalence of IgM antibodies to human herpesvirus 6 early antigen (p41/38) in patients with chronic fatigue syndrome.* Journal of Infectious Diseases, 1995. **172**(5): p. 1364-7.
207. Sairenji, T., et al., *Antibody responses to Epstein-Barr virus, human herpesvirus 6 and human herpesvirus 7 in patients with chronic fatigue syndrome.* Intervirology, 1995. **38**(5): p. 269-73.
208. Ablashi, D.V., et al., *Frequent HHV-6 reactivation in multiple sclerosis (MS) and chronic fatigue syndrome (CFS) patients.* Journal of Clinical Virology, 2000. **16**(3): p. 179-191.
209. Enbom, M., A. Linde, and B. Evengård, *No Evidence of Active Infection with Human Herpesvirus 6 (HHV-6) or HHV-8 in Chronic Fatigue Syndrome.* Journal of Clinical Microbiology, 2000. **38**(6): p. 2457.
210. Wallace, H.n., et al., *Human herpesviruses in chronic fatigue syndrome.* Clinical and Diagnostic laboratory immunology, 1999. **6**(2): p. 216-23.
211. Reeves, W.C., et al., *Human Herpesviruses 6 and 7 in Chronic Fatigue Syndrome: A Case-Control Study.* Clinical Infectious Diseases, 2000. **31**(1): p. 48-52.
212. Koelle, D.M., et al., *Markers of Viral Infection in Monozygotic Twins Discordant for Chronic Fatigue Syndrome.* Clinical Infectious Diseases, 2002. **35**(5): p. 518-525.
213. Cameron, B., et al., *Serological and virological investigation of the role of the herpesviruses EBV, CMV and HHV-6 in post-infective fatigue syndrome.* Journal of Medical Virology, 2010. **82**(10): p. 1684-1688.
214. Buchwald, D., et al., *Viral serologies in patients with chronic fatigue and chronic fatigue syndrome.* Journal of Medical Virology, 1996. **50**(1): p. 25-30.
215. Caselli, E. and D. Di Luca, *Molecular biology and clinical associations of Roseoloviruses human herpesvirus 6 and human herpesvirus 7.* The New Microbiologica, 2007. **30**(3): p. 173-87.
216. Frenkel, N., et al., *Isolation of a new herpesvirus from human CD4+ T cells.* Proceedings of the National Academy of Sciences, 1990. **87**(2): p. 748-752.
217. Lusso, P., et al., *CD4 is a critical component of the receptor for human herpesvirus 7: Interference with human immunodeficiency virus.* Proc Natl Acad Sci USA, 1994. **91**(9): p. 3872-3876.
218. Kempf, W., et al., *Persistence of Human Herpesvirus 7 in Normal Tissues Detected by Expression of a Structural Antigen.* Journal of Infectious Diseases, 1998. **178**(3): p. 841-845.
219. Emery, V.C. and D.A. Clark, *HHV-6A, 6B, and 7: persistence in the population, epidemiology and transmission*, in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*, A. Arvin, et al., Editors. 2007, Cambridge University Press: Cambridge.
220. Wyatt, L.S. and N. Frenkel, *Human herpesvirus 7 is a constitutive inhabitant of adult human saliva.* Journal of Virology, 1992. **66**(5): p. 3206-3209.
221. Black, J.B., et al., *Frequent isolation of human herpesvirus 7 from saliva.* Virus Research, 1993. **29**(1): p. 91-98.
222. Di Luca, D., et al., *Human herpesviruses 6 and 7 in salivary glands and shedding in saliva of healthy and human immunodeficiency virus positive individuals.* Journal of Medical Virology, 1995. **45**(4): p. 462-468.

223. Miyake, F., et al., *Latent infection of human herpesvirus 7 in CD4+ T lymphocytes.* Journal of Medical Virology, 2006. **78**(1): p. 112-116.
224. Katsafanas, G.C., et al., *In vitro activation of human herpesviruses 6 and 7 from latency.* Proceedings of the National Academy of Sciences, 1996. **93**(18): p. 9788-9792.
225. Tanaka, K., et al., *Human herpesvirus 7: Another causal agent for roseola (exanthem subitum).* The Journal of Pediatrics, 1994. **125**(1): p. 1-5.
226. Drago, F., et al., *Human herpesvirus 7 in patients with pityriasis rosea. Electron microscopy investigations and polymerase chain reaction in mononuclear cells, plasma and skin.* Dermatology, 1997. **195**(4): p. 374-8.
227. Drago, F., et al., *Human herpesvirus 7 in pityriasis rosea.* The Lancet, 1997. **349**(9062): p. 1367-1368.
228. Chuh, A.A.T., H.H.L. Chan, and V. Zawar, *Is human herpesvirus 7 the causative agent of pityriasis rosea? – a critical review.* International Journal of Dermatology, 2004. **43**(12): p. 870-875.
229. Kumar, D., *Emerging viruses in transplantation.* Current Opinion in Infectious Diseases, 2010. **23**(4): p. 374-378 10.1097/QCO.0b013e32833bc19d.
230. Secchiero, P., et al., *Biological and Molecular Characteristics of Human Herpesvirus 7: In Vitro Growth Optimization and Development of a Syncytia Inhibition Test.* Virology, 1994. **202**(1): p. 506-512.
231. Chapenko, S., et al., *Association of Active Human Herpesvirus-6, -7 and Parvovirus B19 Infection with Clinical Outcomes in Patients with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome.* Advances in Virology, 2012. **2012**: p. 7.
232. Yoshida, M., et al., *Comparison of antiviral compounds against human herpesvirus 6 and 7.* Antiviral Research, 1998. **40**(1–2): p. 73-84.
233. De Clercq, E., et al., *Antiviral agents active against human herpesviruses HHV-6, HHV-7 and HHV-8.* Reviews in Medical Virology, 2001. **11**(6): p. 381-395.
234. Humar, A., et al., *An Assessment of Herpesvirus Co-infections in Patients with CMV Disease: Correlation with Clinical and Virologic Outcomes.* American Journal of Transplantation, 2009. **9**(2): p. 374-381.
235. Razonable, R.R., D.M. Zerr, and A.S.T.I.D.C.o.P. the, *HHV-6, HHV-7 and HHV-8 in Solid Organ Transplant Recipients.* American Journal of Transplantation, 2009. **9**: p. S97-S100.
236. Ryan, F.P., *Human endogenous retroviruses in health and disease: a symbiotic perspective.* JRSM, 2004. **97**(12): p. 560-565.
237. Stoye, J.P., *Studies of endogenous retroviruses reveal a continuing evolutionary saga.* Nat Rev Micro, 2012. **10**(6): p. 395-406.
238. Sutkowski, N., et al., *An Epstein-Barr Virus-associated Superantigen.* The Journal of Experimental Medicine, 1996. **184**(3): p. 971-980.
239. Vouloumanou, E.K., P.I. Rafailidis, and M.E. Falagas, *Current diagnosis and management of infectious mononucleosis.* Current Opinion in Hematology, 2012. **19**(1): p. 14-20 10.1097/MOH.0b013e32834daa08.
240. Herman, A., et al., *SUPERANTIGENS: Mechanism of T-Cell Stimulation and Role in Immune Responses.* Annual Review of Immunology, 1991. **9**: p. 745-72.
241. Huber, B.T., P.N. Hsu, and N. Sutkowski, *Virus-encoded superantigens.* Microbiological Reviews, 1996. **60**(3): p. 473-82.
242. Tai, A. and B. Huber, *Viral superantigens in mouse and man*, in *Superantigens: molecular basis for their role in human diseases*, M. Koth and J. Fraser, Editors. 2008. p. 59-76.
243. Sutkowski, N., et al., *Epstein-Barr Virus Transactivates the Human Endogenous Retrovirus HERV-K18 that Encodes a Superantigen.* Immunity, 2001. **15**(4): p. 579-589.

244. Hasuike, S., et al., *Isolation and localization of an IDDMK1,2-22-related human endogenous retroviral gene, and identification of a CA repeat marker at its locus.* J Hum Genet, 1999. **44**(5): p. 343-347.
245. Thorley-Lawson, D.A., et al., *Epstein-Barr virus superinduces a new human b cell differentiation antigen (B-LAST 1) expressed on transformed lymphoblasts.* Cell, 1982. **30**(2): p. 415-425.
246. Conrad, B., et al., *A Human Endogenous Retroviral Superantigen as Candidate Autoimmune Gene in Type I Diabetes.* Cell, 1997. **90**(2): p. 303-313.
247. Stauffer, Y., et al., *Interferon-α-Induced Endogenous Superantigen: A Model Linking Environment and Autoimmunity.* Immunity, 2001. **15**(4): p. 591-601.
248. Sicat, J., N. Sutkowski, and B.T. Huber, *Expression of human endogenous retrovirus HERV-K18 superantigen is elevated in juvenile rheumatoid arthritis.* The Journal of Rheumatology, 2005. **32**(9): p. 1821-1831.
249. Marguerat, S., et al., *Association of Human Endogenous Retrovirus K-18 Polymorphisms With Type 1 Diabetes.* Diabetes, 2004. **53**(3): p. 852-854.
250. Ramos-Lopez, E., et al., *Neither an intronic CA repeat within the CD48 gene nor the HERV-K18 polymorphisms are associated with type 1 diabetes.* Tissue Antigens, 2006. **68**(2): p. 147-152.
251. Tai, A., et al., *Human endogenous retrovirus-K18 Env as a risk factor in multiple sclerosis.* Multiple Sclerosis, 2008. **14**(9): p. 1175-1180.
252. Afari, N. and D. Buchwald, *Chronic Fatigue Syndrome: A Review.* American Journal of Psychiatry, 2003. **160**(2): p. 221-236.
253. Sutkowski, N., et al., *Epstein-Barr Virus Latent Membrane Protein LMP-2A Is Sufficient for Transactivation of the Human Endogenous Retrovirus HERV-K18 Superantigen.* Journal of Virology, 2004. **78**(14): p. 7852-7860.
254. Tai, A.K., et al., *HHV-6A infection induces expression of HERV-K18-encoded superantigen.* Journal of Clinical Virology, 2009. **46**(1): p. 47-48.
255. Turcanova, V.L., B. Bundgaard, and P. Höllsberg, *Human herpesvirus-6B induces expression of the human endogenous retrovirus K18-encoded superantigen.* Journal of Clinical Virology, 2009. **46**(1): p. 15-19.
256. Hadinoto, V., et al., *The Dynamics of EBV Shedding Implicate a Central Role for Epithelial Cells in Amplifying Viral Output.* PLoS Pathog, 2009. **5**(7): p. e1000496.
257. Schlaberg, R., et al., *XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors.* Proc Natl Acad Sci USA, 2009. **106**: p. 16351 - 16356.
258. Hsiao, F.C., et al., *Cutting Edge: Epstein-Barr Virus Transactivates the HERV-K18 Superantigen by Docking to the Human Complement Receptor 2 (CD21) on Primary B Cells.* The Journal of Immunology, 2006. **177**(4): p. 2056-2060.
259. Karlsson, T., et al., *Development of a new quantitative real-time HHV-6-PCR and monitoring of HHV-6 DNAaemia after liver transplantation.* Journal of Virological Methods, 2012. **181**(1): p. 25-36.
260. Lou, J., et al., *Subtype-specific, probe-based, real-time PCR for detection and typing of human herpesvirus-6 encephalitis from pediatric patients under the age of 2 years.* Diagnostic Microbiology and Infectious Disease, 2011. **70**(2): p. 223-229.
261. Fernandez, C., et al., *Quantitation of HHV-7 genome by real-time polymerase chain reaction assay using MGB probe technology.* Journal of Virological Methods, 2002. **106**(1): p. 11-16.
262. Qiu, X., et al., *Characterization of antibodies elicited by XMRV infection and development of immunoassays useful for epidemiologic studies.* Retrovirology, 2010. **7**(1): p. 68.

263. Chalder, T., et al., *Development of a fatigue scale.* Journal of Psychosomatic Research, 1993. **37**(2): p. 147-53.
264. Jason, L., et al., *A screening scale for chronic fatigue syndrome: Reliability and validity.* Journal of Chronic Fatigue Syndrome, 1997. **3**: p. 39-59.
265. Taylor, R.R., *Quality of Life and Symptom Severity for Individuals With Chronic Fatigue Syndrome: Findings From a Randomized Clinical Trial.* The American Journal of Occupational Therapy, 2004. **58**(1): p. 35-43.
266. Cohen, S., T. Kamarck, and R. Mermelstein, *A Global Measure of Perceived Stress.* Journal of Health and Social Behavior, 1983. **24**(4): p. 385-96.
267. Goldberg, D., *Manual of the General Health Questionnaire*. 1978, NFER Publishing: Windsor, England.
268. Frankel, W., et al., *A linkage map of endogenous murine leukemia proviruses.* Genetics, 1990. **124**: p. 221 - 236.
269. Jern, P., J. Stoye, and J. Coffin, *Role of APOBEC3 in genetic diversity among endogenous murine leukemia viruses.* PLoS Genet, 2007. **3**: p. 2014 - 2022.
270. Tomonaga, K. and J. Coffin, *Structures of endogenous nonecotropic murine leukemia virus (MLV) long terminal repeats in wild mice: implication for evolution of MLVs.* J Virol, 1999. **73**: p. 4327 - 4340.
271. Lo, S., et al., *Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors.* Proc Natl Acad Sci USA, 2010. **107**(36): p. 15874 - 15879.
272. Huber, B.T. *EBV and IFN-a activation of the human endogenous retrovirus HERV-K18 and CFS*. in *The 6th International Conference on HHV-6 & 7*. 2008. Baltimore, Maryland, USA.
273. Hickie, I., et al., *Post-infective and chronic fatigue syndromes precipitated by viral and non-viral pathogens: prospective cohort study.* BMJ, 2006. **333**(7568): p. 575.
274. Galbraith, S., et al., *Peripheral blood gene expression in postinfective fatigue syndrome following from three different triggering infections.* J Infect Dis, 2011. **204**(10): p. 1632-40.
275. Gow, J., et al., *A gene signature for post-infectious chronic fatigue syndrome.* BMC Med Genomics, 2009. **2**(38).