

NO-CFS, stage one, regarding human gammaretrovirus



**THE NORWEGIAN STUDY OF CFS,
NO-CFS, Stage 1:
Confirmatory study
for the detection of
Gamma-retrovirus related
Gene Sequences.**

BACKGROUND

Chronic fatigue syndrome

Chronic fatigue syndrome (CFS) was first defined in 1988, with a revised criteria in 1994 (ref 1) and 2003 (ref 2). The disease is defined through clinical characterizations. The major symptoms as defined by the last criteria are exhaustion, often post-exertion, pains and sleep disorders. Other neuroendocrine, autonomic and immune manifestations are also imperative.

The prevalence is not clear, however one has assumed 0,3% and 3 times as many women as men (ref 3).

Subgroup of extremely disabled patients

Of these patients a small amount have an extremely disabling variety where they are bed-bound, needing extensive amounts of help day and night. These patients are often extremely light and sound sensitive and extremely sensitive to the slightest amount of stress. Some of these patients have extreme pain; others have sleeping disorders so extreme that they over years never seem to find relief in sleep. Some patients are tube-fed, as they cannot swallow their own food, or need to be fed by caretakers. In extreme cases, patients have been lying in bed in the same position for years without bearing to be washed or cared for, as this would produce unbearable amounts of pain.

These patients are often given up on by their local GP. Most have been through quite extensive diagnostics without any certain pathological findings. Some have been classified as psychiatric diseases.

A few of these patients do over time recover to a certain degree, but it is very seldom a patient returns to normal life after being so sick. Many barely get out of bed, but function to a better degree. However some patients are bed-ridden for years and years without any sign of any recovery whatsoever. These patients are often more or less over-looked by health authorities and often no physician has visited them for years. They are often, by family and caretakers, kept at home as they are too sick to be able to cope with hospital stressors, giving a huge amount of stress to family who looks after them, often for years.

In Norway there is no certainty to the number of bed-bound disabled ME/CFS patients, however, we believe to find 50-100 all over Norway with 20-40 others having recovered to a certain degree.

Etiology

The etiology to this mysterious disease is still unknown. There are different hypotheses including pathogenetic mechanisms within immunology, genetics, virology, endocrinology and even psychiatry (ref 4-9). A multifactor etiology has been considered, including both host and environmental factors. There is a need for understanding etiology and pathogenesis, so one can develop biomarkers and diagnostic tests. This will lead to specific therapeutic revenues.

One of the main arguments concerning etiology of ME/CFS is immune deregulation, often after an external stimulus, often a viral infection (ref 10-18). In 2009 two

researchers at Haukeland Sykehus, Bergen Norway, published a pilot study on 3 ME/CFS patients treated with rituximab, a B cell depletory (ref 19). On this background they have a hypothesis concerning ME/CFS being an immunological disease where especially the B cells are involved in the pathogenesis. This can mean there is a certain autoimmune process going on in these patients, however an alternative theory is be that the B-cells are directly involved in a retroviral infection.

In October 2009 a study was published showing the presence of a novel retrovirus, XMRV (ref 20). Later studies could not reproduce these findings (ref 21-22). New publications are supporting the findings (ref 23), however, the need for a specific PRC analysis for accurate diagnostics seems imperative at this stage.

Hypothesis

Patients with severe, disabling ME/CFS have an ongoing MLV-related virus infection that is the main reason for their disease. Close relatives may or may not also have a latent retroviral infection. We want to look further into differences between extremely disabled patients, healthy relatives, and healthy controls in retroviral prevalence.

We will also look into markers for immune dysfunction including cytokine profiles, and also antibody detection. We believe it is possible to find biomarkers that can be used to follow the disease.

We believe it is possible that the retroviral reservoir is the B cells. This can explain the positive effect of rituximab. Mature B cells expressing the CD20 will then have to carry the major retroviral load. However, as immature B cells emerge from stem cells in the bone marrow and plasma cells, the increasing retroviral load contributes to the increasing symptoms reappearing as the effect of rituximab lessens over time.

Importance of this study

As the study will be multi-centered with a completely independent cohort of patients, the findings in two different research centers will be truly relevant and extremely important for later studies.

For the pharmaceutical industry to take interest in bigger clinical trials with relevant retroviral medication, we have to prove prevalence and disease association with the virus. If this can be proven by this study, studies on relevant anti-retroviral will be started as soon as possible. As the disabled patients have no hope of therapeutic intervention at this point, this study is of great importance to them.

The diagnostic procedure developed through this study will be vital for ALL patients diagnosed with ME/CFS.

We believe a new disease definition will need to be looked into as we map out prevalence of MLV related viruses and disease association in these patients.

AIMS

Primary aim:

See if the Murine Leukemia Viruses (MLV) viruses related gene sequences can be found in extremely disabled patients diagnosed with ME/CFS in Norway.

Secondary aims:

Look for other characteristics in retroviral cytotoxicity.

Look into disease association and prevalence in healthy relatives and healthy controls.

Look for the major viral load in live PBMC.

Detect antibodies.

Look for characteristic cytokine profiles and develop biomarkers for monitoring disease activity.

DESIGN AND METHODS

Overview

A total of 30 individuals, who fit the criteria of the Canadian Consensus Criteria of 2003, will go through extensive blood work. All blood will be drawn in one setting and prepared for both research centers and blood bank.

Patients will be recruited through Lillestrom Helseklinikk. We want to include only patients who are or have been extremely disabled, that is Karnofsky score 40 and below.

Recruitment, inclusion, exclusion

The disabled

Lillestrom Helseklinikk treats a total of 600 patients every month. Mostly patients with exhaustion and pain disorders, come for treatment, however patients with chronic gastrointestinal disorders and other chronic disorders as psoriasis and arthritis are also seen.

The clinic has over the years had contact with many disabled patients with ME/CFS. We also have an extensive contact network that will be used to contact these patients.

For inclusion and exclusion, see table 1. We will strive for a correct diagnostic inclusion of patients so that only patients fulfilling the Canadian Consensus Criteria of 2003 are included.

Only patients that are or have been extremely disabled (Karnofsky score (KS) of 40 or below) are included. Exclusion criteria are secondary disorders including secondary bacterial and or viral infections, and other autoimmune disorders. Ongoing antiviral

therapy will also lead to exclusion. This is to rule out any situation that might make the diagnostics and PCR development more difficult. For this study we will not set any age limits.

The controls

We will include 2 sets of controls, 15 in each group, totaling 30 all in all. Both have to be completely healthy. One group will be in close contact with the disabled and/or close family member, the other will be without any contact at all with disabled or chronically ill patients without a family history of chronic disease.

Family/close contact control (FC):

One healthy relative will be included in the project. This individual will have to fill a certain set of criteria. We will primarily be looking for healthy children over the age of 12 or adults as closest relatives (that is parents or siblings), with no ongoing relevant medication, and no antibiotics the last 4 weeks before the trial. There has to be no sign of autoimmune disease. We will not exclude low-grade allergies that are under control nor any cardiovascular disease that is under control. Should the criteria not be met by any close relative, we will strive to find controls who are in contact with the patient but completely healthy.

Non-family/close contact control (nFC):

We will include healthy controls that have all criteria for good health (see over) including no contact with anyone with a chronic disease and also no chronic diseases in the family.

	Inclusion criteria	Exclusion criteria
CFS/ME patients	Canadian Consensus criteria of 2003, see below	Exclusion criteria as seen in the Canadian consensus criteria of 2003
	Karnofsky score 40 or below for 1 year or more. There is no age limit.	Secondary/parallel disorders as infections and autoimmune disease. Use of antiviral medication, antibiotics and or any kind of immune modulating therapy the last 4 weeks.

Table 2. Healthy controls: criteria for inclusion and exclusion		
	Inclusion criteria	Exclusion criteria
Healthy control, FC:	18 years or older or paired with the disabled.	Any kind of chronic disease that is disabling in everyday life.
	Closest of relative, that is sibling or parent.	Use of antiviral medication and/or antibiotics the last 4 weeks.
Healthy control, nFC:	18 years or older or paired with the disabled.	Any kind of chronic disease that is disabling in everyday life.
		Use of antiviral medication and/or antibiotics the last 4 weeks.
		More than 2 in close family history with cancer, CVD, autoimmune disease or any other relevant chronic disease
		No close contact with any person with chronic disease.

Overview Canadian Consensus Criteria of 2003, see:
http://www.mefmaction.net/documents/ME_Overview.pdf

Long version: <http://www.mefmaction.net/Portals/0/docs//ME-CFS-Consensus-Document.pdf>

POWER CALCULATION

The last study on ME/CFS found MLV related viruses in 86,5% of the patients and 6,8% in normal controls. This is quite close to recent findings at the Whittemore-Peterson-Institute (direct communication with Judy Mikovits).

With a positive test we do mean positive PCR and positive cell culture. Serology can be negative in cell culture. Should we find many positive serology tests and negative PCR or cell culture, we will have to discuss this. As of today there seems to be an understanding that either the serology or the cell culture tests are positive, usually not both.

We will assume that of the 30 disabled, 60% have positive tests for MLV related viruses. From the control group one might imagine between 4% (reflecting the study in ref 20) and 7% (reflecting the study in ref 23). However, by direct communication with Mikovits, we are told there is a significantly higher level of positives in family members and people in close contact with ME/CFS patients. For this reason we stipulate a higher degree of positives in the family/contact control group, a total of 25% positive tests for MLV related viruses in this whole group.

With 30 subjects per group the study will have power of 80%. This means that there is an 80% likelihood that the study will yield a statistically significant effect, and allow us

to conclude that the percentage of subjects in "MLV positive" differs for disabled versus controls.

However, as our cohort consists of patients with a greater disability than earlier cohorts, it is possible that the amount of positives will be higher than 60%. In that case, the statistical power will be greater than 80%.

The computation of sample size is based on the following assumptions and decisions.

The expected pattern of responses for the disabled is as follows: MLV positive 60%, MLV negative 40%.

The expected pattern for responses for controls is as follows: MLV positive 25%, MLV negative 75%.

In computing the sample size we assume that there will be no data missing.

Conclusion:

The study will enroll 30 people per group for a total of 60 people. With this sample size there is an 80% likelihood that the study will yield a statistically significant result, and allows us to conclude that the percentage of subjects in "MLV positive" is different for the disabled than for controls.

PRACTICALITIES

We will be sending the following blood samples (per participant):

Blood sampling

Blood sampling will be performed through Lillestrom Helseklinikk where that is possible. As many patients live far away and are extremely disabled, some blood testing will have to be performed through the patients GP. Lillestrom Helseklinikk will have to receive the blood samples within 6 (8) hours for preparation. Detailed written information will be sent out to ensure correct samples.

1. To the WPI Institute by Judy Mikovits:

- 2 plasma aliquots of 1 ml each (frozen to minus 80 degrees)
- 1 tube viable PBMC 5-10 mill cells (sendt within 4 weeks frozen to minus 80 degrees)
- 1 plasma aliquot processed from refrigerated blood, 1ml (frozen to minus 80 degrees)

2. To The San Raffaele Scientific Institute by Mauro Malnati:

- 3 plasma aliquots of 1 ml (frozen to minus 80 degrees)
- 2 PBMC Dry pellets 1-2 mill cells each (frozen to minus 80 degrees)
- 1 tube viable PBMC 5-10 mill cells (frozen to minus 80 degrees)
- 2 aliquots of snap frozen all blood (200ul each) taken before plasma separation (frozen to minus 80 degrees)

- 1 plasma aliquot processed from refrigerated blood, 1ml (frozen to minus 80 degrees)

All blood samples will be prepared in cooperation with the Department of Pediatric Research, Laboratory, Rikshospitalet, Oslo, within a time frame of 4 weeks.

Other blood samples:

We will also include following blood samples performed at Akershus Universitetets Sykehus:

Hb, Lkc diff, IgA IgG IgM total, IgG subclasses.

All blood samples will be prepared in cooperation with the Department of Pediatric Research, Laboratory, Rikshospitalet, Oslo, within a time frame of 4 weeks.

Relevant questionnaires:

1. Symptom assessments:
 - Karnofsky score
 - Canadian consensus criteria
 - Symptom assessment
2. Assessment relevant to etiology:
 - The start of symptoms/length of disability
 - Any relevant disorders before they became disabled
 - Any traveling earlier (Asia, Africa, south Europe, South America and North America, special attention to any disease acquired on these travels)
 - Genetics, how many relatives have autoimmune/chronic diseases
 - How many children and whether or not they have following disease: allergies, digestion problems, ADHD, ADD, other chronic diseases.
 - Degree of self-assessed stress earlier on in life.
 - Vaccines
 - Any other relevant information that can explain any trigger, or lack of trigger.

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Prof. Dr. Judy A. Mikovits, Whittemore-Peterson Institute, Reno, USA:

Institute: Whittemore-Peterson Institute (WPI), Director of Research and former staff member NCI.

Profile: PhD in Biochemistry and Molecular Biology, formally trained as a cell biologist, molecular biologist and virologist: Dr. Mikovits spent more than 20 years at the National Cancer Institute in Frederick MD during which time she received her PhD in Biochemistry and Molecular Biology, investigating mechanisms by which retroviruses dysregulate the delicate balance of cytokines in the immune response. This work led to the discovery of the role aberrant DNA methylation plays in the pathogenesis of HIV. Later in her career at the NCI, Dr. Mikovits directed the Lab of Antiviral Drug Mechanisms (LADM) - a section of the NCI's Screening Technologies Branch in the Developmental Therapeutics Program. The LADM's mission was to identify, characterize and validate molecular targets and to develop high-throughput cell-based, genomic and epigenomic screens for the development of novel therapeutic agents for AIDS and AIDS-associated malignancies (Kaposi's sarcoma).

Dr. Mikovits has studied the immune response to retroviruses and herpes viruses including HIV, SIV, HTLVI, HERV, HHV6 and HHV8 with a special emphasis on virus host cell interactions in cells of the hematopoietic system including hematopoietic stem cells (HSC). Dr. Mikovits' commercial experience includes serving as a senior scientist and group leader at Biosource International, where she led the development of proteomic assays for the Luminex platform that is used extensively for cytokine activity assessment in therapy development. She also served as Chief Scientific Officer and VP of Drug Discovery at Epigenx Biosciences, where she led the development and commercialization of cell and array-based methylation assays for drug discovery and diagnostic development. Dr. Mikovits has co-authored more than 40 peer-reviewed publications that address fundamental issues of viral pathogenesis, hematopoiesis and cytokine biology.

Expertise: Specialist in XMRV virus, cancer, retrovirology, virology, molecular and cell biology

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Dr. Med. Mauro Malnati (colleague of Prof. Dr. Paulo Lusso), Milan, Italy

Institute: San Raffaele Scientific Institute, Milan, Italy. In 2001, the San Raffaele Institute was acknowledged by the Health Minister as the IRCCS for Molecular Medicine.

Profile: Doktor, researcher and group leader In Human Virology. He is also a top expert in the design and validation of molecular diagnostic assays for human viruses based on TaqMan real-time PCR. Deputy Chief of Human Virology at the DIBIT - S. Raffaele Scientific Institute.

Dr. Mauro Malnati is designing a calibrated Real-Time PCR assay based on the combination of TaqMan PCR and "calibrator" technologies. The TaqMan technology is the most reliable and widely tested technology developed for Real-Time PCR as it displays an outstanding degree of specificity, accuracy, as well as cost and time efficiency. The "calibrator" technology, developed and patented in Dr. Malnati's laboratory, is standardized for the detection of false-negative results and provides absolute quantification. Because of the improved accuracy of quantification, it also decreases the degree of inter-sample variability. Finally, the calibrator technology helps to determine a cut-off value sensitivity for each negative sample which would provide clinicians with a more accurate interpretation of the negative result and thus a more complete patient evaluation. Dr. Malnati intends to test various HHV-6A and B isolates to see that results are consistent. The isolates will be provided through the Foundation repository

About Malnati and the PCR test for retrovirus aids:
<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC516357/>

Expertise: PCR expert especially specialist in human virology testing/retrovirus HIV. Advise and develop new PCR technique for the XMRV virus in coop with Dr. Mikovits.

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