DIAGNOSI CFS/ME
RIFERIMENTI BIBLIOGRAFICI

RNase-L

Englebienne P, Herst CV, De Smet K, *et al.*
Interactions between RNase L Ankyrin-Like Domain and ABC Transporters as a Possible Origin for Pain, Ion Transport, CNS and Immune Disorders of Chronic Fatigue Immune Dysfunction Syndrome.
J Chronic Fatigue Syndrome 2001;8:83-102.

E Demettre, L Bastide, A D'Haese et al.
Ribonuclease L Proteolysis in Peripheral Blood Mononuclear Cells of Chronic Fatigue Syndrome Patients
The Journal of Biological Chemistry, Vol. 277, No. 38, Issue of September 20, pp. 35746–35751, 2002
Abstract: A 37-kDa binding polypeptide accumulates in peripheral blood mononuclear cell (PBMC) extracts from chronic fatigue syndrome (CFS) patients and is being considered as a potential diagnostic marker (De Meirleir, K., Bisbal, C., Campine, I., De Becker, P., Salehzada, T., Demettre, E., and Lebleu, B. (2000) *Am. J. Med.* 108, 99–105). We establish here that this low molecular weight 2-5A-binding polypeptide is a truncated form of the native 2-5A-dependent ribonuclease L (RNase L), generated by an increased proteolytic activity in CFS PBMC extracts. RNase L proteolysis in CFS PBMC extracts can be mimicked in a model system in which recombinant RNase L is treated with human leukocyte elastase. RNase L proteolysis leads to the accumulation of two major fragments with molecular masses of 37 and 30 kDa. The 37-kDa fragment includes the 2-5A binding site and the N-terminal end of native RNase L. The 30-kDa fragment includes the catalytic site in the C-terminal part of RNase L. Interestingly, RNase L remains active and 2-5A-dependent when degraded into its 30- and 37-kDa fragments by proteases of CFS PBMC extract or by purified human leukocyte elastase. The 2-5A-dependent nuclease activity of the truncated RNase L could result from the association of these digestion products, as suggested in pull down experiments.

M Fremont, K El Bakkouri et al.
2V,5V-Oligoadenylate size is critical to protect RNase L against proteolytic cleavage in chronic fatigue syndrome
Experimental and Molecular Pathology 78 (2005) 239– 246
Abstract: A dysregulation in the 2V,5V-oligoadenylate (2-5A)-dependent RNase L antiviral pathway has been detected in peripheral blood mononuclear cells (PBMC) of chronic fatigue syndrome (CFS) patients, which is characterized by upregulated 2-5A synthetase and RNase L activities, as well as by the presence of a low molecular weight (LMW) 2-5A-binding protein of 37-kDa related to RNase L. This truncated protein has been shown to originate from proteolytic cleavage of the native 83-kDa RNase L by m-calpain and human leukocyte elastase (HLE). We investigated the possible role of 2-5A oligomers in the proteolytic action toward the endonuclease and show that incubation of CFS PBMC extracts with 2-5A trimer and tetramer, but not with the dimer, results in a significant protection of the native 83-kDa RNase L against cleavage by endogenous and purified proteases. Similar results are obtained with a purified recombinant RNase L. An analysis of the size of 2-5A oligomers produced by the catalytic activity of the 2-5A synthetase present in PBMC extracts further shows that samples containing the 37-kDa RNase L preferentially produce 2-5A dimers instead of higher oligomers. Taken together, our results indicate that homodimerization of RNase L by 2-5A oligomers higher than the dimer prevents its cleavage by proteolytic enzymes. The presence of the truncated 37-kDa RNase L in PBMC extracts is therefore likely to result, not only from the abnormal activation of inflammatory proteases, but also from a dysregulation in 2-5A synthetase induction or activation towards the preferential production of 2-5A dimers.

J Nijs, K De Meirleir
Impairments of the 2-5A Synthetase/RNase L Pathway in Chronic Fatigue Syndrome
Anticancer Research 25: 1013-1022 (2005)
Abstract. This paper provides an overview of the evidence addressing the impairments of the 2'-5' oligoadenylate (2-5

A) synthetase/RNase L pathway in Chronic Fatigue Syndrome (CFS) patients. The 2-5A synthetase/RNase L pathway in CFS patients appears to be both up-regulated (i.e. increased levels of bioactive 2-5A synthetase and increased activity of the RNase L enzyme) and deregulated (elastase and calpain initiate 83 kDa RNase L proteolysis, generating two major fragments with molecular masses of 37 and 30 kDa, respectively). The deregulation of the 2-5A synthetase/RNase L pathway in CFS accompanies decreased NK-function and deregulation of apoptotic pathways. Since various components of the pathway appear to be related to performance during a graded exercise stress test, some evidence supportive of the clinical importance of the impaired pathway in CFS patients has been provided. Studies addressing the treatment of the deregulation of the 2-5A synthetase/RNase L pathway in CFS are warranted.

J Nijs, M Fremont

Intracellular immune dysfunction in myalgic encephalomyelitis/chronic fatigue syndrome: state of the art and therapeutic implications.

encephalomyelitis (ME)/chronic fatigue syndrome (CFS) is accumulating, but few studies have addressed intracellular immunity as a potential therapeutic target. OBJECTIVE: To provide an overview of our present understanding of intracellular immunity in ME/CFS, to relate the intracellular immune dysfunctions to other aspects of the illness like decreased natural killer cell function, the presence of infections and poor exercise performance, and to point to potential therapeutic targets. METHODS: An in-depth review of the scientific literature of intracellular immunity in people with ME/CFS was performed. RESULTS/CONCLUSION: From the scientific literature it is concluded that proteolytic cleavage of the native RNase L enzyme is characteristic of the dysregulation of intracellular immunity in people with ME/CFS, but the origin of the dysregulation is speculative. There is increasing evidence for immune cell apoptosis and upregulation of various aspects of the 2'-5' oligoadenylate (2-5A) synthetase/RNase L pathway in ME/CFS. This review provides the theoretical rationale for conducting studies examining the effectiveness of direct or indirect drug targeting of the 2-5A synthetase/RNase L pathway in ME/CFS patients.

NAGALASE

Paul R. Cheney MD, PhD

Nagalase Activity is Inversely Correlated with CFS Clinical Status (KPS)

IACFS/ME – Biennial International Conference : Translating Evidence into Practice; Ottawa, Ontario, Canada, Sept. 22-25, 2011

INTRODUCTION: The sera of patients with HIV and cancer possess alpha-N-acetylgalatosidase enzyme activity known as Nagalase activity. This glycosidase activity appears to reside in the proteolytically cleaved gp160 envelope protein of HIV and also found in the similarly cleaved envelope protein of influenza virus and possibly possessed by other classes of virus that probably induces viral virulence as this glycosidase activity appears to be important in both cellular entry by virus-to-membrane fusion and immunosuppression. Nagalase activity specifically destroys the Gc protein (VDBP) precursor capacity for GcMAF activity leading to direct immunosuppression. Cancer cells also secret Nagalase activity that may come from genomic activation of HERV's within these cells or other viruses active within such cancer cells. In metastatic breast cancer, Nagalase activity correlates with tumor burden and Nagalase values dropping to control ranges near zero correlate with eradication of tumor cells resulting in a prolonged cancer free state lasting years. Nagalase has also correlated even better than CD4 counts for clinical status of HIV patients. METHODS: We measured Nagalase activity in 50 consecutive CFS cases with an average age of 47.7 years. There were 20 males and 30 females from a national CFS referral center meeting the 1994 CDC CFS case definition. Serum Nagalase activity was measured in nmoles/min/mg protein by a commercial laboratory (ELN Labs, NJ). The idea to measure Nagalase was suggested by the detection of XMRV in the great majority of patients in this national practice (> 75% positive for XMRV with one measurement and > 95% if measured more than once at a CLIA certified laboratory, VIP Dx, NV). All patients during their office visits at this clinic are routinely given a physician assigned functional capacity score known as the Karnofsky Performance Score or KPS which has been well validated in both CFS and other chronic diseases. This clinic has had two decades of experience using this physician applied functional score including FDA sponsored clinical trials. Patients were sent kits for Nagalase testing and then assigned a KPS score in their charts. Inter-assay measures of KPS typically can vary plus or minus 5 KPS units over time by chance alone in CFS unless there is a significant shift in clinical status which usually occurs slowly over time. KPS is not a symptom score and expresses what the patient can and cannot do with respect to activities of daily living. RESULTS: The Nagalase activity of 50 consecutive CFS cases reported here averaged 3.0 nmoles/min/mg protein, range 0.8 – 6.7. The Nagalase mean of CFS cases is comparable to HIV and comparable to breast cancer in respect to both mean and range. Average KPS was 59, range 40-90. Correlation statistics were developed for Nagalase vs. KPS. KPS was found to be negatively correlated with an r-square of 0.3, $p < 0.00005$, $N = 50$. The only two CFS cases with a KPS > 80 were at control values for Nagalase and one was our best responder to GcMAF (see GcMAF Abstract). XMRV detection rate in this 50 patient cohort was 77%, mostly single measures by culture and/or serology. CONCLUSION: Nagalase activity has been

previously demonstrated to be an excellent clinical status marker in HIV and cancer. This data supports the hypothesis that Nagalase activity is also a good clinical status marker for CFS. The origin of Nagalase activity in CFS remains unknown but its finding in all disabled cases to date and that it correlates with clinical status along with the finding that almost all of the same cases are XMRV positive supports the hypothesis that XMRV may be the cause or contributes to Nagalase activity in CFS.

K De Meirleir, C. Rowe, M. Fremont

Nagalase Activity is A Good Marker For ME/CFS

OBJECTIVES: The GcProtein or Vit. D binding protein is naturally transformed in the body by intervention of sialidase of T cells and betagalactosidase of B cells thus removing 2 sugars from the Gc's protein's trisaccharide group, leaving a single sugar at the threonine location. The protein is now GcMAf and can activate macrophages. Previous research has shown that the enzyme alpha-Nacetylgalactosaminidase, called Nagalase, removes the entire trisaccharide group.

Deglycosylated GcProtein cannot activate macrophages. Because ME/CFS patients often have reduced macrophage / NK cell function we hypothesized that Nagalase activity would be elevated in these patients. METHODS: Serum Nagalase activity was determined in 395 ME/CFS patients who met both the Canadian clinical criteria for ME (2003) and the Fukuda criteria for CFS (1994). The Nagalase assay was performed on serum. The blood was centrifuged within one hour of venous blood drawl and serum was frozen immediately till assayed. The assay method used is described in J. Med. Virol. 81:p.9 (2009) by Yamamoto et al. Healthy control sera exhibit very low enzyme activities. STATISTICAL ANALYSIS: A one-sided t test was used to test the hypothesis that the mean value of the ME group is significantly different from the middle of the normal range (representing the normal population). RESULTS: Average serum Nagalase activity was 1.72 nmol/min/mg (range 0.28-4.0). This is significantly higher compared to levels in normal controls (0.35-0.68 nmol/min/mg) (Yamamoto, 2009). Only 12 of 395 patients had a Nagalase activity below 0.69 nmol/min/mg, or 3 % of the study population. CONCLUSION: When tested in a large cohort of ME/CFS patients, serum Nagalase is increased in 97 % of the study population. Irrespectively of the cause of these findings, serum Nagalase activity is a good marker.

IACFS/ME – Biennial International Conference : Translating Evidence into Practice; Ottawa, Ontario, Canada, Sept. 22-25, 2011.

Nagasawa H, Sasaki H, Uto Y, Kubo S, Hori H.

Association of the macrophage activating factor (MAF) precursor activity with polymorphism in vitamin D-binding protein.

Anticancer Res. 2004 Sep-Oct;24(5C):3361-6

Abstract: BACKGROUND: Serum vitamin D-binding protein (Gc protein or DBP) is a highly expressed polymorphic protein, which is a precursor of the inflammation-primed macrophage activating factor, GcMAF, by a cascade of carbohydrate processing reactions. In order to elucidate the relationship between Gc polymorphism and GcMAF precursor activity, we estimated the phagocytic ability of three homotypes of Gc protein, Gc1F-1F, Gc1S-1S and Gc2-2, through processing of their carbohydrate moiety. MATERIALS AND METHODS: We performed Gc typing of human serum samples by isoelectric focusing (IEF). Gc protein from human serum was purified by affinity chromatography with 25-hydroxyvitamin D3-sepharose. A phagocytosis assay of Gc proteins, modified using beta-glycosidase and sialidase, was carried out. RESULTS: The Gc1F-1F phenotype was revealed to possess Galbeta1-4GalNAc linkage by the analysis of GcMAF precursor activity using beta1-4 linkage-specific galactosidase from jack bean. The GcMAF precursor activity of the Gc1F-1F phenotype was highest among three Gc homotypes. CONCLUSION: The Gc polymorphism and carbohydrate diversity of Gc protein are significant for its pleiotropic effects.

P R Cheney

Compassionate Use Treatment of CFS with GcMAF

IACFS/ME – Biennial International Conference : Translating Evidence into Practice; Ottawa, Ontario, Canada, Sept. 22-25, 2011.

INTRODUCTION: GcMAF is a partially deglycosylated vitamin D binding protein (VDBP) also known as Gc protein. The functional change in the Gc protein caused by deglycosylation is known as GcMAF. GcMAF is extremely potent and will at very low concentrations activate, regulate and expand macrophages which are the central processing unit of the immune system and capable of modulating and controlling both the innate and cognate immune systems. METHODS: Twenty-five CFS patients meeting the 1994 CDC criteria were selected from a national referral practice and under informed consent were self-treated with a semi-synthetic GcMAF administered by sub-lingual route. Previous reports from clinicians in The Netherlands using this commercial-grade version of human GcMAF suggested significant bioactivity and promising clinical responses in CFS cases in The Netherlands. Patients were monitored for blood chemistry, CBC, active and non-active forms of vitamin D as well as Nagalase activity. VDR polymorphisms determined from restriction enzyme products of BsmI and FokI were determined and a clinical instrument for symptom assessment of the seven key CFS symptoms was used to evaluate patient response. The protocol called for administering initially low doses of GcMAF at 20 ng SL every five days for the first 30 days followed by a q 5 day ramp to 100 ng SL using 20 ng increments. The study length was scheduled for 5 months but is being extended on a

case-by-case basis. RESULTS: Results are reported here for those eighteen CFS patients who have received a minimum of two months of sub-lingual GcMAF. 6/18 or 33.3% had a significant to clinically resolved response in at least two of seven critical CFS symptoms and two of those were functional cures at 80 KPS units or better. 5/18 or 27.8% failed to respond at all or even got worse over those same seven key symptoms. The remainder or 38.9% (7/18) had a mild to moderate improvement in two or more significant symptoms. A total of 72.2% (13/18) responded to GcMAF. VDR polymorphism data in 11 of 18 patients are known and the balance pending. Of these 11 with known VDR results, 3 of 4 who were non-responders were either BB or ff genotypes suggesting that if you had a BB or ff, you had a 75% chance of being a non-responder. On the other hand, there were no BB's in the best responding group of four suggesting that if you had a BB, there was no chance at all of being a significant responder or cure though there was a 33% chance of being a mild to moderate responder but only a 14% chance of being a member of the combined response group (1 chance in 7). Analysis of calcitriol levels demonstrated that there were three response patterns to GcMAF that were predictive of clinical response. All those with low to normal calcitriol to start with who then had a modest rise in calcitriol in response to GcMAF responded clinically (6/6). All those whose calcitriol was initially low to normal that did not respond at all to initial doses of GcMAF failed to respond clinically (4/4). In those with elevated initial calcitriol (>68) had a mixed response with 5 of 7 responding and 2 of 7 not responding. Initial D3 levels were variable and did not predict response nor did their response to GcMAF predict response. Nagalase activity was elevated in all study participants (average 3.4, range 1.3-6.5). 87.5% of the patients tested for XMRV were positive (14/16). In 6 of 8 with known response data, Nagalase activity declined with therapy and one patient declined to zero and is one of the recovered patients (KPS > 80). CONCLUSIONS: GcMAF appears to be a relatively benign and generally effective treatment for CFS. Most patients, however, had early though short bouts of what appeared to be exacerbations of CFS symptoms regardless of the eventual outcome. Two patients who were deemed responders developed clinical vitamin D toxicity later in their treatment heralded by a rapid rise in calcitriol above 90 as Nagalase dropped but responded very well to GcMAF dose reduction or elimination with no lasting effect on their previously good responses. Nagalase activity generally fell especially in the best responders and initial calcitriol response was predictive of outcome in most patients. VDR genomic data appears to also be a predictor of the relative chance of response vs. non-response to GcMAF.

ME/CFS AND INFECTIOUS DISEASES

J K S Kia et al.
Chronic Clamydia Pneumoniae infection : a treatable cause of chronic fatigue syndrome
Clin Infect Dis. 1999; 29: 452-453

K Ikuta, T Yamada, T Shimomura et al
Diagnostic evaluation of 2', 5'-oligoadenylate synthetase activities and antibodies against Epstein–Barr virus and *Coxiella burnetii* in patients with chronic fatigue syndrome in Japan
Microbes and Infection 5 (2003) 1096–1102
Abstract: To investigate the association of viral infections with chronic fatigue syndrome (CFS), we assayed 2', 5'-oligoadenylate synthetase (2-5AS) activities in peripheral blood mononuclear cells from CFS patients in Japan. These patients were diagnosed in two hospitals, H1 and H2, located in different areas of the country. The activities were detected in 19 (86%) and 7 (32%) of each of the 22 patients in H1 and H2, respectively, while they were detected in only four (11%) out of the 38 healthy controls. IFN-a was similarly detected in a few CFS patients and healthy controls.We also assayed the antibody titers against Epstein–Barr virus (EBV) and *Coxiella burnetii* in these patients. The EBV anti-EA-IgG antibodies were detected in two (9%) and seven (32%) of each of the 22 patients in H1 and H2, respectively.Anti-*C. burnetii* IgG antibodies were detected in six (27%) out of 22 patients in H1 but not in 22 patients in H2, while they were detected in one (11%) of the nine healthy controls. Some CFS patients may be associated with EBV or *C. burnetii* infection. There were some statistical correlations between the 2-5AS activities and antibody titers of EA-IgG ($P < 0.05$, Student's *t*-test) but not to the antibody titers of *C. burnetii*. The up-regulation of 2-5AS activities suggests immunological dysfunctions with some virus infections in the CFS patients. Our results indicate that 2-5AS activities are useful for a diagnostic marker of CFS and for exploring the complicated pathogenesis of CFS.

Nicolson GL, Gan R, Haier J.
Multiple co-infections (*Mycoplasma, Chlamydia*, human herpes virus-6) in blood of chronic fatigue syndrome patients: association with signs and symptoms.
APMIS 2003;111:557–66.

Garth L. Nicolson,1 PhD, Marwan Y. Nasralla,2 PhD, Kenny De Meirleir,3 MD, PhD,
Robert Gan,2 MB, PhD and Joerg Haier,4 MD, PhD
Evidence for Bacterial (Mycoplasma, Chlamydia) and Viral (HHV-6) Co-Infections in Chronic Fatigue Syndrome Patients

Journal of Chronic Fatigue Syndrome 2003; 11(2): 7-19.

S. Chapenko, A. Krumina, S. Kozireva et al.
Activation of human herpesviruses 6 and 7 in patients with chronic fatigue syndrome
Journal of Clinical Virology, vol. 37, supplement 1, pp. S47–S51, 2006.

J K S Kia, A Y Kia
Chronic fatigue syndrome is associated with chronic enterovirus infection of the stomach
J Clin Pathol. 2007, 10; 1-6.

J R Kerr
Enterovirus infection of the stomach in chronic fatigue syndrome/myalgic encephalomyelitis.
J Clin Pathol. 2008, 6, 1

B. Cameron, L. Flamand, H. Juwana 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, vol. 82, no. 10, pp. 1684–1688, 2010.

Martin Lerner, Safedin Beqaj
A paradigm linking herpesvirus immediate-early gene expression apoptosis and myalgic encephalomyelitis chronic fatigue syndrome
Virus Adaptation and Treatment 2011:3 19–24
Abstract: There is no accepted science to relate herpesviruses (Epstein–Barr virus [EBV], uman cytomegalovirus [HCMV], and human herpesvirus 6 [HHV6]) as causes of myalgic encephalomyelitis (ME)/chronic fatigue syndrome (CFS). ME/CFS patients have elevated serum immunoglobulin (Ig)G serum antibody titers to EBV, HCMV, and HHV6, but there is no herpesvirus DNA-emia, herpesvirus antigenemia, or uniformly elevated IgM serum antibody titers to the complete virions. We propose that herpesvirus EBV, HCMV, and HHV6 immediate-early gene expression in ME/CFS patients leads to host cell dysregulation and host cell apoptosis without lytic herpesvirus replication. Specific antiviral nucleosides, which alleviate ME/CFS, namely valacyclovir for EBV ME/CFS and valganciclovir for HCMV/HHV6 ME/CFS, inhibit herpesvirus DNA polymerases and/or thymidine kinase functions, thus inhibiting lytic virus replication. New host cell recruitment thus ceases. In the absence of new herpesvirus, nonpermissive herpesvirus replication stops, and ME/CFS recovery ensues.

S Chapenko, A Krumina, I Logina, 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, Volume 2012, Article ID 205085.
Abstract: Frequency of active human herpesvirus-6, -7 (HHV-6, HHV-7) and parvovirus B19 (B19) infection/coinfection and its association with clinical course of ME/CFS was evaluated. 108 ME/CFS patients and 90 practically healthy persons were enrolled in the study. Viral genomic sequences were detected by PCR, virus-specific antibodies and cytokine levels—by ELISA, HHV-6 variants—by restriction analysis. Active viral infection including concurrent infection was found in 64.8% (70/108) of patients and in 13.3% (12/90) of practically healthy persons. Increase in peripheral blood leukocyte DNA HHV-6 load as well as in proinflammatory cytokines' levels was detected in patients during active viral infection.Definite relationship was observed between active betaherpesvirus infection and subfebrility, lymphadenopathy and malaise after exertion, and between active B19 infection and multijoint pain. Neuropsychological disturbances were detected in all patients. The manifestation of symptoms was of more frequent occurrence in patients with concurrent infection. The high rate of active HHV-6, HHV-7 and B19 infection/coinfection with the simultaneous increase in plasma proinflammatory cytokines' level as well as the association between active viral infection and distinctive types of clinical symptoms shows necessity of simultaneous study of these viral infections for identification of possible subsets of ME/CFS.

A. S. Bansal, A. S. Bradley, K. N. Bishop, S. Kiani-Alikhan, and B. Ford
Chronic fatigue syndrome, the immune system and viral infection
Brain, Behavior, and Immunity, vol. 26, no. 1, pp. 24–31, 2012.

PERFORIN IN CFS

V R Falkenberg, T Whistler, et al.
Promoter DNA Methylation and Expression of Perforin in CFS and Controls
IACFS/ME – Biennial International Conference : Translating Evidence into Practice; Ottawa, Ontario, Canada, Sept. 22-25, 2011.
OBJECTIVES: Perforin plays a key role in immune surveillance and several studies report decreased perforin protein

and mRNA in peripheral blood of patients with chronic fatigue syndrome (CFS). Factors that modulate gene-environmental interaction and thus the pathophysiology of disease include gene silencing by DNA methylation. The objectives of this study were to determine the pattern of perforin gene methylation in conjunction with perforin gene expression and whether these features were altered in CFS. METHODS: Subjects (34 CFS and 47 non-fatigued, NF) selected from a population based study underwent the Trier Social Stress Test (TSST), a standardized psychosocial test that induces stress, and is known to influence cortisol secretion. Blood samples were collected prior to (10:30am, T1), and after the TSST (3:05 pm, T2). DNA extracted from peripheral blood mononuclear cells (PBMC) was used to examine site-specific CpG methylation levels in the methylation sensitive region (MSR) of the promoter (sites -876, -776, -744, -720, -691, -670 and -650). This was quantified by bisulfite treatment of DNA followed by pyrosequencing.

RNA from PBMCs collected at the same time points was used to quantify perforin mRNA expression by LightCycler real-time RT-PCR. Total RNA from peripheral blood collected at the same time points was used in the Affymetrix Human Exon Array 1.0 platform. RESULTS: Methylation of the MSR ranged from 38%-79% and no differences in CpG site-specific methylation of perforin was detected between CFS and NF at T1 or T2. In PBMC, there was no difference in the perforin expression between CFS and NF at T1 but expression was significantly higher in CFS than NF (1.4 fold, p=0.02) at T2. NF subjects had reduced perforin expression (0.8 fold, p=0.008) and methylation levels were increased by 4% (range 2.6-4.3, p= 0.01-0.05) at four CpG sites (-876, -744, -691, and -670) at T2 compared to T1. However in CFS subjects, methylation levels were increased by 6% (range 4.7-6.8, p=0.02-0.03) at T2 compared to T1 at two positions (-776 and -744) without a corresponding change in expression. Expression results by real-time RT-PCR and exon arrays were concordant. CONCLUSION: While increased promoter DNA methylation correlated with reduced perforin expression in NF, this relationship was not seen in CFS. The small but statistically significant differences in methylation were detected over the course of the day were different for the NF and CFS groups. Further studies are needed to confirm these results and to evaluate explanations (changes in cell population, circadian rhythm or stress) for the observed dynamics in perforin DNA methylation and expression.

L Bateman, A.R. Light, et al.

Gene Expression Of Sensory Ion Channels, Adrenegic Receptors and Cytokines: Potential Biomarkers for CFS and Fibromyalgia

IACFS/ME – Biennial International Conference : Translating Evidence into Practice; Ottawa, Ontario, Canada, Sept. 22-25, 2011.

OBJECTIVES: To determine whether baseline and/or post-exercise expression of genes involved in signaling and modulating sensory fatigue and muscle pain are potential biomarkers for distinguishing patients with Chronic Fatigue Syndrome (CFS) and Fibromyalgia Syndrome (FM) from healthy controls. METHODS: Forty eight Patients with CFS-only or CFS with comorbid FM, 18 Patients with FM that did not meet criteria for CFS, and 49 healthy Controls underwent moderate exercise (25 min at 70% of age-predicted maximum heart rate on Air-Dyne). Blood samples were taken before and 0.5, 8, 24, and 48 hours after exercise. Leukocytes were immediately isolated in buffer, number coded for blind processing, and flash frozen. Using real-time, quantitative PCR, the amount of mRNA for 13 genes (relative to control gene) involved in sensory ion channel, adrenergic, and immune functions was compared between groups at baseline and following exercise. Visual-analogue measures of fatigue and pain were taken before, during, and after exercise, including concurrently with all blood samples. Changes in amounts of mRNA were correlated with these measures, with history of orthostatic intolerance and with blinded ratings of disorder severity by the treating physician derived from multiple clinics. RESULTS: No gene expression changes occurred following exercise in Controls except for inconsistent increases in β-1 adrenergic receptor. In 71% of CFS patients, moderate exercise increased most sensory ion channels and adrenergic receptors and one cytokine gene for 48 hours. These post-exercise increases correlated with numerical ratings of fatigue and pain, and greater increases were shown by patients with higher physician ratings of disorder severity. In contrast, for the other 29% of CFS patients, adrenergic α-2A receptor expression was decreased at all time points after exercise; other genes were not altered. History of orthostatic intolerance was significantly more common in the α-2A decrease subgroup. FM only patients showed no post-exercise alterations in gene expression, but their pre-exercise baseline mRNA for two sensory ion channels and one cytokine were significantly higher than Controls. CONCLUSIONS: At least two subgroups of CFS patients can be identified by gene expression changes following exercise. The larger subgroup showed increases in mRNA for sensory ion channels and adrenergic receptors and a cytokine. Both self-rated and physician-rated symptom severity was associated with greater post-exercise increases in these genes. The smaller subgroup contained most of the CFS patients with orthostatic intolerance, showed no post-exercise increases in any gene, and was defined by decreases in mRNA for α-2A adrenergic receptor. FM only patients can be identified by baseline increases in 3 genes. Post-exercise increases for 4 genes meet published criteria as an objective biomarker for CFS, and could be useful in guiding treatment selection for different subgroups.

M Frémont, D Coomans, K De Meirleir

High-Throughput 16s rDNA Sequencing Reveals Alterations of Intestinal Microflora in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome Patients

IACFS/ME – Biennial International Conference : Translating Evidence into Practice; Ottawa, Ontario, Canada, Sept. 22-25, 2011.

OBJECTIVES: Human intestinal microflora plays an important role in the maintenance of host health by providing

energy, nutrients, and immunological protection. Intestinal dysfunction is a frequent complain in ME-CFS patients, and previous reports suggest that dysbiosis, i.e. the overgrowth of abnormal populations of bacteria in the gut, is linked to the pathogenesis of the disease. Recently developed technologies are able to provide a comprehensive overview of the gut bacterial populations (metagenomics approach). We used high-throughput 16s rDNA sequencing to investigate the presence of specific alterations in the gut flora of MECFS patients from Belgium and Norway. METHODS: 39 ME-CFS patients and 35 healthy controls were included in the study. Bacterial DNA was extracted from stabilized stool samples and PCR amplification was performed on conserved 16S rDNA regions. PCR amplicons were then sequenced using Roche FLX 454 genome sequencer (6000-10000 sequences per sample). Bacteria were classified by phylum, family and genus; diversity indexes (Chao and Shannon) were also calculated. Data were analyzed using Mann-Whitney test and step-wise linear discriminant analysis. RESULTS: ME-CFS patients presented altered levels of specific bacterial populations: Prevotella, Asaccharobacter, Lactonifactor, Eubacterium. Linear discriminant analysis showed that a significant ($p<0,001$) discrimination between control and patient populations could be achieved by using a combination of Asaccharobacter, Turicibacter, Ruminococcus and Enterococcus as variables. Differences could be seen between males and females, as well as between people from different geographical origins (Belgium vs. Norway). CONCLUSIONS: ME-CFS patients present significant alterations of their gut flora composition. More research has to be done to fully understand how intestinal bacteria can contribute to the pathogenesis of the disease (production of toxic metabolites, interaction with host immune cells…), but also how host factors (especially genetic factors) and external factors like diet or viral infections can influence the response of the body to gut bacteria. Metagenomics is a useful tool to diagnose dysbiosis in ME-CFS patients and to help designing treatments based on gut flora modulation (antibiotics, pre- and probiotics supplementation).

OTHER BIOMARKERS

M A Fletcher, N G Klimas
Biomarkers in CFS/ME
IACFS/ME – Biennial International Conference : Translating Evidence into Practice; Ottawa, Ontario, Canada, Sept. 22-25, 2011.
OBJECTIVES: Validated laboratory tests are essential for diagnosis and for monitoring therapy of CFS/ME. Diagnosis using the case definition [Fukuda, et al, 1994] requires the exclusion of any other medical explanation for these symptoms, yielding an inefficient, slow, error prone process. This is also costly because the current clinical diagnosis typically involves tertiary care specialists. The search for biomarkers included lymphocyte functions as well as molecules associated with lymphocyte activation, with stress and with inflammation. METHODS: CFS/ME patients were drawn from the University of Miami (UM) Miller School of Medicine CFS/ME and Immunodeficiency Clinic. All were participants in funded studies (NIH, DOD, Chronic Fatigue Immunodeficiency Syndrome Association (CFIDS) or the Veterans Afairs Merit grant). Prospective biomarkers included natural killer cell cytotoxicity (NKCC), T lymphocyte proliferation in vitro in response to mitogen (LPA), lymphocyte activation markers (CD26, CD38), 16 plasma cytokines and neuropeptide Y. All laboratory evaluations of prospective biomarkers were done in the UM/VA clinical immunology laboratories. The diagnostic accuracy of biomarkers was assessed in terms of true positive (sensitivity) versus true negative (specificity) rates using nonparametric receiver operating characteristics (ROC) curve analyses RESULTS: These studies provided credible biomarker status for NKCC, LPA, and markers of lymphocyte activation in CFS/ME. A significant elevation in the relative amounts of 4 of 5 pro-inflammatory cytokines in peripheral blood plasma of patients with CFS/ME was found when compared with the controls Only tumor necrosis factor (TNF)a was unchanged. In cases, lymphotoxin (LT)a was elevated by 257% and IL-6 by 100% over the controls. Both interleukin (IL)-4 and IL-5 were elevated in CFS/ME, with the median of IL-4 240% and of IL-5 95% higher in cases over controls. The anti-inflammatory cytokine IL-13 was significantly lower (15%) in CFS/ME patients while IL-10 was not different. Plasma levels of IL-2 and IFN. in CFS were similar to those in controls. However, IL-12 was significantly elevated (120%) and IL-15 decreased 15% in cases compared to controls. IL-8 (CXCL8) was 42% lower in the CFS/ME patients. IL-17 and IL-23 were not significantly different in CFS cases compared to controls. ROC analyses calculating area under the curve (AUC) for IL-5 (0. 84), LTa (0.77), IL-4 (0.77), IL-12 (0.76) indicated good biomarker potential. The AUC of IL-6 (0.73), IL-15 (0.73), IL-8 (0.69), IL-13 (0.68) IL-1a (0.62), IL-1ß (0.62) showed fair potential as biomarkers. The stress hormone, NPY, was elevated in plasma of CFS/ME cases and positively correlated with perceived stress, anger, depression, negative thoughts and maladaptive coping. ROC analysis indicated that the predictive ability of plasma NPY was significantly better than chance alone in distinguishing patients with CFS/ME from healthy controls. CONCLUSIONS: Fifteen useful biomarkers were identified in these studies. The differences of these markers in CFS/ME compared to controls also give important information regarding the pathophysiology of the disorder. The association of low LPA response, elevated proportion of activated CD4 and CD8 T cells, defective NKCC, elevated TH2 cytokines with CFS/ME cases suggests that T cells are metabolically limited in performing their helper function. All but one of the inflammatory cytokines measured were elevated as was the stress hormone, NPY – supporting the hypotheses that inflammation and abnormal stress responses are important components in the pathophysiology of CFS/ME.

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Assessment of Natural Killer Cell Function in Chronic Fatigue Syndrome/ME

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OBJECTIVE: Immunological abnormalites are recognized as an important components of Chronic Fatigue Syndrome (CFS). Natural Killer (NK) cell dysfunction is the most common immunological finding across studies. It has been suggested that these reductions in NK cell function is caused by decreases in the expression pattern of perforin and granzyme molecules. However, other factors may be involved in NK cell dysfunction.. Hence the purpose of this study is to investigate other potential mechanisms of NK cell dysfunction in CFS/ME. METHODS: This study examined samples collected from 20 CFS/ME subjects and 5 normal controls. CFS participants were pre-selected by demonstration of low NK cell function and diminished VO2 max on stress testing. Using flow cytometry and real time quantitative PCR, samples were assessed for levels of cytokines, lytic molecules and expression of miRNAs. RESULTS: Preliminary data demonstrated differential expression of cytokines, miRNAs and cytotoxic molecules in the CFS/ME participants compared to healthy controls. Additionally, cytokines, perforin and granzymes were differentially expressed between groups for both the serum and CSF. CONCLUSION: These results confirm the observation of impaired NK cell function in, CFS/ME patients which may be related to alterations in cytokins and lytic proteins.

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ROLE OF CELLULAR PRION PROTEINS (PrPc) IN CFS/ME AND OTHER CHRONIC DISEASES

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OBJECTIVE: Cellular prion proteins (PrPc) are small glycoproteins attached to the outer leaflet of the plasmamembrane of mammalian cells by a glycosylphosphatidyl anchor. The isoform of the prion protein is expressed in hematopoietic stem cells, neuronal cells, T and B lymphocytes, natural killer cells, muscle, intestinal tract, spleen, adrenal glands, endothelial cells, platelets. PrPc binds copper, plays a role in calcium uptake, protects cells against oxidative stress, prevents cells from apoptosis, interacts with viruses (binds gp-120), is involved in neuroprotection and plays an important role in immune and angiogenic responses. Therefore we estimated that hallmarks of CFS/ME such as oxidative stress, calcium channelopathy, T-cell dysfunction, copper uptake changes, altered red blood cells and oxygen transport, coagulation and hormonal responses (HPA-axis) as well as viral entry could be attributed to aberrant PrPc function which could than ultimately explain the “multi-system” character of the CFS/ME disorder. In order to investigate PrPc function in CFS/ME, we first needed to develop a test allowing to measure “activity” of PrPc in “real time”. METHODS: We have developed a cell-based chemiluminometric (CL) assay accordingly to the following principle: cells or tissue under study are incubated in an appropriate buffer in the presence of a chemiluminometric probe (CLP). Next a PrPc redox-trigger is added that stimulates PrPc-mediated reactive oxygen species (ROS) production which is proportional to the active state of the PrPc and which ROS react with CLP to produce a basal glow of light (Lb) that can be detected in front of a photomultiplier. Next, to an identical sample and CLP an additional trigger is added that stimulates cells to produce ROS at maximum capacity, producing maximum glow type chemiluminescence (Lmax). Lmax/Lb defines a PrPc functional stimulation index (SI) that can be compared for different tissues and cells obtained from controls and patient populations. RESULTS: Peripheral blood mononuclear cells (PBMC’s) obtained from CFS/ME patients show aberrant SI’s (extremely low SI<3 or extremely high SI>20 compared to controls (SI=10 +/- 3). In addition we could demonstrate the influence of heparin, minocyclin, metals (copper, mercury) and other agents on PrPc function by means of this luminometric technique Conclusion: PrPc functionality of PBMC’s is altered in CFS/ME. Chemiluminometric analysis provides a useful tool to further develop and explore PrPc functional tests and PrPc drug interaction platforms (drug discovery) in CFS/ME and other chronic diseases (fibromyalgia, rheumatoid arthritis, autism, cancer).

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GVDR-Fok1 and GVDR-Bsm1 Polymorphisms in ME/CFS Patients

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OBJECTIVES: Several researchers have demonstrated abnormalities in Vitamin D metabolism in ME/CFS patients. The individual degree of responsiveness of Vit. D binding protein – macrophage activating factor (GcMAF) is according to Ruggiero et al. dependent on Vit. D receptor (VDR) gene polymorphism, which can be identified by Bsm1 and Fok1, two SNPs (single nucleotide polymorphisms). VDR is also involved in skeletal metabolism, modulation of immune response and regulation of cell proliferation and differentiation. Fok1 is a T-C polymorphism; the T allele leads to a protein which is less effective in transduction of the Vit. D signal, the C allele with a higher response. Bsm1 is a C-T polymorphism. The C allele is associated with Th1 suppression and breast cancer, the T allele with SLE and RA. Given the published scientific studies on the immune system in ME/CFS, we hypothesized that both for Fok1 and Bsm1, the incidence of low responders to GcMAF is higher than in the general population, thus predisposing to a lower natural defense to viruses, intracellular bacteria, mycoses and parasites (low Th1/Th2 ratio).

Methods:
185 ME/CFS patients were included in this study.
GVDR-Fok1 & Bsm1 were determined.
Based on Ruggiero's work we know that related to GcMAF:

Fok1:
C/C genotype: high responder (FF genotype)
T/C genotype: moderate responder (Ff genotype)
T/T genotype: low responder (ff genotype)

Bsm1:
C/C genotype: high responder (bb genotype)
T/C genotype: moderate responder (Bb genotype)
T/T genotype: low responder (BB genotype)

Results:

GVDR (VDR polymorphism) Analysis in 185 ME/CFS patients					
FOK1			BSM1		
	% patients	% control		% patients	% control
FF (high responder)	24	37	bb (high responder)	28	35
Ff (moderate responder)	46	43	Bb (moderate responder)	43	43
ff (low responder)	30	20	BB (low responder)	29	22

A one sided t-test was used to test the hypothesis that the mean % of the Bsm1 BB/Bb/bb and Fok1 FF/Ff/ff groups is statistically different from the average in the normal population according to reference percentages. For Fok1/Bsm1 ff/BB fenotypes (low responder) were significantly higher than in controls and FF/bb (high responder) were significantly higher in controls ($p < 0.001$). DISCUSSION: When compared to the general population, more ME/CFS patients seem to be genetically low GcMAF responders, possibly explaining higher susceptibility for persistent infection. This finding can be added to the list of genetic predisposition factors which may predispose to the development of ME/CFS.