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# Evidence for T-helper 2 shift and association with illness parameters in chronic fatigue syndrome (CFS)

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## Abstract

Few immunological markers have been consistently reported in CFS. However, a shift to a T-helper 2 (Th2) type immune response has been hypothesized for individuals with CFS. The current study investigated whether individuals with CFS who exhibited a stronger shift towards a Th2 type of immune response would also exhibit more severe symptoms, poorer neurocognitive functioning, and poorer physical and psychosocial functioning. The current investigation measured the percentage of Th1-like and Th2-like memory cells using cell surface flow cytometry in 114 individuals with CFS. The associations between the ratio of Th1 and Th2 memory cells and various illness parameters measures were then examined, including symptom severity, psychiatric functioning, neurocognitive functioning, salivary cortisol levels, and chronic pain status. Results indicated that individuals who exhibited a more extreme shift towards a Th2 immune response also exhibited poorer sleep and high levels of basal salivary cortisol. The implications of these findings are discussed.

### Keywords

chronic fatigue syndrome; t-helper 2 shift; immunology; salivary cortisol; cognitive functioning

## Introduction

Chronic fatigue syndrome is an illness characterized by unexplained severe, disabling fatigue lasting six months or more, along with the presence of additional symptoms that may include sore throat, headaches, lymph node pain, joint pain, muscle pain, post-exertional malaise, unrefreshing sleep, and memory and concentration difficulties (Fukuda et al., 1994). Investigations into the etiology of CFS have not yet been able to pinpoint the specific underlying physiological changes that underlie this illness, and to date, no diagnostic marker has been found. However, research into this illness has suggested that dysfunction of the immune, endocrine, and neurological systems may be involved. Individuals often report that the experience of CFS begins after infectious illness, and flu-like symptoms (i.e. sore throat, headaches, muscle, joint, and lymph node; memory and concentration problems) are a feature of this illness. In particular, because this illness includes flu-type symptoms, the role

of viral infection and immune activation has been suspected to underlie the pathology of CFS (Natelson, Haghihi, & Ponzio, 2002). However, to date, little research has attempted to examine the associations between the self-reported symptomatology of CFS, particularly neurocognitive symptoms, and immunological status.

Evidence for multiple immunological abnormalities has been frequently reported in the CFS literature, leading some to conclude that strong evidence exists for immune dysfunction in this illness (Maher, Klimas, & Fletcher, 2003). However, many immunological abnormalities reported in the literature have not been consistently replicated. This may be due to limitations in some studies due to low statistical power, and variations in the application of the definitional criteria for CFS resulting in variable sampling from this population. The cross-sectional design of most studies and the lack of reporting of phase or duration of illness at time of sampling may lead to conflicting results, as these illness variables may change over time in individuals with CFS (Maher, Klimas, & Fletcher, 2003). Further, deficiencies in laboratory methodologies and variations in results due to methodological parameters such as sampling time, shipping conditions, transit times, and processing methods, may have also led to conflicting results in many of these studies (Maher, Klimas, & Fletcher, 2003).

Despite these methodological challenges, some immunological findings have been more consistently reported across studies when comparing individuals with CFS with healthy controls. The more consistently reproduced findings include the impaired functioning of the natural killer cells, antinuclear antibody positivity, and the percentage of lymphocytes bearing the CD4 and CD45RA cell surface markers, and shifts in cytokine profiles to a Th2 profile (Maher, et al., 2003; Natelson, Haghighi, & Ponzio, 2002; Patarca-Montero, Antoni, Fletcher, & Klimas, 2001).

The Th1 and Th2 phenotype refers to the pattern of cytokines secreted by the T-helper cell upon activation of naïve T-helper cells (Th0) (Schwarz, Chiang, Muller & Ackenheil, 2001). Th1 cells secrete mainly IFN-γ, IL-2, IL-12, IL-18, and TNF-β, while Th2 cells secrete primarily IL-4, IL-5, IL-6, IL-10, IL13, and TGF-β (Schwarz, et al., 2001). IL-12 is the primary growth cytokine for Th1 cells, and IL-4 is the primary growth cytokine for Th2 cells (Schwarz, et al., 2001). IL-4 promotes allergy- associated inflammatory responses which result from the activation of mast cells and other effector cell types during a Th2 type immune response (Hanson, Gause, & Natelson, 2001). In addition, cytokine of either pathway also antagonize the production of the other type of helper cell (Schwarz, et al., 2001). The Th1 pathway promotes immune responses against intracellular pathogens and the cell-mediated immune response; and the Th2 pathway promotes B cell maturation and the proliferation of IgE and IgA antibodies (Schwarz, et al., 2001). The induction of either the Th1 or Th2 pathway is influenced by the antigen-presenting cells, including dendritic cells and macrophages, which are part of the innate immune system response, and by activated B cells, which are part of the adaptive immune response, in response to the types of pathogens encountered (Leonard, 2003).

Several researchers have found evidence for a shift from Th1 to Th2 type cytokine profiles in some individuals with CFS. Skowera and colleagues (2004) examined the frequency of type 1, type 2, and regulatory CD4 and CD8 T cells in 35 patients with CFS using flow cytometry to study intracellular cytokine production by CD and CD8 T lymphocytes. These researchers found a bias towards Th2 type immune responses in CFS. In particular, IL-4 production following polyclonal stimulation was higher in CFS patients when compared with healthy controls. In contrast, levels of IFN-γ, IL-2, and IL-10 were similar in both groups (Skowera, et al., 2004). Hanson, Gause, and Natelson (2001) also reported evidence for a type 2 cytokine pattern. These researchers used neural network analysis in order to

identify immunological differences between 127 individuals with CFS and 87 healthy, sedentary control subjects. Neural network models examined a range of immunological subsets with a range of cell surface markers and levels of various cytokines, including IFN-α, TNF-α, IL-2, IL-4, IL-6, IL10, and IL-12. These models found that only IL-4, a Th2 type cytokine, contributed significantly to the correct classification of participants to either the CFS or the control group. The authors note that this finding provides evidence for the hypothesis of the type 2 cytokine pattern (Hanson, et al., 2001).

In addition to differences in Th1 and Th2 cytokine profiles, one of the more consistent immunological findings in this illness is the dysfunction of natural killer cell cytotoxicity (Klimas, Salvato, Morgan, & Fletcher, 1990; Natelson, Haghighi, & Ponzio, 2002; Maher, et al., 2003), and this also may be related to the shift to a Th2 type immune response. Maher, Klimas, and Fletcher (2003) found that the majority of studies (8) reviewed found evidence for impaired functioning of the natural killer cells, and only one study did not, thus supporting the idea that a clear majority of studies demonstrate that individuals with CFS have reduced NKC cytotoxicity when compared to controls. Similarly, Natelson, Haghighi, & Ponzio (2002), in their extensive review of immunological findings in CFS, also found that natural killer cell cytotoxicity was one of the more consistently reported findings in the CFS literature. Because NK cells play a role in the generation of Th1 immune responses, the loss of NK activity might be related to the presence of a Th2 bias and persistent viral activation and chronic infection (Skowera, et al., 2004).

Finally, Hanson, Gause, and Natelson (2001), in the neural network analysis reported above, found that individuals with CFS showed evidence for selective T cell activation in CFS patients. In particular, they found a proportional increase in memory/effector CD45RO cells relative to naïve CD45RA cells and an increase in CD28 T cells in addition to an overall increase in CD3 T cells. They propose that the finding of a positive interaction with IL-4 suggests that this Th2 cytokine is also associated with this observed selective T cell expansion.

They also suggest that an increase in CD45 RO memory cells would be expected if CFS were caused by immune system activation.

Although few studies have examined the presence of a shift to Th2 immune response in CFS, this hypothesis holds much promise in explaining the differing profiles in cytokine patterns, impaired natural killer cell functioning, and specific T cell activation that has been observed frequently observed in persons with CFS. Most research that examined this shift towards Th2 immune responses have examined individuals with CFS and compared them to controls in order to identify diagnostic markers with CFS. However, some studies have also examined the association between Th2 type cytokines and illness parameters in CFS. It has been suggested that proinflammatory cytokines can produce sickness behaviors and fever (Eskanderi & Sternberg, 2002, Dantzer, et al., 1998; Dantzer, 2001).

When looking at individuals with CFS, several researchers have found associations between cytokine profiles and illness symptoms. Skowera and colleagues (2004) found no correlation between non-stimulated or polyclonally stimulated IFN-γ, IL2, or IL-4 cells and fatigue, psychiatric status, history of psychiatric illness, disability, age, duration of illness, or weight. However, they did report a weak correlation between polyclonally stimulated IL-10 cells and age and duration of illness; but the authors urged caution in interpretation of the results due to the possibility of Type I error (Skowera, et al., 2004). Patarca, Lugtendorf, Antoni, Klimas and Fletcher (1994) found elevations of IL-1α in 70 CFS patients, and the most disabled patients had the highest elevations of this cytokine. Kozora, Ellison, Sorenson, DuBray, & Jones (2005) found that higher levels of IL-1β was related to lower verbal

fluency, although better performance in visuomotor speed, visuomotor sequencing, and visual learning were related to higher levels of Il-1β, IL-6, and IFN-α. Finally, Patarca-Montero, Antoni, Fletcher, and Klimas (2001) reported the results of an experimental therapy using lymph node extraction, ex vivo cell culture, and an autologous cell reinfusion as a treatment strategy for CFS patients The lymph node cells were cultured for 10 to 12 days with anti-CD3 and IL-2. After reinfusion of these cells, 9 of 11 participants demonstrated cognitive improvements, including improvements in visual scanning, and an increase in the patients' ability to mentally track and rapidly shift cognitive set. These researchers also observed improvements in illness severity, as measured by increased in Karnofski scores and in the Quality of Life scale from the Sickness Impact Profile (Patarca-Montero, et al., 2001). These researchers suggest that the degree of cellular immune activation is associated with the severity of CFS-related physical symptoms, cognitive, complaints, and perceived illness burden (Patarca-Montero, et al., 2001). In contrast, Swanink et al. (1996) examined levels of IL-1β and TNF-α, and did not find that these correlated with fatigue severity or psychologic well-being scores.

In summary, some researchers have suggested that illness behavior and symptoms, particularly neurocognitive symptoms, in CFS may be related to a shift from Th1 to Th2 type immune responses. This study was an attempt to examine whether individuals with CFS who demonstrate a bias toward Th2 type immune response exhibit increased illness burden and poorer overall physical, psychosocial, or neurocognitive functioning. This hypothesis was tested by examining the ratio of Th2 memory helper cells (CD4+CD62L +CD45RA-) to Th1 memory helper cells (CD4+CD62L-CD45RA-). Cluster of differentiation (CD) markers provide a means of identifying the propensity of various groups of T cells. The presence of the CD4 marker (CD4+) indicates T helper cells, whereas the absence of CD45RA (−) indicates the cell has been exposed to antigen and has a memory phenotype. The presence or absence of CD62L, also known as lymph note homing receptor or L-Selectin is reported to allow for distinguishing between Th1 (CD62L-) and Th2 (Cd62L+) cytokine producing cells (Kanegane et al., 1996, Matsuzaki et al., 2005). Through the use of tagged antibodies to these markers, flow cytometry can determine general predisposition of peripheral T cells.

While some researchers have examined markers for Th2 type immune responses and neurocognitive testing, the relationship between a Th2 immune system bias and other aspects of the illness is not known, such as the experience of the physical symptoms including subjective reports of fatigue, or other physiological processes such as impaired cortisol production. This investigation examined whether a bias towards Th2 immune response in a group of individuals diagnosed with CFS is associated with other key features of this illness. It was expected that a shift towards Th2 immune response would be associated with more severe flu-type symptoms, more neurocognitive difficulties, and impaired physical and psychological functioning in individuals with CFS.

## Method

Prior to implementation of the study protocol, all procedures outlined below were reviewed and found to meet all relevant ethical guidelines for research with human subjects by the DePaul University Institutional Review Board. All participants signed detailed informed consent forms prior to collection of any data.

### Participant Recruitment

Study participants were recruited to participate in a larger treatment trial investigating the effectiveness of cognitive behavior therapy for individuals with chronic fatigue syndrome. Participants were recruited from a variety of sources, including physician referrals.

Information about the non-pharmacologic treatment trial study was disseminated to medical colleagues through mailings and phone communication. In addition, study announcements for new participants were placed in local newspapers and recruitment offers were made at local CFS support group meetings. These efforts were continued throughout the study period until the target enrollment numbers were achieved. One hundred and fourteen individuals were recruited. Of the 114 individuals, 46% were referred by physicians, 34% were recruited by media (newspapers, TV, radio, etc.), and 20% stemmed from other sources (e.g., heard about the study from a friend, family member, person in the study, etc.). Twenty-four additional individuals who were screened were excluded due to a variety of reasons (i.e., lifelong fatigue, less than 4 Fukuda symptoms, BMI > 45, melancholic depression or bipolar depression, alcohol or substance abuse disorder, autoimmune thyroiditis, cancer, lupus, rheumatoid arthritis).

### Initial Screening

All participants were required to be at least 18 years of age, not pregnant, able to read and speak English, and considered to be physically capable of attending the scheduled sessions. Bedridden and wheelchair bound patients were excluded due to the practical difficulties of making appointments. Referrals to local physicians who treat CFS and to support groups were offered to these individuals. After a detailed informed consent form was signed, prospective participants were initially screened by the third author, using a structured questionnaire. Because CFS is a diagnosis of exclusion, prospective participants were screened for identifiable psychiatric and medical conditions that may explain CFS-like symptoms. These measures were completed at DePaul University and took approximately two hours. After the initial interview was completed, the patients' information was reviewed to ensure that they met all eligibility requirements. If found to be eligible for the study, participants attended a medical appointment with the study physician in order to confirm the diagnosis of chronic fatigue syndrome. After confirmation that the individual fully met the criteria for CFS according to the Fukuda et al. (1994) case definition, individuals completed a battery of baseline measures (described below). They were also assigned randomly to one of 4 treatment conditions, and completed measures at three follow-up testing periods. However, only the data obtained at baseline will be considered in the current investigation.

One hundred fourteen individuals were found to be eligible and enrolled in the study. Of the participants, 16.7 % were male and 83.3 % were female. The average age at baseline was 43.8 years. Regarding ethnicity, 87.7 % were Caucasian, 4.4 % were African-American, 4.4 % were Latino, and 3.5% were Asian-American. As for marital status, 49.1 % were married/ living with someone, 33.3 % were single, and 17.6 % were either divorced or separated.

## Measures

### The CFS Questionnaire

This screening scale was initially validated by Jason, et al. (1997). This scale is used to collects demographic, health status, medication usage, and symptom data, and it uses the definitional symptoms of CFS (Fukuda et al., 1994). Hawk, Jason, and Torres-Harding (in press) recently revised this CFS Questionnaire, and administered the questionnaire to three groups (those with CFS, Major Depressive Disorder, and healthy controls). The revised instrument, which was used in the present study, evidences good test-retest reliability and has good sensitivity and specificity (Hawk, Jason, & Torres-Harding, in press). For each Fukuda et al. (1994) case definition symptom, rate the intensity of each symptom they endorsed on a scale of 0 to 100, where 0 = no problem and 100 = the worst problem possible.

### The Structured Clinical Interview for DSM-IV (SCID) (First et al., 1996) Axis I

This interview was used to establish psychiatric diagnoses. The professionally administered SCID allows for clinical judgment in the assignment of symptoms to psychiatric or medical categories, a crucial distinction in the assessment of symptoms that overlap between CFS and psychiatric disorders, such as fatigue, concentration difficulty, and sleep disturbance (Friedberg & Jason, 1998). A psychodiagnostic study (Taylor & Jason, 1998) validated the use of the SCID in a sample of CFS patients.

### Medical Examination

The physician screening evaluation included a general and neurological physical examination. Laboratory tests in the battery were the minimum necessary to rule out other illnesses (Fukuda et al., 1994). Laboratory tests included a chemistry screen (which assesses liver, renal, and thyroid functioning), complete blood count with differential and platelet count, erythrocyte sedimentation rate, arthritic profile (which includes rheumatoid factor and antinuclear antibody), hepatitis B, Lyme Disease screen, HIV screen and urinalysis. A tuberculin skin test was also performed. If the TB skin test was positive, a follow-up chest x-ray was conducted to rule out tuberculosis. The project physician performed a detailed medical examination to detect evidence of diffuse adenopathy, hepatosplenomegaly, synovitis, neuropathy, myopathy, cardiac or pulmonary dysfunction. This medical examination was used to confirm the diagnosis of chronic fatigue syndrome, according to the Fukuda et al. (1994) criteria and to rule out exclusionary medical conditions.

### Medical Outcomes Study-Short Form-36. (MOS-SF-36)

The MOS-SF-36, a 36 item broadly-based self-report measure of functional status related to health. Subscales include Physical Functioning, Role Functioning-Physical, Role Functioning-Emotional, Bodily Pain, General Health, Vitality, Mental Health, and Health Transition (Ware & Sherbourne, 1992). A higher score indicates better health or less impact of health on functioning. Test construction studies for the SF-36 (McHorney, Ware & Raczek, 1993; McHorney, Ware, Lu, & Sherbourne, 1994) have shown adequate internal consistency, significant discriminate validity among subscales, and substantial differences between patient and non-patient populations in the pattern of scores. The SF-36 has also indicated sufficient psychometric properties as a measure of functional status in a CFS population (Buchwald, Pearlman, Umali, Schmaling, & Katon, 1996). A behavioral treatment study of CFS patients showed that the MOS-SF-36 is sensitive to treatment changes (Deale, Chalder, Marks, & Wessely, 1997). The MOS Physical Composite Score (PCS) and Mental Composite scores (MCS) were utilized in the present investigation as combined measures of the MOS.

### Fatigue Scale (FS)

Krupp et al.'s (1989) Fatigue Severity Scale was used to measure fatigue. This scale includes 9 items rated on 7-point scales and is sensitive to different aspects and gradations of fatigue severity. Previous findings have demonstrated the utility of the Fatigue Severity Scale (Krupp et al., 1989) to discriminate between individuals with CFS, MS, and primary depression (Pepper et al., 1993). In addition, the Fatigue Severity Scale (Krupp et al., 1989) was normed on a sample of individuals with MS, SLE, and healthy controls. A study by Taylor, Jason and Torres (2000) found that, within a CFS-like group, the Fatigue Severity Scale (Krupp et al., 1989) was associated with severity ratings for the eight Fukuda et al. (1994) CFS symptoms.

### Beck Depression Inventory (BDI-II)

Because depression is the most commonly diagnosed psychiatric disorder in CFS (Friedberg, 1996), a quantitative measure of depression severity was used. Depressive symptomatology was measured with the BDI-II (Beck, Steer, & Brown, 1996), a 21-item self-report. The BDI-II is the only depression rating scale to be empirically tested and interpreted for both depressed and non-depressed patients with CFS (Johnson, DeLuca & Natelson, 1996). Also the Beck Depression Inventory has shown sensitivity to treatment changes in two cognitive behavioral treatment studies of CFS (Deale et al., 1997).

### Perceived Stress Scale

The Perceived Stress Scale (PSS) is a four-item revised version of a previous 14-item measure of global perceived stress. The time period that this instrument measured was the previous month (Cohen, Kamarck, & Mermelstein, 1983). The authors report a coefficient alpha reliability of .72 for the four-item short version. The Total Stress score was used in the present study. It has a range from 0–16, with higher scores indicating more stress.

### Brief Pain Inventory

The Brief Pain Inventory (Cleeland & Ryan, 1994) was administered to measure the intensity of pain (pain severity) and the interference of pain in the patient's life (pain interference). Higher scores indicate more severe levels of persistent pain and higher levels of interference with functioning. This measure exhibits adequate levels of reliability to assess pain in noncancer samples, with coefficient alphas of .70 and above, also evidences good concurrent validity with other generic pain measures, and has been shown to be sensitive to changes in pain status over time (Keller et al., 2004).

### Beck Anxiety Inventory (BAI)

Anxiety symptoms was measured with the BAI, a 21-item self-report measure with established and replicated construct validity (Hewitt & Norton, 1993; Steer, Clark, Beck & Ranieri, 1995). Factor analysis of the BAI and BDI yielded a first-order factor labeled anxiety that had salient loadings for all 21 items on the BAI, but only one item on the BDI. Anxiety symptoms at intake was a predictor of treatment outcome in two cognitive behavioral treatment studies of CFS (Sharpe, 1996).

### Quality of Life Scale (Burckhardt & Anderson, 2003)

This scale measures satisfaction with different life activities for individuals with various chronic illnesses. The scale consists of 16 items answered on a Likert type 1 to 7 scale which measure six conceptual domains of quality of life: material and physical well-being, relationships with other people, social, community and civic activities, personal development and fulfillment, and recreation, and independence. Higher scores mean more overall life satisfaction. This scale demonstrated high test-retest reliability for this 16-item scale, and convergent and discriminate construct validity in groups of individuals with various stable chronic illness, including post-ostomy surgery, osteoarthritis, rheumatoid arthritis, fibromyalgia, COPD, and insulin-dependent diabetes (Burckhardt & Anderson, 2003).

### California Verbal Learning Test—Second Edition ( Delis, Kramer, Kaplan, & Ober, 2000)

This test provides a measure of a participant's ability to learn and remember verbal information. It requires that the participant learn and remember a 16-item word list after repeated presentations, both immediately and after a delay of approximately 20 minutes. This test includes free recall, cued recall, and word recognition of the 16 item list. Split-half reliabilities have been estimated at above .90 with age groups, and r = .94 for the

standardization sample; and test-retest reliabilities have been reported to be .82 after a mean of 21 days (Delis, et al., 2000).

### Digit Span

Digit Span is one of the subtests from the Wechsler Adult Intelligence Scales-Third Edition (WAIS-III). This test measures attention and short-term attention. This subtest includes two parts, digits forward, and digits backward. According to the Technical Manual for the WAIS-III (Wechsler, 1997) test-retest reliability over a 2–12 week interval for digit span for all age groups (16–89), ranged from .83 to .89. Reliability coefficients for the reference group on Digit Span ranged from a low of .84 (85–89 year olds), to a high of .93 (55–69 year olds), with an average of .91.

### Rey-Osterreith Figure Drawing

This test involves the reproduction of an abstract visual stimuli. This test measures visuo-spatial, visual organizational and integration abilities, motor abilities, and immediate and delayed memory for complex visual stimuli. Administration involves copying a complex figure by hand. Next, the participant is asked to reproduce the figure from memory three months after the first trial; and again at 30 minutes after the first trial. This test measures visuo-spatial, visual organizational and integration abilities, motor abilities, and immediate and delayed memory for complex visual stimuli (Lezak, 1995).

### NES-2 Continuous Performance Test

The continuous performance test is a subtest of the Neurobehavioral Evaluation System (Letz & Baker, 1988), a computer-administered test of neurocognitive functioning that has been used extensively in occupational health studies (Arcia & Otto, 1992). The continuous performance test consists of a test where lettesr flash briefly on the screen (for approximately 50 ms), at the rate of one per second. The participant is instructed to press the response button when the letter ‘S’ flashes on the screen, but not for any other letter. Cognitive abilities measured on this test include, response speed and attention. The mean latency of response for all responses (in milliseconds) was examined. The continuous performance test mean latency response evidenced acceptable test-retest reliability (.66) (Arcia & Otto, 1992).

### Trailmaking Test, Trails A and B

This is a brief, easily administered test of attention, sequencing, mental flexibility, visual search, and motor functioning (Spreen & Strauss, 1998). This test consists of two parts (A and B). The participant is first instructed to connect consecutively numbered circles on a worksheet (part A), and then is asked to consecutively connect numbered and lettered circles in order, alternating between the numbers and the letters (part B). The participant is encouraged to work as quickly as they can. The scoring used on this measure is the seconds to completion for part A and for part B. Reliability coefficients for this test generally fall between .60 to .90, with most falling into the .80 range (Spreen & Strauss, 1998).

### Grooved Pegboard

The grooved pegboard is a manipulative dexterity test. It consists of a unit pegboard containing 25 holes, and the individual must place the pegs into randomly positioned slots on the pegboard. It measures complex visual motor coordination, manual dexterity, and processing speed. The pegboard is completed twice, once with each hand. The score is time to completion in seconds. Good test-retest reliability has been found for this test (Lezak, 1995).

### Sleep Difficulties

Sleep disturbances were examined by using the Pittsburgh Sleep Quality Index, which was developed to measure sleep quality in psychiatric research (Buysse, et al., 1989). This index measures sleep disruptions and sleep quality. There are nineteen questions (on 0–3 scale) which generate an overall score. Acceptable measures of internal homogeneity, consistency (test-retest reliability), and validity have been reported for this measure. A global PSQI score greater than 5 yielded a diagnostic sensitivity of 89.6% and specificity of 86.5% (kappa = 0.75, $p < 0.001$) in distinguishing good and poor sleepers (Buysse, et al. 1989).

### Salivary cortisol

Individuals also completed 5 samples of salivary cortisol. Saliva was collected using Salivettes® brand collection tubes. Over the course of one day, samples were collected immediately upon first awakening and 45 minutes afterward; and at 9 AM, 4 PM, and 9 PM. Salivary cortisol. Individuals also provided 5 samples of salivary cortisol. Saliva was collected using Salivettes® brand collection tubes. Over the course of one day, samples were collected immediately upon first awakening and 45 minutes afterward; and at 9 AM, 4 PM, and 9 PM. The kit consists of cotton swabs inside small plastic tubes, which are placed into a storage container.

Patients were instructed how to properly collect saliva samples. They first were shown how to place the cotton swab in their mouth and gently chew for 30–45 seconds. Participants were then instructed to deposit the moistened swab into its plastic tube and the tube into the container. The container recorded the exact time that they placed the plastic tube into it. Samples were held at −20°C prior to assay and shipped on dry ice to Dr. Kevin Maher at the University of Miami for laboratory analysis. On the day of assay, samples were thawed, vortexed and centrifuged at 1500 RPM for 15 minutes. Salivary cortisol was determined by immunoassay using the Salimetrics high sensitivity kit (State College, PA). This kit is designed to measure cortisol levels in saliva with the calibrator in a saliva-like matrix. A built-in pH indicator warns the technologist of acidic or basic samples. Cortisol values from samples with pH values < 3.5 or > 9.0 may be artificially inflated or lowered. This assay was run using the Biomek 2000 robotic system. Control samples with high and low concentrations of cortisol were included in each assay. Using this assay, the AM range for healthy adults is 0.94 to 1.551 μg/dL. The PM range is from not detectible (i.e., below sensitivity of the assay) to 0.359μg/dL.

Three methods were then used to statistically examine basal salivary cortisol. First, salivary cortisol mean scores were calculated by simply averaging the available time points for each study participant, in order examine the average daily output of cortisol. Next, mean regression slope of the different sampling times were computed, as cortisol levels tend to be highest upon first awakening, peak 45 minutes afterwards, and then decrease throughout the waking hours. Finally, each participant's individual daily cortisol results were examined by an independent physician who was blinded to the identity of the study participant. The physician, Dr. Tony Lu, who is board-certified in internal medicine, then classified whether individuals had abnormal or normal cortisol levels using clinical judgment.

### Flow Cytometry

Levels of CD4+CD62L+CD45RA-(Th2 Memory) and CD4+CD62L-CD45RA-(Th1 memory) were measured using flow cytometry. All specimens were shipped to the University of Miami Clinical Immunology Laboratory by overnight courier. Helper/inducer (CD3+CD4+) T-cell, cytotoxic (CD3+CD8+) T-cell, B-cell (CD19+), and natural killer (CD3-CD56+) cell counts were determined using four-color flow cytometry. Briefly, one hundred microliters of heparinized whole blood was incubated for 15 minutes at room

temperature with TetraOne reagents from Beckman Coulter, and process in the Q-Prep to lyse and fix the stained blood preparations. Optimal concentrations of fluorochrome conjugated antibodies. CD45-FITC, CD14-RD1, CD45RA-FITC, CD62L-PE, CD4-ECD, CD8-PC5 and isotype controls were added in 4 color combinations for 15 minutes at 25oC. Samples were then fixed and lysed with Optilyse-C reagent, followed by analysis on an XL-MCL flow cytometer. All cytometric determinations were represented as percent of lymphocytes with the exception of CD45RA and CD62L, whose subsets were represented as percent of the CD4 population. All reagents and instrumentation were from Beckman Coulter Corporation, Hialeah, Florida.

Analyses were performed by collecting 2500 events in the lymphocyte region. All determinations were corrected for purity by dividing by the percent CD45+CD14- events in the lymphocyte gate. Absolute count for each of the subsets was calculated by multiplying the percent positive for each marker by the lymphocyte count determined from the automated complete blood count (CBC). CBC was performed on a Coulter MAX-M (Coulter Corporation, Hialeah, FL). Accuracy and precision of analyses were optimized through the adherence to the CDC's recommendations for flow cytometric analyses Centers for Disease Control and Prevention (1997). Lymphocyte, monocyte and granulocyte populations were determined using light scatter and back gating on fluorescence for the CD45 bright and CD14 negative population. The isotype controls were the reference for negative events. Spectral compensation was established daily. Quality control included the optimization for lymphocyte recovery, purity of the gate of analysis and lymphosum.

### Natural Killer Cell Cytotoxicity (NKCC)

A whole blood, chromium release assay was utilized since it more closely reproduces physiological conditions. The assay was done in triplicate at 4 effector to target cell ratios. The NKCC was expressed as % of target cells (51Cr-labeled erythroleukemia cell line, K562) killed at an effector to target cell ratio of 1:1. Effector cells were defined as CD3-CD56+ lymphocytes, determined by flow cytometry on each sample. Detailed methods have been published (Fletcher, Baron, Ashman, & Klimas, 1987; Patarca, Fletcher, & Podack, 1997).

## Statistical Analyses

The ratio of Th2/Th1 shift was computed by dividing the percentage of Th2 type memory helper cells to Th1 type memory helper cells. The association between this Th2/Th1 ratio was then compared in separately analyses with a range of other physical and psychosocial outcome variables, using Pearson's R correlation test statistic or the $X^2$ test statistic, where appropriate. The Spearman's correlation test statistic was used when the outcome variable to be examined was not normally distributed. Due to the number of analyses conducted, the p value for significance was set at .01 in order to minimize type 1 error. Results

First, the Th2/Th1 ratio score was correlated with the following psychosocial measures, using the Pearson's correlation coefficient in separate analyses: the MOS-SF 36: Standardized physical component scale (PCS), the MOS-SF 36: standardized mental component scale (MCS), Beck Depression Inventory Total score, Beck Anxiety Inventory Total score, the Krupp Fatigue Severity Scale score, the Perceived Stress Scale total, the Brief Pain Inventory: Severity and Interference subscale scores, The Pittsburgh Sleep Quality Inventory: Global score, and the Quality of Life Scale total score. The correlation between the Th2/Th1 ratio and the Pittsburgh Sleep Quality Inventory score was significant ($r = .34$, $p < .01$). No other analyses were significant, although the Brief Pain Inventory severity ($r = .19$, $p = .046$) and interference ($r = .20$, $p < .041$) scale scores approached significance.

Next, t-tests were used to examine whether psychiatric and employment status were associated with higher Th2/Th1 ratios. Separate t-tests were conducted with current psychiatric status, lifetime psychiatric status as the independent variables, and the Th2/Th1 ratio variable as the dependent variable. None of these analyses were significant; however, the analyses examining whether the presence of a current psychiatric disorder predicted higher levels of Th2/Th1 ratio approached significance ($t = -2.07$, $df = 112$, $p = .046$).

Next, the Th2/Th1 ratio was correlated with the neurocognitive measures using the Pearson's correlation coefficient. The neurocognitive tests included the California Verbal Learning Test total score, short delay free recall score, and long delay free recall score; the NES-2 total median latency and mean response time; the Rey-Osterreith Figure Drawing copy scaled score, immediate recall scaled score, and delayed recall scaled score; the Grooved Pegboard dominant hand and non-dominant hand scale scores; the Wechsler Adult Intelligence Scale Digit Span- scaled score, and the Trailmaking Test Trails A and B, time in seconds. None of the neurocognitive tests were significantly correlated with the Th2/Th1 ratio.

Next, salivary cortisol levels were examined to determine whether an association existed between cortisol levels and the Th2/Th1 ratio. First, the mean daily cortisol levels and the raw regression slope for the daily cortisol levels were separately correlated with the Th2/Th1 ratio, using Pearson's correlation coefficient. The mean daily cortisol levels were significantly correlated with the Th2/Th1 ratio ($r = .29$, $p < .01$). In addition, the physician's clinical classification of cortisol results into either 'normal' or 'abnormal' were examined to see whether they predicted higher levels of the Th2/Th1 ratio. A t-test was run, using the clinical cortisol classification as the predictor variable, and the Th2/Th1 ratio as the dependent variable. This t-test analysis was nonsignificant.

Next, the association between the Th2/Th1 ratio and symptom severity rated on a 0–100 point scale was examined using Pearson correlation coefficients. Fatigue, sore throat, lymph node pain, joint pain, muscle pain, unrefreshing sleep, memory and concentration difficulties, headaches, and post-exertional malaise, muscle weakness, nausea, and fever/chills were examined in these analyses. None of the Pearson correlation coefficients between the symptom severity ratings and the Th2/Th1 ratio were significant.

Finally, the association between the Th2/Th1 ratio was compared to various other illness parameters. Duration of CFS in years was compared separately to the Th2/Th1 ratio using the Pearson correlation coefficient, and these analyses were not significant. Further, whether a person had a sudden (i.e. one month or less) or gradual onset of illness and whether they met the criteria for fibromyalgia were used as independent variables in separate t-tests, with the Th2/Th1 ratio as the dependent variables. While these t-tests were not statistically significant, the analysis examine whether the presence of fibromyalgia predicted higher levels of Th2/Th1 ratio approached significance ($t = -2.049$, $df = 112$, $p = .043$).

## Discussion

This study attempted to examine evidence of a shift toward a Th2 immune response, as defined by cell surface phenotyping. Higher levels of the Th2/Th1 ratio were used to indicate the presence of a shift of general immune responsiveness towards the Th2 pathway, as measured by the high levels of Th2 memory cells in comparison to the Th1 memory cells. After examining the relationship between the Th2/Th1 ratio and a wide range of psychosocial and physical variables, few associations were found. Contrary to what was hypothesized, there was no relationship between the Th2 shift and neurocognitive findings.

Further, a shift towards Th2 was not associated with other key illness parameters including stress, disability level, depression, anxiety, or symptom severity.

However, higher mean salivary cortisol levels and a shift towards Th2 immune responsiveness were found to be associated. This finding is consistent with other research studies that found cortisol levels to be related to higher Th2 functioning in individuals with respiratory infections (Pinto, Arredondo, Bono, Gaggero, & Diaz, 2006) and tuberculosis (Kelestimur, 2004). Similarly, Capuron & Dantzer (2003) noted that, among depressed patients, proinflammatory cytokines have been associated with increased activity of the hypothalamic-pituitary-adrenal axis.

In addition, higher levels of sleep difficulties were associated with the shift towards the Th2 pathway. This indicates that individuals with a shift towards Th2 response might have more severe sleep difficulties because of an interrelationship between sleep and immune parameters. For example, some researchers have noted that, in animals, proinflammatory cytokines such as IL-2, IL-1β, and TNF-α may enhance sleep; and immunosuppressive cytokines, including IL-4 and IL-10, may decrease sleep (Marshall & Born, 2002). In the current investigation, the global score on the Pittsburgh Quality Sleep Inventory was used to a measure of severity of sleep difficulties. This global score may include many different types of sleep disturbances, including insomnia, hypersomnia, frequent awakening, or unrefreshing sleep, so whether a Th2 shift was associated with increased or decreased sleep is not clear. However, many individuals with CFS report an increased need for sleep, including frequent naps, even though this sleep is not refreshing or restorative; further, this increased need to sleep is perceived to be disruptive and may affect their ability to function physically. Thus, these findings may be consistent with the research literature, in that individuals with CFS with a higher Th2 shift may be experiencing an increased amount of disturbed sleep. However, future research should more fully explore the link between sleep and these immune parameters.

In addition, adrenal corticosteroids are known to be associated with sleep (Dimitrov, Lange, Fehm, & Born, 2004; Buckley & Schatzberg, 2005). For example, some researchers note that acute cortisol administration increases slow-wave sleep and inhibits REM sleep (Steiger, 2002; Breslau, 2006). Also, cortisol production occurs on a circadian rhythm. Basal cortisol production is highest 45 minutes after first awakening, decreases throughout the day, and tends to be very low at the point of sleep onset (Buckley & Schatzberg, 2005). Thus, there may be an interrelationship among the three variables of sleep, cortisol levels, and Th2 immune responsiveness, although the direction of associations cannot be fully ascertained in the current study because of its cross-sectional design.

Endrocrine abnormalities have consistently been reported among individuals with CFS. The most frequently reported abnormality is reduced cortisol production; however, the current study found that higher, not lower, levels of cortisol output were associated with immune changes. This may have been due to the heterogeneity of the CFS diagnosis. This study did not subgroup individuals with CFS, and it might be that subgroups exists such that some individuals with poorer immunological functioning might have more intact HPA axis functioning, and those with reduced cortisol production might have fewer immunological abnormalities. An alternative explanation might that, even within the same individual, immunological and endocrine changes may be vary over time and some changes might be more prominent at different phases of illnesses. Further, if these changes occur in a cyclical manner, which might be expected given that both cytokine production and hormonal shifts occur using feedback mechanisms, statistical methods that attempt to discover relatively simple linear relationships would not be adequate to uncover more complex, nonlinear relationships.

Next, the findings suggest there might be a weak association between a Th2 shift and pain issues, and Th2 and psychiatric status, as these findings approached the significance level. The associations between the Th2/Th1 level and pain severity, pain interference, and fibromyalgia status all approached significance. Taken together, these suggest that there may be a weak association between pain and Th2 responding. Individuals who are more prone toward Th2 response may be experiencing more general pain. Another finding that approached statistical significance was that individuals with a current psychiatric disorder also exhibited a more pronounced Th2 shift. This is consistent with research studies that found an association between the Th2 shift and psychiatric disorders such as major depression (Schwartz, et al., 2001), but these results are not consistent with Skowera et al. (2004) who found that a Th2 shift was not related to psychiatric status in CFS patients. However, these findings should be interpreted with caution, as they only approached, but did not meet, the a priori .01 significance; further research should examine whether there is indeed a connection between pain or psychiatric disorders and a shift towards Th2 responsiveness.

Contrary to the study hypotheses, the Th2/Th1 ratio was not associated with any of the neurocognitive testing results. This is not consistent with results reported by Kozora et al. (2005) and Patarca-Montero, Antoni, Fletcher, and Klimas (2001). However, it should be noted that Kozora et al. (2005) reported mixed results, in that higher levels of IL-1β was related to lower verbal fluency, but better performance in visuomotor speed, visuomotor sequencing, and visual learning were related to higher levels of Il-1β, IL-6, and IFN-α. While this study did include measures of visuomotor speed, visuomotor sequencing, and visual learning, it did not include a measure of verbal fluency. Further, a limitation of this study was that cytokines such as IL-1β, IL-6, or IFN- α were not measured directly, such that changes in neurocognitive functioning might be tied more directly towards specific cytokine levels and less to more global measures of a shift towards Th2 responsiveness.

Finally, contrary to what was hypothesized, individuals who demonstrated a more extreme Th2 shift did not exhibit more severe levels of fatigue or other key symptoms of CFS; nor did they exhibit poorer overall physical functioning. Similarly, duration and onset of illness was not associated with a shift toward Th2 responsiveness. Little is known about whether this bias towards immune responsiveness might change over time; and these results suggest that this Th2 shift might not vary over time; however, in order to be statistically significant, these results would have had to have demonstrated a linear relationship. As noted above, if changes in Th2 bias change over time in a cyclical or non-linear fashion, then these changes would not have been reflected in the current results. Longitudinal studies examining changes in the Th1/Th2 pathway over time are needed to more fully explore how immune system changes might occur during the course of the illness.

In the future, it would be of value to investigate cytokine production in this population in the face of an apparent Th2 bias. The continued production of IL-4 by Th2 cells could reinforce a Th2 bias through suppressing effects on cytokines necessary to convert cells to a pro-inflammatory state (namely IL-12). Continued expression of Il-4 could also contribute the production of antibody and immunoglobulin. This relationship however is speculative without evidence as to the cytokine/chemokine profile of these cells.

Several limitations exist that may affect the generalizability of results. First, the number of comparisons conducted during the statistical analyses increased the possibility of type I error. In order to minimize type I error, the significant level was set at .01, but it is still possible that the significant results observed in this study occurred by chance. In addition, the present investigation did not directly measure the cytokines that are part of the Th2 or Th1 pathway. The current investigation examined indirect evidence of Th2 shift using T-

helper memory cells, but less is known about how T-helper memory cells are activated and maintained when compared to the knowledge regarding the production of cytokines. In addition, the current study did not investigate whether subgroups of patients existed in the current sample—the heterogeneity of the study participants might have made it harder to obtain statistically significant results, as it is possible that some of the proposed hypothesis in fact might have been applicable only to specific subsets of persons with CFS.

Finally, the current investigation did not look examine changes in these illness parameters over time; longitudinal studies using repeated sampling of these measures likely would provide a more accurate description of how these variables might be related to one another, and would also describe how these abnormalities might change over time.

In summary, these findings provide evidence for a link between immune system responding and cortisol production. Further, these findings suggest that immune system response might be associated with the observed illness parameters, including sleep difficulties. Given the exploratory nature of this study, further research is needed to more fully understand the potenteial associations between Th2 immune response with sleep difficulties and cortisol output to determine the physiological parameters that might underlie these findings.

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