

Edith Cowan University
Research Online

ECU Publications Post 2013

2016

Reductions in circulating levels of IL-16, IL-7 and VEGF-A in myalgic encephalomyelitis/chronic fatigue syndrome

Abdolamir Landi

David Broadhurst

Edith Cowan University, d.broadhurst@ecu.edu.au

Suxanne D. Vernon

D. Lorne J. Tyrell

Michael Houghton

Follow this and additional works at: <https://ro.ecu.edu.au/ecuworkspost2013>



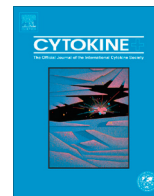
Part of the [Investigative Techniques Commons](#)

[10.1016/j.cyto.2015.11.018](https://ro.ecu.edu.au/ecuworkspost2013/2016)

Landi, A., Broadhurst, D., Vernon, S. D. Tyrell, S. L. J., & Houghton, M. (2016). Reductions in circulating levels of IL-16, IL-7 and VEGF-A in myalgic encephalomyelitis/chronic fatigue syndrome. *Cytokine*. 78. 27-36. Available [here](#)

This Journal Article is posted at Research Online.

<https://ro.ecu.edu.au/ecuworkspost2013/2016>



Reductions in circulating levels of IL-16, IL-7 and VEGF-A in myalgic encephalomyelitis/chronic fatigue syndrome



Abdolamir Landi^{a,*}, David Broadhurst^b, Suzanne D. Vernon^c, D. Lorne J. Tyrrell^a, Michael Houghton^{a,*}

^a Li Ka Shing Institute of Virology, Department of Medical Microbiology and Immunology, Canada

^b Department of Medicine, Katz Group Centre for Pharmacy & Health, University of Alberta, Edmonton, AB T6G 2E1, Canada

^c Bateman Horne Center, 1002 E. South Temple, Suite 408, Salt Lake City, UT 84102, USA

ARTICLE INFO

Article history:

Received 13 August 2015

Received in revised form 2 November 2015

Accepted 15 November 2015

Available online 28 November 2015

Keywords:

Chronic fatigue syndrome

Cytokine

Growth factor

Chemokine

Diagnostic

ABSTRACT

Recently, differences in the levels of various chemokines and cytokines were reported in patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) as compared with controls. Moreover, the analyte profile differed between chronic ME/CFS patients of long duration versus patients with disease of less than 3 years. In the current study, we measured the plasma levels of 34 cytokines, chemokines and growth factors in 100 chronic ME/CFS patients of long duration and in 79 gender and age-matched controls. We observed highly significant reductions in the concentration of circulating interleukin (IL)-16, IL-7, and Vascular Endothelial Growth Factor A (VEGF-A) in ME/CFS patients. All three biomarkers were significantly correlated in a multivariate cluster analysis. In addition, we identified significant reductions in the concentrations of fractalkine (CX3CL1) and monokine-induced-by-IFN- γ (MIG; CXCL9) along with increases in the concentrations of eotaxin 2 (CCL24) in ME/CFS patients. Our data recapitulates previous data from another USA ME/CFS cohort in which circulating levels of IL-7 were reduced. Also, a reduced level of VEGF-A was reported previously in sera of patients with Gulf War Illness as well as in cerebral spinal fluid samples from a different cohort of USA ME/CFS patients. To our knowledge, we are the first to test for levels of IL-16 in ME/CFS patients. In combination with previous data, our work suggests that the clustered reduction of IL-7, IL-16 and VEGF-A may have physiological relevance to ME/CFS disease. This profile is ME/CFS-specific since measurement of the same analytes present in chronic infectious and autoimmune liver diseases, where persistent fatigue is also a major symptom, failed to demonstrate the same changes. Further studies of other ME/CFS and overlapping disease cohorts are warranted in future.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Myalgic encephalomyelitis (ME) or chronic fatigue syndrome (CFS) is a complex and severely debilitating disease characterized by profound fatigue lasting for more than six months, post-exercise malaise, unrefreshing sleep, chronic pain, and cognitive dysfunction. It is more common in middle-age women with an estimated prevalence of 0.8 million people in the USA and 0.4–1% in the world [1,2]. There is no laboratory test for diagnosis and no approved cure for the illness. Diagnosis has been difficult,

subjective and controversial leaving an urgent need for an objective laboratory-based diagnostic test for this very challenging illness. Currently, the disease is diagnosed solely by subjective clinical symptoms [3] and evidence for an infectious etiology has been controversial [4–8]. There is some evidence suggesting that the patients might suffer from a neuro-immune disorder [9] with neuroinflammation in the brain [10]. Alterations in the frequency and function of immune cells such as B cells and NK cells have been also reported [11,12]. Abnormalities in the concentrations of some circulating cytokines and chemokines have been previously reported [13–18] including three very recent studies of plasma and cerebrospinal fluid (CSF) samples from ME/CFS patients [19–21]. However, so far, there has been little confirmation of these abnormal analytes between different ME/CFS patient groups. The objective of the current study was to measure the levels of various chemokines, cytokines and growth factors in the plasma of 100 ME/CFS patients from the USA along with 79 gender

* Corresponding authors at: Li Ka Shing Institute of Virology, Department of Medical Microbiology & Immunology, University of Alberta, Faculty of Medicine & Dentistry, 6-010 Katz Group-Rexall Centre for Health Research, T6G 2E1 Edmonton, AB, Canada.

E-mail addresses: landi@ualberta.ca (A. Landi), michael.houghton@ualberta.ca (M. Houghton).

and age-matched controls to see if any consensus with previous studies could be identified.

2. Materials and methods

2.1. Patients and sample collection

The collection and analysis of clinical information and biological samples by the Solve ME/CFS BioBank was ethically approved by the Genetic Alliance ethics review board, which approved all procedures for obtaining written informed consent from all subjects to participate in this study. A total of 100 samples from ME/CFS patients and 79 from healthy controls were available from a bigger cohort [22] in the Solve ME/CFS BioBank and studied in blinded fashion. The Solve ME/CFS BioBank samples were selected from five, geographically distinct clinical sites from long-term patients of expert clinician, who are specialized in the diagnosis and management of ME/CFS. A subject was excluded if they had a body mass index >40, an immunosuppressive disorder including, but not limited to cancer, severe infections, HCV, or HIV. In addition, subjects were excluded if they had a history of substance or alcohol abuse <2 years before onset of ME/CFS or were mentally or legally incapacitated at the time of collection. Most of the exclusionary factors are a part of ME/CFS case definition criteria. Healthy control participants were geographically co-localized (same neighborhood or region; but not residing in the same household or of close relation to a ME/CFS subject); they were also matched to ME/CFS subjects by age, sex, race and BMI. Patients with primary mental disorders (depression, bipolar mental disorder, and schizophrenia) were excluded as per ME/CFS case definition. ME/CFS diagnosis was based on the Fukuda and/or the Canadian Consensus clinical case definition [23,24].

The blood was collected in heparinized tubes and was sent to the Rutgers University Cell and DNA Repository at ambient temperature via overnight shipping. Plasma was collected after centrifugation, dispensed into 0.2 ml aliquots and stored at -80°C until analysis. Processing of blood samples followed guidelines approved by Rutgers University (Newark, NJ, USA). Analytes were also measured in chronic infectious and autoimmune liver diseases where persistent fatigue is also a major symptom.

2.2. Analysis of cytokines/chemokines and growth factors

Pre-coated multiplex ELISA plates from Meso Scale Discovery (MSD, Gaithersburg, MA, USA) were selected for assays after a rigorous comparison with other technologies for their sensitivity at low concentrations, linearity and high dynamic range of the standard curves, as well as inter-plate low variability. MSD Human V-PLEX Plus Kits employed in this study included Chemokine Panel 1, Cytokine Panel 1, and Pro-inflammatory Panel 1; In addition, Human Eotaxin-2 Kit, a custom-designed 3-Plex kit, and a custom-designed 1-Plex kit were used. The list included eotaxin 1 (CCL11), eotaxin 2 (CCL24), eotaxin-3 (CCL26), IL-8, interferon gamma-induced protein 10 (IP-10; CXCL10), monocyte chemoattractant protein-1 (MCP-1; CCL2), monocyte chemoattractant protein-4 (MCP-4; CCL13), macrophage-derived chemokine (MDC; CCL22), macrophage inflammatory protein-1-alpha (MIP-1 α ; CCL3), macrophage inflammatory protein-1-beta (MIP-1 β ; CCL4), thymus and activation regulated chemokine (TARC; CCL17), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL12/23p40, IL-15, IL-16, IL-17A, IL-1 α , IL-5, IL-7, tumor necrosis factor beta (TNF- β), Vascular Endothelial Growth Factor A (VEGF-A), interferon (IFN)- γ , IL-10, IL-12p70, IL-13, IL-1 β , IL-2, IL-4, IL-6, TNF- α , fractalkine (CX3CL1), monokine induced by IFN- γ (MIG; CXCL9), macrophage inflammatory protein 3 beta (MIP-3 β ; CCL19), and

β_2 microglobulin. Frozen plasma samples from healthy controls and CFS patients were assayed in duplicate immediately after the first thaw. All experiments were performed according to the manufacturer's instructions with minimal modifications and optimization as described previously [25]. Briefly, 50 μl of each 1:2 diluted sample was added to each well of the pre-coated 96-well plate and incubated at room temperature (RT) for 2.5 h with continuous counter-clockwise shaking. The plates were then washed three times with $1 \times$ Wash Buffer (MSD) and Sulfotag Detection Antibody Cocktail (MSD) was then added to each well and the plates then incubated for an additional 2 h with shaking at RT. Finally, the plates were washed again, and were scanned by a SECTOR[®] Imager 6000 Reader (MSD) after adding 150 μl of $2 \times$ Read Buffer (MSD).

2.3. Data pre-processing

To accurately quantify the concentrations of each analyte in the test samples, a four-point logarithmic standard curve is produced using the MSD Discover Workbench V4 software as developed by the Meso Scale Discovery Company. According to the plate manufacturer and software developer, this kind of curve is optimized to produce the most accurate standard curves for the cytokine study. The validation and optimization was done in line with a commonly accepted method of "Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement" [26], which is published and recommended by major diagnostic companies.

Intra-CVs were calculated for each plate using the values from all samples duplicates and the average of all the plates are reported for each analyte. To measure the Inter-CVs, we added four control samples at different concentrations to each plate. The LOD was calculated based on the mean of negative control samples +2.5 standard deviations (SD). The LLOQ was set to the lowest standard sample value on the linear part of the standard curve, where its "Mean Recovery Value" was within 20% of the actual value (accuracy of 20%) with a CV of 20% (precision of 20%) for the given standard curve point. When the signals obtained by the scanner were plotted against the calculated concentrations for each standard and control sample (data not shown), any adverse matrix effect was ruled out as the concentration curves for both standard and control samples were highly correlated, and comparable with the signals reported by the scanner. The determined concentrations were also corrected for dilution prior to statistical analysis.

2.4. Statistical analyses: A. Hypothesis testing

The data in each group (ME/CFS & Controls) for individual analytes were not normally distributed, thus for each analyte in turn, the null hypothesis that there was no difference in sample median values between ME/CFS and control groups was tested using the Mann-Whitney U test. Correction for multiple comparisons was performed using Storey's FDR methodology [27]. Both p -values and corrected p -values (q -values) are reported. The linear correlation between all reproducibly measured analytes was calculated using the non-parametric pairwise Pearson's correlation coefficient. The resulting correlation matrix is presented in the form of a spring-embedded correlation plot [28]. Here a network of "nodes" and "spring-edges" are constructed such that each node represents each of the tested analytes and the spring constant of each edge is proportional to the correlation coefficient between two connected nodes. The size of each node is proportional to significance of that variable; the larger the node the lower the q -value (the corrected p -values). Edges were only included in the network if the correlation coefficient was positive, and significant at a critical p -value of 0.05. Once the network is constructed it is allowed to "relax". That is, the connected spring-edges compete against each other to pull the nodes in a given direction based on the

Table 1
Number, gender, age, and MFI/RAND36 scores of subjects in the study.

	Controls	CFS/ME
Number of subjects	79	100
Gender (F/M)	62/17	72/28
Age (mean ± SD)	50.4 ± 12.6	50.5 ± 12.6
MFI (mean ± SD)	37.4 ± 14.5	77.8 ± 12.7
RAND36 (mean ± SD)	87.1 ± 9.2	37.9 ± 13.9

Controls = Healthy controls, **ME/CFS** = Myalgic encephalomyelitis/chronic fatigue syndrome, **SD** = Standard deviation, **M** = Male, and **F** = Female, **MFI** = Multidimensional fatigue inventory score (the higher, the more fatigue), **RAND36** = The RAND 36-item health survey (The higher, the healthier).

spring constant (the higher the correlation, the stiffer the spring, and hence the more power organizing the clustering of the node). Once relaxed (i.e. the model is in a low energy configuration) the spring embedded plot can be viewed as a simple multivariate cluster analysis, where nodes that cluster close to each other can be considered to be highly correlated in a multivariate sense. Node color directly maps both the level of significance and whether the median ME/CFS concentration was higher or lower than median control (Red = $p < 0.05$ and ME/CFS > Control; Orange = $p < 0.1$ and ME/CFS > Control; Blue = $p < 0.05$ and Control > ME/CFS; Light Blue = $p < 0.1$ and Control > ME/CFS; nodes were colored gray when their corresponding p -value was > 0.1). Networks were constructed using the graph visualization software–Graphviz (www.graphviz.org) using the ‘neato’ virtual physics model [29].

Table 2
Detailed information and statistics for the thirty-four analytes that are investigated. Gray rows are the biomarkers with significant changes after p -value correction. The q -values refer to the corrected p -value for gender and age in multiple comparisons.

	p -value	q -value	Median value (pg/ml)		Up/Down in CFS	Intra CV (%)	Inter CV (%)	Lower limit of detection		Lower limit of quantification	
			CFS/ME	Control				Value (pg/ml)	% <LLOD	Value (pg/ml)	% <LLOQ
IL-16	9.38E-09	2.35E-07	403	767	Down	8	11	2	0	19	0
IL-17A	2.79E-05	3.49E-04	1.07	1.54	Down	24	19	2.2	84	2.4	87
IL-7	2.74E-04	0.002	10.9	12.6	Down	9	9	0.4	0	1.4	0
TNF-β	0.003	0.02	0.1	0.13	Down	23	14	0.12	53	1.2	100
CCL24	0.007	0.03	1530	1172	Up	6	8	2.4	0	24	0
CXCL9	0.006	0.03	339	427	Down	9	18	0.4	0	4.8	0
CX3CL1	0.01	0.04	3095	3482	Down	9	6	21.6	0	195.4	0
VEGF-A	0.01	0.05	88.4	107	Down	8	10	2.4	0	7.6	0
CCL19	0.04	0.10	97	89.3	Up	8	5	0.2	0	4.8	0
CCL11	0.05	0.11	714	646	Up	3	8	3.6	0	24.6	0
IL-1β	0.04	0.11	0.42	0.81	Down	23	7	0.2	27	1	64
TNF-α	0.06	0.12	3.42	4.03	Down	8	8	0.12	0	0.6	0
CCL3	0.10	0.19	34.3	50.1	Down	5	10	4.8	0	18	12
CCL17	0.14	0.25	105	116	Down	4	6	0.6	0	1.6	0
CCL2	0.22	0.36	292	276	Up	6	9	0.2	0	0.6	0
IFN-γ	0.27	0.42	5.45	6.46	Down	8	15	0.8	1	2.6	7
IL-15	0.34	0.50	0.92	0.99	Down	12	9	0.4	2	1.4	91
CCL26	0.44	0.58	275	272	Up	7	18	3.8	0	20.4	0
IL-6	0.43	0.60	1.15	1.37	Down	13	23	0.2	2	1.6	56
IL-12/23p40	0.92	0.70	115	114	Up	6	12	0.6	0	1.4	0
CCL22	0.98	0.70	1209	1217	Down	5	9	9	0	44.2	0
IL-5	0.97	0.71	0.34	0.34	Up	36	10	0.8	92	6.2	100
CCL13	0.91	0.71	354	377	Down	3	8	3.4	0	2.6	0
IL-1α	0.91	0.73	0.26	0.26	Down	23	10	10	99	0.6	86
CCL4	0.89	0.74	181	199	Down	5	8	0.8	0	4.6	0
GM-CSF	0.84	0.75	0.21	0.24	Up	30	11	0.4	83	2	97
IL-10	0.87	0.75	0.5	0.57	Down	19	19	0.08	0	0.6	56
IL-4	0.82	0.76	0.11	0.08	Down	29	11	0.1	61	0.4	94
IL-13	0.72	0.79	4.2	4.72	Down	26	9	1.8	22	4.2	50
IL-2	0.80	0.80	0.32	0.33	Down	44	8	0.2	37	0.8	78
CXCL10	0.70	0.80	283	289	Down	5	22	0.2	0	2.8	0
IL-12p70	0.68	0.81	0.35	0.27	Down	32	9	0.4	63	3.4	99
IL-8	0.79	0.83	331	374	Down	4	10	0.16	0	1.4	0
β2M	0.68	0.84	2.51	2.66	Down	9	15	0.02	7	0.04	7

2.5. B. Multivariate data analysis

To investigate the potential utility of combining multiple analytes into a single model predictive of ME/CFS, two techniques were compared: LASSO-LR and CART. Logistic Regression is a type of probabilistic statistical classification model commonly used for predicting the outcome of a categorical dependent variable (in this case ME/CFS vs. Control), and can be considered as a special case of a generalized linear model such that, $logit(p_i) = \beta_0 + \beta_1 x_{1,i} + \beta_2 x_{2,i} \dots + \beta_m x_{m,i}$ (where, p_i is the predicted probability of positive classification for the i th patient, $x_{1,i} \dots x_{m,i}$ are the m analyte measurements for the i th patient, is the regression constant, and $\beta_1 \dots \beta_m$ are regression coefficients indicating the relative influence of a particular analyte on the outcome). In complete contrast, CART is a non-parametric decision tree model that produces (in this case) a classification tree as a predictive model. A decision tree can be translated into a simple set of logical rules for classification such as: *if* ($x_{1,i} > a$) *AND* ($x_{2,i} < b$) *then* $y_i = 1$, *else* $y_i = 0$.

Both methodologies were optimized in order to produce a robust and parsimonious model. The LR model was optimized using LASSO regularization [30], and the CART model was optimized using tree “pruning” [31]. For each method, 5-fold cross-validation with 100 Monte Carlo repetitions was performed during the optimization to ensure the avoidance of “over fitting” (i.e. ensuring the model is generalizable for future testing with new independent samples). The resulting optimal classifier models were assessed using Receiver Operator Characteristic (ROC) curve analyses. This allows determination *a posteriori* of the optimal

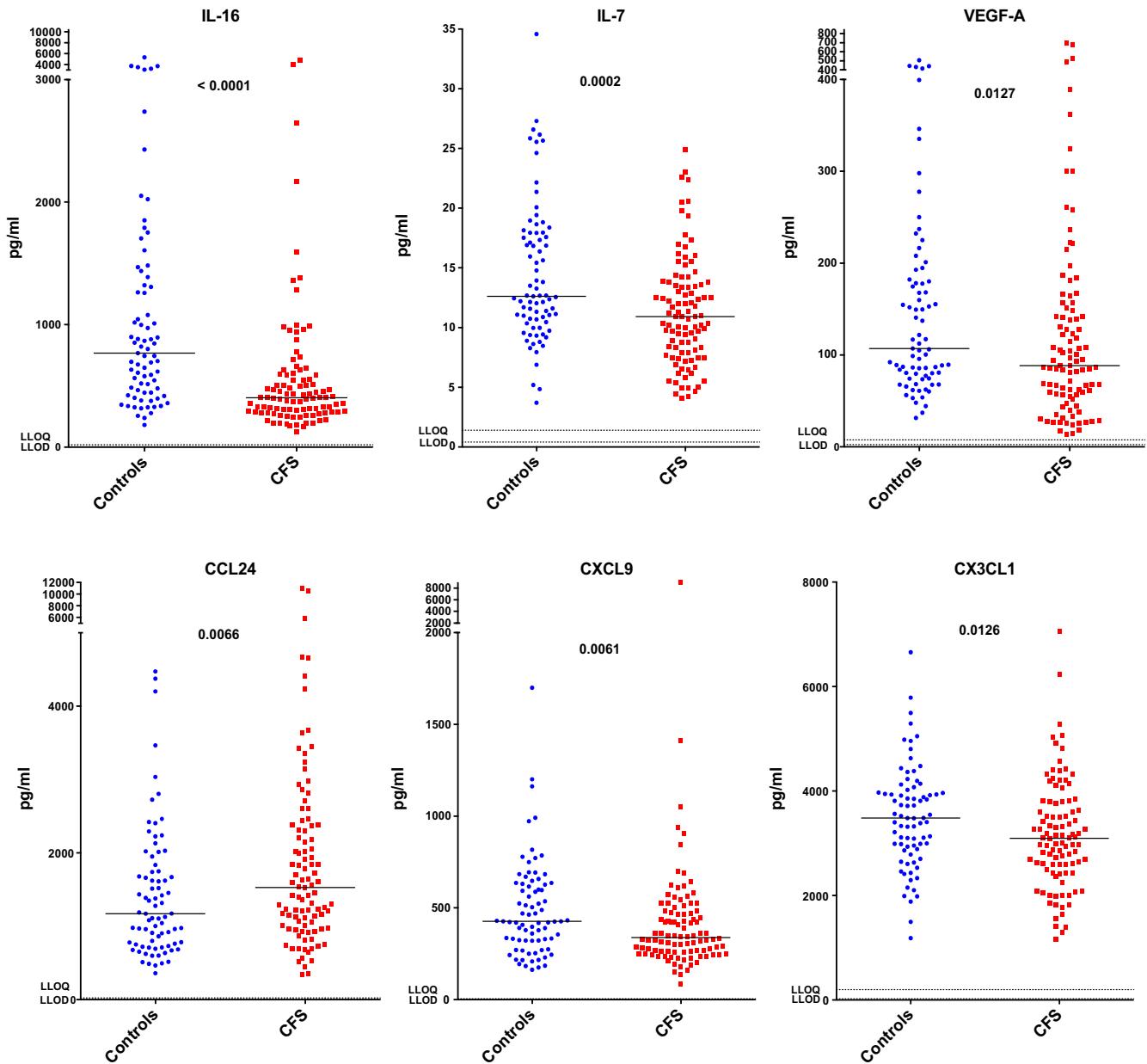


Fig. 1. The level of cytokines, chemokines and growth factors that are significantly altered in the patient group as compared to healthy controls. The horizontal lines are medians. The numbers show the p value for each analyte. CFS = Myalgic encephalomyelitis/chronic fatigue syndrome; LLOD = Lower level of detection; LLOQ = Lower level of quantification.

“decision boundary” (the predictive score determining whether a sample is classified as ME/CFS rather than control) and the associated optimal classification sensitivity and specificity. The AUC is used as a generalized non-parametric estimate of biomarker utility (AUC = 1 implies a perfect classifier; AUC = 0.5 implies a model which is no better than flipping a coin to determine outcome). Bootstrap resampling was performed ($n = 500$) to estimate the 95% confidence interval (CI) for both the AUC, and a given model's optimal sensitivity given a fixed specificity.

All statistical analysis was performed using Matlab[®] scientific scripting language, version R2014b (<http://www.mathworks.com>). The Logistic Regression model was verified using STATA[®] statistical software, version 13 (<http://www.stata.com>).

3. Results

3.1. Study population

Plasma samples from 100 USA patients diagnosed with ME/CFS and 79 gender- and age-matched healthy controls were tested in this study. The details of the patient's characteristics are summarized in Table 1. There were no significant differences in gender or age between these two groups. As expected, the multidimensional fatigue inventory (MFI) and RAND 36, a 36-item health survey identical to that of MOS SF-36, which provides health status [32] scores were significantly different in ME/CFS patients compared to healthy controls (p values of <0.0001) (Table 1).

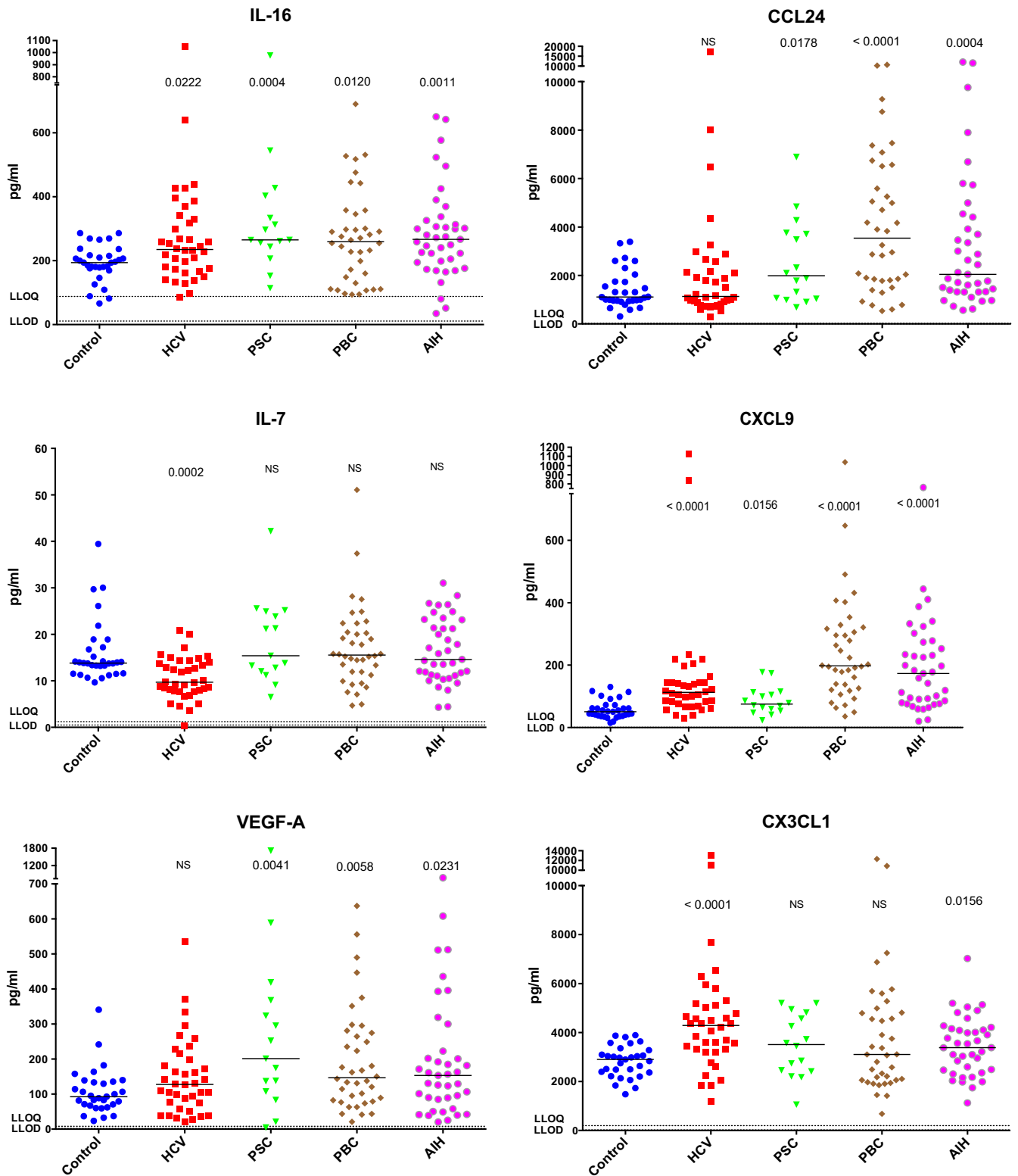


Fig. 2. The level of cytokines, chemokines and growth factors in chronic liver diseases. The horizontal lines are medians. The numbers above each group show the *p* value for each analyte in comparison to control group. NS = Not significant; HCV = Chronic hepatitis C; PSC = Primary sclerosing cholangitis; PBC = Primary biliary cholangitis; AIH = Autoimmune hepatitis; LLOD = Lower level of detection; LLOQ = Lower level of quantification.

3.2. Univariate analysis

The concentration of 34 plasma cytokines, chemokines and growth factors were measured in each test subject using Multiplex Elisa Assays. Table 2 describes each measured analyte, the measured median concentrations, the statistical difference

between ME/CFS and control samples, the intra- and inter-assay coefficient of variance (Intra-/Inter-CVs) and the lower level of detection and quantification (LLOD and LLOQ).

Analytes with measured concentrations below the LLOQ were excluded from subsequent data analyses. After correction for multiple comparisons, univariate hypothesis testing revealed 6

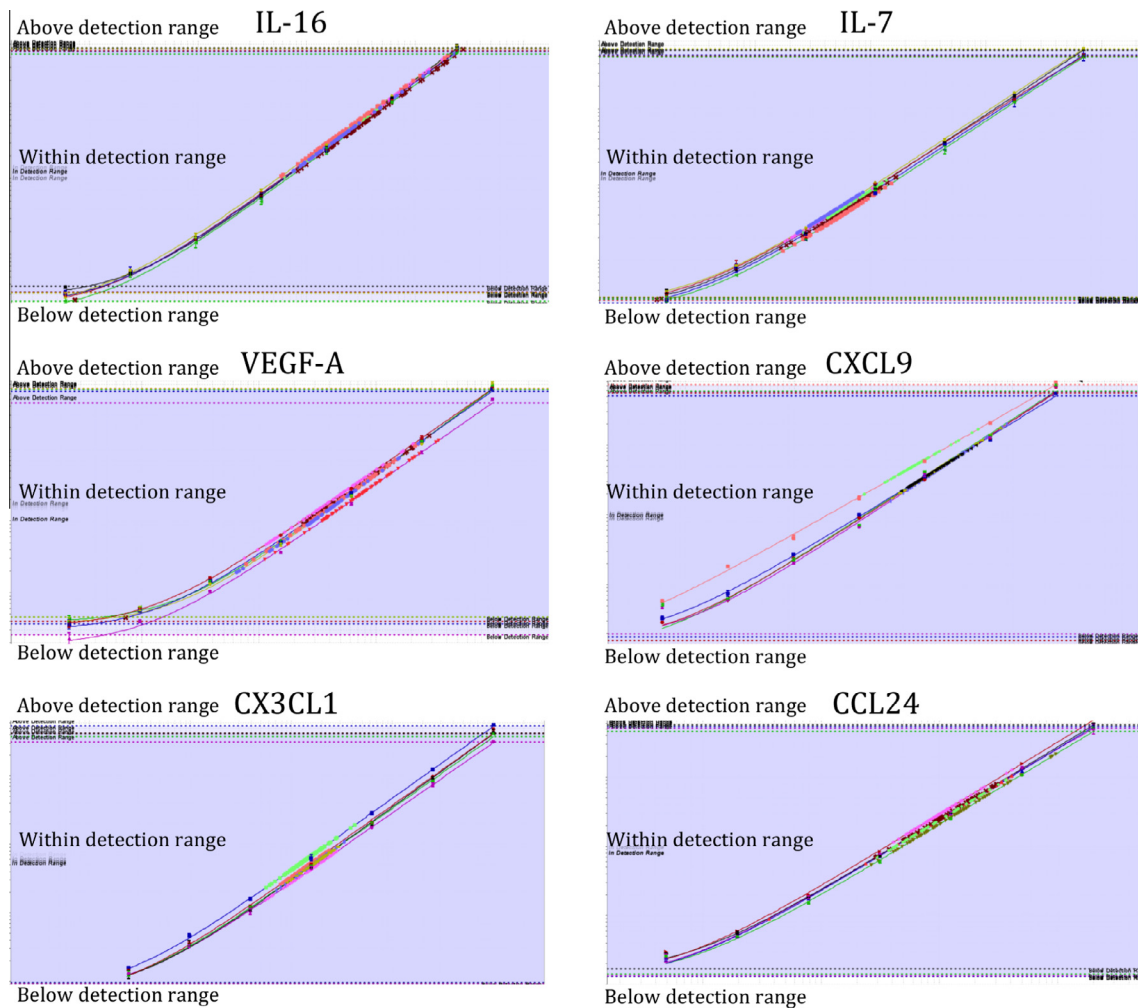


Fig. 3. The overlapped standard curves from individual plates show minimal inter-plate variation and support the validity and accuracy of comparing the measured concentrations from different plates between study groups. The data point for each replicate are also shown that are located within the detection range of the standard curves. The above and bottom dotted lines are “Above Detection Range” and “Below Detection Range” limits, respectively.

analytes that had significantly different median group values (Fig. 1). Five analytes had significantly decreased levels in our ME/CFS patients compared with matched controls (IL-16, IL-7, VEGF-A, CXCL9, CX3CL1) and one analyte that was significantly increased (CCL24). When the same six analytes were measured in control patients with chronic HCV infections or autoimmune liver diseases, such as primary sclerosing cholangitis (PSC), primary biliary cirrhosis (cholangitis) (PBC), and autoimmune hepatitis (AIH), the pattern observed in ME/CFS patients was unique (Fig. 2). The standard curves calculated from different assay plates were very well overlapped indicating minimal inter-plates variation and the highly reproducible nature of the data (Fig. 3). Moreover, signal analysis showed a high level of consistency between plates with very low variations consistent with a minimal matrix effect in the assay (data not shown). This was confirmed by the very low Inter-CVs of 5–18% for the different measurements and the Intra-CVs values were also very low, varying from 3% to 9% for different analytes (Table 2).

3.3. Multivariate analysis

A spring-embedded correlation analysis of the 18 analytes above the LLOQ showed that the significantly different analytes associated into 3 clusters (Fig. 4). In particular, IL-16, IL-7 and VEGF-A were significantly down regulated and the most tightly correlated. The other two clusters had less significance; a cluster

comprising CCL24 and CCL11 and a cluster comprising CXCL9, CX3CL1, and CCL19.

Logistic Regression optimized by LASSO regularization (LASSO-LR) produced an optimal model using three analytes (IL-16, IL-7, and CCL24) that resulted in the following diagnostic regression model:

$$\text{logit}(y) = 3.98 - 1.24 \times \ln(\text{IL-16}) - 0.92 \times \ln(\text{IL-7}) + 0.89 \times \ln(\text{CCL24})$$

The corresponding Receiver Operator Characteristic (ROC) curve had an AUC (area under the ROC curve) of 0.79 (95% CI: 0.71–0.83) (Fig. 5a). However, for a fixed specificity of 94%, the corresponding sensitivity for predicting ME/CFS was 41% (95% CI: 0.29–0.49). The model statistics are described in Table 3.

The Classification And Regression Trees (CART) analysis produced two optimal decision trees using two series of three analytes. In the first one (CART-1), a combination of IL-16, CXCL9, and CCL19 was used that for a fixed specificity of 96%, the corresponding sensitivity for predicting ME/CFS was 43% (95% CI: 0.35–0.51) (Fig. 5b). The second CART model (CART-2) was based on IL-16, IL-7, and VEGF-A and showed a sensitivity of 46% for predicting ME/CFS for a specificity of 96% (Fig. 5c). These low sensitivities may reflect a broad underlying heterogeneity of ME/CFS disease as discussed elsewhere [33,34].

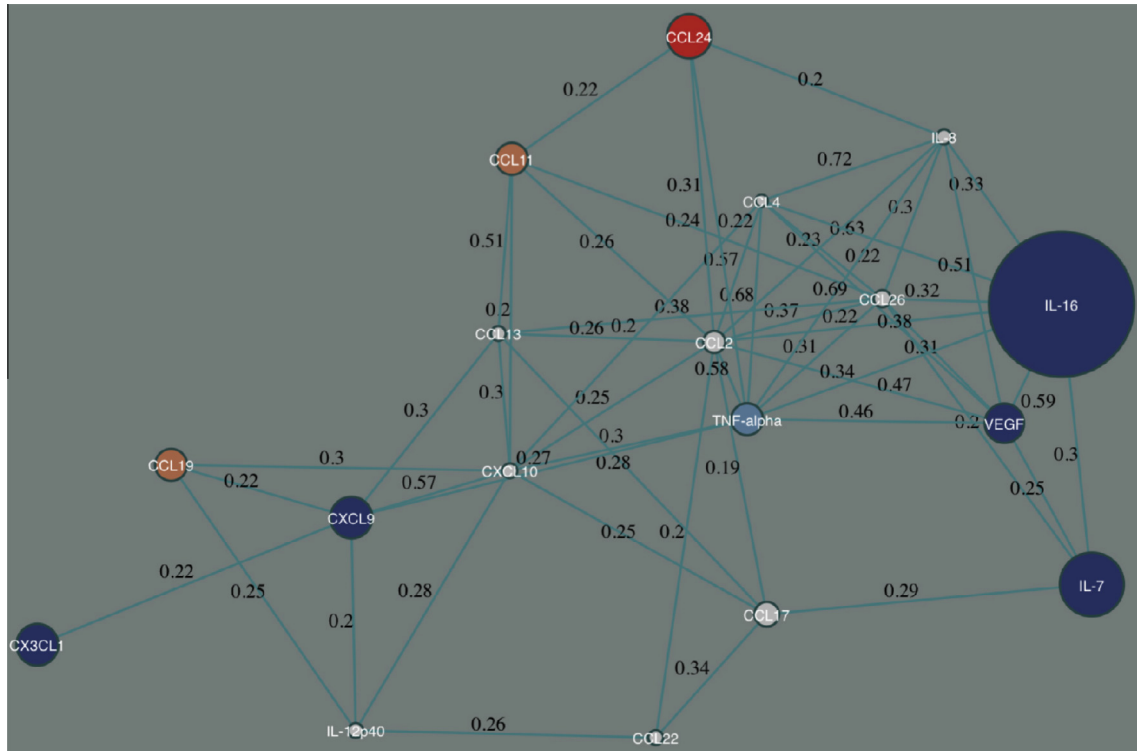


Fig. 4. The spring-embedded correlation plot as a simple multivariate cluster analysis. The larger the node, the lower the p -value and the higher the correlation, the stiffer the spring. Red = $p < 0.05$ and ME/CFS > Control; Orange = $p < 0.1$ and ME/CFS > Control; Blue = $p < 0.05$ and Control > ME/CFS; Light Blue = $p < 0.1$ and Control > ME/CFS; nodes were colored gray when their corresponding p -value was > 0.1.

4. Discussion

Our study revealed six analytes that were altered significantly in our ME/CFS patients versus gender- and age-matched controls. Of these, the most significant changes were reductions in the plasma concentrations of IL-16 and IL-7, both of which were significantly correlated with reductions in VEGF-A plasma concentrations in a multivariate cluster analysis (Fig. 4). These changes were specific to ME/CFS since they were not observed in patients suffering from chronic HCV infections or chronic autoimmune liver diseases where fatigue is also a major symptom (Fig. 2). While to the best of our knowledge, we are the first to test for IL-16 levels in ME/CFS patients, previous reports have also shown a reduction in circulating levels of IL-7 in a different USA cohort of ME/CFS patients [16] and very recently, VEGF-A was shown to be reduced in sera of patients with Gulf War Illness (a discrete subset of ME/CFS) [35] and in CSF samples from a different cohort of USA ME/CFS patients [20]. While the clustered reduction of these three analytes needs to be confirmed in plasma and CSF samples (logistically challenging) from other ME/CFS cohorts including other diseases cohorts, such as patients with primary depression and other mental disorders as controls, it is tempting to speculate on the possible physiological significance of our observed changes. We intend to conduct a study as soon as we can logistically collect enough CSF samples from ME/CSF patients.

Interleukin-16 is a unique pro-inflammatory cytokine with little sequence homology to other cytokines/chemokines. It is chemotactic for CD4⁺ T lymphocytes, monocytes, eosinophils, and is produced by epithelial cells, mast cells, lymphocytes, macrophages, synovial fibroblasts, eosinophils, and residing microglia in brain. Expression of CD4 receptor is required for mediating

IL-16 function. In the periphery, this interaction can specifically initiate an increase in intra-cytoplasmic inositol tri-phosphate (IP₃) and calcium, both of which are involved in muscle contraction [36]. IL-16 mRNA is constitutively expressed in CD4⁺ and CD8⁺ T cells, which is further induced upon exposure to antigens. It has been reported that IL-16 may repress HIV-1 replication and its serum level may drop during disease progression [37]. In addition, an important role for IL-16 in the early development of the human immune system has been described [38]. Low concentrations of IL-16 have also been suggested to be correlated to the impaired development of B cells within the bone marrow of thymic-deprived nude and old mice [39]. In contrast, high expression of IL-16 is linked to pro-inflammatory diseases such as asthma, rheumatoid arthritis, systemic lupus erythematosus, colitis, atopic dermatitis and MS [40–42]. This may indicate that the circulating level of IL-16 is under a rigorous control and its increase or reduction may result in immunopathology or immunodeficiency, respectively.

With respect to the central nervous system (CNS), the role of IL-16-secreting microglial cells in the development of the human fetal brain has suggested a critical role for IL-16 in neuronal development [43]. However, reports on neuroprotection by IL-16 is controversial as the microglial IL-16 up-regulation has been reported under inflammatory and degenerative conditions, while its constitutive, but low expression of that in normal brain has been reported [10,38]. Altogether, the lower plasma levels that we have observed in ME/CFS patients may possibly reflect a neuronal dysfunction possibly reflective of the well-known cognitive dysfunction and mental fog symptoms associated with ME/CFS disease [44].

IL-7 is a hematopoietic growth factor that can be secreted by a wide range of cells including stromal cells in the bone marrow,

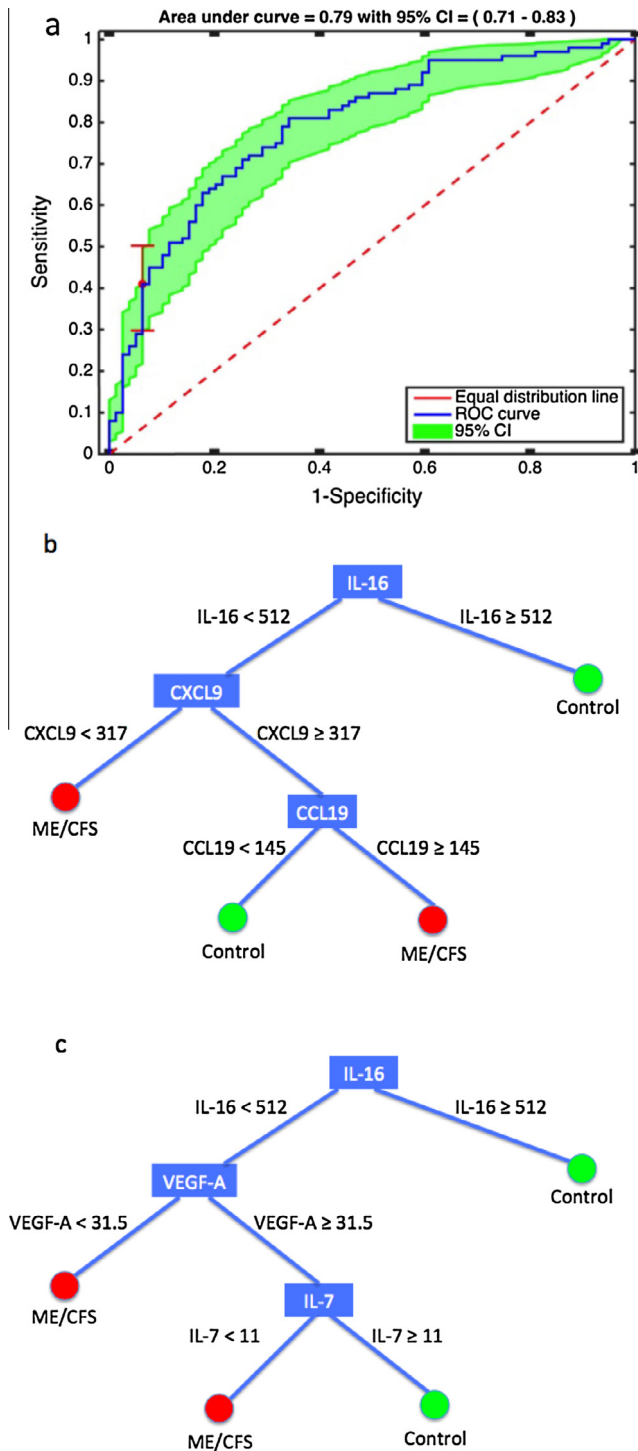


Fig. 5. Multivariate data analysis: (a) The corresponding Receiver Operator Characteristic (ROC) curve for Logistic Regression optimized by LASSO regularization (LASSO-LR) model shows an AUC (area under the ROC curve) of 0.79 with confidence interval of 95% (range of 0.71–0.83). (b) The optimal decision tree that is produced by Classification And Regression Trees (CART) model using three analytes (IL-16, CXCL9, and CCL19). (c) The optimal decision tree that is produced by Classification And Regression Trees (CART) model using three analytes (IL-16, IL-7, and VEGF-A).

thymus, hepatocytes, epithelial cells, and lymphocytes [45–47]. It is an indispensable interleukin for T cell, B cell, and NK cell proliferation and survival [48,49]. Age-related thymic atrophy and decreased thymopoiesis has been correlated with lower levels of IL-7 [50,51]. These processes result in a decline in T lymphocyte

Table 3
Logistic regression (LASSO-LR) model parameters and significance test.

Analyte	Coefficient	S.E.	z	P > z	[95% Conf. Interval]	
IL-16	−1.24	0.27	−4.53	5.84E−06	−1.78	−0.70
IL-7	−0.92	0.45	−2.04	0.041	−1.80	−0.04
CCL24	0.89	0.29	3.02	0.002	0.31	1.47
Constant	3.98	2.56	1.56	0.12	−1.04	9.01

output, lower peripheral T cells, and lower number of T cells in lymph nodes, which, in turn, may compromise the immune response and promote immunosenescence [52–54]. Indeed, the anti-apoptotic effects of IL-7 [55] may be responsible for the increase in the number and performance of T cells in animal models [56]. Higher levels of IL-7 are seen in chronic hepatitis C possibly indicating the chronic activation of the immune response. Interestingly, IL-7 is also expressed in developed brain neurons that may indicate its potential role in CNS-related diseases [57], especially as lower IL-7 has been correlated with cognitive decline during aging [50,51]. In turn, its reduction in ME/CFS may indicate a reduction in immune activation at least in a subgroup of ME/CFS patients along with a potential neuropathology that could mimic the process of aging.

VEGF-A is known to be produced by contractile pericyte cells that are wrapped around the endothelial cells of capillaries and venules and promote the survival and stability of endothelial cells [58]. It is a signaling protein known initially for its roles in stimulating vascular angiogenesis and muscle growth; however, subsequent findings have indicated an important neurotrophic and neuroprotective role for VEGF-A [59–62]. The elevated level of VEGF-A has been suggested as a surrogate marker for peripheral vascular disease in contrast to its angiogenesis effect [63]. However, a recent study has suggested the existence of different isoforms of VEGF-A, VEGF-A_{165a} and VEGF-A_{165b} that may explain these different biological effects. The elevated level of VEGF-A_{165b} that is seen in peripheral artery disease may have anti-angiogenic characteristics, which is opposite to the known angiogenic feature of VEGF-A that may be related to the VEGF-A_{165a} isoform [64]. The levels of VEGF-A mRNA and protein have also been shown to be significantly increased after exercise in humans and mice which may be due to the increase in blood flow in muscles [65]. This may suggest a role for VEGF-A in the post-exertional malaise and fatigue in ME/CFS. In addition to its peripheral effects, later findings have indicated an important neurotrophic and neuroprotective role for VEGF-A in both the periphery and in the central nervous system [59–62] suggesting that its depletion in ME/CFS may also contribute to the neurobiological phenotype of disease. An effect of VEGF-A on the blood brain barrier facilitating the entry of immunomodulators into the central nervous system has also been reported [66]. Interestingly, VEGF-A promotes neurogenesis by stimulating epithelial cells to release neurogenic signals such as brain-derived neurotrophic factor (BDNF) [67]; a factor that has recently been shown to be suppressed in PBMCs from ME/CFS patients in comparison to that of healthy controls [68]. As BDNF is also neuroprotective [69], the reduction of BDNF due to a decline of VEGF-A may inhibit the neurogenesis process. Administration of VEGF-A has also been shown to revert the cognitive impairment induced by focal traumatic brain injury [70] and to restore impaired memory behavior in a mouse model of Alzheimer's disease [71]. Down-regulation of VEGF-A mRNA in the CSF and PBMCs from patients with primary and secondary MS has also been reported [72,73].

Despite the urgent need for a serologic diagnostic for ME/CFS, no blood test that is reproducible and validated has yet been

developed for ME/CFS patients. The three algorithms described herein produced very similar predictive abilities (LASSO-LR: specificity of 94% and sensitivity of 41%; CART-1: specificity of 96% and sensitivity of 43%; CART-2: specificity of 96% and sensitivity of 46%), even though they are based on different components of the cytokine/chemokine/growth factor profile. The LASSO-LR model used analytes IL-16, IL-7, and CCL24, whereas the CART-1 model used analytes IL-16, CXCL9, and CCL19. The CART-2 model uses analytes IL-16, IL-7, and VEGF-A. When mapped on to the correlation plot (Fig. 4), the LASSO-LR model focuses on both the IL-16/IL-7/VEGF-A cluster and the CCL24/CCL11 cluster; whereas the CART model focused on the IL-16/IL-7/VEGF cluster and the CCL19/CXCL9/CX3CL1 cluster. It is clear that a central component of the ME/CFS biomarker profile is IL-16. If confirmed in other ME/CFS cohorts, these algorithms could greatly aid the diagnosis of some ME/CFS patients and potentially aid in their clinical management. To our knowledge, this is the first study that describes algorithms with defined sensitivities and specificities in predicting ME/CFS patients and there are no other studies to compare our sensitivities and specificities with.

In conclusion, our study shows significant changes in the circulating cytokine, chemokine and growth factor profile of ME/CFS patients from the USA and is consistent with previous studies of USA ME/CFS cohorts that also showed reductions in the circulating levels of IL-7 & VEGF-A levels [16,20,35]. In this study, we have taken great care to report LLOD, LLOQ, Intra-assay CV, and Inter-assay CV values for our studied analytes and to discuss interpretations only when our measured values are consistently reproducible with low variance and above the LLOQ. Not all studies have done this, which could account for some of the apparent variations in the literature [74]. In addition, we have detected reductions in IL-16 that clustered statistically with IL-7 & VEGF-A levels. While it will be important in the future to investigate these analytes in blood and CSF samples from other ME/CFS cohorts from around the world and in patients exhibiting overlapping symptoms, our data suggests that there could be physiological relevance in the observed reductions of these three linked analytes in ME/CFS disease which if confirmed, could open up new diagnostic and therapeutic avenues for this challenging disease.

Acknowledgements

We are very grateful to the many patients and healthy volunteers who donated blood samples. We would like to thank Gloria Smith, the Solve CFS BioBank coordinator, for consenting and managing the Solve ME/CFS BioBank. Finally, we thank the Solve ME/CFS Initiative for providing the samples and information used in this study. This work has been supported by Li Ka Shing Institute of Virology and Applied Virology Institute and Canadian Excellence Research Chair (CERC) grant, for which MH is the holder. AL is supported by fellowships from the Alberta Innovates-Health Solutions (AIHS), Canadian Institutes of Health Research (CIHR), Canadian Association of Gastroenterology (CAG), and Takeda Canada Inc.

References

- [1] J.M. Coffin, J.P. Stoye, *Virology*. A new virus for old diseases?, *Science* 326 (2009) 530–531.
- [2] C. King, L.A. Jason, Improving the diagnostic criteria and procedures for chronic fatigue syndrome, *Biol. Psychol.* 68 (2005) 87–106.
- [3] L.A. Jason, M. Sunnquist, B. Kot, A. Bron, Unintended consequences of not specifying exclusionary illnesses for systemic exertion intolerance disease, *Diagnosics* (2015).
- [4] T. Paprotka, K.A. Delviks-Frankenberry, O. Cingoz, A. Martinez, H.J. Kung, C.G. Tepper, et al., Recombinant origin of the retrovirus XMRV, *Science* (2011).
- [5] K. Knox, D. Carrigan, G. Simmons, F. Teque, Y. Zhou, J. Hackett Jr., et al., No evidence of murine-like gammaretroviruses in CFS patients previously identified as XMRV-infected, *Science* (2011).
- [6] G. Simmons, S.A. Glynn, A.L. Komaroff, J.A. Mikovits, L.H. Tobler, J. Hackett Jr., et al., Failure to confirm XMRV/MLVs in the blood of patients with chronic fatigue syndrome: a multi-laboratory study, *Science* (2011).
- [7] I. Steffen, D.L. Tyrrell, E. Stein, L. Montalvo, T.H. Lee, Y. Zhou, et al., No evidence for XMRV nucleic acids, infectious virus or anti-XMRV antibodies in Canadian patients with chronic fatigue syndrome, *PLoS ONE* 6 (2011) e27870.
- [8] H.J. Alter, J.A. Mikovits, W.M. Switzer, F.W. Ruscetti, S.C. Lo, N. Klimas, et al., A multicenter blinded analysis indicates no association between chronic fatigue syndrome/myalgic encephalomyelitis and either xenotropic murine leukemia virus-related virus or polytropic murine leukemia virus, *mBio* (2012) 3.
- [9] G. Morris, M. Maes, A neuro-immune model of Myalgic Encephalomyelitis/Chronic fatigue syndrome, *Metab. Brain Dis.* 28 (2013) 523–540.
- [10] Y. Nakatomi, K. Mizuno, A. Ishii, Y. Wada, M. Tanaka, S. Tazawa, et al., Neuroinflammation in patients with chronic fatigue syndrome/myalgic encephalomyelitis: an 11C-(R)-PK11195 PET study, *J. Nucl. Med.: Offic. Publ., Soc. Nucl. Med.* (2014).
- [11] E.W. Benu, T.K. Huth, S.L. Hardcastle, K. Fuller, M. Kaur, S. Johnston, et al., Role of adaptive and innate immune cells in chronic fatigue syndrome/myalgic encephalomyelitis, *Int. Immunol.* (2014).
- [12] M. Curriu, J. Carrillo, M. Massanella, J. Rigau, J. Alegre, J. Puig, et al., Screening NK-, B- and T-cell phenotype and function in patients suffering from Chronic Fatigue Syndrome, *J. Transl. Med.* 11 (2013) 68.
- [13] G. Broderick, J. Fuite, A. Kreitz, S.D. Vernon, N. Klimas, M.A. Fletcher, A formal analysis of cytokine networks in chronic fatigue syndrome, *Brain Behav. Immun.* 24 (2010) 1209–1217.
- [14] R. Patarca-Montero, M. Antoni, M.A. Fletcher, N.G. Klimas, Cytokine and other immunologic markers in chronic fatigue syndrome and their relation to neuropsychological factors, *Appl. Neuropsychol.* 8 (2001) 51–64.
- [15] M.A. Fletcher, X.R. Zeng, Z. Barnes, S. Levis, N.G. Klimas, Plasma cytokines in women with chronic fatigue syndrome, *J. Transl. Med.* 7 (2009) 96.
- [16] V.C. Lombardi, K.S. Hagen, K.W. Hunter, J.W. Diamond, J. Smith-Gagen, W. Yang, et al., Xenotropic murine leukemia virus-related virus-associated chronic fatigue syndrome reveals a distinct inflammatory signature, *In vivo* 25 (2011) 307–314.
- [17] T. Nakamura, S.K. Schwander, R. Donnelly, F. Ortega, F. Togo, G. Broderick, et al., Cytokines across the night in chronic fatigue syndrome with and without fibromyalgia, *Clin. Vaccine Immunol.* CVI 17 (2010) 582–587.
- [18] B.H. Natelson, M.H. Haghghi, N.M. Ponzio, Evidence for the presence of immune dysfunction in chronic fatigue syndrome, *Clin. Diagn. Lab. Immunol.* 9 (2002) 747–752.
- [19] D. Peterson, E.W. Benu, G. Gottschalk, S. Ramos, T. Nguyen, D. Staines, et al., Cytokines in the cerebrospinal fluids of patients with chronic fatigue syndrome/myalgic encephalomyelitis, *Mediators Inflamm.* (2015).
- [20] M. Hornig, G. Gottschalk, D.L. Peterson, K.K. Knox, A.F. Schultz, M.L. Eddy, et al., Cytokine network analysis of cerebrospinal fluid in myalgic encephalomyelitis/chronic fatigue syndrome, *Mol. Psychiatry* (2015).
- [21] M. Hornig, J.G. Montoya, N.G. Klimas, S. Levine, D. Felsenstein, L. Bateman, et al., Distinct plasma immune signatures in ME/CFS are present early in the course of illness, *Sci. Adv.* (2015).
- [22] D.M. Irlbeck, S.D. Vernon, K.K. McCleary, L. Bateman, N.G. Klimas, C.W. Lapp, et al., No association found between the detection of either xenotropic murine leukemia virus-related virus or polytropic murine leukemia virus and chronic fatigue syndrome in a blinded, multi-site, prospective study by the establishment and use of the SolveCFS BioBank, *BMC Res. Notes* 7 (2014) 461.
- [23] B.M. Carruthers, Definitions and aetiology of myalgic encephalomyelitis: how the Canadian consensus clinical definition of myalgic encephalomyelitis works, *J. Clin. Pathol.* 60 (2007) 117–119.
- [24] K. Fukuda, S.E. Straus, I. Hickie, M.C. Sharpe, J.G. Dobbins, A. Komaroff, The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group, *Ann. Intern. Med.* 121 (1994) 953–959.
- [25] A. Landi, T.J. Weismuller, T.O. Lankisch, D.M. Santer, D.L. Tyrrell, M.P. Manns, et al., Differential serum levels of eosinophilic eotaxins in primary sclerosing cholangitis, primary biliary cirrhosis, and autoimmune hepatitis, *J. Interferon Cytokine Res.* 34 (2014) 204–214.
- [26] J.W. Lee, V. Devanarayan, Y.C. Barrett, R. Weiner, J. Allinson, S. Fountain, et al., Fit-for-purpose method development and validation for successful biomarker measurement, *Pharm. Res.* 23 (2006) 312–328.
- [27] J.D. Storey, A direct approach to false discovery rates, *J. Roy. Stat. Soc. Series B* 64 (2002) 479–498.
- [28] D. Broadhurst, D.B. Kell, Statistical strategies for avoiding false discoveries in metabolomics and related experiments, *Metabolomics: Official J. Metabolomic Soc.* 2 (2006) 171–196.
- [29] J. Ellson, E.R. Gansner, E. Koutsofos, S.C. North, G. Woodhull, *Graphviz and Dynagraph – Static and Dynamic Graph Drawing Tools*, Springer-Verlag, Berlin/Heidelberg, 2004.
- [30] T. Hastie, R. Tibshirani, J. Friedman, *The Elements of Statistical Learning*, second ed., Springer, New York, 2008.
- [31] L. Breiman, J. Friedman, R. Olshen, C. Stone, *Classification and Regression Trees*, CRC Press, Boca Raton, FL, 1984.
- [32] J.E. Ware Jr., C.D. Sherbourne, The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection, *Med. Care* 30 (1992) 473–483.
- [33] E. Aslakson, U. Vollmer-Conna, W.C. Reeves, P.D. White, Replication of an empirical approach to delineate the heterogeneity of chronic unexplained fatigue, *Popul. Health Metrics* 7 (2009) 17.

- [34] L.A. Jason, K. Corradi, S. Torres-Harding, R.R. Taylor, C. King, Chronic fatigue syndrome: the need for subtypes, *Neuropsychol. Rev.* 15 (2005) 29–58.
- [35] S.F. Khaiboullina, K.L. DeMeirleir, S. Rawat, G.S. Berk, R.S. Gaynor-Berk, T. Mijatovic, et al., Cytokine expression provides clues to the pathophysiology of Gulf War illness and myalgic encephalomyelitis, *Cytokine* 72 (2014) 1–8.
- [36] A.P. Somlyo, A.V. Somlyo, Signal transduction and regulation in smooth muscle, *Nature* 372 (1994) 231–236.
- [37] C. Amiel, E. Darcissac, M.J. Truong, J. Dewulf, M. Loyens, Y. Mouton, et al., Interleukin-16 (IL-16) inhibits human immunodeficiency virus replication in cells from infected subjects, and serum IL-16 levels drop with disease progression, *J. Infect. Dis.* 179 (1999) 83–91.
- [38] C.A. Thornton, J.A. Holloway, J.K. Shute, J.W. Holloway, N.D. Diaper, J.O. Warner, Human mid-gestation amniotic fluid contains interleukin-16 bioactivity, *Immunology* 126 (2009) 543–551.
- [39] P. Szabo, K. Zhao, I. Kirman, J. Le Maoult, R. Dyall, W. Cruikshank, et al., Maturation of B cell precursors is impaired in thymic-deprived nude and old mice, *J. Immunol.* 161 (1998) 2248–2253.
- [40] L.R. Lard, B.O. Roep, R.E. Toes, T.W. Huizinga, Enhanced concentrations of interleukin 16 are associated with joint destruction in patients with rheumatoid arthritis, *J. Rheumatol.* 31 (2004) 35–39.
- [41] Y. Kageyama, T. Ozeki, M. Suzuki, T. Ichikawa, T. Miura, S. Miyamoto, et al., Interleukin-16 in synovial fluids from cases of various types of arthritis, *Joint, Bone, Spine: Revue du Rhumatisme* 67 (2000) 188–193.
- [42] D.S. Skundric, J. Cai, W.W. Cruikshank, D. Gveric, Production of IL-16 correlates with CD4+ Th1 inflammation and phosphorylation of axonal cytoskeleton in multiple sclerosis lesions, *J. Neuroinflamm.* 3 (2006) 13.
- [43] J.M. Schwab, H.J. Schluesener, K. Seid, R. Meyermann, IL-16 is differentially expressed in the developing human fetal brain by microglial cells in zones of neurogenesis, *Int. J. Develop. Neurosci.: Official J. Int. Soc. Develop. Neurosci.* 19 (2001) 93–100.
- [44] L. Shanks, L.A. Jason, M. Evans, A. Brown, Cognitive impairments associated with CFS and POTS, *Front. Physiol.* 4 (2013) 113.
- [45] T.J. Fry, C.L. Mackall, Interleukin-7: from bench to clinic, *Blood* 99 (2002) 3892–3904.
- [46] Y. Sawa, Y. Arima, H. Ogura, C. Kitabayashi, J.J. Jiang, T. Fukushima, et al., Hepatic interleukin-7 expression regulates T cell responses, *Immunity* 30 (2009) 447–457.
- [47] M. Watanabe, Y. Ueno, T. Yajima, Y. Iwao, M. Tsuchiya, H. Ishikawa, et al., Interleukin 7 is produced by human intestinal epithelial cells and regulates the proliferation of intestinal mucosal lymphocytes, *J. Clin. Invest.* 95 (1995) 2945–2953.
- [48] K.S. Schluns, L. Lefrancois, Cytokine control of memory T-cell development and survival, *Nat. Rev. Immunol.* 3 (2003) 269–279.
- [49] D. Andrew, R. Aspinall, IL-7 and not stem cell factor reverses both the increase in apoptosis and the decline in thymopoiesis seen in aged mice, *J. Immunol.* 166 (2001) 1524–1530.
- [50] I. Tsuboi, K. Morimoto, Y. Hirabayashi, G.X. Li, S. Aizawa, K.J. Mori, et al., Senescent B lymphopoiesis is balanced in suppressive homeostasis: decrease in interleukin-7 and transforming growth factor-beta levels in stromal cells of senescence-accelerated mice, *Exp. Biol. Med.* 229 (2004) 494–502.
- [51] D. Andrew, R. Aspinall, Age-associated thymic atrophy is linked to a decline in IL-7 production, *Exp. Gerontol.* 37 (2002) 455–463.
- [52] A.L. Gruver, L.L. Hudson, G.D. Sempowski, Immunosenescence of ageing, *J. Pathol.* 211 (2007) 144–156.
- [53] L. Lazzardi, B. Jenewein, A.M. Wolf, G. Pfister, A. Tzankov, B. Grubeck-Loebenstein, Age-related loss of naive T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes, *Immunology* 114 (2005) 37–43.
- [54] J. Ongradi, V. Kovacs, Factors that may impact on immunosenescence: an appraisal, *Immun. Ageing: 1 & A* 7 (2010) 7.
- [55] K. Kim, C.K. Lee, T.J. Sayers, K. Muegge, S.K. Durum, The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways, *J. Immunol.* 160 (1998) 5735–5741.
- [56] J. Pido-Lopez, N. Imami, D. Andrew, R. Aspinall, Molecular quantitation of thymic output in mice and the effect of IL-7, *Eur. J. Immunol.* 32 (2002) 2827–2836.
- [57] M.D. Michaelson, M.F. Mehler, H. Xu, R.E. Gross, J.A. Kessler, Interleukin-7 is trophic for embryonic neurons and is expressed in developing brain, *Develop. Biol.* 179 (1996) 251–263.
- [58] N. Reinmuth, W. Liu, Y.D. Jung, S.A. Ahmad, R.M. Shaheen, F. Fan, et al., Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival, *FASEB J.* 15 (2001) 1239–1241.
- [59] T. Licht, I. Goshen, A. Avital, T. Kreisel, S. Zubedat, R. Eavri, et al., Reversible modulations of neuronal plasticity by VEGF, *Proc. Natl. Acad. Sci. USA* 108 (2011) 5081–5086.
- [60] Y. Zhu, K. Jin, X.O. Mao, D.A. Greenberg, Vascular endothelial growth factor promotes proliferation of cortical neuron precursors by regulating E2F expression, *FASEB J.* 17 (2003) 186–193.
- [61] E. Storkebaum, D. Lambrechts, P. Carmeliet, VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection, *BioEssays* 26 (2004) 943–954.
- [62] L. Cao, X. Jiao, D.S. Zuzga, Y. Liu, D.M. Fong, D. Young, et al., VEGF links hippocampal activity with neurogenesis, learning and memory, *Nat. Genet.* 36 (2004) 827–835.
- [63] A. Stehr, I. Topel, S. Muller, K. Unverdorben, E.K. Geissler, P.M. Kasprzak, et al., VEGF: a surrogate marker for peripheral vascular disease, *Eur. J. Vasc. Endovasc. Surg.: Official J. Eur. Soc. Vasc. Surg.* 39 (2010) 330–332.
- [64] J.M. Boucher, V.L. Bautch, Antiangiogenic VEGF-A in peripheral artery disease, *Nat. Med.* 20 (2014) 1383–1385.
- [65] P.D. Wagner, The critical role of VEGF in skeletal muscle angiogenesis and blood flow, *Biochem. Soc. Trans.* 39 (2011) 1556–1559.
- [66] D.H. Dobrogowska, A.S. Lossinsky, M. Tarnawski, A.W. Vorbodt, Increased blood-brain barrier permeability and endothelial abnormalities induced by vascular endothelial growth factor, *J. Neurocytol.* 27 (1998) 163–173.
- [67] A. Louissaint Jr., S. Rao, C. Leventhal, S.A. Goldman, Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain, *Neuron* 34 (2002) 945–960.
- [68] M. Sorenson, L. Jason, J. Peterson, J. Herrington, H. Mathews, Brain derived neurotrophic factor is decreased in chronic fatigue syndrome and multiple sclerosis, *J. Neurol. Neurophys.* (2014) S12.
- [69] D.H. Lee, E. Geyer, A.C. Flach, K. Jung, R. Gold, A. Flugel, et al., Central nervous system rather than immune cell-derived BDNF mediates axonal protective effects early in autoimmune demyelination, *Acta Neuropathol.* 123 (2012) 247–258.
- [70] N. Ortuzar, I. Rico-Barrio, H. Bengoetxea, E.G. Argandona, J.V. Lafuente, VEGF reverts the cognitive impairment induced by a focal traumatic brain injury during the development of rats raised under environmental enrichment, *Behav. Brain Res.* 246 (2013) 36–46.
- [71] P. Religa, R. Cao, D. Religa, Y. Xue, N. Bogdanovic, D. Westaway, et al., VEGF significantly restores impaired memory behavior in Alzheimer's mice by improvement of vascular survival, *Sci. Reports* 3 (2013) 2053.
- [72] E. Tham, A.W. Gielen, M. Khademi, C. Martin, F. Piehl, Decreased expression of VEGF-A in rat experimental autoimmune encephalomyelitis and in cerebrospinal fluid mononuclear cells from patients with multiple sclerosis, *Scand. J. Immunol.* 64 (2006) 609–622.
- [73] E. Iacobaeus, P. Amoudruz, M. Strom, M. Khademi, L. Brundin, J. Hillert, et al., The expression of VEGF-A is down regulated in peripheral blood mononuclear cells of patients with secondary progressive multiple sclerosis, *PLoS ONE* 6 (2011) e19138.
- [74] S. Blundell, K.K. Ray, M. Buckland, P.D. White, Chronic fatigue syndrome and circulating cytokines: a systematic review, *Brain Behav. Immun.* (2015).