Brain, Behavior, & Immunity - Health 36 (2024) 100720

Contents lists available at ScienceDirect

Brain, Behavior, & Immunity - Health

journal homepage: www.editorialmanager.com/bbih/default.aspx

Identification of CD8 T-cell dysfunction associated with symptoms in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and Long COVID and treatment with a nebulized antioxidant/anti-pathogen agent in a retrospective case series

Anna Gil [a], George E. Hoag [b], John P. Salerno [b,c], Mady Hornig [d], Nancy Klimas [e], Liisa K. Selin [a,*]

[a] University of Massachusetts Chan Medical School, Department of Pathology, Worcester, MA, USA
[b] Inspiritol, Inc., Fairfield, CT, USA
[c] The Salerno Center for Complementary Medicine, New York, USA
[d] Columbia University Mailman School of Public Health, New York, USA
[e] Nova Southeastern University, Fort Lauderdale, Florida, USA

ARTICLE INFO

Keywords:
Inspiritol
CD8 T-cell dysfunction
Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS)
Long COVID
Immune dysregulation
Nebulized
Antioxidant
T cell exhaustion

ABSTRACT

Background: Patients with post-acute sequelae of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection (PASC, i.e., Long COVID) have a symptom complex highly analogous to many features of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), suggesting they may share some aspects of pathogenesis in these similar disorders. ME/CFS is a complex disease affecting numerous organ systems and biological processes and is often preceded by an infection-like episode. It is postulated that the chronic manifestations of illness may result from an altered host response to infection or inability to resolve inflammation, as is being reported in Long COVID. The immunopathogenesis of both disorders is still poorly understood. Here, we show data that suggest Long COVID and ME/CFS may be due to an aberrant response to an immunological trigger-like infection, resulting in a dysregulated immune system with CD8 T-cell dysfunction reminiscent of some aspects of T-cell clonal exhaustion, a phenomenon associated with oxidative stress. As there is an urgent need for diagnostic tools and treatment strategies for these two related disabling disorders, here, in a retrospective case series, we have also identified a potential nebulized antioxidant/anti-pathogen treatment that has evidence of a good safety profile. This nebulized agent is comprised of five ingredients previously reported individually to relieve oxidative stress, attenuate NF-κB signaling, and/or to act directly to inhibit pathogens, including viruses. Administration of this treatment by nebulizer results in rapid access of small doses of well-studied antioxidants and agents with anti-pathogen potential to the lungs; components of this nebulized agent are also likely to be distributed systemically, with potential to enter the central nervous system.

Methods: and Findings: We conducted an analysis of CD8 T-cell function and severity of symptoms by self-report questionnaires in ME/CFS, Long COVID and healthy controls. We developed a CD8 T-cell functional assay, assessing CD8 T-cell dysfunction by intracellular cytokine staining (ICS) in a group of ME/CFS ($n = 12$) and Long COVID patients ($n = 8$), comparing to healthy controls (HC) with similar age and sex ($n = 10$). Magnet-enriched fresh CD8 T-cells in both patient groups had a significantly diminished capacity to produce both cytokines, IFNγ or TNFα, after PMA stimulation when compared to HC. The symptom severity questionnaire showed similar symptom profiles for the two disorders. Fortuitously, through a retrospective case series, we were able to examine the ICS and questionnaire data of 4 ME/CFS and 4 Long COVID patients in conjunction with their treatment (3–15 months). In parallel with the treatment pursued electively by participants in this retrospective case series, there was an increase in CD8 T-cell IFNγ and TNFα production and a decrease in overall self-reported symptom severity score by 54%. No serious treatment-associated side effects or laboratory anomalies were noted in these patients.

Conclusions: Here, in this small study, we present two observations that appear potentially fundamental to the pathogenesis and treatment of Long COVID and ME/CFS. The first is that both disorders appear to be

* Corresponding author.
E-mail address: liisa.selin@umassmed.edu (L.K. Selin).

https://doi.org/10.1016/j.bbih.2023.100720
Received 2 May 2023; Received in revised form 29 November 2023; Accepted 18 December 2023
Available online 27 December 2023

characterized by dysfunctional CD8 T-cells with severe deficiencies in their abilities to produce IFNγ and TNFα. The second is that in a small retrospective Long COVID and ME/CFS case series, this immune dysfunction and patient health improved in parallel with treatment with an immunomodulatory, antioxidant pharmacological treatment with anticipated anti-pathogen activity. This work provides evidence of the potential utility of a biomarker, CD8 T-cell dysfunction, and suggests the potential for benefit from a new nebulized antioxidant/anti-pathogen treatment. These immune biomarker data may help build capacity for improved diagnosis and tracking of treatment outcomes during clinical trials for both Long COVID and ME/CFS while providing clues to new treatment avenues that suggest potential efficacy for both conditions.

Abbreviations

ALB	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	Analysis of variance
AST	aspartate aminotransferase
BUN	blood urea nitrogen
2B4	Natural killer cell receptor 2B4
CBC	Complete Blood Count
CMP	Comprehensive Metabolic Panel
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
CMV	cytomegalovirus
COVID-19	Coronavirus Disease 2019
CRTN	creatinine
EBV	Epstein-Barr Virus
EBNA	Epstein-Barr Nuclear Antigen
FCSB	Flow Cytometry Staining Buffer
FACS	Fluorescence-Activated Cell Sorting
GI	gastrointestinal
HC	healthy control
HC/Unstim	healthy control unstimulated
HC/Stim	healthy control stimulated
HHV6	human herpes virus 6
HSV	herpes simplex virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ICS	intracellular cytokine staining
IL	Interleukin
IFN	interferon
LAG3	Lymphocyte-Activation Gene 3
LC/Unstim	Long COVID unstimulated
LC/Stim	Long COVID stimulated
ME/CFS	myalgic encephalomyelitis/chronic fatigue syndrome
ME/Unstim	myalgic encephalomyelitis/chronic fatigue syndrome unstimulated
ME/Stim	myalgic encephalomyelitis/chronic fatigue syndrome stimulated
NF-κB	Nuclear Factor Kappa B
OD	once daily
PBMC	peripheral blood mononuclear cells
PLT	platelet count
PMA	Phorbol-12-myristate-13-acetate
PD1	Programmed cell death protein 1
PC-L1	programmed cell death ligand 1
PEM	post-exertional malaise
POTS	postural orthostatic tachycardia syndrome
RNS	reactive nitrogen species
ROS	reactive oxygen species
RBC	red blood cell
RT	room temperature
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SOB	shortness of breath
STD	standard deviation
SEM	standard error of the mean
TCR	T-cell receptor
TENS	transcutaneous electrical nerve stimulation
TID	three times per day
TGF-β	Transforming Growth Factor beta
TNFα	tumor necrosis factor alpha
Tx	treatment
ULN	upper limit normal
UMass Chan	University of Massachusetts Chan Medical School
VCA	Viral Capsid Antigen
VZV	varicella zoster virus
WBC	white blood cell

1. Introduction

Severe and long-lasting immunopathologic nervous system and systemic sequelae of acute viral infections have been known to occur for decades (Choutka et al., 2022). Long COVID and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), two highly similar disorders, have recently come to the forefront of medicine following the recent acute coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (Davis et al., 2023). There are reports that 20–30% of people who had acute COVID-19 infection develop a prolonged syndrome now known as Long COVID (Post-acute sequelae of SARS-CoV-2 infection (PASC)), wherein patients note severe fatigue interfering with daily living and ability to work (95%), post-exertional malaise (PEM) (90%), and many other symptoms associated with ME/CFS, including postural orthostatic tachycardia syndrome (POTS), cognitive difficulties (often referred to as "brain fog"), and unrefreshing sleep (Davis et al., 2021; Hill et al., 2022; Al-Aly et al., 2021; Taquet et al., 2021). This condition appears to begin either as a continuation of the acute COVID-19 episode, or starts a month or more later, often in individuals with mild or moderately severe acute illness who were never hospitalized. Long COVID predominantly affects people between the ages of 20–60 years (Davis et al., 2021; Al-Aly et al., 2021; Hill et al., 2022; Taquet et al., 2021). Half of the individuals with mild or moderate acute COVID-19 symptoms who were still ill after 6 months were recently reported to meet the criteria for ME/CFS (Kedor et al., 2022). Considering that acute SARS-CoV-2 infection causes major dysregulation in the immune system (Huang IP, 2020) it would not be too surprising that the immune system may have difficulty in re-establishing homeostasis in some individuals (Phetsouphanh et al., 2022).

If some Long COVID develops into ME/CFS, it is predicted that there will be an increase of up to 22 million cases of ME/CFS. At this time, it is unknown whether those with Long COVID will ever fully recover from this disorder, or what will be the long-term health consequences including risk of increases mortality. What is without doubt is that this new secondary pandemic will be devastating to the medical system and

economy without intervention.

ME/CFS is a debilitating disease with the prevalence in the United States estimated between 519 and 1038 per 100,000 people impacting between 1.7 and 3.7 million people (based on 2017 population data) and as many as 34 million worldwide (Valdez et al., 2019) At least 75% of those diagnosed with ME/CFS are unable to work, 25% report being housebound and 10% are bedbound (Unger et al., 2017; Pendergrast et al., 2016). Currently, an estimated 80% of ME/CFS cases remain undiagnosed (Bested and Marshall, 2015).

A single specific, causative pathogen in ME/CFS has not been found, although certain infectious agents like Epstein-Barr and other herpes viruses, in addition to enteroviruses, have more frequently been considered as potential triggers of ME/CFS (Beyond Myalgic Encephalomyelitis, 2015). Multiple molecular processes appear to be perturbed, especially immunological and metabolic pathways, but the causes of these perturbations, and the reason they are variable between patients and within patients across time, remains unknown (Hornig et al., 2015; Fluge et al., 2016; Armstrong et al., 2014; Naviaux et al., 2016a). Inflammatory processes are implicated in many studies, with evidence of altered inflammatory cytokines in ME/CFS patient serum and plasma; although this indicates significant immune dysregulation, it does not identify the immunopathogenic mechanism and serum cytokines' high degree of lability limits their utility as biomarkers (Hornig et al., 2015; Broderick et al., 2010; Russell et al., 2016; Fletcher et al., 2009; Hardcastle et al., 2015; Maes et al., 2012a, 2012b). It has been suggested that there is a general pattern of increased inflammation early in the disease and a more immunosuppressed state after long-term illness (Hornig et al., 2015; Russell et al., 2016).

Dysfunctional T cells have been reported in ME/CFS and Long COVID patients as well as during infection with persistent virus such as EBV, HIV and HCV in humans and LCMV in mice (Blank et al., 2019; McLane et al., 2019). During persistent viral infections and cancers in humans and in animal models this CD8 T-cell dysfunction has been well studied and described as CD8 T-cell clonal exhaustion (Blank et al., 2019; McLane et al., 2019). Clonally exhausted CD8 T-cells are characterized by a progressive loss of effector functions, high and sustained inhibitory receptor expression, metabolic dysregulation, poor memory recall and homeostatic self-renewal and distinct transcriptional programs (McLane et al., 2019). The nature of the CD8 T-cell dysfunction in ME/CFS and Long COVID has not been well characterized.

Many ME/CFS patients have had this disease for decades, while Long COVID patients currently have a maximum duration of illness of 3.5 years, given the timing of the SARS-CoV-2 pandemic. Effective therapies for Long COVID patients are critically needed now to help prevent this disorder from becoming a decades-long chronic disease, as with ME/CFS. Inspiritol, a nebulized antioxidant/anti-pathogen agent, was developed, in part, as a broad spectrum, multi-mechanism biopharmaceutical. It is administered as a nebulized liquid therapeutic to address derangements of reactive oxygen species (ROS), aberrant production of type I and II IFNs, and high levels of pro-inflammatory cytokines, key factors involved in the pathogenesis of pulmonary inflammation and cell death that accompany acute respiratory distress syndrome (Maes et al., 2011, 2012a, 2012b, 2021; Dennis et al., 2021; Kumar et al., 2020; Wu, 2020; Al-Hakeim et al., 2023; Fukuda et al., 1994; Paul et al., 2021; Takemoto et al., 2021; Gould et al., 2010, 2011, 2015; Prousky, 2007; Rusznak et al., 2000; Buhl et al., 1990; Dekhuijzen, 2004; Straface et al., 2000; Bridgeman et al., 1991; Hagiwara et al., 2000; Paulin et al., 2017; Lima et al., 2013; Juergens et al., 2003, 2004, 2018, 2020; Rhoden et al., 2004; Kennedy-Feitosa et al., 2016; Dahham et al., 2015; Machado et al., 2018; Kim et al., 2015). These oxidative stress and immune anomalies are well-documented in patients with chronic obstructive pulmonary disease (COPD), asthma, various respiratory viral diseases, ME/CFS, and more recently, in both hospitalized and non-hospitalized COVID-19 and Long COVID patients (). Oxidative stress, cytokine and free radical storms, and T-cell abnormalities, including T-cell dysfunction, are associated with immune system dysregulation, resulting in systemic impacts that include the respiratory, neurocognitive, gastrointestinal, heart, lung, liver, kidney and pancreatic sequelae documented in Long COVID patients (Huang IP, 2020; Phetsouphanh et al., 2022). This nebulized treatment was initially designed for use in patients with COPD, asthma and, more recently, acute COVID, Long COVID and ME/CFS.

The pathogenesis of illness in some subjects with Long COVID may overlap with at least a subset of ME/CFS. A common theme in Long COVID and ME/CFS may be an aberrant response to an immunological trigger, like infection, resulting in a sustained dysregulated immune system because of overactivation of CD8 T-cells and subsequent dysfunction, reminiscent of CD8 T-cell clonal exhaustion. Compounds in the nebulized antioxidant/anti-pathogen agent can act to attenuate Nuclear Factor Kappa B (NF-κB) signaling (Kim et al., 2015; Greiner et al., 2013; Francomano FC et al., 2019; Oka et al., 2000; Sudhoff et al., 2015; Yadav and Chandra, 2017; Cantin et al., 2000; Lou and Kaplowitz, 2007; Xu et al., 2016), potentially decreasing CD8 T-cell abnormalities, including T-cell dysfunction. Clonal T-cell exhaustion can lead to further oxidative stress, and compounds in this nebulized treatment have been shown to decrease oxidative stress by scavenging oxygen and other free radical species (Maes et al., 2011, 2012a, 2012b, 2021; Dennis et al., 2021; Kumar et al., 2020; Wu, 2020; Al-Hakeim et al., 2023; Fukuda et al., 1994; Paul et al., 2021; Takemoto et al., 2021; Gould et al., 2010, 2011, 2015; Prousky, 2007; Rusznak et al., 2000; Buhl et al., 1990; Dekhuijzen, 2004; Straface et al., 2000; Bridgeman et al., 1991; Hagiwara et al., 2000; Paulin et al., 2017; Lima et al., 2013; Juergens et al., 2003, 2004, 2018, 2020; Rhoden et al., 2004; Kennedy-Feitosa et al., 2016; Dahham et al., 2015; Machado et al., 2018; Kim et al., 2015). Both oxidative stress and T-cell dysfunction can lead to poor control of latent and persistent viruses such as herpes viruses, which may all contribute to autonomic instability and neurological abnormalities. Our hypothesis is that immunomodulatory properties in this nebulized antioxidant/anti-pathogen agent may break the cycle of immune system dysfunction and associated oxidative stress responses in ME/CFS and Long COVID by working simultaneously through these multiple mechanisms.

To partially test this hypothesis and to potentially develop a tool for tracking response to therapy, we developed a CD8 T-cell functional assay, specifically assessing CD8 T-cell dysfunction by ICS in a group of ME/CFS and Long COVID patients and comparing them to healthy controls with similar age and sex. To help in future studies designed to track therapy response we also developed a self-report symptom severity questionnaire that further confirmed these two disorders had similar symptom profiles. Then, in a case series of 8 patients treated with the nebulized antioxidant/anti-pathogen agent, we retrospectively reviewed ICS and questionnaire data in 4 ME/CFS and 4 Long COVID patients for evidence of changes in function of their T-cell immune response and symptoms. First, we present a detailed retrospective description during treatment for one individual from each group to highlight the characteristics and severity of these poorly understood illnesses, and to illustrate changes in individuals in parallel with therapy over 15 months. Then, we present retrospective data from the two diagnostic subsets from the biomarker study (ME/CFS, LC) that show significant increases in CD8 T-cell IFNγ and TNFα production and decreased patient-reported symptom severity ratings, without side effects, over 3–15 months of treatment.

2. Materials and methods

Human subjects: All participants were recruited from University of Massachusetts Chan Medical School (UMass Chan) community, UMass Memorial Health Center and from The Salerno Center for Complementary Medicine, New York, New York. Written informed consent was obtained from all participants. ME/CFS patients in this study had previously been diagnosed by Institute of Medicine criteria by their physicians (Beyond Myalgic Encephalomyelitis, 2015). As there are no

standardized criteria for Long COVID, we applied a modified CDC definition of PASC (Hill et al., 2022), diagnosing Long COVID patients based on a history of more than 2 months of protracted fatigue unrelieved by rest since having acute COVID-19, and of persistent major symptoms such as brain fog, poor sleep and post-exertional malaise. At each blood testing visit, questionnaires (Supplemental Fig. S9 or S10) were completed by all study participants. Subjects were tracked over time if possible, returning every 1–6 months for symptom review and based on their availability. In order to not be so tiring for these very ill patients and to facilitate the patient and research experience, the UMass Chan Symptom Severity Questionnaire (Suppl Fig. S9&S10) was a more simplified version of the dominant symptoms identified in the more extensive questionnaires frequently used in the assessment of patients for ME/CFS (Unger et al., 2017). Symptom presence, and severity when present, was scored from 0 to 10, with 10 being the most severe; this widened range of scoring options appeared to increase patients' ability to document gradual changes in severity. The research presented here was approved by the Institutional Review Board committee at University of Massachusetts Chan Medical School, Worcester, Massachusetts.

A subset of 8 patients that had enrolled in the UMCMS study elected separately to pursue treatment with the nebulized antioxidant/anti-pathogen agent under the auspices of a clinical center for complementary medicine. Each of these patients later provided consent to be included in a published retrospective case series. Each patient either had in-person or telemedicine medical consultations prior to being prescribed treatment. Each patient also signed a Medical Information Release Form (HIPAA Release Form) and provided a signed comprehensive medical history, previous medical records, and testing results. Each patient completed the DePaul Symptom Questionnaire-2 (DSQ-2) questionnaire to assess the presence of symptoms, and their severity, and to evaluate the case definition fulfillment of individuals with ME/CFS; the DSQ-2 was also used in this study for Long COVID patients (Bedree et al., 2019). In this retrospective case series we only included those patients who were already enrolled in the UMass Chan Medical School T-cell biomarker study who had separately elected to pursue, and who had stayed on, treatment for 2 months or more and who had elected to return for follow-up questionnaires and repeat blood work to enable a retrospective review of changes in their T-cell biomarkers and in their clinical parameters over time in parallel with their use of the nebulized agent.

Study population: For the UMass Chan Symptom Severity Questionnaire studies we enrolled 20 ME/CFS (16 females, 4 males; mean age: 53.8 ± 3.1 (range 23–77); mean (±SEM) duration of illness: 23 ± 8 years (yr)) and 16 HC (11 females, 5 males; mean age: 55 ± 4 yr (range 22–72)). For the ICS assay used to determine if CD8 T-cell dysfunction was present, we studied a subset of the questionnaire respondents, 10 ME/CFS (7 females, 3 males; mean age: 53.0 ± 3.1 (range 23–72 yr); duration of illness: 22 ± 7 yr) and 10 HC (7 females, 3 males; mean age: 51 ± 3 yr (range 22–72 yr)). The same 8 Long COVID patients were used for the questionnaire and ICS assay studies (6 females, 2 males; mean age: 53 ± 3.3 yr (range 42–64 yr); mean duration of illness: 7.1 ± 1.8 months).

The retrospective case series of 8 nebulized antioxidant/anti-pathogen-treated patients were a subset of the UMass Chan biomarker and symptom profile study. Both the questionnaire and ICS study populations included the 8 treated patients: 4 ME/CFS (all females; mean age: 55.3 ± 6.9 (range 37–69 yr); duration of illness: 24.3 ± 12.9 yr) and 4 Long COVID (all females; mean age: 54.8 ± 4.5 (range 47–64 yr)); duration of illness: 6.0 ± 3.1 months). Table 1 (ME/CFS) and Table 2 (Long COVID) show detailed characteristics of the treated patients

Table 1
ME/CFS patient characteristics in nebulized antioxidant/anti-pathogen study.

Patient ID	ME patient 1	ME patient 2*	ME patient 3	ME patient 4
Age	67-73	50-55	35-40	60-65
Sex	F	F	F	F
Race	White	Asian/Pacific	White	White
Years with disease**	47	2	2	46
Start treatment	5/6/2021	2/11/2022	3/17/2022	8/30/2021
End treatment	ongoing	ongoing	ongoing	4/17/2022
No. of days on treatment	519	238	204	230
COVID-19 vaccines	1	0	4	2
Antivirals treatment	VLX, SPL	0	VLX	0
Viral Serology***	VZV IgG AB 598 HSV2 IgG ND HSV1 IgG 4.85 EBV IgG VCA 726 EBV IgM VCA <36 EBV EBNA IgG >600 HHV6 IgG ND CMV IgG ND	EBV IgG VCA >600 EBV IgM VCA <36 EBV EBNA IgG 48 CMV IgG 6.1 CMV IgM <30	VZV IgG 3335 HSV2 IgG ND HSV1 IgG ND EBV IgG VCA 158 EBV IgM VCA <36 EBV EBNA IgG 582 EBV Early Ag NEG HHV6 1:10 CMV NEG	VZV IgG AB 1210 VZV IgM <0.91 CMV IgM <8.00 CMV IgG <0.20 HSV1 IgG 0.45 HSV2 IgG 14.60 EBV IgG VCA >750 EBV IgM VCA <10 EBV EBNA IgG 74.40 EBV EarlyAg IgG >150 HHV6 IgG 1:320 HHV6 IgM <1:20

VLX - valacyclovir, VLC - valganciclovir, SPL - spironolactone, ND - Not Detected, NEG - Negative, POS -Positive, VZV - Varicella-zoster virus, HSV - Herpes simplex virus,, HHV6 - Human herpesvirus 6, EBV - Epstein Barr Virus, EBV EBNA - EBV Nuclear Antigen, EBV EarlyAg - EBV Early Antigen, CMV - Cytomegalovirus

* Least compliant patient with treatment dosing, stopping and starting treatment multiple times and often taking only 1ml/day

** number of years with disease before starting nebulized treatment

*** Red color indicates antibody titers that were considered high

Table 2
Long-COVID patient characteristics in nebulized antioxidant/anti-pathogen study.

Patient ID	LC patient 1	LC patient 2	LC patient 3	LC patient4
Age	59-64	45-50	45-50	62-67
Sex	F	F	F	F
Race	White	White	White	White
Months with disease*	5	2	2	15
Start treatment	5/6/2021	12/21/2021	12/29/2021	7/14/2021
End treatment	ongoing	3/11/2022	3/11/2022	11/15/2021
No. of days on treatment	519	80	72	124
COVID-19 vaccines	2	4	0	3
Antivirals treatment	VLX, VLC, SPL	0	0	0
Viral serology **	VZV IgG AB 1428 HSV1 IgG <90 HSV2 IgG <90 EBV IgG VCA >750 EBV IgM VCA 63.3 EBV EBNA IgG .>600 EBV EA IgG >150 HHV6 IgM <1:20 HHV6 IgG 1:160 CMV IgM 31.2 CMV IgG >10	EBV IGG VCA 503 EBV IGM VCA <10 EBV EBNA IgG 299 EBV EA IGG 11.4 HHV6 IgG 1.89 CMV IgM 8.7 CMV IgG 9.3	EBV IgG VCA 25.6 EBV IgM VCA <36 EBV EBNA IgG 55.1 EBV EA IgG <9.0	VZV IgG AB 1179 VZV IgM 1.93 HSV2 IgG 14.6 EBV IgG VCA 242 EBV IgM VCA <10 EBV EBNA IgG 302 EBV EarlyAg IgG <5.0 HHV6 IgM <1:20 HHV6 IgG 2.71

VLX - valacyclovir, VLC - valganciclovir, SPL - spironolactone, ND - Not Detected, NEG - Negative, POS -Positive, VZV - Varicella-zoster virus, HSV - Herpes simplex virus, HHV6 - Human herpesvirus 6, EBV - Epstein Barr Virus, EBV EBNA - EBV Nuclear Antigen, EBV EarlyAg - EBV Early Antigen, CMV - Cytomegalovirus
* Number of months with disease before starting nebulized treatment
** Red color indicates antibody titers that were considered high

including: gender, age, ethnicity, how long they had been ill with ME/CFS or Long COVID prior to treatment, all dates of treatment with the nebulized agent, the number of COVID-19 vaccines a patient had received prior to and including the period of treatment with the nebulized agent, and whether any antiviral medicines were additionally taken in parallel with their elective use of the nebulized agent. Antibody titers for herpes viruses were performed for each patient (Tables 1 and 2). All patients were female and all but one, who is Asian/Pacific, were white. Patients took the nebulized agent for different periods of time, dependent upon the rate of improvement in symptom severity. Long COVID patients were treated for 72–519 days, with 1 of 4 of those patients continuing treatment as of 10/6/2022. ME/CFS patients were treated for 204–519 days and 3 of 4 patients continue to take treatment as of 10/6/2022. Fortuitously, as part of the UMass Chan study, each patient returned for follow-up visits for blood tests and questionnaires every 1–6 months based on their availability while electing to pursue treatment with the nebulized agent. Based on viral blood test results and symptom profiles, LC patient 1, ME patient 1 and ME patient 3 additionally took certain antiviral medicines prescribed by their regular providers during their elective treatment with the nebulized agent, as noted in Tables 1 and 2 Several patients also took doxycycline during the treatment period for either acute Lyme disease or other infections.

Treatment with a nebulized antioxidant/anti-pathogen agent: The nebulized antioxidant/anti-pathogen agent, Inspiritol, is a proprietary nebulized, multi-mechanism medication, initially developed to treat major symptoms of respiratory distress through an agent with combined antioxidant, anti-inflammatory, and broad-spectrum antiviral, antifungal and antibacterial properties. It is a sterile isotonic pH-balanced liquid suspension with four ingredients that are US Pharmacopeia and Drug Master File (DMF)-listed: two endogenously produced compounds (glutathione and methylcobalamin); one repurposed prescription drug that is a pro-drug to an endogenously produced amino acid (N-acetylcysteine); one plant extract monoterpene cyclic ether (1,8-cineole) and one natural bicyclic sesquiterpene (β-caryophyllene), in a proprietary liquid carrier. These five compounds are present in low concentrations (i.e., total of 8.1% (w/w)) in a 90.9% (w/w) saline solution with a DMF-listed emulsifier.

All the active compounds in the nebulized agent are naturally found in foods common in the diet, and each has established values for daily dietary intake. Each of the five active ingredients have individually undergone human clinical trials via the inhalation pathway and have demonstrated efficacy and safety in their use (Prousky, 2007; Buhl et al., 1990; Holroyd et al., 1993; Calabrese et al., 2015; Homma et al., 2012; Bishop et al., 2005; Panahi et al., 2023; Li et al., 2018; Yamada et al., 2023; Williams, 2013; Smith et al., 1952; Kim et al., 2014; Jager et al., 1996). At present, the nebulized antioxidant/anti-pathogen agent is manufactured as a compounded medicine exclusively for a single complementary medicine center (Salerno Center, New York) and is not available through any other source. Inspiritol Inc. has filed documents with the U.S. Food and Drug Administration seeking approval to conduct human clinical trials and has U.S. and foreign patent applications pending. The treatment is inhaled using commercially available nebulizers.

The nebulized agent was prepared as a refrigeration-stable suspension and shipped directly overnight in coolers (to maintain cold temperatures) to patients electing the treatment. The recommended prescribed dose of this nebulized treatment was 5.0 mL TID, taken morning, afternoon and evening. Generally, patients tolerated the treatment well, except for a mild cough in some patients, controlled by decreasing the rate of administration. In retrospective review, patient compliance with this dose was generally good, however, some patients skipped occasional doses or had intermittent use over the course of treatment. Significant deviations from recommended dosing are noted in the Results and Discussion sections.

Clinical laboratory tests for treated patients: In the course of their clinical care, each patient in the retrospective case series had peripheral blood samples tested for complete blood count (CBC) with automated differential; comprehensive metabolic panel (CMP) including liver enzymes and kidney function, thyroid function and herpes family viral serology panels (IgM and IgG for EBV VCA, IgG for EBV EBNA, IgM and IgG for CMV, IgM and IgG for VZV, IgG for HHV6, IgG for HSV1 and HSV2). The above blood tests were conducted at licensed commercial testing companies, including UMass Memorial Health, Accu Reference Medical Laboratory, Quest Diagnostics, Mayo Clinic Department of Laboratory Medicine and Pathology, Lab Corp, Prohealth Laboratory and others. Each patient provided peripheral blood test results for at least two years prior to consultation at the complementary medicine center recruiting for the UMass Chan biomarker study. Each patient included in the retrospective case series had at least one additional peripheral blood test conducted during or after the treatment period.

Peripheral blood mononuclear cell (PBMC) isolation: PBMCs from healthy subjects and patients were isolated from fresh blood samples, processed within 24 h of being drawn, using Ficoll density gradient centrifugation (Ficoll-Paque Plus purchased from GE Healthcare BioSciences, Pittsburgh, PA). The cells were spun at 1800 rpm for 40 min with no brake at room temperature (RT). Most isolated cells were immediately used for CD8 T-cell positive enrichment, and plasma and/or serum samples were subjected to antibody testing. The rest of the cells were cryopreserved in freezing medium +10% DMSO at $10x10^6$ cells/ml at -150^oC in liquid nitrogen.

CD8 T-cell isolation: PBMC from fresh blood, processed within 24 h of being drawn, were counted and re-suspended in anti-CD8 microbeads (Miltenyi Biotech, Auburn, CA) and MACS buffer, 4 °C phosphate-buffered saline, 2.5 g of bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 2 ml 0.5M EDTA (pH 8.0) (Invitrogen, Grand Island, NY), and then degassed with sterile mesh filter following the manufacturer's protocol as previously described (Aslan et al., 2017). After a 20-min incubation in the dark at 4 °C, PBMCs were washed with MACS buffer and the $CD8^+$ T-cells were enriched using Miltenyi Biotech MACS system as previously described (Aslan et al., 2017).

Extracellular staining: Fresh magnet-enriched CD8 T-cells, processed within 24 h of being drawn, were incubated with surface markers for 30 min at RT and subsequently washed twice with flow cytometry staining buffer (FCSB) (500 ml Hank's Balanced Salt Solution with 2% Fetal Calf Serum) and fixed using 100 μl of Cytofix (Becton Dickinson Biosciences, San Jose, CA) for 5 min in the dark at RT. The cells were washed with and re-suspended in FCSB and analyzed by fluorescence-activated cell sorting (FACS) on the LSRII (Becton Dickinson, Waltham, MA). All immunophenotyping surface marker antibodies were purchased from BD Bioscience (CA, USA): CD3 (clone: UCT1), CD4 (clone: SK4), CD8 (clone: SK1). Cells were washed and re-suspended in 300 μl FCSB and analyzed on the LSRII (Becton Dickinson, Waltham, MA).

Intracellular cytokine staining (ICS): The following protocol allows for simultaneous analysis of cell surface and intracellular markers at the single cell level (singlets) by flow cytometry, as previously described (Aslan et al., 2017). To induce production of cytokines IFNγ and TNFα, isolated fresh CD8 T-cells were stimulated for 5 h with PMA/ionomycin in presence of protein transport inhibitors (monensin or brefeldin A solution; BD Biosciences). After staining with cell surface markers (CD3, CD8 and CD4 purchased from BD Biosciences), cells were permeabilized with Cytofix/Cytoperm buffer (BD Biosciences) in accordance with the manufacturer's protocol. Anti-IFNγ (clone B27) and anti-TNFα (clone D-21-1351) antibodies (BD Biosciences) were subsequently used for intracellular staining. Flow cytometry was done with LSRII (Becton Dickinson, Waltham, MA). Control samples to assess for the presence of spontaneous production of cytokines were left unstimulated during the assay and otherwise processed identically in parallel with the PMA-stimulated samples. All ICS data presented in this study are the actual values following stimulation without subtracting spontaneous cytokine production, if any was present.

Statistics: Statistical analyses included multivariate ANOVA with Tukey's multiple comparison test and with corrections for multiple comparisons, paired Student's *t*-test (two-tailed) and presentation of symptom severity scores and clinical lab test by radar plot.

3. Results

Development of a functional T-cell assay directly *ex vivo* demonstrating CD8 T-cell dysfunction in both ME/CFS and Long COVID patients. We tested whether CD8 T-cells were dysfunctional in both ME/CFS and Long COVID patients using an assay examining the ability of CD8 T-cells to produce IFNγ or TNFα following stimulation with PMA and ionomycin. We designed a classic ICS assay to provide a direct measure of the functional capabilities of magnet-enriched fresh CD8 T-cells in a format that would be easy to adapt to clinical testing. These functional ICS assays showed that CD8 T-cells of ME/CFS and Long COVID patients had a significantly diminished capacity to produce both cytokines, IFNγ or TNFα, after PMA stimulation when compared to HC as seen in representative FACS plots (Fig. S1) and following statistical analysis of multiple individuals from each group (Fig. 1). HC produced 3.5- or 3.4-fold more IFNγ (HC: 26.1 ± 5.9; Long COVID: 7.4 ± 1.7; ME/CFS: 7.6 ± 2.3) and 3.2- or 4.7-fold more TNFα (HC: 32.2 ± 6.8; Long COVID: 10.2 ± 4.6; ME/CFS: 6.9 ± 2.6) upon stimulation than either the Long COVID or ME/CFS patient group, respectively (Fig. 1). The ME/CFS and Long COVID patients produced a small amount of IFNγ and TNFα, but it was not significantly different from the unstimulated CD8 T-cells. These results are consistent with significant immune dysregulation within CD8 T-cells suggesting that they are highly dysfunctional, although it is not yet clear exactly what persistent antigen, virus, bacteria, fungi or auto-antigen, is keeping these cells in a highly activated state resulting in this dysfunctional state (Cornberg et al., 2013).

ME/CFS and Long COVID patients have similar symptoms. Based on the UMass Chan Symptom Severity questionnaire ME/CFS and Long COVID patients exhibited similar symptoms and severity scores on their first visit (Fig. 2). Both groups scored at similar severity in fatigue, PEM, sleep problems, headaches, postural hypotension, difficulty concentrating and dysnomia (difficulty retrieving words), the pathognomonic features of ME/CFS diagnosis. Interestingly, self-reported overall scores for the severity of illness were similar for Long COVID (mean: 7.2 ± 0.9; n = 20) and ME/CFS patients (6.7 ± 1, n = 8). Those with Long COVID did report greater severity in muscle and joint pain, chills, and cough. All symptom severity scores for both patient groups were significantly different from HC ($p < 0.05$) except for vertigo and shortness of breath for both patient groups. Also, ME/CFS patients did not significantly differ from HC in low grade fever and sore throat severity, while Long COVID did not differ from HC in allergy severity (Fig. 2).

Both the results of the similarity of CD8 T-cell immune dysregulation with T-cell dysfunction and the similarity in symptoms suggested that a similar immunopathologic process may be occurring in these two disorders. Thus, a similar treatment may be beneficial in both. Therefore, we decided to examine in a retrospective case series the ICS and questionnaire data we had obtained over time on the subset of ME/CFS and Long COVID patients that chose to be treated at a complementary medicine center with a nebulized antioxidant/anti-pathogen agent. These patients appeared to be self-reporting significant improvement while on treatment. This treatment, as outlined above, has ingredients that would relieve oxidative stress (Maes et al., 2011, 2012a, 2012b, 2021; Dennis et al., 2021; Kumar et al., 2020; Wu, 2020; Al-Hakeim et al., 2023; Fukuda et al., 1994; Paul et al., 2021; Takemoto et al., 2021; Gould et al., 2010, 2011, 2015; Prousky, 2007; Rusznak et al., 2000; Buhl et al., 1990; Dekhuijzen, 2004; Straface et al., 2000; Bridgeman et al., 1991; Hagiwara et al., 2000; Paulin et al., 2017; Lima et al., 2013; Juergens et al., 2003, 2004, 2018, 2020; Rhoden et al., 2004; Kennedy-Feitosa et al., 2016; Dahham et al., 2015; Machado et al., 2018; Kim et al., 2015), known to be disturbed in ME/CFS and Long COVID;

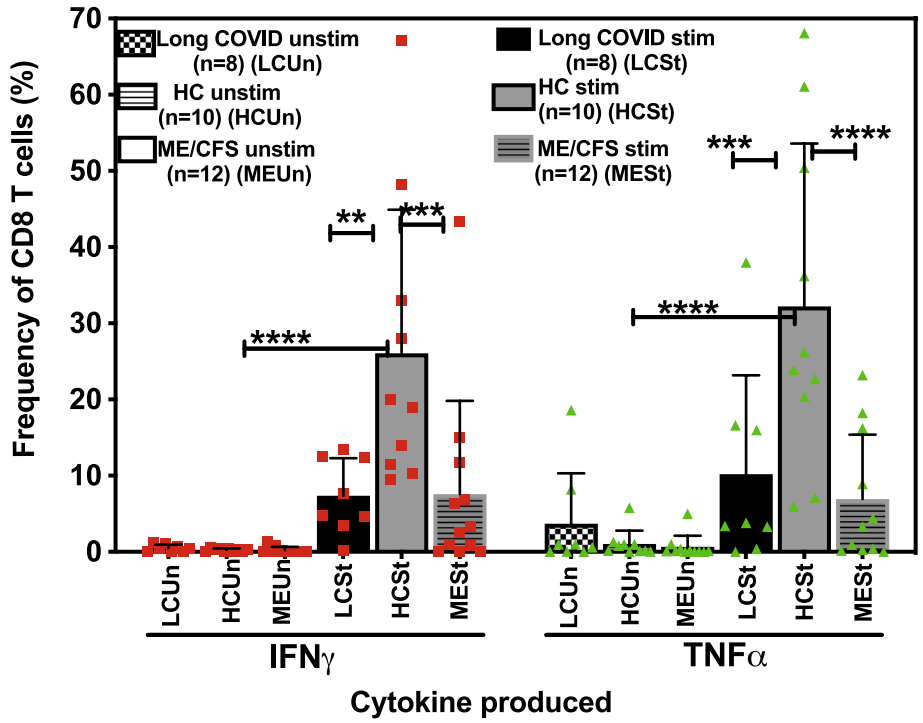


Fig. 1. Dysfunction of CD8 T cells in Long COVID & ME/CFS patients compared to HC. Intracellular cytokine assay (ICS) shows decreased ability of magnet-enriched fresh CD8 T cells of Long COVID & ME/CFS patients to produce IFNγ or TNFα following stimulation with PMA (stim). Individual data points and positive STD are shown. Unstimulated control (unstim). Multivariate ANOVA with adjusted p value for multiple comparisons: **p<0.01, ***p<0.001, ****p<0.0001.

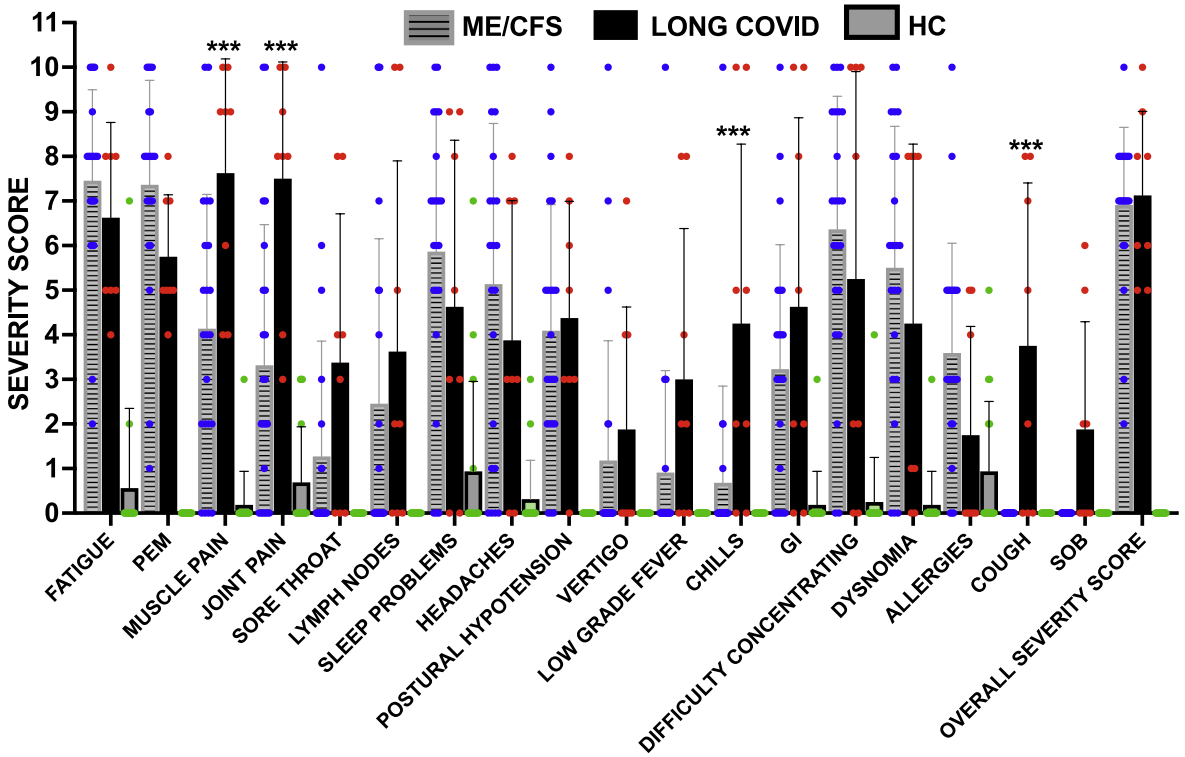


Fig. 2. Severity scoring of symptoms in ME/CFS and Long Covid patients were similar. Patients scored these symptoms on a scale from 0 (no symptom) to 10 (most severe symptom) in questionnaire. ME/CFS n = 20 (blue dots), Long COVID n = 8 (red dots), HC n = 16 (green dots). Individual data points and positive STD are shown. Multivariate ANOVA with adjusted p value for multiple comparisons: ***p < 0.001.

attenuate NF-κB signaling (Kim et al., 2015; Greiner et al., 2013; Francomano FC et al., 2019; Oka et al., 2000; Sudhoff et al., 2015; Yadav and Chandra, 2017; Cantin et al., 2000; Lou and Kaplowitz, 2007; Xu et al., 2016), which would decrease T-cell receptor (TCR) signaling and potentially reverse CD8 T-cell dysfunction; and act directly against pathogens, including viruses, bacteria and fungi (Dahham et al., 2015;

Holroyd et al., 1993; Nencioni et al., 2003; Roederer et al., 1990; Geiler et al., 2011; Yang et al., 2010; Li et al., 2016; Astani et al., 2011; Pajaro-Castro et al., 2015; Cavar Zeljkovic et al., 2022; Kim et al., 2022; Jimenez-Guardeno et al., 2022), which we suspect are involved in driving ME/CFS and Long COVID.

To better enhance understanding of these two related complex disorders, we first describe in more detail (Supplemental text) and briefly summarize below the disease and response to therapy for one ME/CFS (ME patient 1) and one Long COVID patient (LC patient 1). These were also the first two patients with these disorders that received this nebulized treatment, who later became part of the retrospective case series; thus, adjustments initially needed to be made in dosage. Perhaps as a result, these two patients have been on the treatment the longest time (more than 15 months). These detailed descriptions of the changes in disease symptoms are compiled from the patients' self-reported summaries over time.

Case 1: ME/CFS patient improves on a nebulized antioxidant/anti-pathogen agent after relapsing into fourth severe episode of ME/CFS. At presentation, ME/CFS patient, ME patient 1, was a 69-year-old female with ME/CFS diagnosed 47 years previously, with 3 severe episodes of ME/CFS during her lifetime after viral infections (April 1974, March 1984, April 1996). This fourth recent severe episode began following the first dose of the Pfizer-BioNTech COVID-19 vaccine on April 24, 2021. ME patient 1 previously had significant exacerbations often for 2–4 months of her ME/CFS symptoms following vaccines such as tetanus (2009) and influenza (2014), after which she decided not to take any more vaccines. However, she decided because of the severity of acute COVID-19 disease and as a requirement of her employment to take the first dose of vaccine, with devastating consequences. She has no other major medical problems.

ME patient 1 was highly functional working full time as a professional by following a very strict protocol of supplements and pacing. Her self-reported overall severity score for her ME/CFS symptoms prior to vaccination was 2 (Fig. 3A). Similar to our data in ME/CFS patients, her immunological profile showed CD8 T-cell dysfunction as seen in the representative FACS plot (Fig. S1B) and in the mean of 3 different time points taken over 3 years with only 2.3 ± 0.7% (n = 3) of her CD8 T-cells producing IFNγ and 0.01 ± 0.01% (n = 3) producing TNFα upon PMA stimulation compared to 34 ± 10.9% and 25.5 ± 2.8%, respectively, the mean observed of three different time points over 2 years in a healthy individual (HC 1) (Fig. 4, Fig. S1A representative FACS plot). This >90% reduction in cytokine production illustrates the profound dysfunction of her CD8 T-cells.

Within 48 h after the SARS-CoV-2 vaccine her left arm was extremely sore and swollen (where she received the vaccine), with biceps muscle fasciculations, and left axillary adenopathy. Within one week she had massive total body extremely tender lymphadenopathy (10+), muscle and joint pain, increased bruising and bilateral conjunctivitis, a splitting headache worsened by positioning her head downwards, extreme fatigue and PEM, sleeping most of the time, and narcolepsy following eating. She progressively worsened such that by day 10 post vaccine (5/4/21) she felt like she was in a cytokine storm, barely able to speak because of brain fog and word retrieval problems, wobbly with a wide-based gait, as she was not certain of her footing and her head began bobbing much like a patient with Parkinson's Disease. At this time, the patient self-reported an overall severity score of 10/10. She also scored PEM, lymph nodes, sleep problems, headaches, difficulty concentrating and dysnomia (difficulty retrieving words) at 10/10 in severity (Fig. 3A and B). Instead of calling an ambulance she start a nebulized antioxidant/anti-pathogen agent. Within 1½ hours of her first nebulizer treatment most of her very severe symptoms decreased (headache, brain fog, difficulty walking, head bobbing and passing out after eating), and she felt some increase in energy. This improvement was such that she did not need hospitalization and she was able to function well enough that she could just care for herself while living alone (Fig. 3).

In order to receive an exemption at her workplace from a second dose of COVID vaccine she was required to see a neurologist. The neurologist diagnosed her with a neuro-inflammatory condition based on her symptoms and findings. The major clinical finding at this time was evidence of complete inability to feel the vibration of a tuning fork in both feet (impaired vibration sense) and her initial acute onset post vaccine of inability to tell where she was placing her feet, consistent with possible either central or peripheral neuropathology and involvement of the proprioception pathway. (At two years post vaccine while still on the nebulizing therapy the patient had return of her ability to feel the tuning fork in both feet and improved ability to tell the position of her feet more like her normal prior to the vaccine). The magnetic resonance imaging studies showed multiple neuro-inflammatory lesions bilaterally in the subcortical regions of her brain. At this time the patient's cognitive function was still impaired as she could not play Sudoku at all, nor could

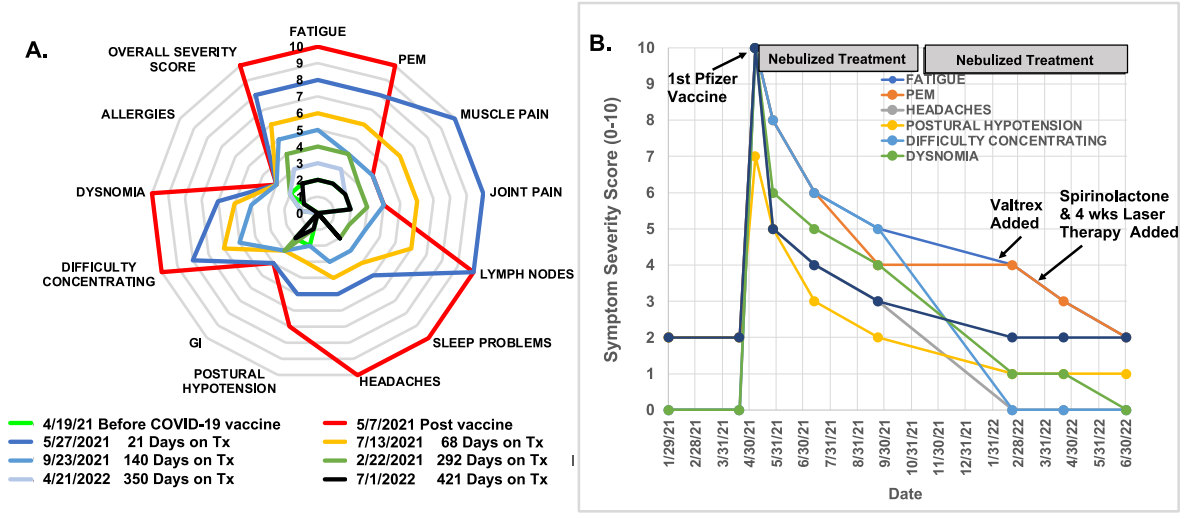


Fig. 3. Symptoms improve over time in ME/CFS patient (ME 1) during nebulized antioxidant/anti-pathogen therapy. A. Radar plot shows the change in self-reported score of each symptom at various times over the therapy including overall symptom severity. B. Line plot shows how the most common symptoms of ME/CFS improve over time on therapy and indicate when antiviral and laser therapy were added to treat chronic shingles (VZV), cold sores (HSV-1) and EBV reactivation. Nebulized antioxidant/anti-pathogen treatment (Tx) period5/3/2021 to present. Patient scored these symptoms on a scale from 0 (no symptoms) to 10 (most severe symptoms). First Pfizer-BioNTech vaccine 4/24/2021. Valacyclovir −1/24/2022 to the present. Spironolactone (SPLT) started 3/21/22 to the present. PEM-post exertional malaise, GIgastrointestinal, dysnomia (difficulty retrieving words).

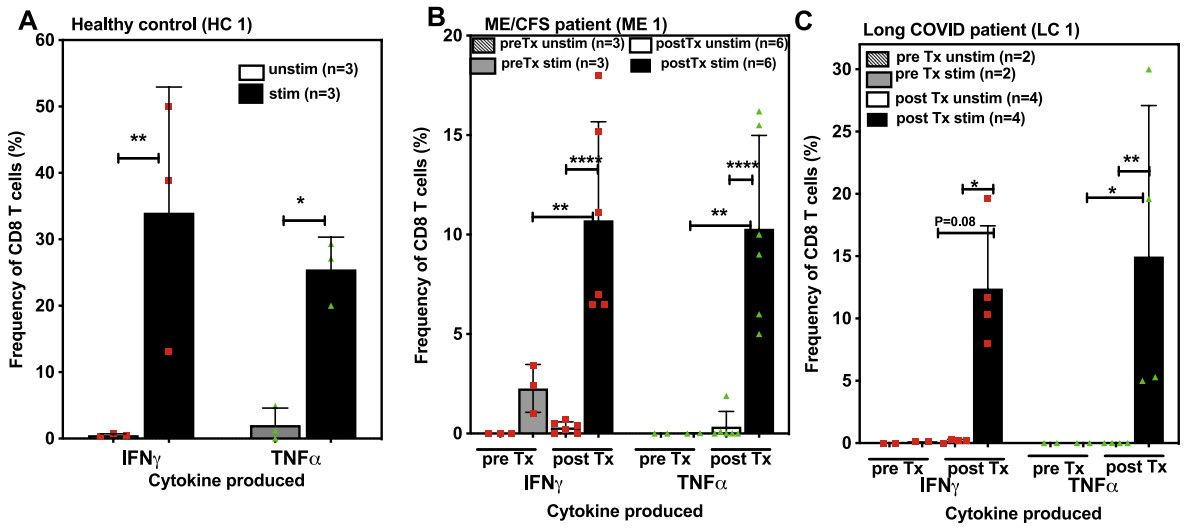


Fig. 4. ME/CFS (ME 1) (B) and Long COVID (LC 1) (C) patients show reversal of CD8 T cell dysfunction as evidenced by significantly improved IFNγ and TNFα production following PMA stimulation (stim) in ICS assay. Data shows the mean of ICS assays which were done sequentially 2–3 times before nebulized antioxidant/antipathogen treatment and 5–6 times post-treatment (Tx) over a 15-month period (between 5/26/21-7/1/22). Mean of 3 different time points over 2 years of healthy control (HC 1) (A) shows that ICS findings are consistent in HC. Unstimulated control (unstim). Pre = Pre-treatment, post = post-treatment. Individual data points and positive STD are shown. Multivariate ANOVA with adjusted p values for multiple comparisons *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

she write papers and these abilities did not return until 7 months on therapy following the vaccine.

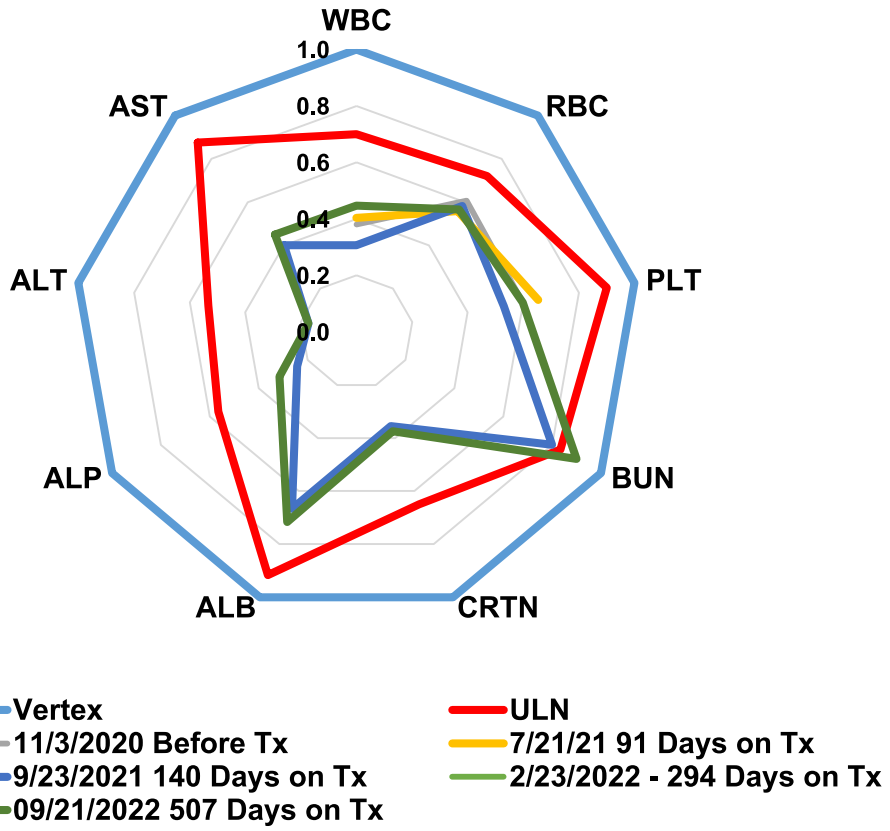
She was gradually improving on the nebulized therapy when in November 10–24th, 2021 (week 31–32) the treatment was stopped for 2 weeks due to issues with getting ingredients at the compounding pharmacy. During this time the patient greatly regressed with increased fatigue, and headaches, unable to work. During this interruption in therapy she also developed symptoms of chronic shingles on the right side of her back, presenting with burning and tingling feeling with very mild redness and induration (no vesicles) ("zoster sine herpete"), which is more common in immune-compromised individuals, and chronic cold sores on the right side of her upper lip. She had elevated levels of VZV IgG antibodies, HSV1 antibodies, and EBV VCA IgG and EBNA IgG, consistent with reactivation of these viruses as well as her clinical diagnosis of chronic shingles and cold sores (Table 1). She restarted the nebulized treatment and started valacyclovir 1000 mg three times a day for 2 weeks and then 1000 mg once daily (OD). She also started spironolactone on March 3rd, 2022 based on the findings of Swaminathan and colleagues (Verma et al., 2020) that this mild diuretic could also inhibit EBV lytic protein transcription and thus prevent reactivation of the virus.

The patient continued to gradually improve on this triple therapy of nebulized antioxidant/anti-pathogen agent, valacyclovir and spironolactone; after 15 months she was close to her pre-vaccine level of symptom severity. Fig. 3A summarizes in a radar plot the gradual decrease in severity of her main symptoms, such as fatigue, PEM, muscle and joint pain, lymph node enlargement and tenderness, sleep quality, headaches, postural hypotension, difficulty concentrating, dysnomia, and allergy-type symptoms in 8 visits over 15 months. Her overall severity score of 10 prior to treatment returned to her baseline of 2 (Fig. 3A). Fig. 3B in a linear scatter plot shows how the severity of her more neurological-type symptoms, including fatigue, headache, difficulty concentrating, sleep problems, PEM, postural hypotension and dysnomia, had decreased to scores of 2–5 at 5 months after therapy, from initial scores of 8–10 prior to treatment. By 15 months of triple therapy these symptom scores were at a level of 0–2. Her chronic shingles and cold sores still continue to slowly resolve.

Throughout her symptom improvement she had a consistent improvement in the CD8 T-cell dysfunction assay employed here (Fig. 4, Fig. S1B). The representative FACS plot shows that her CD8 T-cell IFNγ production upon PMA stimulation prior to treatment was 2.9% and increased to 11.1% post-treatment, while her TNFα went from 0.06% to 16.1%. She had a sustained 5-fold increase in her production from 2.3 ± 0.7% (mean of 3 time points over 3 years prior to treatment) pre-treatment to 10.7 ± 2.0% (mean of 6 different time points over 15 months) post treatment, and her TNFα production had gone from essentially zero pre-treatment to 10.3 ± 2.3% post-treatment (mean of 6 time points over 15 months) (Fig. 4). These results suggest the patient may have been able to recover from a severe episode of ME/CFS brought on by the COVID vaccine. In fact, her pre-vaccine CD8 T-cell dysfunction, which may have contributed to her severe reaction to the vaccine, has at least partially reversed while she continues treatment.

Serological studies showed that following the first COVID vaccine, ME/CFS patient ME patient 1 did develop antibodies by ELISA to the SARS-CoV-2 spike protein but had no evidence of neutralizing antibody. This patient has no history of having acute COVID-19, her viral tests by PCR have always been negative and she had no SARS-CoV-2 nucleocapsid antibodies by ELISA, which only the virus infection can induce (not the current mRNA vaccines). Thus, this ME/CFS patient shows evidence that the potential immunological biomarkers that we have identified in this disease may parallel symptomatic improvement during treatment and appear to be moving in the direction of more normal responses as the patient improves. This patient had no side effects from the treatment and her routine blood work (CBC with complete differential, liver and kidney function, thyroid function) remained normal following 15 months of continuous treatment with the nebulized agent described here (Fig. 5).

Case 2: Long COVID patient (LC patient 1) improves on a nebulized antioxidant/anti-pathogen agent. This case involves a 61-year-old female who was healthy until she suffered symptomatic acute COVID-19 infection on November 23, 2020, with severe fatigue, chills, cough, headache, laryngitis, loss of smell and extreme muscle and joint pain. She recovered at home over 2 weeks, but within 4 weeks after acute COVID she developed symptoms of Long COVID with ongoing severe fatigue, brain fog, post exertional malaise, headache, laryngitis, feeling freezing cold, and extreme muscle and joint pain, increased bruising and difficulty walking and suddenly passing out and sleeping for hours after eating, like the clinical phenomena described for ME patient 1. She was a fully self-employed professional who was unable to work and support herself after the acute COVID-19 infection. In 3/3/21



	WBC	RBC	PLT	BUN	CRTN	ALB	ALP	ALT	AST
Center	0	0	0	0	0	0	0	0	0
Vertex	15.0	7.50	500.0	30	2.0	6.0	260	75	40
Units	10**3/µL	10**6/µL	10**3/µL	mg/dL	mg/dL	g/dL	U/L	U/L	U/L
ULN	10.5	5.40	450.0	25	1.3	5.5	147	40	35

Fig. 5. Blood chemistry and CBC of ME/CFS patient (ME 1) remains normal throughout nebulized antioxidant/anti-pathogen treatment (Tx) as shown on a radar plot. WBC- white blood cell, RBC –Red Blood Cell, PLT – Platelet Count, BUN – Blood Urea Nitrogen, CRTN – Creatinine, ALB – Albumin, ALP – Alkaline Phosphatase, ALT – Alanine Aminotransferase, AST - Aspartate Aminotransferase, ULN- upper limit of normal.

her severity scores for overall symptom severity and for the individual symptoms of dysnomia, low grade fever, cough, and sore throat were 8/10; her severity scores for difficulty concentrating, chills, lymph nodes, gastrointestinal symptoms, muscle and joint pain were 10/10; and her headaches, PEM, postural hypotension and sleep difficulty symptom severity scores were between 3 and 5.

She then had a severe immune overactivation following the first dose of the Pfizer-BioNTech COVID-19 vaccine on April 23, 2021, further aggravating her Long COVID symptoms. This was accompanied by development of marked total body tender lymphadenopathy, very similar to ME patient 1. It is thought that immune overactivation like that described above can lead to T-cell dysfunction as discussed earlier (McLane et al., 2019). Her overall symptom severity score increased from her Long COVID baseline of 8–10. Her headaches, PEM, postural hypotension and sleep difficulty severity scores increased to 6–8 (Fig. 6). At this time, 12 days after receiving the SARS-CoV-2 vaccine, her CD8 T-cells were extremely dysfunctional producing essentially no IFNγ or TNFα upon stimulation (Fig. 4, Fig. 1C representative FACS plot).

Twelve days (5/5/21) after the vaccine she started the nebulized therapy. Within 1½ hours of the first dose, she had a dramatic decrease in many of her severe symptoms including brain fog, headache, wobbly walk and her laryngitis. Most of her other symptoms gradually improved over the next few weeks, including passing out after eating, and the severe cold feeling. On May 26, 2021 (Day 21 on treatment) the patient reported that her joint and muscle pain severity had decreased from 10 to 5; muscle tightness had decreased from 10 to 5; brain fog decreased from 9 to 4, and headaches decreased from intermittent daily to infrequent and mild/manageable (Fig. 6). She was able to resume some work, such as client meetings within two weeks of starting the nebulized treatment.

This patient has continued to use the nebulized antioxidant/anti-pathogen agent through to the present time and has reported her symptoms have continued to improve.

However, in November (7 months after beginning treatment), when the nebulized agent was not available for 2 weeks, the patient had a significant exacerbation of all her symptoms like ME patient 1. In fact, she had difficulty when re-starting the nebulized agent, as it did not give her the same level of increase in energy that she had previously. She had more problems tolerating the treatment, particularly with cough and a difficult to describe unpleasant feeling. She continued treatment as it did increase her energy level. She was found to be IgM + for EBV VCA and CMV; HHV6 and VZV IgG titers were also elevated (Table 2). This would suggest that treatment with the nebulized agent may have been helping to keep these viruses under some control whereas temporary discontinuation of treatment for 2 weeks may have allowed these latent viruses to profoundly reactivate.

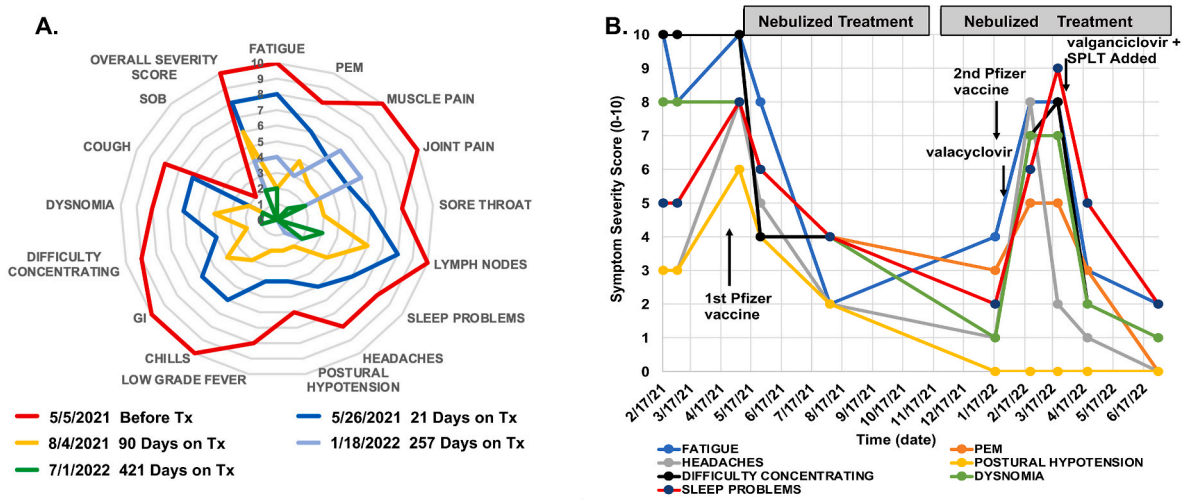


Fig. 6. Symptoms improve over time in Long COVID patient (LC 1) during nebulized antioxidant/anti-pathogen therapy with exacerbation during second COVID-19 vaccine. A. Radar plot shows the change in self reported score of each symptom at various times over therapy. B. Line plot shows how the most common symptoms of ME/CFS and Long COVID improve over time on therapy and indicate when vaccines were given, and antiviral therapies to treat reactivated herpes viruses VZV, EBV and CMV. Nebulized treatment (Tx) period-5/5/2021 to present with 2 wk break 11/10/21-11/24/21. Patient had acute COVID-19 on 11.23.20 and there after developed Long COVID. Patient scored these symptoms on a scale from 0 (no symptoms) to 10 (most severe symptoms). First PfizerBioNTech vaccine was given 4/23/2021 and second BioNTech-Pfizer COVID-19 vaccine on 1/21/22. Valacyclovir - for 2 weeks (1/26/2022 to 2/9/2022). Spironolactone (SPLT) started 3/21/22 to the present. valganciclovir 3/21/2022 to 7/1/2022. PEM-post exertional malaise, GI-gastrointestinal.

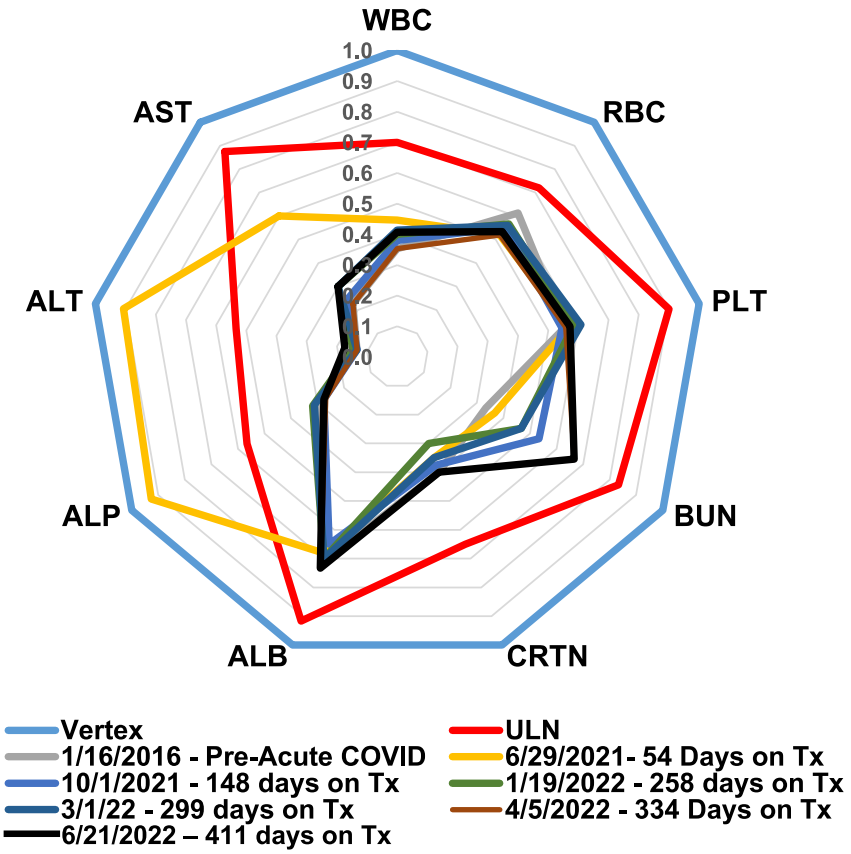
Prior to being treated with anti-viral agents LC Patient 1 decided to take her second dose of the Pfizer-BioNTech COVID-19 vaccine. The vaccine caused all her symptoms of Long COVID to exacerbate although not as severely as before (Fig. 6). Her symptoms also improved much more rapidly than after the first vaccine as she had continued the nebulized treatment at the time the second vaccine dose was administered. She was treated with valganciclovir 900 mg daily for 15 weeks for her apparent CMV and HHV6 reactivation, along with spironolactone 12.5 mg daily for EBV reactivation. Within a week of starting the valganciclovir she found that the nebulized agent began working as well as it had before and she subsequently returned to work almost full time. Serological studies showed that following the first COVID vaccine, LC patient 1 did develop good neutralizing antibody responses to SARS-CoV-2.

Like ME patient 1, during treatment with the nebulized agent, while her symptoms were gradually improving, LC patient 1 had a sustained increase in her CD8 T-cell IFNγ production, going from essentially none pre-treatment to 12.4 ± 2.5% post-treatment (mean of 5 different time points over 15 months), and her TNFα production went from levels below detection pre-treatment to 15 ± 6% post-treatment (mean of 5 different time points over 15 months) (Fig. 4C, Fig. S1 representative FACS plot). Thus, we found evidence over the course of treatment of LC patient 1 that the potential immunological biomarker that we have identified in this disorder, CD8 T-cell dysfunction, may be tracked as it normalizes during treatment becoming more normal as the patient improves. This patient had no side effects from the treatment other than some cough from the nebulizer, which was controlled by nebulizing at a slower rate, and a short spell of an unpleasant feeling when restarting the nebulized therapy following a 2-week break due to production issues, during which she experienced apparent reactivation of multiple herpesviruses. Her routine blood work (CBC with differential, liver and kidney function, thyroid function) following 15 months of continuous nebulized treatment remains normal (Fig. 7). In fact, her liver function studies, which were slightly elevated by the acute COVID infection, were normal after treatment.

ME/CFS and Long COVID patients show reversal of CD8 T-cell dysfunction and symptomatic improvement during treatment with a nebulized antioxidant/anti-pathogen agent.

The above retrospective results suggest these two patients (ME patient 1, LC patient 1) were able to largely recover from their disorder, one with chronic ME/CFS that developed into a severe episode following an aberrant immune response to a SARS-CoV-2 mRNA vaccine, and the other recovering from severe Long COVID that was further exacerbated twice after administration of COVID mRNA vaccines. At the same time both patients had evidence that a potential immunological biomarker that we have identified in these disorders, CD8 T-cell dysfunction, decreased in parallel with treatment as the patients symptomatically improved. There is an urgent need for treatment for both disorders; based on these results and the absence of major side effects we retrospectively examined the ICS and questionnaire data of the 6 other patients, 3 ME/CFS and 3 Long COVID patients, who had elected to use the nebulized antioxidant/anti-pathogen therapy and for whom we had sequential symptom severity score and immunological data (Tables 1 and 2). These other 6 patients have been treated for a shorter period than ME patient 1 and LC patient 1, ranging from 3 to 9 months; all were on the higher dose of 5 ml TID. The length of time these patients were symptomatic with LC post-acute COVID-19, prior to treatment is listed in Table 2. All 6 of these other patients showed improvement in symptoms with some variation in which symptoms improved to a greater degree for any individual (detailed radar plots of symptom severity scores of each individual shown in Supplemental Figs. S2–S7). They also had no significant side effects other than mild cough with nebulizer use in some patients, and their metabolic profiles and CBC remained normal (Supplemental Figs. S2–S7).

The data of all 8 patients treated with the nebulized agent (4 ME/CFS and 4 Long COVID; includes ME patient 1 and LC patient 1) showed a mean of 4.4 points (out of 10 points) in self-reported overall symptom severity scores after treatment (54% decrease) (Fig. 8A). In one of the most common symptoms of both disorders, PEM, there was a mean decrease of 3.9 points following treatment (54% decrease) (Fig. 8B). Two self-reported symptoms included dysnomia (Fig. S8B) and difficulty concentrating (Fig. S8B) were also reduced after treatment with the nebulized agent, with a mean decrease of 3.8 points (64% decrease) (Fig. S8A) and mean of 3.9 points (73.8% decrease) following treatment, respectively. Numbers in this retrospective case series were small; however, future work in controlled clinical trials needs to address whether Long COVID patients respond more rapidly to treatment, versus the possibility that some Long COVID patients may improve without



	WBC	RBC	PLT	BUN	CRTN	ALB	ALP	ALT	AST
Center	0	0	0	0	0	0	0	0	0
Vertex	15.0	7.50	500.0	30	2.0	6.0	260	75	40
Units	10**3/µL	10**6/µL	10**3/µL	mg/dL	mg/dL	g/dL	U/L	U/L	U/L
ULN	10.5	5.40	450.0	25	1.3	5.5	147	40	35

Fig. 7. Blood chemistry and CBC of Long COVID patient (LC 1) remains normal throughout nebulized antioxidant/antipathogen treatment (Tx) as shown on a radar plot. Elevated ALT and ALP on 6/29/2022 from acute COVID-19 infection, subsequently decreased while on inhalation therapy. WBC- white blood cell, RBC –Red Blood Cell, PLT – Platelet Count, BUN – Blood Urea Nitrogen, CRTN – Creatinine, ALB – Albumin, ALP – Alkaline Phosphatase, ALT – Alanine Aminotransferase, AST - Aspartate Aminotransferase, ULN- upper limit of normal.

treatment (Fig. 9).

There also was evidence of improvement in CD8 T-cell dysfunction, with a 3.6-fold increase ($p = 0.006$, $n = 7$) in the frequency of CD8 T-cells producing IFNγ by a mean difference of 9.5 ± 2.3.0% going from 3.7 ± 0.9% pre-treatment to 13.2 ± 1.7% post-treatment. We also saw a 2.5-fold increase ($p = 0.016$, $n = 7$) in the frequency of CD8 T-cells producing TNFα by a mean 8.8 ± 2.6% going from 5.9 ± 2.3% pre-treatment to 14.8 ± 1.1% post-treatment (Fig. 10). Post-treatment, these values are not yet reaching the mean observed in HC but are approaching levels seen in some individual healthy controls at the lower range of HC, who had a mean of 26.1 ± 6.0% of IFNγ-producing CD8 T-cells and a mean of 32.2 ± 6.8% of TNFα-producing CD8 T-cells (Fig. 1). This retrospective case series suggests that not only was there an improvement in the severity of individual and overall symptoms in both ME/CFS and Long COVID patients; in addition, we noted an improvement in immune dysregulation (decrease in CD8 T-cell dysfunction) that paralleled treatment with the nebulized agent described above.

4. Discussion

These retrospective case series results suggest there may be both symptomatic relief and improvement of a type of CD8 T-cell dysfunction, in subjects with two disorders with similar manifestations, ME/CFS and Long COVID, over the course of treatment with a multi-mechanism nebulized agent with antioxidant, anti-pathogen and likely immunomodulatory properties. These findings provide evidence of a potential immunological biomarker, CD8 T-cell dysfunction, for diagnosis and for monitoring of symptoms and treatment outcomes in both ME/CFS and Long COVID. There was no evidence of serious adverse events or laboratory anomalies with this treatment. It is noteworthy that 8/8 patients had a positive response, some within hours of treatment. Intervening in Long COVID patients at earlier stages after the inciting infection potentially holds greater potential to reverse the course of disease, as compared with later stages or with more chronic ME/CFS patients. The therapy may also allow ME/CFS and Long COVID patients to have increased functionality in their daily life, as shown by the setbacks that occurred when some of the patients (ME1 and LC1) temporarily stopped treatment. This suggests that even if many Long COVID patients were to eventually improve without treatment, the nebulized therapy would give them symptomatic relief that would allow them to return to a more normal active life sooner. The preliminary safety, efficacy and biomarker data presented here on the use of this nebulized agent in patients with ME/CFS and Long COVID, in the face of the absence of defined treatments for either disorder, also support moving

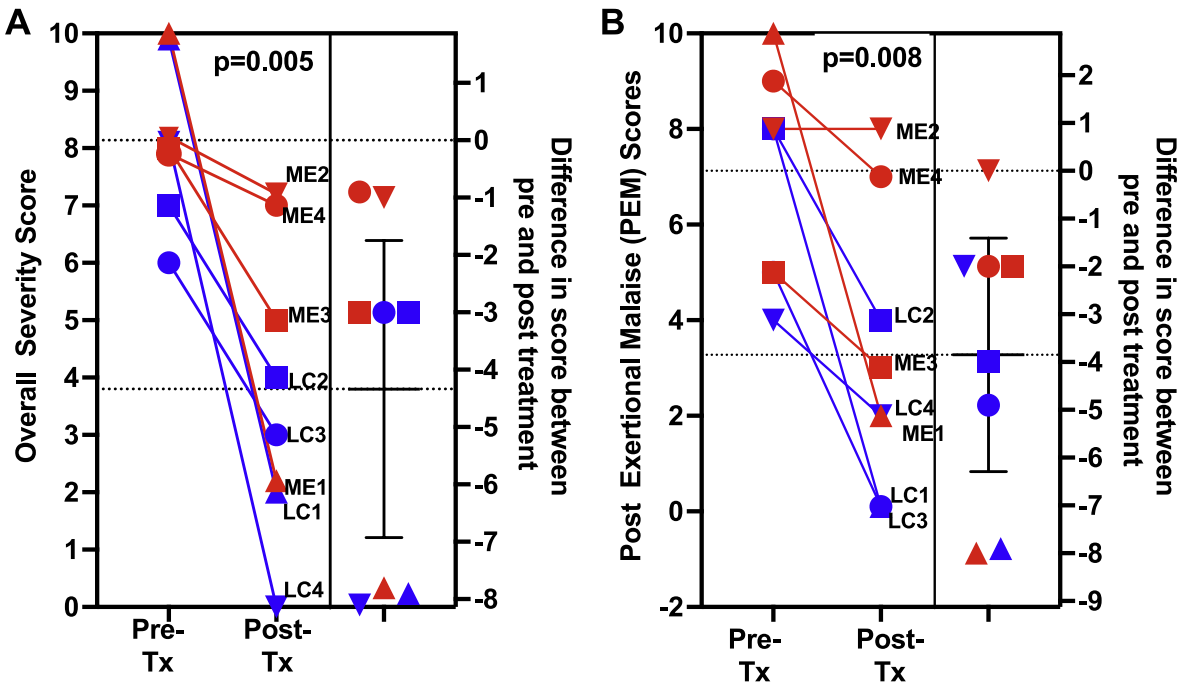


Fig. 8. Significant decreases in self-reported overall symptoms (A) and PEM (B) scores in ME/CFS (red symbols, n = 4) and Long COVID (blue symbols, n = 4) patients following nebulized antioxidant/anti-pathogen treatment. Patients scored these symptoms on a scale from 0 (no symptoms) to 10 (most severe symptoms). Paired *t*-test (two-tailed) indicates significant differences pre- and post-treatment. Right side of graph shows the difference in score between pre- and post-treatment (Tx) for each patient. PEM-post exertional malaise.

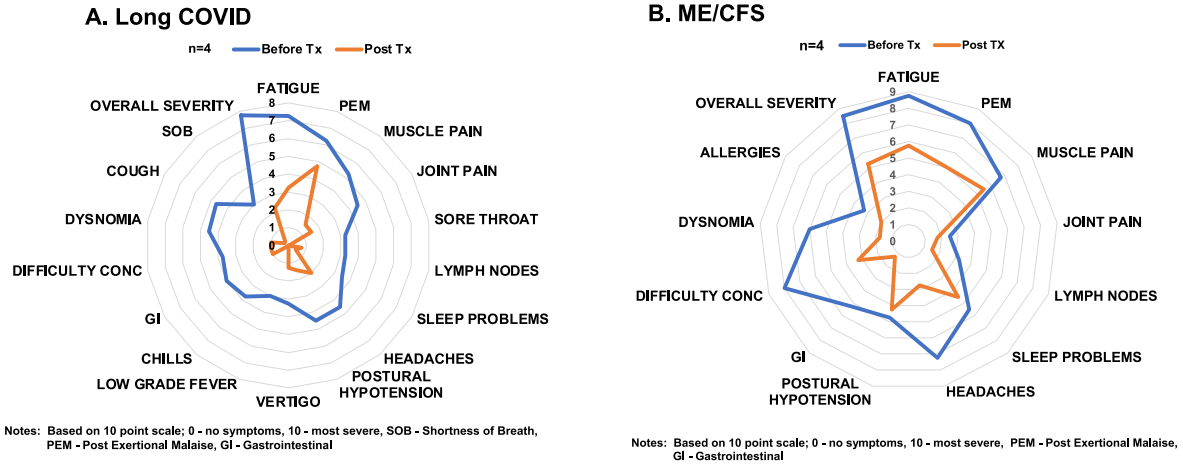


Fig. 9. Mean value of Long-COVID (A) and ME/CFS (B) patient symptom scores pre and post nebulized antioxidant/anti-pathogen treatment (Tx). Patients scored these symptoms on a scale from 0 (no symptoms) to 10 (most severe symptoms). Long COVID and ME/CFS patients had an overall mean reduction of all symptoms equally weighted of 75% and 52%, respectively. MECFS n = 4, long COVID n = 4.

this novel treatment strategy into appropriately powered, controlled clinical trials to address both disabling conditions.

Most patients in the retrospective case series reported improvement right away in sleep with this nebulized treatment, a factor considered crucial for recovery from ME/CFS. Though data are limited from this small case series, the individual profiles show that there appears to be variability as to which symptoms improve, when, and at what rate. This may relate to the individual immunological compensations each patient has made as their immune systems struggle to control their individual specific set of persistent pathogens, and depending on their overall immune reserves at the time the nebulized treatment was begun. However, we do not know if some patients with more protracted and severe immune dysregulation may require continued treatment, much like individuals with diabetes require insulin to continually correct metabolic defects. Our results, though based on a small number of patients, nonetheless suggest that the earlier we intervene with treatment in Long COVID patients, and in ME/CFS patients with recent onset of disease, the greater their chances of recovery.

A nebulized antioxidant/anti-pathogen agent with potential immunomodulatory properties was used in this small retrospective case series of ME/CFS and Long COVID patients, as it contains multiple components that can act against the multiple mechanisms postulated to underlie these complex disorders. All compounds in this treatment have antioxidant properties, which have been shown to decrease oxidative stress and inflammatory processes, a process previously demonstrated to be a major problem in COVID-19, ME/CFS and Long-COVID (Maes et al., 2011, 2012a, 2012b, 2021; Dennis et al., 2021; Kumar et al., 2020; Wu, 2020; Al-Hakeim et al., 2023; Fukuda et al., 1994; Paul et al., 2021;

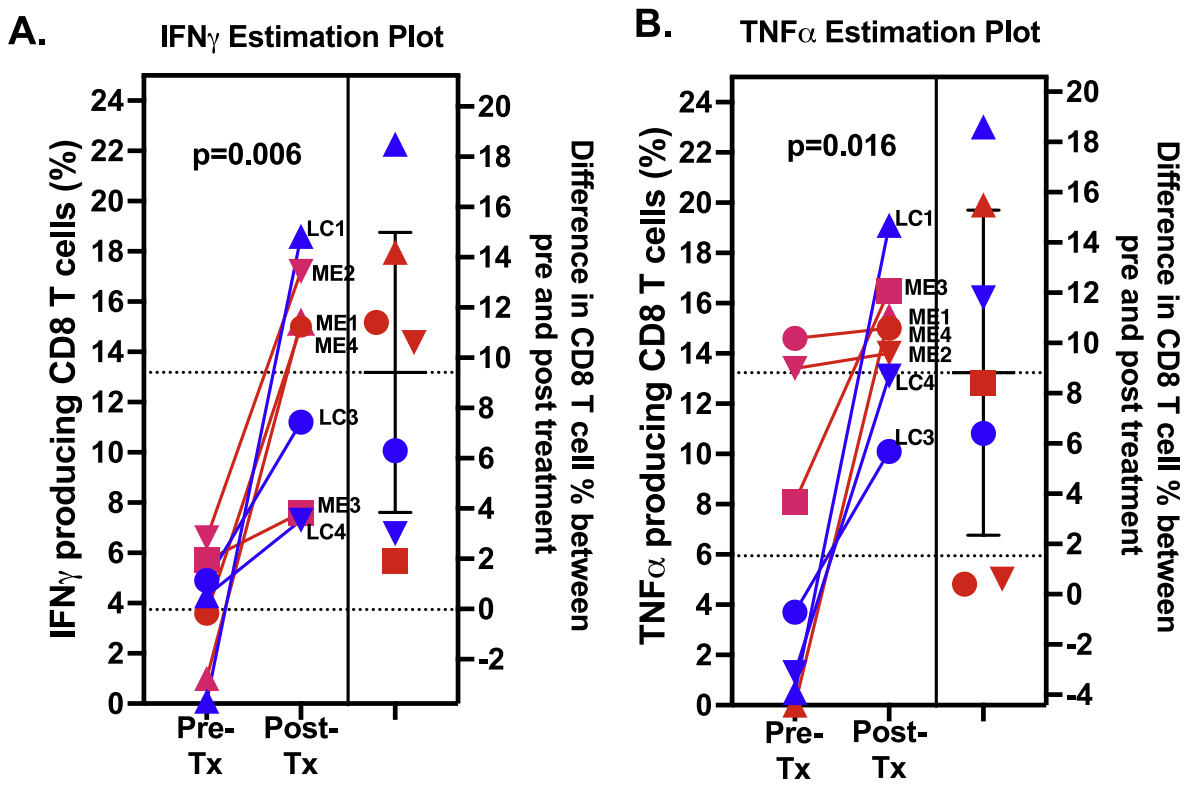


Fig. 10. Reversal of CD8 T cell dysfunction evident from increase in INF-γ (A) and TNF-α (B) cytokine production in ICS following PMA stimulation in 4 ME/CFS (red symbols) and 3 Long COVID (blue symbols) patients following nebulized antioxidant/anti-pathogen treatment. Red dots and blue dots show results of ME/CFS and Long COVID patients, respectively. Paired *t*-test (two-tail) analyses. Right side of graph shows the difference in CD8 T cell % between pre- and post-treatment (Tx) for each patient. Long COVID patient (LC 2) data not available as blood sample was degraded during shipping. (only one selected time point pre- and post-treatment is shown for each patient).

Takemoto et al., 2021; Gould et al., 2010, 2011, 2015; Prousky, 2007; Rusznak et al., 2000; Buhl et al., 1990; Dekhuijzen, 2004; Straface et al., 2000; Bridgeman et al., 1991; Hagiwara et al., 2000; Paulin et al., 2017; Lima et al., 2013; Juergens et al., 2003, 2004, 2018, 2020; Rhoden et al., 2004; Kennedy-Feitosa et al., 2016; Dahham et al., 2015; Machado et al., 2018; Kim et al., 2015). Although the underlying mechanism for ME/CFS pathogenesis is not known, some research studies suggest that ME/CFS is a disease of chronic inflammation driven by factors such as mitochondrial dysfunction and oxidative/nitrosative stress (Pall, 2003; Morris et al., 2016). Metabolomics studies suggest *hypometabolism* occurs in ME/CFS, including evidence of downregulated glycolysis (Fluge et al., 2016; Armstrong et al., 2014; Naviaux et al., 2016a, 2016b)). Other studies suggest that disruption of the TCA cycle with impaired pyruvate dehydrogenase (an enzyme in the mitochondrial matrix that catalyzes the TCA cycle (Fluge et al., 2016)) and elevated lactate and low citrate play a role in ME/CFS (Yamano et al., 2016). Previous clinical trials in both ME/CFS (Wood et al., 2021) and Gulf War Illness (Golomb et al., 2014; Baraniuk et al., 2013; Hodgin et al., 2021; Van Doren et al., 2022), a disorder with some shared clinical features, suggest that antioxidants could be beneficial in these conditions.

All the components in this nebulized agent have been reported to have broad direct anti-viral properties against several viruses, including several that routinely produce respiratory symptoms, including parainfluenza-1, influenza A (H5N1), herpes simplex virus type-1 (HSV-1) and type-2 (HSV-2), human immune deficiency virus (HIV-1), and dengue virus 2 (DENV-2) (Holroyd et al., 1993; Nencioni et al., 2003; Roederer et al., 1990; Geiler et al., 2011; Yang et al., 2010; Li et al., 2016; Astani et al., 2011; Pajaro-Castro et al., 2015). 1,8-cineole, β-caryophyllene and methylcobalamin each have antiviral properties to SARS-CoV-2 (Cavar Zeljkovic et al., 2022; Jimenez-Guardeno et al., 2022; Kim et al., 2022). β-caryophyllene has broad spectrum antibacterial properties (in vitro) against several bacterial species, including S. aureus, K. pneumoniae, and P. aeruginosa, and in vivo for *M. pneumoniae* (Dahham et al., 2015). β-caryophyllene also has broad spectrum antifungal properties against several fungal species (Dahham et al., 2015). As many different persistent pathogens have been associated with ME/CFS as potentially causative or contributing to disease via reactivation, viruses such as EBV, CMV, HHV-6, HHV-7, HHV-8, (Unger et al., 2017; Buchwald et al., 1992; Ablashi et al., 2000), human parvovirus B19 (B19V), enteroviruses (O'Neal and Hanson, 2021; Chia and Chia, 2008), lentivirus, and bacteria (Beyond Myalgic Encephalomyelitis, 2015) such as mycoplasma, lyme and Q-fever, this nebulized agent could potentially help dampen pathogen load of multiple infectious agents at the same time. This nebulized treatment also has multiple ingredients capable of acting as immune modulators through their ability to attenuate NF-κB signaling, a process essential for signaling through the TCR, thus decreasing over-activation and potentially averting subsequent dysfunctional CD8 T-cells (Kim et al., 2015; Greiner et al., 2013; Francomano FC et al., 2019; Oka et al., 2000; Sudhoff et al., 2015; Yadav and Chandra, 2017; Cantin et al., 2000; Lou and Kaplowitz, 2007; Xu et al., 2016; Wenzel et al., 2021; Attiq et al., 2021; Davies et al., 2021).

We propose that an inappropriate immune response to an infection triggers Long COVID and at least a subset of ME/CFS, resulting in chronically dysregulated immune responses with CD8 T-cell dysfunction. For instance, inappropriate immune responses to viruses can occur in the presence of heterologous immunity and activation of cross-reactive CD8 T-cells (Welsh and Selin, 2002; Selin et al., 2011). Cross-reactive activation of memory influenza A (IAV)-specific memory CD8 T-cells in HLA-A2+ patients during acute EBV infections result in the highly dysregulated immune response and syndrome of acute infectious mononucleosis, wherein there is massive over-expansion of

functionally exhausted CD8 T-cells in conjunction with high viral load persisting for life (Aslan et al., 2017; Clute et al., 2005). A highly dysregulated immune response may lead to viral persistence, CD8 T-cell dysfunction and/or T-cell clonal exhaustion.

Clonally exhausted CD8 T-cells are characterized by a progressive loss of effector functions, high and sustained inhibitory receptor expression, metabolic dysregulation, poor memory recall and homeostatic self-renewal and distinct transcriptional programs (McLane et al., 2019). For CD8 T-cell clonal exhaustion to occur there needs to be persistent antigen from virus or tumor that drives hyperactivation of T-cells, leading to sustained co-expression of multiple inhibitory receptors on T-cells *e.g.*, programmed cell death protein 1 (PD1), cytotoxic T-lymphocyte associated protein 4 (CTLA4), natural killer cell receptor 2B4 (2B4), lymphocyte-activation gene 3 (Lag 3). These inhibitory receptors provide negative costimulatory signals to CD8 T-cells that prevent optimal CD8 T-cell effector responses. However, responses to persistent antigen, virally infected cells, immunoregulatory cells, antigen presenting cells, and tumors can contribute to a chronic state of inflammation by producing both inflammatory cytokines, like interferon alpha/beta (IFN α/β) and inhibitory cytokines, like interleukin-10 (IL-10) or transforming growth factor beta (TGFβ), further driving exhaustion by eliciting negative regulatory signals on T-cells, either directly or indirectly. The ability to reinvigorate exhausted CD8 T-cells through inhibitory receptor blockade (check-point inhibitors) such as anti-PD1, anti-programmed cell death ligand 1 (PDL1) and anti-CTLA4 highlights the therapeutic potential of better understanding dysfunctional T-cells (Barber et al., 2006; Pauken et al., 2019; Mellman et al., 2016). Thus, we will focus in future work on extending current findings of T-cell dysfunction to determine whether other features associated with T-cell clonal exhaustion are also present in ME/CFS and Long COVID, through investigation of specific markers such as PD1 and RNA seq expression patterns. We used a general trigger, PMA, in the well-established ICS assay system in studies here to examine over all functionality of the CD8 T-cells, which has the advantage of not being antigen or HLA-restricted making it easier to utilize as a clinical biomarker assay. Studies interrogating T-cell responses to specific pathogenic triggers (SARS-CoV-2, EBV, others) are also likely to be informative about the disease process.

Similar to our results on CD8 T-cell dysfunction, a recent report based on 215 patients, using extensive state-of-the-art single cell RNA-sequencing immune profiling, found evidence consistent with CD8 T-cell clonal exhaustion and evidence of herpes virus reactivation to be two of the hallmark findings in the immunopathogenesis of Long COVID (Klein et al., 2022). A recent study from Hanson and colleagues that examined T-cell metabolism and cytokine associations found that ME/CFS CD8 T-cells had reduced mitochondrial membrane potential compared to healthy controls (Mandarano et al., 2020). CD8 T-cells from ME/CFS patients had reduced glycolysis both at rest and following activation. These disturbances in T-cell metabolism are highly consistent with findings in chronic viral infections and CD8 T-cell clonal exhaustion (McLane et al., 2019).

CD8 T-cell dysfunction, or a state of clonal T-cell exhaustion, in turn can lead to difficulty in controlling many of the persistent pathogens that are already nearly universally present in adult humans, such as EBV, CMV and HHV6; as these viruses become under poorer control, reactivating more frequently, they may further contribute to the immune dysregulation. It is becoming increasingly recognized that immune system dysregulation of acute COVID-19 and Long COVID may enable reactivation of latent viruses already present in their systems (Lehner et al., 2020; Le Balc'h et al., 2020; Gold et al., 2021; Proal and VanElzakker, 2021). Early studies and case histories demonstrate herpes virus reactivation in some acute COVID-19 patients (Le Balc'h et al., 2020; Garcia-Martinez et al., 2020). ME/CFS patients have been reported to have increased levels of antibodies to persistent viruses in the herpes family, like EBV, HHV6, CMV, VZV and HSV-1; treatment with antivirals, such as acyclovir and valacyclovir and valganciclovir, has had success in some patients (Henderson, 2014; Watt et al., 2012). There are also recent reports of finding herpes viruses by PCR in tissues like the brain (Kasimir et al., 2022). Patients treated with anti-TNF have also been reported to develop shingles (symptomatic VZV reactivation), requiring treatment, and have been found to have EBV and CMV by PCR in the stool (Kim and Solomon, 2010; Santella et al., 2022). In fact, other T-cell mediated autoimmune diseases, such as inflammatory bowel disease has been associated with reactivation of herpes viruses, as EBV has been found in the mucosal lining of the gut in some of these patients (Zhang et al., 2022; Wang et al., 2022). Dysregulated immune responses to EBV have also been strongly linked to the immunopathogenesis of multiple sclerosis, as well as finding evidence of EBV in the brain (Pender et al., 2017, 2018). Symptomatic herpes virus reactivation, such as VZV, has been previously reported following vaccination (Walter et al., 1999). It would, therefore, not be too surprising that ME patient 1, who did not produce any TNFα from her CD8 T-cells prior to vaccination, might develop chronic shingles (VZV) and cold sores (HSV-1) following vaccination.

It is likely other pathogens known to persist in humans, including RNA viruses such as enteroviruses, or bacteria such as *M. pneumonia*, or fungi such as *C. albicans,* that are controlled by CD8 T-cell responses may also be reactivated and be contributing to T-cell dysfunction. Identifying which persistent pathogens are reactivated in any one patient and determining if they need to be treated will be challenging, but possible. The nebulized agent described here has anti-viral, anti-bacterial and anti-fungal properties. It is thus useful to help treat multiple pathogens at the same time. However, anti-pathogen drugs such as anti-viral or antibiotic medications may be needed in addition to this nebulized therapy as seen in some of the patients in this study.

In patients with chronic ME/CFS, studies suggest that subsequent new infections and antigenic challenges, such as with vaccines, may exacerbate their symptoms (Beyond Myalgic Encephalomyelitis, 2015). In the case of ME patient 1, viral infections or the SARS-CoV-2 vaccine appeared to trigger all 4 major relapses of severe ME/CFS over the course of decades. Some ME/CFS patients have been reported to have exacerbation of their symptoms following SARS-CoV-2 vaccination (ANZMES, 2021; Johnson, 2022; Johnson, 2023). A recent study shows that people infected with SARS-CoV-2 prior to vaccination produced spike-specific CD8 T-cells at considerably lower levels—and with more dysfunctional response—than vaccinated people who had never been infected (Gao et al., 2023). Similar to our results, these findings suggest that SARS-CoV-2 infection damages the CD8 T-cell response, an effect similar to that observed in previous studies showing long-term damage to the immune system after infection with hepatitis C or HIV1, both persistent viruses that can induce T-cell clonal exhaustion (McLane et al., 2019). Unfortunately, established clinical testing by serology, and PCR of serum/plasma for viremia, is limited, making it difficult to determine if there is impairment in immune system control over existing persistent herpesviruses. It is likely that compensatory immune mechanisms keep some patients from becoming markedly viremic, so the presence of virus in peripheral blood or circulating immune cells, if any, may not be picked up by PCR.

Recent studies also suggest that reactivation of persistent viruses may be difficult to detect as they mostly reside within tissues, such as muscle, or even in the gastrointestinal (GI) tract and/or in stool, as seen in patients immunosuppressed by anti-TNF therapy (Santella et al., 2022; Solomon et al., 2022). SARS-CoV-2 is also thought to linger in the body such as the GI tract for long periods after initial infection, particularly in the neurons of the myenteric plexus (Gray-Rodriguez et al., 2022; Peluso et al., 2023). CMV is known to infect endothelial cells lining the vasculature (Takatsuka et al., 2003) and thus could potentially be replicating throughout the body, including the blood vessels in the brain. Currently, clinicians will need to maintain a high index of suspicion regarding reactivation of persistent agents and may find it useful to perform the herpesvirus serology panel outlined here. If the patient harbors IgM antibodies targeting any of these persistent viruses, this

suggests reactivation, as in LC patient 1, or has high titers and clinical symptoms of chronic shingles or recurrent cold sores, as in ME patient 1, then the patient should be treated with the appropriate antivirals. In these two patients, introducing antivirals at 11–12 months into treatment with the nebulized agent (at which time overall severity scores had already decreased from 10 to 4–5) may have contributed to greater clinical improvement, such that overall severity scores decreased further (e.g., scores dropping from 4 to 5 to 2).

Other clinical factors may need to be considered before embarking on added anti-viral treatment if high titers are found for antibodies specific for multiple herpesviruses. Nonetheless, high antibody titers even against multiple herpesviruses at the same time, are strongly suggestive of significant CD8 T-cell impairment and potentially T-cell clonal exhaustion leading to difficulties controlling persistent infections. In the present retrospective study, only 3 of 8 patients were also treated with anti-viral therapy while on this nebulized treatment. Larger, controlled clinical trials are needed to determine whether adjunct treatment with antivirals during treatment with this nebulized agent could further enhance therapeutic outcome resulting in more rapid and permanent reversal of CD8 T-cell dysfunction, with greater control over persistent pathogens and more rapid improvement of symptoms.

Using functional ICS assays to examine CD8 T-cell cytokine production led to identification of CD8 T-cell dysfunction as a possible underlying immunopathologic pathway in some patients with ME/CFS and Long COVID. This type of assay may prove useful as a diagnostic tool and biomarker to track disease outcome during therapy. It is a relatively simple and well-established technology that has the potential to be adapted for use by clinical labs. Although altered inflammatory cytokines in ME/CFS patient serum and plasma have been previously reported to indicate significant immune dysregulation, circadian rhythms, sex and other individual differences, and various stressors can introduce variance. Measuring serum cytokine levels also is not generally useful as biomarkers, as serum cytokines are generally highly labile, with very short half-lives often due to the fact that other immune cells consume them rapidly and they are also very sensitive to how they are handled in the lab (Hornig et al., 2015; Broderick et al., 2010; Russell et al., 2016; Fletcher et al., 2009; Hardcastle et al., 2015; Maes et al., 2012a, 2012b). This can often lead to differing results between studies. Because one cannot identify which immune cell subsets are producing (or failing to produce) these immune molecules, these peripheral immune signatures also do not point clearly to the underlying immunopathogenic mechanisms (Hornig et al., 2015; Broderick et al., 2010; Russell et al., 2016; Fletcher et al., 2009; Hardcastle et al., 2015; Maes et al., 2012a, 2012b). Single cell RNA sequencing assays on multiple immune cells at the same time may someday be useful for diagnostic assays, but at present are still more of a research tool.

This is only the beginning of our research into immune dysregulation and its role in the immunopathogenesis of ME/CFS and Long COVID. In our ongoing research there appears to be evidence of other compensatory immune mechanisms for control of persistent pathogens in these patient populations. At present, we have demonstrated the utility of a CD8 T-cell functional assay for IFNγ and TNFα production following stimulation with a generic T-cell stimulus as a first logical and clinically feasible step in assessing CD8 T-cell dysfunction for diagnosis and tracking of responses to therapy. With further research we hope to be able to identify treatment-responsive subsets of patients, predict outcome and severity and be better able to understand the pathogenesis of ME/CFS and Long COVID and the mechanism of action of this nebulized antioxidant, anti-pathogen, and potentially immunomodulatoryagent in treatment of these disabling disorders.

Funding

This study was funded by NIH R01 AI159314-01 (LKS), Ramsey Award (SolveME, Inc.) (LKS), private donations (LKS) and Patient -Led Research Collaborative (LSK).

CRediT authorship contribution statement

Anna Gil: Conceptualization, Formal analysis, Investigation, Methodology, Project administration. **George E. Hoag:** Formal analysis, Writing – review & editing. **John P. Salerno:** Project administration, Resources. **Mady Hornig:** Writing – review & editing, Validation. **Nancy Klimas:** Validation, Writing – review & editing. **Liisa K. Selin:** Conceptualization, Formal analysis, Funding acquisition, Investigation.

Declaration of competing interest

The authors have conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

We would like to thank Drs. R. Welsh, E. Szomolanyi-Tsuda and N. Aslan for editorial help with the manuscript; R. Solomon, J. Perrin, A. Joliat and the Massachusetts ME/CFS Association for their assistance in helping us move this research ahead.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2023.100720.

References

Ablashi, D.V., Eastman, H.B., Owen, C.B., Roman, M.M., Friedman, J., Zabriskie, J.B., et al., 2000. Frequent HHV-6 reactivation in multiple sclerosis (MS) and chronic fatigue syndrome (CFS) patients. J. Clin. Virol. 16 (3), 179–191.

Al-Aly, Z., Xie, Y., Bowe, B., 2021. High-dimensional characterization of post-acute sequelae of COVID-19. Nature 594 (7862), 259–264.

Al-Hakeim, H.K., Al-Rubaye, H.T., Al-Hadrawi, D.S., Almulla, A.F., Maes, M., 2023. Long-COVID post-viral chronic fatigue and affective symptoms are associated with oxidative damage, lowered antioxidant defenses and inflammation: a proof of concept and mechanism study. Mol. Psychiatr. 28 (2), 564–578.

ANZMES Preliminary survey findings, n.d. Nov 15, 2021. https://anzmesorgnz/anzmes-preliminary-survey-findings/. 2021.

Armstrong, C.W., McGregor, N.R., Butt, H.L., Gooley, P.R., 2014. Metabolism in chronic fatigue syndrome. Adv. Clin. Chem. 66, 121–172.

Aslan, N., Watkin, L.B., Gil, A., Mishra, R., Clark, F.G., Welsh, R.M., et al., 2017. Severity of acute infectious mononucleosis correlates with cross-reactive CD8 T-cell receptor repertoires. mBio 8, e01841-17. https://doi.org/10.1128/mBio.01841-17.

Astani, A., Reichling, J., Schnitzler, P., 2011. Screening for antiviral activities of isolated compounds from essential oils. Evid Based Complement Alternat Med 2011, 253643.

Attiq, A., Yao, L.J., Afzal, S., Khan, M.A., 2021. The triumvirate of NF-kappaB, inflammation and cytokine storm in COVID-19. Int. Immunopharm. 101 (Pt B), 108255.

Baraniuk, J.N., El-Amin, S., Corey, R., Rayhan, R., Timbol, C., 2013. Carnosine treatment for gulf war illness: a randomized controlled trial. Global J. Health Sci. 5 (3), 69–81.

Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., et al., 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 439 (7077), 682–687.

Bedree, H., Sunnquist, M., Jason, L.A., 2019. The DePaul symptom questionnaire-2: a validation study. Fatigue 7 (3), 166–179.

Bested, A.C., Marshall, L.M., 2015. Review of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: an evidence-based approach to diagnosis and management by clinicians. Rev. Environ. Health 30 (4), 223–249.

Beyond myalgic encephalomyelitis/chronic fatigue syndrome: redefining an illness. Mil. Med. 180 (7), 2015, 721–723.

Bishop, C., Hudson, V.M., Hilton, S.C., Wilde, C., 2005. A pilot study of the effect of inhaled buffered reduced glutathione on the clinical status of patients with cystic fibrosis. Chest 127 (1), 308–317.

Blank, C.U., Haining, W.N., Held, W., Hogan, P.G., Kallies, A., Lugli, E., et al., 2019. Defining 'T cell exhaustion'. Nat. Rev. Immunol. 19 (11), 665–674.

Bridgeman, M.M., Marsden, M., MacNee, W., Flenley, D.C., Ryle, A.P., 1991. Cysteine and glutathione concentrations in plasma and bronchoalveolar lavage fluid after treatment with N-acetylcysteine. Thorax 46 (1), 39–42.

Broderick, G., Fuite, J., Kreitz, A., Vernon, S.D., Klimas, N., Fletcher, M.A., 2010. A formal analysis of cytokine networks in chronic fatigue syndrome. Brain Behav. Immun. 24 (7), 1209–1217.

Buchwald, D., Cheney, P.R., Peterson, D.L., Henry, B., Wormsley, S.B., Geiger, A., et al., 1992. A chronic illness characterized by fatigue, neurologic and immunologic

disorders, and active human herpesvirus type 6 infection. Ann. Intern. Med. 116 (2), 103–113.
Buhl, R., Vogelmeier, C., Critenden, M., Hubbard, R.C., Hoyt Jr., R.F., Wilson, E.M., et al., 1990. Augmentation of glutathione in the fluid lining the epithelium of the lower respiratory tract by directly administering glutathione aerosol. Proc. Natl. Acad. Sci. U. S. A. 87 (11), 4063–4067.
Calabrese, C., Tosco, A., Abete, P., Carnovale, V., Basile, C., Magliocca, A., et al., 2015. Randomized, single blind, controlled trial of inhaled glutathione vs placebo in patients with cystic fibrosis. J. Cyst. Fibros. 14 (2), 203–210.
Cantin, A.M., Paquette, B., Richter, M., Larivee, P., 2000. Albumin-mediated regulation of cellular glutathione and nuclear factor kappa B activation. Am. J. Respir. Crit. Care Med. 162 (4 Pt 1), 1539–1546.
Cavar Zeljkovic, S., Schadich, E., Dzubak, P., Hajduch, M., Tarkowski, P., 2022. Antiviral activity of selected lamiaceae essential oils and their monoterpenes against SARS-Cov-2. Front. Pharmacol. 13, 893634.
Chia, J.K., Chia, A.Y., 2008. Chronic fatigue syndrome is associated with chronic enterovirus infection of the stomach. J. Clin. Pathol. 61 (1), 43–48.
Choutka, J., Jansari, V., Hornig, M., Iwasaki, A., 2022. Unexplained post-acute infection syndromes. Nat. Med. 28 (5), 911–923.
Clute, S.C., Watkin, L.B., Cornberg, M., Naumov, Y.N., Sullivan, J.L., Luzuriaga, K., et al., 2005. Cross-reactive influenza virus-specific CD8+ T cells contribute to lymphoproliferation in Epstein-Barr virus-associated infectious mononucleosis. J. Clin. Invest. 115 (12), 3602–3612.
Cornberg, M., Kenney, L.L., Chen, A.T., Waggoner, S.N., Kim, S.K., Dienes, H.P., et al., 2013. Clonal exhaustion as a mechanism to protect against severe immunopathology and death from an overwhelming CD8 T cell response. Front. Immunol. 4, 475.
Dahham, S.S., Tabana, Y.M., Iqbal, M.A., Ahamed, M.B., Ezzat, M.O., Majid, A.S., et al., 2015. The anticancer, antioxidant and antimicrobial properties of the sesquiterpene beta-caryophyllene from the essential oil of aquilaria crassna. Molecules 20 (7), 11808–11829.
Davies, D.A., Adlimoghaddam, A., Albensi, B.C., 2021. The effect of COVID-19 on NF-kappaB and neurological manifestations of disease. Mol. Neurobiol. 58 (8), 4178–4187.
Davis, H.E., Assaf, G.S., McCorkell, L., Wei, H., Low, R.J., Re'em, Y., et al., 2021. Characterizing long COVID in an international cohort: 7 months of symptoms and their impact. EClinicalMedicine 38, 101019.
Davis, H.E., McCorkell, L., Vogel, J.M., Topol, E.J., 2023. Long COVID: major findings, mechanisms and recommendations. Nat. Rev. Microbiol. 1–14.
Dekhuijzen, P.N., 2004. Antioxidant properties of N-acetylcysteine: their relevance in relation to chronic obstructive pulmonary disease. Eur. Respir. J. 23 (4), 629–636.
Dennis, A., Wamil, M., Alberts, J., Oben, J., Cuthbertson, D.J., Wootton, D., et al., 2021. Multiorgan impairment in low-risk individuals with post-COVID-19 syndrome: a prospective, community-based study. BMJ Open 11 (3), e048391.
Fletcher, M.A., Zeng, X.R., Barnes, Z., Levis, S., Klimas, N.G., 2009. Plasma cytokines in women with chronic fatigue syndrome. J. Transl. Med. 7, 96.
Fluge, O., Mella, O., Bruland, O., Risa, K., Dyrstad, S.E., Alme, K., et al., 2016. Metabolic profiling indicates impaired pyruvate dehydrogenase function in myalgic encephalopathy/chronic fatigue syndrome. JCI Insight 1 (21), e89376.
Francomano FC, A., Barbarossa, A., Fazio, A., La Torre, C., Ceramella, J., Mallamaci, R., Saturnino, C., Iacopetta, D., Sinicropi, M.S., 2019. β-Caryophyllene: a sesquiterpene with countless biological properties. Appl. Sci. 9, 5420.
Fukuda, K., Straus, S.E., Hickie, I., Sharpe, M.C., Dobbins, J.G., Komaroff, A., 1994. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group. Ann. Intern. Med. 121 (12), 953–959.
Gao, F., Mallajoysula, V., Arunachalam, P.S., van der Ploeg, K., Manohar, M., Roltgen, K., et al., 2023. Spheromers reveal robust T cell responses to the Pfizer/BioNTech vaccine and attenuated peripheral CD8(+) T cell responses post SARS-CoV-2 infection. Immunity.
Garcia-Martinez, F.J., Moreno-Artero, E., Jahnke, S., 2020. SARS-CoV-2 and EBV coinfection. Med. Clin. 155 (7), 319–320.
Geiler, J., Michaelis, M., Sithisarn, P., Cinatl Jr., J., 2011. Comparison of pro-inflammatory cytokine expression and cellular signal transduction in human macrophages infected with different influenza A viruses. Med. Microbiol. Immunol. 200 (1), 53–60.
Gold, J.E., Okyay, R.A., Licht, W.E., Hurley, D.J., 2021. Investigation of long COVID prevalence and its relationship to epstein-barr virus reactivation. Pathogens 10 (6).
Golomb, B.A., Allison, M., Koperski, S., Koslik, H.J., Devaraj, S., Ritchie, J.B., 2014. Coenzyme Q10 benefits symptoms in Gulf War veterans: results of a randomized double-blind study. Neural Comput. 26 (11), 2594–2651.
Gould, N.S., Min, E., Gauthier, S., Chu, H.W., Martin, R., Day, B.J., 2010. Aging adversely affects the cigarette smoke-induced glutathione adaptive response in the lung. Am. J. Respir. Crit. Care Med. 182 (9), 1114–1122.
Gould, N.S., Min, E., Gauthier, S., Martin, R.J., Day, B.J., 2011. Lung glutathione adaptive responses to cigarette smoke exposure. Respir. Res. 12 (1), 133.
Gould, N.S., Min, E., Huang, J., Chu, H.W., Good, J., Martin, R.J., et al., 2015. Glutathione depletion accelerates cigarette smoke-induced inflammation and airspace enlargement. Toxicol. Sci. 147 (2), 466–474.
Gray-Rodriguez, S., Jensen, M.P., Otero-Jimenez, M., Hanley, B., Swann, O.C., Ward, P. A., et al., 2022. Multisystem screening reveals SARS-CoV-2 in neurons of the myenteric plexus and in megakaryocytes. J. Pathol. 257 (2), 198–217.
Greiner, J.F., Muller, J., Zeuner, M.T., Hauser, S., Seidel, T., Klenke, C., et al., 2013. 1,8-Cineol inhibits nuclear translocation of NF-kappaB p65 and NF-kappaB-dependent transcriptional activity. Biochim. Biophys. Acta 1833 (12), 2866–2878.
Hagiwara, S.I., Ishii, Y., Kitamura, S., 2000. Aerosolized administration of N-acetylcysteine attenuates lung fibrosis induced by bleomycin in mice. Am. J. Respir. Crit. Care Med. 162 (1), 225–231.
Hardcastle, S.L., Brenu, E.W., Johnston, S., Nguyen, T., Huth, T., Ramos, S., et al., 2015. Serum immune proteins in moderate and severe chronic fatigue syndrome/myalgic encephalomyelitis patients. Int. J. Med. Sci. 12 (10), 764–772.
Henderson, T.A., 2014. Valacyclovir treatment of chronic fatigue in adolescents. Adv. Mind Body Med. 28 (1), 4–14.
Hill, E., Mehta, H., Sharma, S., Mane, K., Xie, C., Cathey, E., et al., 2022. Risk Factors Associated with Post-Acute Sequelae of SARS-CoV-2 in an EHR Cohort: A National COVID Cohort Collaborative (N3C) Analysis as Part of the NIH RECOVER Program. medRxiv.
Hodgin, K.S., Donovan, E.K., Kekes-Szabo, S., Lin, J.C., Feick, J., Massey, R.L., et al., 2021. A placebo-controlled, pseudo-randomized, crossover trial of botanical agents for gulf war illness: resveratrol (polygonum cuspidatum), luteolin, and fisetin (rhus succedanea). Int. J. Environ. Res. Publ. Health 18 (5).
Holroyd, K.J., Buhl, R., Borok, Z., Roum, J.H., Bokser, A.D., Grimes, G.J., et al., 1993. Correction of glutathione deficiency in the lower respiratory tract of HIV seropositive individuals by glutathione aerosol treatment. Thorax 48 (10), 985–989.
Homma, S., Azuma, A., Taniguchi, H., Ogura, T., Mochiduki, Y., Sugiyama, Y., et al., 2012. Efficacy of inhaled N-acetylcysteine monotherapy in patients with early stage idiopathic pulmonary fibrosis. Respirology 17 (3), 467–477.
Hornig, M., Montoya, J.G., Klimas, N.G., Levine, S., Felsenstein, D., Bateman, L., et al., 2015. Distinct plasma immune signatures in ME/CFS are present early in the course of illness. Sci. Adv. 1 (1).
Huang IP, R., 2020. Lymphopenia in severe coronavirus disease-2019 (COVID-10): systematic review and meta-analysis. Journal of Intensive Care 8.
Jager, W., Nasel, B., Nasel, C., Binder, R., Stimpfl, T., Vycudilik, W., et al., 1996. Pharmacokinetic studies of the fragrance compound 1,8-cineol in humans during inhalation. Chem. Senses 21 (4), 477–480.
Jimenez-Guardeno, J.M., Ortega-Prieto, A.M., Menendez Moreno, B., Maguire, T.J.A., Richardson, A., Diaz-Hernandez, J.I., et al., 2022. Drug repurposing based on a quantum-inspired method versus classical fingerprinting uncovers potential antivirals against SARS-CoV-2. PLoS Comput. Biol. 18 (7), e1010330.
Johnson, C., 2022. The ME/CFS Coronavirus Vaccine Side Effects Poll Update + the New Severely Ill Vaccine Side-Effects Poll. Health Rising. https://www.healthrising.org/blog/2021/03/17/coronavirus-vaccine-poll-chronic-fatigue-fibromyalgia-severely-ill/.
Johnson, C., 2023. "Long Vax" (Coronavirus Vaccine-Induced Injury) Starting to Make Inroads in the Medical Community. Health Rising. https://www.healthrising.org/blog/2023/08/01/long-vax-coronavirus-vaccine-injury-inroads-medical-community/.
Juergens, U.R., Dethlefsen, U., Steinkamp, G., Gillissen, A., Repges, R., Vetter, H., 2003. Anti-inflammatory activity of 1.8-cineol (eucalyptol) in bronchial asthma: a double-blind placebo-controlled trial. Respir. Med. 97 (3), 250–256.
Juergens, U.R., Engelen, T., Racke, K., Stober, M., Gillissen, A., Vetter, H., 2004. Inhibitory activity of 1,8-cineol (eucalyptol) on cytokine production in cultured human lymphocytes and monocytes. Pulm. Pharmacol. Ther. 17 (5), 281–287.
Juergens, L.J., Tuleta, I., Stoeber, M., Racke, K., Juergens, U.R., 2018. Regulation of monocyte redox balance by 1,8-cineole (eucalyptol) controls oxidative stress and pro-inflammatory responses in vitro: a new option to increase the antioxidant effects of combined respiratory therapy with budesonide and formoterol? Synergy 7, 1–9.
Juergens, L.J., Worth, H., Juergens, U.R., 2020. New perspectives for mucolytic, anti-inflammatory and adjunctive therapy with 1,8-cineole in COPD and asthma: review on the new therapeutic approach. Adv. Ther. 37 (5), 1737–1753.
Kasimir, F., Toomey, D., Liu, Z., Kaiping, A.C., Ariza, M.E., Prusty, B.K., 2022. Tissue specific signature of HHV6 infection in ME/CFS. Front. Mol. Biosci. 9, 1044964.
Kedor, C., Freitag, H., Meyer-Arndt, L., Wittke, K., Hanitsch, L.G., Zoller, T., et al., 2022. A prospective observational study of post-COVID-19 chronic fatigue syndrome following the first pandemic wave in Germany and biomarkers associated with symptom severity. Nat. Commun. 13 (1), 5104.
Kennedy-Feitosa, E., Okuro, R.T., Pinho Ribeiro, V., Lanzetti, M., Barroso, M.V., Zin, W. A., et al., 2016. Eucalyptol attenuates cigarette smoke-induced acute lung inflammation and oxidative stress in the mouse. Pulm. Pharmacol. Ther. 41, 11–18.
Kim, S.Y., Solomon, D.H., 2010. Tumor necrosis factor blockade and the risk of viral infection. Nat. Rev. Rheumatol. 6 (3), 165–174.
Kim, K.Y., Seo, H.J., Min, S.S., Park, M., Seol, G.H., 2014. The effect of 1,8-cineole inhalation on preoperative anxiety: a randomized clinical trial. Evid Based Complement Alternat Med 2014, 820126.
Kim, K.Y., Lee, H.S., Seol, G.H., 2015. Eucalyptol suppresses matrix metalloproteinase-9 expression through an extracellular signal-regulated kinase-dependent nuclear factor-kappa B pathway to exert anti-inflammatory effects in an acute lung inflammation model. J. Pharm. Pharmacol. 67 (8), 1066–1074.
Kim, E.H., Lee, B.W., Ryu, B., Cho, H.M., Kim, S.M., Jang, S.G., et al., 2022. Inhibition of a broad range of SARS-CoV-2 variants by antiviral phytochemicals in hACE2 mice. Antivir. Res. 204, 105371.
Klein, J., Wood, J., Jaycox, J., Lu, P., Dhodapkar, R.M., Gehlhausen, J.R., et al., 2022. Distinguishing features of Long COVID identified through immune profiling. medRxiv.
Kumar, A., Kumar, P., Dungdung, A., Kumar Gupta, A., Anurag, A., Kumar, A., 2020. Pattern of liver function and clinical profile in COVID-19: a cross-sectional study of 91 patients. Diabetes Metabol. Syndr. 14 (6), 1951–1954.
Le Balc'h, P., Pinceaux, K., Pronier, C., Seguin, P., Tadie, J.M., Reizine, F., 2020. Herpes simplex virus and cytomegalovirus reactivations among severe COVID-19 patients. Crit. Care 24 (1), 530.

Lehner, G.F., Klein, S.J., Zoller, H., Peer, A., Bellmann, R., Joannidis, M., 2020. Correlation of interleukin-6 with Epstein-Barr virus levels in COVID-19. Crit. Care 24 (1), 657.
Li, Y., Lai, Y., Wang, Y., Liu, N., Zhang, F., Xu, P., 2016. 1, 8-cineol protect against influenza-virus-induced pneumonia in mice. Inflammation 39 (4), 1582–1593.
Li, X., Wei, X., Chen, C., Zhang, Z., Liu, D., Hei, Z., et al., 2018. N-Acetylcysteine inhalation improves pulmonary function in patients received liver transplantation. Biosci. Rep. 38 (5).
Lima, P.R., de Melo, T.S., Carvalho, K.M., de Oliveira, I.B., Arruda, B.R., de Castro Brito, G.A., et al., 2013. 1,8-cineole (eucalyptol) ameliorates cerulein-induced acute pancreatitis via modulation of cytokines, oxidative stress and NF-kappaB activity in mice. Life Sci. 92 (24–26), 1195–1201.
Lou, H., Kaplowitz, N., 2007. Glutathione depletion down-regulates tumor necrosis factor alpha-induced NF-kappaB activity via IkappaB kinase-dependent and -independent mechanisms. J. Biol. Chem. 282 (40), 29470–29481.
Machado, K.D.C., Islam, M.T., Ali, E.S., Rouf, R., Uddin, S.J., Dev, S., et al., 2018. A systematic review on the neuroprotective perspectives of beta-caryophyllene. Phytother Res. 32 (12), 2376–2388.
Maes, M., Kubera, M., Uytterhoeven, M., Vrydags, N., Bosmans, E., 2011. Increased plasma peroxides as a marker of oxidative stress in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). Med. Sci. Mon. Int. Med. J. Exp. Clin. Res. 17 (4), SC11–S15.
Maes, M., Twisk, F.N., Kubera, M., Ringel, K., 2012a. Evidence for inflammation and activation of cell-mediated immunity in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS): increased interleukin-1, tumor necrosis factor-alpha, PMN-elastase, lysozyme and neopterin. J. Affect. Disord. 136 (3), 933–939.
Maes, M., Twisk, F.N., Johnson, C., 2012b. Myalgic Encephalomyelitis (ME), Chronic Fatigue Syndrome (CFS), and Chronic Fatigue (CF) are distinguished accurately: results of supervised learning techniques applied on clinical and inflammatory data. Psychiatr. Res. 200 (2–3), 754–760.
Maes, M., Kubera, M., Stoyanova, K., Leunis, J.C., 2021. The reification of the clinical diagnosis of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) as an immune and oxidative stress disorder: construction of a data-driven nomothethic network and exposure of ME/CFS subgroups. Curr. Top. Med. Chem. 21 (16), 1488–1499.
Mandarano, A.H., Maya, J., Giloteaux, L., Peterson, D.L., Maynard, M., Gottschalk, C.G., et al., 2020. Myalgic encephalomyelitis/chronic fatigue syndrome patients exhibit altered T cell metabolism and cytokine associations. J. Clin. Invest. 130 (3), 1491–1505.
McLane, L.M., Abdel-Hakeem, M.S., Wherry, E.J., 2019. CD8 T cell exhaustion during chronic viral infection and cancer. Annu. Rev. Immunol. 37, 457–495.
Mellman, I., Hubbard-Lucey, V.M., Tontonoz, M.J., Kalos, M.D., Chen, D.S., Allison, J.P., et al., 2016. De-risking immunotherapy: report of a consensus workshop of the cancer immunotherapy consortium of the cancer research Institute. Cancer Immunol. Res. 4 (4), 279–288.
Morris, G., Walder, K., Puri, B.K., Berk, M., Maes, M., 2016. The deleterious effects of oxidative and nitrosative stress on palmitoylation, membrane lipid rafts and lipid-based cellular signalling: new drug targets in neuroimmune disorders. Mol. Neurobiol. 53 (7), 4638–4658.
Naviaux, R.K., Naviaux, J.C., Li, K., Bright, A.T., Alaynick, W.A., Wang, L., et al., 2016a. Metabolic features of chronic fatigue syndrome. Proc. Natl. Acad. Sci. U. S. A. 113 (37), E5472–E5480.
Naviaux, R.K., Naviaux, J.C., Li, K., Bright, A.T., Alaynick, W.A., Wang, L., et al., 2016b. Reply to Vogt et al.: metabolomics and chronic fatigue syndrome. Proc. Natl. Acad. Sci. U. S. A. 113 (46), E7142–E7143.
Nencioni, L., Iuvara, A., Aquilano, K., Ciriolo, M.R., Cozzolino, F., Rotilio, G., et al., 2003. Influenza A virus replication is dependent on an antioxidant pathway that involves GSH and Bcl-2. FASEB J 17 (6), 758–760.
O'Neal, A.J., Hanson, M.R., 2021. The enterovirus theory of disease etiology in myalgic encephalomyelitis/chronic fatigue syndrome: a critical review. Front. Med. 8, 688486.
Oka, S., Kamata, H., Kamata, K., Yagisawa, H., Hirata, H., 2000. N-acetylcysteine suppresses TNF-induced NF-kappaB activation through inhibition of IkappaB kinases. FEBS Lett. 472 (2–3), 196–202.
Pajaro-Castro, N.F.M.C., Ocazionez, R., Stashenko, E., Olivero-Verbel, J., 2015. Potential interaction of components from essential oils with dengue virus proteins. Bol. Latinoam. Caribe Plantas Med. Aromat. 14, 141–155.
Pall, M.L., 2003. Elevated nitric oxide/peroxynitrite theory of multiple chemical sensitivity: central role of N-methyl-D-aspartate receptors in the sensitivity mechanism. Environ. Health Perspect. 111 (12), 1461–1464.
Panahi, Y., Ghanei, M., Rahimi, M., Samim, A., Vahedian-Azimi, A., Atkin, S.L., et al., 2023. Evaluation the efficacy and safety of N-acetylcysteine inhalation spray in controlling the symptoms of patients with COVID-19: an open-label randomized controlled clinical trial. J. Med. Virol. 95 (1), e28393.
Pauken, K.E., Dougan, M., Rose, N.R., Lichtman, A.H., Sharpe, A.H., 2019. Adverse events following cancer immunotherapy: obstacles and opportunities. Trends Immunol. 40 (6), 511–523.
Paul, B.D., Lemle, M.D., Komaroff, A.L., Snyder, S.H., 2021. Redox imbalance links COVID-19 and myalgic encephalomyelitis/chronic fatigue syndrome. Proc. Natl. Acad. Sci. U. S. A. 118 (34).
Paulin, F.V., Zagatto, A.M., Chiappa, G.R., Muller, P.T., 2017. Addition of vitamin B12 to exercise training improves cycle ergometer endurance in advanced COPD patients: a randomized and controlled study. Respir. Med. 122, 23–29.
Peluso, M.J., Ryder, D., Flavell, R., Wang, Y., Levi, J., LaFranchi, B.H., et al., 2023. Multimodal Molecular Imaging Reveals Tissue-Based T Cell Activation and Viral RNA Persistence for up to 2 Years Following COVID-19. medRxiv.
Pender, M.P., Csurhes, P.A., Burrows, J.M., Burrows, S.R., 2017. Defective T-cell control of Epstein-Barr virus infection in multiple sclerosis. Clin Transl Immunology 6 (1), e126.
Pender, M.P., Csurhes, P.A., Smith, C., Douglas, N.L., Neller, M.A., Matthews, K.K., et al., 2018. Epstein-Barr virus-specific T cell therapy for progressive multiple sclerosis. JCI Insight 3 (22).
Pendergrast, T., Brown, A., Sunnquist, M., Jantke, R., Newton, J.L., Strand, E.B., et al., 2016. Housebound versus nonhousebound patients with myalgic encephalomyelitis and chronic fatigue syndrome. Chron. Illness 12 (4), 292–307.
Phetsouphanh, C., Darley, D.R., Wilson, D.B., Howe, A., Munier, C.M.L., Patel, S.K., et al., 2022. Immunological dysfunction persists for 8 months following initial mild-to-moderate SARS-CoV-2 infection. Nat. Immunol.
Proal, A.D., VanElzakker, M.B., 2021. Long COVID or post-acute sequelae of COVID-19 (PASC): an overview of biological factors that may contribute to persistent symptoms. Front. Microbiol. 12, 698169.
Prousky, J., 2007. The treatment of pulmonary diseases and respiratory-related conditions with inhaled (nebulized or aerosolized) glutathione. Evid. base Compl. Alternative Med. 5 (1), 27–35.
Rhoden, C.R., Lawrence, J., Godleski, J.J., Gonzalez-Flecha, B., 2004. N-acetylcysteine prevents lung inflammation after short-term inhalation exposure to concentrated ambient particles. Toxicol. Sci. 79 (2), 296–303.
Roederer, M., Staal, F.J., Raju, P.A., Ela, S.W., Herzenberg, L.A., Herzenberg, L.A., 1990. Cytokine-stimulated human immunodeficiency virus replication is inhibited by N-acetyl-L-cysteine. Proc. Natl. Acad. Sci. U. S. A. 87 (12), 4884–4888.
Russell, L., Broderick, G., Taylor, R., Fernandes, H., Harvey, J., Barnes, Z., et al., 2016. Illness progression in chronic fatigue syndrome: a shifting immune baseline. BMC Immunol. 17, 3.
Rusznak, C., Mills, P.R., Devalia, J.L., Sapsford, R.J., Davies, R.J., Lozewicz, S., 2000. Effect of cigarette smoke on the permeability and IL-1beta and sICAM-1 release from cultured human bronchial epithelial cells of never-smokers, smokers, and patients with chronic obstructive pulmonary disease. Am. J. Respir. Cell Mol. Biol. 23 (4), 530–536.
Santella, C., Bitton, A., Filliter, C., Bessissow, T., Vutcovici, M., Lakatos, P.L., et al., 2022. Anti-TNF therapy and the risk of herpes zoster among patients with inflammatory bowel disease. Inflamm. Bowel Dis. 28 (2), 176–182.
Selin, L.K., Wlodarczyk, M.F., Kraft, A.R., Nie, S., Kenney, L.L., Puzone, R., et al., 2011. Heterologous immunity: immunopathology, autoimmunity and protection during viral infections. Autoimmunity 44 (4), 328–347.
Smith, F.J., Monto, R.W., Rebuck, J.W., 1952. B12 inhalation therapy in pernicious anemia. Trans. Am. Clin. Climatol. Assoc. 64, 27–39.
Solomon, B.J., Loong, H.H., Summers, Y., Thomas, Z.M., French, P., Lin, B.K., et al., 2022. Correlation between treatment effects on response rate and progression-free survival and overall survival in trials of targeted therapies in molecularly enriched populations. ESMO Open 7 (2), 100398.
Straface, E., Matarrese, P., Gambardella, L., Forte, S., Carlone, S., Libianchi, E., et al., 2000. N-Acetylcysteine counteracts erythrocyte alterations occurring in chronic obstructive pulmonary disease. Biochem. Biophys. Res. Commun. 279 (2), 552–556.
Sudhoff, H., Klenke, C., Greiner, J.F., Muller, J., Brotzmann, V., Ebmeyer, J., et al., 2015. 1,8-Cineol reduces mucus-production in a novel human ex vivo model of late rhinosinusitis. PLoS One 10 (7), e0133040.
Takatsuka, H., Wakae, T., Mori, A., Okada, M., Fujimori, Y., Takemoto, Y., et al., 2003. Endothelial damage caused by cytomegalovirus and human herpesvirus-6. Bone Marrow Transplant. 31 (6), 475–479.
Takemoto, Y., Kishi, C., Sugiura, Y., Yoshioka, Y., Matsumura, S., Moriyama, T., et al., 2021. Distribution of inhaled volatile beta-caryophyllene and dynamic changes of liver metabolites in mice. Sci. Rep. 11 (1), 1728.
Taquet, M., Dercon, Q., Luciano, S., Geddes, J.R., Husain, M., Harrison, P.J., 2021. Incidence, co-occurrence, and evolution of long-COVID features: a 6-month retrospective cohort study of 273,618 survivors of COVID-19. PLoS Med. 18 (9), e1003773.
Unger, E.R., Lin, J.S., Tian, H., Natelson, B.H., Lange, G., Vu, D., et al., 2017. Multi-site clinical assessment of myalgic encephalomyelitis/chronic fatigue syndrome (MCAM): design and implementation of a prospective/retrospective rolling cohort study. Am. J. Epidemiol. 185 (8), 617–626.
Valdez, A.R., Hancock, E.E., Adebayo, S., Kiernicki, D.J., Proskauer, D., Attewell, J.R., et al., 2019. Estimating prevalence, demographics, and costs of ME/CFS using large scale medical claims data and machine learning. Front Pediatr 6, 412.
Van Doren, W.W., Iqbal, U.H., Helmer, D.A., Litke, D.R., Simon, J.E., Wu, Q., et al., 2022. Changes in polyphenol serum levels and cognitive performance after dietary supplementation with Concord grape juice in veterans with Gulf War Illness. Life Sci. 292, 119797.
Verma, D., Church, T.M., Swaminathan, S., 2020. Epstein-Barr virus co-opts TFIIH component XPB to specifically activate essential viral lytic promoters. Proc. Natl. Acad. Sci. U. S. A. 117 (23), 13044–13055.
Walter, R., Hartmann, K., Fleisch, F., Reinhart, W.H., Kuhn, M., 1999. Reactivation of herpesvirus infections after vaccinations? Lancet 353 (9155), 810.
Wang, W., Chen, X., Pan, J., Zhang, X., Zhang, L., 2022. Epstein-barr virus and human cytomegalovirus infection in intestinal mucosa of Chinese patients with inflammatory bowel disease. Front. Microbiol. 13, 915453.
Watt, T., Oberfoell, S., Balise, R., Lunn, M.R., Kar, A.K., Merrihew, L., et al., 2012. Response to valganciclovir in chronic fatigue syndrome patients with human herpesvirus 6 and Epstein-Barr virus IgG antibody titers. J. Med. Virol. 84 (12), 1967–1974.
Welsh, R.M., Selin, L.K., 2002. No one is naive: the significance of heterologous T-cell immunity. Nat. Rev. Immunol. 2 (6), 417–426.

Wenzel, J., Lampe, J., Muller-Fielitz, H., Schuster, R., Zille, M., Muller, K., et al., 2021. The SARS-CoV-2 main protease M(pro) causes microvascular brain pathology by cleaving NEMO in brain endothelial cells. Nat. Neurosci. 24 (11), 1522–1533.
Williams, T.L.J., 2013. A comparison of nebulized vitamin B12 versus oral vitamin B12 supplementation (Ph.D. Dissertation.). Theses and Dissertations.Ph.D. Dissertation.
Wood, E., Hall, K.H., Tate, W., 2021. Role of mitochondria, oxidative stress and the response to antioxidants in myalgic encephalomyelitis/chronic fatigue syndrome: a possible approach to SARS-CoV-2 'long-haulers'. Chronic Dis Transl Med 7 (1), 14–26.
Wu, J., 2020. Tackle the free radicals damage in COVID-19. Nitric Oxide 102, 39–41.
Xu, J.W.W., Zhong, X.X., Feng, Y., Wei, X., Liu, X.G., 2016. EXPRESS: methylcobalamin ameliorates neuropathic pain induced by vincristine in rats: effect on loss of peripheral nerve fibers and imbalance of cytokines in the spinal dorsal horn. Mol. Pain 12, 1744806916657089.
Yadav, N., Chandra, H., 2017. Suppression of inflammatory and infection responses in lung macrophages by eucalyptus oil and its constituent 1,8-cineole: role of pattern recognition receptors TREM-1 and NLRP3, the MAP kinase regulator MKP-1, and NFkappaB. PLoS One 12 (11), e0188232.
Yamada, K., Toyota, K., Tsunoda, Y., Matahira, Y., Matsumura, S., Yoshioka, Y., et al., 2023. Effects of inhaled beta-caryophyllene on vascular stiffness in smokers: a randomized, double-blind, placebo-controlled trial. Exp. Ther. Med. 25 (1), 57.
Yamano, E., Sugimoto, M., Hirayama, A., Kume, S., Yamato, M., Jin, G., et al., 2016. Index markers of chronic fatigue syndrome with dysfunction of TCA and urea cycles. Sci. Rep. 6, 34990.
Yang, Z., Wu, N., Fu, Y., Yang, G., Wang, W., Zu, Y., et al., 2010. Anti-infectious bronchitis virus (IBV) activity of 1,8-cineole: effect on nucleocapsid (N) protein. J. Biomol. Struct. Dyn. 28 (3), 323–330.
Zhang, H., Zhao, S., Cao, Z., 2022. Impact of Epstein-Barr virus infection in patients with inflammatory bowel disease. Front. Immunol. 13, 1001055.