RESEARCH ARTICLE

Treatable metabolic and inflammatory abnormalities in Post COVID Syndrome (PCS) define the transcriptomic basis for persistent symptoms: Lessons from CIRS

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Acronyms:

ADL	activities of daily living
CIRS	chronic inflammatory response syndrome
CD3D	cluster of differentiation 3D
CD 14	cluster of differentiation 14
CD 48	cluster of differentiation 48
COVID-19	SARS-CoV-2
GENIE	Gene Expression Inflammation Explained
MAPK	mitogen activated protein kinase
MHM	molecular hypometabolism
PCS	Post COVID Syndrome
RNA	ribonucleic acid
RSR	ribosomal stress response
TGF beta-1	transforming growth factor beta-1
TGFBR	transforming growth factor beta-1 receptor
TLR4	Toll receptor 4
VCS	visual contrast sensitivity
VIP	vasoactive intestinal polypeptide
WDB	water damaged building

ABSTRACT

Within three months of the onset of acute SARS-CoV-2 (COVID-19) infections, new and persistent symptoms were noted in survivors. While the world's medical and research communities focus on saving lives following COVID-19 infection, a relentless march of new cases of Post-COVID Syndrome (PCS) continues to spread around the globe as a second COVID-related pandemic. Efforts to define the physiology of PCS, a multisystem, multi-symptom illness, continue without success, in part due to the markedly different case presentations.

Using a transcriptomic assessment of persistently ill cases of PCS, we show the presence of (i) molecular hypometabolism (MHM) and proliferative physiology; (ii) elevated levels of ribosomal stress responses and a concomitant increase in gene activation of TGFBR; and (iii) common co-expression of CD14 and Toll Receptor 4, correlated to exposure of amplified microbial growth in a water-damaged environment, specifically Actinobacteria and endotoxin, respectively, compared to recovered PCS cases. Total symptom scores and visual contrast sensitivity (VCS) results showed statistically significant differences.

The data reported here supports the concept that PCS occurs in patients with additional environmental exposures and enhanced TGF signaling. In a strikingly similar condition called Chronic Inflammatory Response Syndrome (CIRS), named in 2010, the transcriptomic abnormalities were identified to respond to treatment with FDA-cleared medications, with salutary benefits for affected cases. Though sparsely reported, PCS cases share proteomic findings with CIRS. While additional studies are indicated, a new approach to the treatment of PCS is suggested.

Keywords: Chronic inflammatory response syndrome, COVID-19, Post COVID Syndrome, transcriptomics, molecular hypometabolism, proliferative physiology, defective antigen presentation, Actinobacteria, endotoxin, transforming growth factor beta receptors, specific causation.

BACKGROUND

Much has been learned about the molecular biology of the SARS-CoV-2 (COVID-19) virus in a year. The new human-infecting SARS-CoV-2 is a positive-sense single-stranded RNA-enveloped virus belonging to the CoV family. As seen in other coronaviruses, SARS (Severe Acute Respiratory Syndrome) and MERS (Middle East Respiratory Syndrome), the most common symptoms of COVID-19 are fever, fatigue, and respiratory symptoms, including cough, sore throat and shortness of breath[1]. Its single-stranded RNA genome contains 29,891 nucleotides with a 5' cap structure and 3'poly-A tail, encoding for 9860 amino acids. Its genome shows 89% nucleotide identity with bat SARS-like-CoVZXC21 and 79.2% with that of human SARS-CoV[2]. Sequence analysis of SARS-CoV-2-revealed that ORF1 ab located in the first part of the viral genome translates polyproteins pp1 a and pp1 ab that are responsible for encoding

16 nonstructural proteins. The remaining virus genome encodes several accessory proteins and four major structural proteins, including the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein. The S protein is responsible for facilitating the entry of the CoV into the target cells. To infect humans, it binds to angiotensin-converting enzyme 2 (ACE-2) and enters cells[2]. SARS-CoV is the seventh coronavirus (SARS-CoV) that has caused a major public health concern across the globe[3].

One of the earliest groups to recognize PCS as a unique syndrome was the Gemelli Against COVID-19 Post-Acute Study Group from Italy[12]. JAMA published their letter on 8/11/20, based on data of 143 previously hospitalized patients collected from a single site from 4/21/2020 to 5/29/2020. Two months after diagnosis, all study members were PCR negative. 82% had at least one symptom and 55% had three or more persistent symptoms[12]. Even though the study was small and did not report neurologic symptoms, objective biomarkers, or a control group, their findings have been replicated and expanded.

PCS is a symptom-dense cohort not confined to hospitalized survivors and not excluding those with neurologic and psychiatric findings, as shown by a massive retrospective study of over 236,000 patients from the United Kingdom published 4/6/2021[13]. While excluding symptoms other than 14 categories of neurologic and psychiatric diagnoses, the authors report an incidence of neurologic disorders approaching 33% looking at all COVID-19 cases, but the authors noted nearly a 50% incidence if the patients had previously needed intensive care unit treatment. When the results were compared to control groups of (i) influenza patients and (ii) other respiratory viral illnesses, having COVID-19 created significant additional neurologic risks[13]. Again, neither objective biomarkers nor a well-patient control group was reported.

Other studies were symptom-focused as well[5]. Townsend, et al., in an early study of Irish patients with post-viral fatigue, found durable fatigue in over 52% of patients studied 10 weeks or longer after resolution of the acute COVID-19 illness. Fatigue in this study was defined by the Chalder Fatigue Scale and was found to be unrelated to prior severity of COVID-19 illness. Markers of inflammation in adaptive immunity did not separate high or low symptom PCS patients. These included leukocytes, neutrophil or lymphocyte counts, neutrophil-to-lymphocyte ratio, lactate dehydrogenase, C-reactive protein, IL-6, or sCD25. The potential for PCS patients to have a post-viral syndrome, including SARS (SARS-CoV), Middle East Respiratory Syndrome (MERS-CoV), Epstein-Barr Virus (EBV), Ross River Virus (RRV) was reviewed[5] without additional discussion of biomarkers.

A more recent study of post-viral fatigue[14] in 458 non-hospitalized Norwegian COVID-19 patients seen 6-24 weeks after recovery from acute COVID-19 was published 2/19/21. 46% reported ongoing fatigue. Neither a control group nor objective biomarkers were reported.

A study of 124 Dutch patients was reported 11/2020 that included measurements of pulmonary health[15]. Diffusion capacity was reduced in 42% of cases but ground-glass opacities, seen in acute illness, had resolved. 22% had reduced exercise capacity. Executive cognitive abnormalities were found in 34%, matching the later UK study.

In a letter published early after COVID-19 became a pandemic (4/15/2020), Mo and

colleagues[16] alerted the world that reduced diffusion capacity was found in 47% of discharged COVID-19 patients and restrictive lung disease was found in 25%. Unfortunately, no measures of TGF beta-1 or TGF beta-1 receptors were studied. Symptoms were not presented. No controls were presented.

Pulmonary fibrosis was discussed[17] by Vitello in a paper published 8/24/2020. His data suggest that patients with cure of viral infection may have fibrotic lung disease as a residual of acute infection. The paper suggests consideration of anti-fibrotic therapy with pirfenidone; TGF beta-1 is prominently discussed, with TGF beta-1 and VEGF, both also found to be abnormal in CIRS, enhancing the progression of fibrosis. TGF beta-1 signaling was not discussed.

Another early paper[18] looked at residual pulmonary injury in PCS patients. 98% of PCS patients had CT abnormalities seen at 28 days post-hospital discharge, but the authors noted that CT findings do not always correlate with clinical presentation.

An informative study from China was published on 1/8/21[19]. 1733 patients hospitalized with COVID-19 were followed for a median of six months. Persistent fatigue was found in 63%. 23% had anxiety or depression. 24% had reduced exercise tolerance. Diffusion capacity was reduced in 56% of the worst affected patients. No control data was presented and no laboratory analysis for TGF beta-1 was performed.

What is not known about COVID-19 is the link between acute illness and residual symptoms acquired after the acute illness is cleared. Within months after the explosive worldwide outbreak of COVID-19, a new and mysterious ailment was seen in a subpopulation of those who had survived COVID-19: a multisystem, multi-symptom illness that persisted for months after the acute viral and inflammatory syndrome was cleared as shown by negative testing results. Initial attention to the new syndrome largely came from affected patients and social media groups. Symptom rosters included fatigue, executive cognitive issues, respiratory symptoms including cough, shortness of breath, headaches, musculoskeletal problems, gastrointestinal symptoms and unusual neurologic complaints, as well as many others. Patients were variously called "long-haulers" or sufferers of "Post-COVID Syndrome (PCS)" in an effort from providers and patients alike to begin to understand the underlying pathophysiology responsible for the chronic symptoms[4].

The curious unpublished finding is that many patients with PCS noted the progressive onset of symptoms after initial recovery, with a window of time appearing of fewer symptoms shortly after clearance of COVID-19. To some observers, this inexorable worsening, despite negative tests for viral persistence, suggested an acquired, progressive, post-viral syndrome, with metabolic, immune and autoimmune complications leading the list of possible culprits.

Despite the concerted broad-based effort to identify risk factors and objective biomarkers for PCS, none have been identified. In December 2020, the National Institutes of Health announced funding of $1.15 billion to study PCS over four years. Familiar illnesses, such as Chronic Fatigue Syndrome, were mentioned prominently as similar syndromes to PCS, even though no objective biomarkers have been identified for CFS after 35 years of research[5].

Another group of illnesses has not been mentioned in the PCS discussions, namely

Chronic Inflammatory Response Syndromes (CIRS), most commonly seen in patients sickened by exposure to the interior environment of water-damaged buildings (WDB). CIRS has remarkable similarities to PCS as patients with CIRS have (i) a symptom roster nearly identical to PCS; (ii) the chronic illness has prominent measurable inflammatory and metabolic complications; (iii) the illness is progressive, with (iv) worsening upon re-exposure; and (v) confirmed examples of self-healing are rare; (vi) combines published objective biomarkers from proteomics, transcriptomics, VCS, cardiovascular studies and volumetric CNS MRI studies to provide separation of cases from controls; and (vii) shows in published studies results of treatment that correct objective indicators of pathophysiology safely, resulting in clinical benefits[6].

Given the phenotypic similarities between the PCS group and those suffering from CIRS, it is reasonable to investigate whether a common pathophysiologic process underlies both. CIRS has demonstrated effective evidence-based treatment through randomized controlled trials. Therefore, if these conditions are biologically similar, would PCS subjects respond to the same or similar protocol used to treat CIRS? Our hypothesis asserts that these conditions share similar pathophysiology, hence similar overlapping transcriptomic signatures and both respond positively to a similar therapeutic approach.

An observational case-control study was conducted using transcriptomics of differential gene activation in two small cohorts of PCS patients, enabling comparison of patients who did not develop PCS [PCS (-)] to patients developing PCS [PCS (+)] patients; and each PCS cohort to be compared to controls and cases of untreated CIRS. We also determined a majority of PCS (+) subjects demonstrated differential gene activation consistent with the environmental exposure to Actinobacteria and endotoxins commonly found indoors around the world[25], especially in WDB.

Our results support a call for additional investigations to verify these findings, investigate the use of other diagnostic markers used in CIRS in PCS (+), and suggest the evaluation of therapy currently used for CIRS patients be applied to PCS (+) subjects using vasoactive intestinal polypeptide (VIP), currently included under an FDA Emergency Use Authorization for COVID-19[7, 8, 9].

MOLECULAR BIOLOGY STUDIES, INCLUDING TRANSCRIPTOMICS

Jain assessed proteomic and transcriptomic inflammatory responses in 50 acute COVID-19 subjects[20]. Their work identified globally dysregulated immune-related pathways, such as cytokine-receptor signaling, complement and coagulation cascades, JAK-STAT and TGF beta-1 signaling pathways, with worsening in patients with more severe symptoms.

Several metabolic pathways were suppressed, including ribosome and endoplasmic reticulum (ER) protein processing, which suggested a global reduction in the production of proteins related to cellular energy production[20]. The oxidative phosphorylation pathways were mainly downregulated in patients with mild disease, consistent with impairment of mitochondrial oxidative phosphorylation for ATP production in favor of cytosolic aerobic glycolysis[20] and upregulation of IRS2[21]. This paper verifies the observations of Ryan from 2016[7], that there was a suppression of ribosomal mRNA and mRNA of nuclear-

encoded mitochondrial genes, called molecular hypometabolism (MHM), in patients with untreated CIRS. MHM abates in CIRS patients with proper treatment, coinciding with the resolution of symptoms and transcriptomics to equal age-gender matched controls. Of note, the proliferative physiology that often accompanies MHM also was corrected by therapy.

A comparison of the transcriptomic profiles of COVID-19 patients with mild, moderate, and severe disease each separately was made with controls to determine if the cytokine signature, and the identified cytokine storm, varied by disease severity[20]. In addition to the upregulation of immune-related response genes, there was consistent disruption of the ribosome pathway. Complement and coagulation cascades in cases were activated to a higher extent in patients with severe disease, but this correlation was not seen uniformly[20]. Inflammation-induced coagulation pathways, which can be regulated by the complement system, are important in controlling abnormalities associated with inflammatory and metabolic insult[20], such as CIRS, as well as infections. These findings parallel those of Ryan in CIRS.

Jain also showed that the TGF beta-1 pathway was significantly upregulated in patients with severe disease suggesting they were at increased risk for the development of pulmonary fibrosis. Finally, this paper shows that coagulation genes are dysregulated, as shown by Ryan in untreated CIRS, possibly accounting for the observed incidence of clotting seen clinically in COVID-19 patients. How much of the transcriptomic changes seen in acute COVID-19 compared to PCS is not confirmed but the amount and diversity of transcriptomic changes in PCS patients in this study supported the concept that the documented antecedent abnormal gene activity in COVID-19 illness continued into PCS.

An early transcriptomic study (published 3/20) in acute COVID-19 patients[22] looked at three cases and 3 controls to identify inflammatory genes found in blood and bronchoalveolar lavage fluid (BALF). The results reveal distinct host inflammatory cytokine profiles to SARS-CoV-2 infection in patients and highlight the association between COVID-19 pathogenesis and excessive cytokine releases such as CCL2/MCP-1, CXCL 10/IP-10, CCLD/MIP-1A, and CCL4/MIP1B[22].

Of note, viral recognition pathways will usually activate Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs), which in turn will activate protective expression of interferon (IFN) and activation of anti-viral effectors such as natural killer cells, T cells, CD8 + cells and macrophages (22). Coronaviruses, such as SARS-CoV and MERS-CoV, have evolved strategies to dampen or delay IFN production, with such reduction leading to exuberant inflammatory host responses which can lead to severe lung pathology[22]. Whether IFN is abnormally regulated in PCS is not known.

The authors[22] specifically identified several immune pathways and proinflammatory cytokines induced by SARS-CoV-2 infection, including CCL2, CXCL2, CCL8, CXCL1, IL33, CCL3L1 in BALF; and CXCL10, TNFSF10, TIMP1, C5, IL18, AREG, NRG1, IL10 in blood-borne mononuclear cells, showing sustained inflammation and a cytokine storm in the affected patients. Of note, these assays have not been performed in CIRS patients.

The upregulated genes in BALF relate to viral infection-induced changes in various

membrane structures and ER. The most enriched biological processes are co-translational protein targeting to membrane, protein targeting to ER and viral transcription[22]. However, up-regulated genes in mononuclear cells are mainly enriched in complement activation, humoral immune response mediated by circulating immunoglobulin and B-cell mediated immunity[22].

In addition, a group of inflammation-related processes were activated, including regulation of acute inflammatory responses. In contrast, the decreased genes in blood-borne mononuclear cells of patients are involved in other biological processes such as axon guidance and mRNA-related biological processes[22].

Anti-inflammatory cytokines IL-10 and TGF beta-1 were also induced upon SARS-CoV-2 virus infection, resembling the high TGF beta-1 activity found in SARS patients[22]. TGF beta-1 is a multifunctional cytokine that regulates a variety of biological processes including cell proliferation, differentiation, apoptosis and immune responses. TGF beta-1 signaling can promote fibroblast proliferation and myofibroblast differentiation, key factors in the development of pulmonary fibrosis[22]. In our PCS cohort we saw exposure to Actinobacteria and/or bacterial endotoxin resulting in specific immunoreactivity involving TGF beta-1 signaling by virtue of upregulated TGFBR 1, 2, or 3.

The hallmark of severe COVID-19 is a massive cytokine and chemokine release, the so-called "cytokine storm," that provides a basis to study the widespread, uncontrolled dysregulation of host immune defense[1]. A dysregulation in the balance of T reg cells, favoring naïve T-cell activity over regulatory T cells, could contribute to hyperinflammation. A reduction in memory T cells on the other hand could be implicated in COVID-19 relapse since several recurrences have been reported in recovered cases of COVID-19[1]. T memory cells have not been studied in PCS. Deficiency of T reg cells has been reported in CIRS patients with proliferative physiology[21].

It is well-established that upon binding of the viral spike protein to the host cells by the entry receptor ACE2, the viral RNAs act as pathogen-associated molecular patterns (PAMPs), and are detected by the pattern recognition receptors, which include the family of Toll-like receptors (TLRs). In particular, for RNA viruses such as CoVs, viral genomic RNA or the intermediates during viral replication, including dsRNA, are recognized by either the endosomal RNA receptors, TLR3 and TLR7/8, and the cytosolic RNA sensor, retinoic acid-inducible gene (RIG-1)/MDA5[1]. Consistently, such TLRs have been found to activate different signaling pathways in human CD14+ monocytes, correlating with differential type I IFN and cytokine secretion. As a result of virus recognition, downstream transduction pathways, crucial for the proper antiviral response, such as IRF3 (IFN regulatory factor 3), nuclear factor-kB (NFkB), JAK (Janus kinase)/STAT (signal transducer and activator of transcription) signaling pathways, are activated. The identification of the most relevant intracellular signaling pathways involved in the modulation of host immune systems may give important hints on how to overcome SARS-CoV-2[1].

Zhang[23] proposed the possible mechanism of pulmonary fibrosis caused by SARS-CoV-2, based on the published data of COVID-19, citing (i) direct evidence of pulmonary fibrosis was found in autopsy and pulmonary puncture pathology; and (ii) indirect evidence

coming from increased levels of fibrosis-related cytokines, including TGF beta-1, tumor necrosis factor (TNF)-a and interleukin (IL)-6 in peripheral blood. The authors support the potential therapeutic value of two drugs, pirfenidone and nintedanib, for COVID-19-induced pulmonary fibrosis[23]. The role of drug intervention in PCS was not addressed.

Murthy and colleagues[24] further supported the role of TGF beta-1 signaling in PCS patients, focusing deeply on the molecular biology of pulmonary fibrosis. SARS-CoV-2 infection is reported to cause extensive pulmonary damage in affected patients. Given a large number of recovered patients compared to severe or fatal cases, it is important to follow-up the recovered patients searching for apparent lung function abnormalities. However, data on the post-recovery scenario of patients with COVID-19 and the development of secondary complications in PCS are limited.

Some of the characteristic acute presentations in the lung[24] include bilateral and peripheral ground-glass and consolidative pulmonary opacities; unilateral and bilateral pneumonia; moderate to extensive lung lesions; thickening of bronchovascular bundles, pulmonary edema, acute respiratory distress syndrome (ARDS); and pulmonary fibrosis.

Murthy suggests that treatment with histone deacetylase (HDAC) inhibitors are reported to show promising anti-fibrotic effects mainly through suppressing TGF beta-1 signaling.

Pulmonary fibrosis in COVID-19 is characterized by redox imbalance, inflammatory injury to the alveolar epithelium, dysregulated epithelial-to-mesenchymal transformation (EMT), aberrant fibroblast proliferation and differentiation, and excessive accumulation of extracellular matrix molecules in the lung parenchyma[24].

Myofibroblasts are the primary cell type responsible for the fibrotic tissue, which possesses enhanced fibrotic, contractile and migratory activities. Following tissue injury, myofibroblasts can be derived from a variety of cell types including epithelial cells, endothelial cells and fibroblasts. These cells can promote collagen deposition in the extracellular matrix (ECM). The myofibroblast formation coupled with ECM production has been implicated in pulmonary fibrogenesis[24]. The myofibroblasts secrete several mediators, including TGF beta-1, to enhance the fibrotic process.

TGF beta-1 signaling pathway is known to have a central role in the pathogenesis of fibrosis by promoting EMT, fibroblast proliferation and differentiation[24]. TGF beta-1 has been reported to induce lung fibrosis by activating Smad-dependent and Smad-independent pathways. Through Smad signaling pathways, TGF beta-1 has been reported to directly increase the transcription of ECM genes, mainly collagens that favor ECM deposition[24]. In addition, MAP kinases are also involved in TGF beta-1-mediated fibrotic responses. This interplay of MAPK and TGF beta-1 signaling in the presence of an increased abundance of Actinobacteria species is called specific immunoreactivity[25].

TGF beta-1 directly regulates the expression of alpha-smooth muscle actin (a-SMA) in tissue myofibroblasts thereby aiding in tissue granulation[24]. Other matrix proteins regulated by TGF-B during fibrosis are collagen type 1 and III and alpha-tubulin[24].

Aydemir, et al., further investigated TGF beta-1 in COVID-19 by studying genes targeted by microRNA[2]. The authors

constructed an integrative pathway network analysis with putative target genes. They identified 40 SARS-CoV-2 miRNAs and their regulated targets with their analysis showing that targeted genes including NFKB1, NFKBIE, JAK1-2, STAT3-4, STAT5B, STAT6, SOCS1-6, IL2, IL8, IL10, IL17, TGFBR1-2, SMAD2-4, HDAC1-6, JARID1A-C and JARID2 play important roles in NFKB, JAK/STAT and TGF beta-1 signaling pathways, as well as epigenetic regulation pathways[2].

Despite the information obtained from the viral genome structure, many aspects of virus-host interactions are still unknown. The relationship has been reported between small RNA molecule pathways, especially microRNA pathways, and diseases including cancer, cardiovascular disease, neurodegenerative disease and diseases caused by viruses and bacteria[2].

As Aydemir reviews, microRNAs (miRNA) are 19-24 nucleotide, non-coding RNA molecules that post-transcriptionally regulate gene expression by binding to target messenger RNAs (mRNAs). First identified in *Caenorhabditis elegans*, miRNAs are expressed by all eukaryotes and plants, as well as by several DNA and RNA viruses. They function as regulators of cellular processes such as development, differentiation, growth homeostasis, stress responses, apoptosis and immune activation[2].

The authors[2] identified 40 different putative miRNAs encoded from different regions of the SARS-CoV-2 viral RNA genome that were found to be targeting and modulating different human genes involved in host biological processes such as apoptosis, cell cycle and regulation of transcription signaling pathways. The target gene analysis showed that the identified SARS-CoV-2 miRNAs target genes in signaling pathways including NFKB, JAK/STAT and TGF beta-1 signaling pathways. All of these pathways are vital for the cell to be protected from viral infection[2].

Aydemir notes CXCL proteins are pro-inflammatory proteins induced during infections; CXCL9 and CXCL10 stimulate the activation and migration of immune cells to the infected sites, especially activated T cells. Previous studies with human immunodeficiency virus (HIV), human cytomegalovirus, hepatitis C virus (HCV) and avian H5N1 and human pdmH1N1 and H3N2 viruses showed that CXCL8, CXCL9, and CXCL10 are associated with pathogenicity and may serve as a clinical indicator for clinical disease severity and progression and antiviral therapy outcome[2].

METHODS:

21 patients who had a positive test for COVID-19, followed by a negative test performed after treatment for COVID-19 were recruited from the practices of physicians. Patients agreed to undergo testing with a commercial transcriptomic blood test called GENIE in a proof-of-concept study of differential gene activation, understanding that participation in the observational study was not linked to any therapy.

The GENIE test demonstrates quantities of mRNA for 188 genes found to be abnormal in cases of CIRS compared to age-gender matched controls, including ribosomal genes and nuclear-encoded mitochondrial genes. Downregulation of mRNA for these genes has been termed molecular hypometabolism (MHM) since 2016[7]. Additional genes of interest in GENIE that have been reported in the peer-reviewed literature on CIRS include cytokines; TGF beta-1 receptors 1, 2 and 3; coagulation elements; genes involved with apoptosis and necroptosis; CD3D and CD48

associated with T-cell receptors; beta tubulins TUBB1 and TUBA4A; and ribosomal stress response genes, including specific indicators of exposure to Actinobacteria and endotoxins, MAPK and genes CD14 and Toll Receptor 4, respectively. While treatment of CIRS cases with a published protocol results in the resolution of these gene abnormalities, it is not known what role these genes play in PCS cases.

Patients were stratified into two groups. Those without illness that interfered with their activities of daily living (ADL), compared to their pre-COVID state 6 weeks following negative COVID-19 testing were categorized as PCS-recovered (PCS -); those with persistent new symptoms and disruption of ADL compared to pre-COVID state 6 weeks after negative COVID-19 test were called PCS-not recovered (PCS +). Rosters of symptoms, visual contrast sensitivity (VCS), an objective marker for environmental biotoxin exposure used since 2001[10], and a standard transcriptomic assay (GENIE) were compared for PCS (+) to PCS (-), as well as to CIRS cases and age-matched controls.

VCS testing was performed as described in earlier studies[11]. Standard transcriptomic methods used since 3/19 were used in all study subjects. Statistical analysis was performed according to the package in Excel at docs.google.com. An alpha score of $< .05$ was considered significant.

METHODS FOR TRANSCRIPTOMICS ASSAY

RNA extraction

Venous blood was drawn from the arms of subjects into PAXgene RNA blood collection tubes (http://www.preanalytix.com/product-catalog/blood/rna/products/paxgene-blood-rna-tube/), incubated for four hours at room temperature, then frozen at −80 °C until RNA extractions were performed. Total RNA was extracted with the Qiagen PAXgene Blood miRNA System kit according to the manufacturer's protocol. The total RNA was analyzed using an Agilent 2100 bioanalyzer (Agilent Technologies, USA) for RNA integrity and was then quantified using a NanoDrop ND-2000 (Wilmington, DE). Only samples with Agilent RIN scores ≥ 8 were used for sequencing.

Transcriptomic Analysis using Nanostring

We used a Nanostring digital analyzer for measuring gene expression with a custom probe set developed by Progene DX as a Research Use Only assay called GENIE. The GENIE test was designed to assay for Chronic Inflammatory Response Syndrome or CIRS. Specific metabolic gene names were anonymized due to confidentiality restraints. GENIE contains 174 genes of research interest and 14 housekeeping genes for normalization. Roughly half of the research genes (80) on the assay comprise a metabolic panel with multiple probes averaged using a geometric mean to describe gene expression for the following elements, Large ribosomal subunit (17 probes), Small ribosomal subunit (14 probes), Large mitoribosomal subunit (8 probes) Small mitoribosomal subunit (7 probes), ATP synthase (8 probes), Cytochrome C oxidase (8 probes), mitochondrial inner and outer translocases (8 probes), and NADH dehydrogenase; ubiquinone (10 probes). Two hundred nanograms of total RNA were used for input material and the GENIE assay was performed according to standard protocols for the Nanostring digital analyzer platform.

DATA ANALYSIS

Samples for PCS were compared against a control database of 70 healthy, normal, adult

GENIE results. All samples were normalized by using the geometric mean of the 14 housekeeping genes on GENIE. Results of the metabolic panel were achieved by using the simple ratio of PCS patients' metabolic scores (geometric mean of the groups of probes above) over the normal, healthy, control average. For the remaining 94 genes assayed on GENIE, the standard deviation of the control group was used to generate a z score for PCS patients.

RESULTS:

As shown in Table 1 and Table 2, the differences in symptoms, VCS and transcriptomic findings between PCS (-) and PCS (+) were striking. 7 patients were PCS (-) and 14 were PCS (+). PCS (-) had an average of 8.2 symptoms and no VCS abnormalities. PCS (+) had an average of 18.2 symptoms (p =0.013) with 75% showing VCS deficits p =<0.003). No PCS (-) expressed any MHM while all 14 PCS (+) demonstrated MHM (p <0.00001 No PCS (-) subjects had evidence of proliferative physiology compared to 79% of PCS (+); p <0.00001. No PCS (-) had upregulated RIPK1, a marker for necroptosis, while 42% PCS (+) had upregulated RIPK1 (p <0.00001). Coagulation genes were upregulated in 14% of PCS (-) compared to 36% of PCS (+) cases.

Table 1	**SX**	**VCS + %**	**MHM %**	**Prolif Phys %**
Controls	2.7	2	0	0
CIRS	22.3	92	85	88
PCS (-)	8.2	0	0	0
PCS (+)	18.2	75	100	79
p-Value	0.013	0.003	<0.00001	<0.00001

Table 2	**Apoptosis %**	**Defective Apoptosis %**	**Coag %**	**Defensins %**
Controls	0	0	5	25
CIRS	25	15	54	7
PCS (-)	0	0	14	28
PCS (+)	42	42	36	36
p-value	0.008	0.03	.289	.759

In Table 3, 14% of PCS (-) subjects had suppression of CD3D, a marker for defective antigen-presentation from antigen-presenting cells to naïve T-cells, whereas 86% of PCS (+) patients had CD3D suppression (p< 0.001). CIRS biomarkers were abnormal in statistically significantly more PCS (+) patients that PCS (-) and also had increased activity of a PTSD marker.

Table 3	CD3D Suppressed %	CIRS Biomarkers %	PTSD %	Tubulins %
Controls	<5	<5	<5	<5
CIRS	92	80	30	66
PCS (-)	14	14	0	14
PCS (+)	86	56	28	50
p-value	<0.001	0.046	0.04	0.092

Table 4	TGFBR %	Actinobacteria %	Endotoxins %	Mycotoxins %
Controls	5	<5	<5	0
CIRS	52	42	28	7
PCS (-)	14	14	0	0
PCS (+)	57	56	64	21
p-value	0.46	<0.001	<0.001	0.086

In Table 4, ribosomal stress responses (RSR) for exposure to mycotoxins were not found in PCS (-) cells but were found in 21% of PCS (+) patients but this was not statistically significant. RSR for exposure to Actinobacteria were found in 14% of PCS (-) yet positive in 57% of PCS (+) patients ($p < .046$). Furthermore, indicators of exposure to endotoxins were found in no PCS (-) patients but were expressed in 64% of PCS (+) ($p< 0.00032$).

Finally, confirmation of immune reactivity to Actinobacteria species in house dust has been ascribed to elevated TGFB receptor (1, 2, or 3) genes in patients with associated elevated RSR[25]. We found 87.5% in PCS (+) had at least one elevated TGFBR with a (+) RSR for Actinobacteria exposure but none in PCS (-) patients. The p-value for RSR+ with TGFBR+ versus RSR + and TGFBR- was $p <0.0021$ whereas the p-value comparing RSR+ and TGFBR + versus RSR- and TGFBR- was 0.00021. Assays for beta tubulins were upregulated in 14% of PCS (-) cases compared to 50% of PCS (+) cases. No environmental sampling was done in this study. No treatment was prescribed.

Though there were no significant differences between the PCS (+) and PCS (-) groups with the following symptoms, adding both groups together, fatigue was found in 81.8% of all studied patients. Headaches were noted in 81.8% of all patients. Memory loss was found in 72.7%, difficulties with focus and concentration in 63.6% and losing words in 72.7%. Mood lability was noted in 72.7% of all patients post-COVID. Joint pains were described in 54.5% of patients. Astonishingly, blurry vision, sinus problems, shortness of breath, difficulty assimilating new knowledge, night sweats, temperature dysregulation, static shocking and vertigo were each found in 45.5% of patients.

DISCUSSION:

We reviewed current PCS literature to demonstrate state of the art findings regarding (i) symptoms, (ii) inflammatory parameters; and (iii) transcriptomic findings. Our data is novel in the PCS literature. We suggest that confounding environmental exposures add to the inflammatory and metabolic burden demonstrated in PCS (+) patients.

The TGF beta-1 signaling pathway is involved in many cellular processes including cell survival, apoptosis and immunity. Although the TGF beta-1 pathway has important roles, it is often manipulated by viruses as it is a simple pathway[2]. Proteins that play a crucial role in almost every step of this pathway are targeted by SARS-CoV-2 miRNAs. TGFBR1 and TGFBR2, SMAD2, SMAD3, and SMAD4 were all found to be targeted by SARS-CoV-2 miRNAs. SARS-CoV-2 nucleocapsid (N) protein was shown to inhibit the formation of the SMAD3/4 complex, blocking TGF beta-1-induced apoptosis but enhancing tissue fibrosis by SARS-Co-V-infected host cells[2]. Investigation on these miRNAs could shed light on the mechanism of fibrosis seen in COVID-19 patients. It is the overlap of activation of TGF beta-1 signaling in COVID-19 to exposure to Actinobacteria in CIRS activating TGF beta-1 signaling that remains of great interest in the application of treatment of CIRS to treatment of PCS (+) cases.

The infection with an RNA virus leads to the induction of signaling in cascades in the infected host cell. The RNA viruses are recognized by specialized host cell proteins, thus triggering the activation of kinases and transcription factors which in turn mount an antiviral response[2]. In this study many kinases (MAPK1, MAPK3, MAPK4, MAPK6, MAPK7), transcription factors such as E2F1, SP1, EIF4A1, TBP, and tumor suppressor genes including, PTEN, AKT1, RB1, were all targeted by SARS-CoV-2 miRNAs[2]. We await the application of microRNA effects in PCS patients.

Sadeghi[26] looked at T reg cells in COVID-19 patients in a case/control study. Curiously, his findings point to TH17/Treg imbalance with low TGF beta-1. They found a significant increase in the number of Th17 cells, the expression levels of related factors (RAR-related orphan receptor gamma (RORyt), IL-17, and IL-23, and the secretion levels of IL-17 and IL-23 cytokines in COVID-19 patients. In contrast, patients had a remarkable reduction in the frequency of Treg cells, the expression levels of correlated factors (Forkhead box protein P3, (FoxP3)), TGF beta-1, IL-10, and cytokine levels. The ratio of Th17/Treg cells, RORyt/FoxP3, and IL-17/IL-10 had a considerable enhancement in patients compared with the controls and also in dead patients compared with the improved cases[26].

The findings showed that enhanced responses of Th17 cells and decreased responses of Treg cells in COVID-19 patients compared with controls had a strong relationship with hyper inflammation, lung damage, and disease pathogenesis. Also, the high ratio of Th17/Treg cells and their associated factors in COVID-19-dead patients compared with improved cases indicates the critical role of inflammation in the mortality of patients. The variance of low TGF beta-1 in the authors' cohort to high levels presented above is not clear[26].

Part of the problem using gene activation/suppression analyses in illness causation discussion is the complexity of the interaction of transcription factors, microRNAs (miRNAs) and long, non-coding RNAs (LncRNAs) that mostly function post-transcriptionally to regulate gene expression[3.] Although 70% of the genome is transcribed to RNA in humans, only 2% of these transcripts are translated into proteins. The authors used bioinformatic approaches to identify the interactions of SARS-CoV human proteins, miRNAs, and LncRNAs. They found the TGF beta-1 signaling pathway as one of the potential interactive pathways. Altogether, the TGF beta-1 signaling pathway as well as miRNAs, and

LncRNAs involved during SARS-CoV illness can be considered as potential therapeutic targets[3].

By use of transcriptomics we see the role of MHM in PCS (+) patients, likely made worse after environmental exposure to actinomycetes and their secondary products, as well as bacterial endotoxins. Use of salutary protocols for MHM used in CIRS may have benefit in PCS (+) patients. Further studies are planned.

INDOOR AIR CONCERNS FOR COVID-19, PCS AND TGF beta-1

Of major concern regarding specific causation and immunoreactivity to Actinobacteria and endotoxins is whether or not the PCS (+) patients had a known exposure to the interior of a WDB. Given that no environmental testing was done, the source of immunoreactivity will remain unknown in those patients until their attending physicians encourage those patients to assess their dwellings for amplified microbial growth. As also seen in groups with CIRS, the majority of outbreaks involving 3 or more people with COVID-19 have been linked with time spent indoors[27].

Controlling concentrations of indoor aerosols to reduce airborne transmission of infectious/inflammatory agents is critical and can be achieved through source control (masking, physical distancing) and engineering controls, including precipitation of micro-particulates, ventilation and filtration. The problem with success in reducing indoor aerosols is, "With respect to engineering controls, an important flaw exists in how most buildings operate in that the current standards for ventilation and filtration for indoor spaces, except for hospitals, are set for bare minimums and not designed for infection control,"[27].

Reviewing what is known about droplet transmission of COVID-19 indoors, the authors note that larger droplets (>100 um) can settle out of the air due to gravitational forces within 6 feet, but people emit smaller aerosols (<5 um) during talking, breathing, and coughing. Smaller aerosols can stay aloft for 30 minutes to hours and travel well beyond 6 feet[27]. Aerosols containing particulates smaller than 0.3 microns may stay aloft for many days.

Current ventilation standards for most indoor spaces are established by ASHRAE (American Society of Heating, Refrigeration and Air Conditioning Engineers). These standards have been designed to achieve basic levels of acceptable indoor air quality, rather than infection control.

Increasing air exchanges per hour and air filtration is a simplified but important concept that could be deployed to help reduce risk from the within-room, far-field airborne transmission of SARS-CoV-2 and other respiratory infectious diseases. Healthy building controls like higher ventilation and enhanced filtration are a fundamental, but often overlooked, part of risk reduction strategies that could have benefit beyond the current pandemic[27].

As this field of inquiry is advanced, and if indoor environmental concerns in PCS (+) patients are confirmed by larger and wider trials, this field will expand rapidly. We feel that the broader lessons from CIRS will bring new insights into PCS and possibly elsewhere, especially when TGF beta-1 and TGF beta-1 signaling are involved.

CONCLUSIONS:

There is a need to consolidate a pathophysiological framework to organize

the myriad symptoms and biological findings such as neurologic impairments and pulmonary fibrosis in COVID-19, made more urgent by the growing population of PCS (+) individuals. Because COVID-19 is marked by a wide variety of presentations and outcomes, the diversity of clinical illness, ranging from asymptomatic to florid respiratory failure, demands further exploration.

PCS (-) patients had a statistically significantly lower number of symptoms than PCS (+) patients did but had 3 times the number of symptoms of non-CIRS "healthy" control patients. PCS (+) patients trended a higher percentage in 32 of the 37 symptoms than PCS (-). Only 3 of the 32 symptoms were statistically significant though due to i) the low numbers of patients with complete records in this retrospective study; and ii) patients after COVID are still sicker than controls whether PCS (+) or PCS (-). PCS (+) patients also fail VCS testing statistically significantly at a higher rate than PCS (-) patients do and have transcriptomic changes too. A prospective study with larger numbers could discern the difference between PCS (-) and PCS (+) patients to a greater degree. PCS (-) patients, have a number of multisystem, multi-symptom abnormalities which might indicate this damage, occurring after COVID infection, is of metabolic, immune or autoimmune origin or a combination of these factors. Differences between PCS (-) and PCS (+) patients might be explained by exposures to amplified Actinomycetes and/or elevated endotoxins.

Stratifying patients by symptoms alone is not satisfactory for either COVID-19 or PCS. Extrapolating severe PCS to antecedent COVID-19 does not bear out clinically but given worse COVID-19 acutely, the severity of PCS and pulmonary fibrosis are worse. One consideration is to identify evidence of antecedent COVID-19 of variable severity that initiates a secondary innate immune or metabolic response, which progressively disables PCS patients. A way forward is therefore to borrow the CIRS model and apply this to PCS. Our transcriptomic data presented here, while from a small group of patients, hold great promise in comparing PCS with CIRS: i) biotoxin model of illness; ii) wide symptom overlap; iii) failure in VCS tests; iv) transcriptomic findings of abnormal shared patterns; and v) high co-occurrence of reaction to WDB in PCS (+).

Since validated treatments for CIRS have already been published, we hypothesize that this approach may form a pathway to resolution of PCS (+) patients as well, with the caveat that indoor sources of Actinobacteria and bacterial endotoxin are removed. Without documentation of indoor exposure or other failures to remove sources of abnormal TGF beta-1 signaling, we believe treatment failure of PCS will likely follow.

In summary, the use of readily available objective biomarkers in PCS can be drawn from the CIRS literature. Diffusion capacity will stratify a significant number of PCS who have pulmonary fibrosis. Treatment with currently FDA-cleared medications is available to use on a trial basis, including pirfenidone, HDAC inhibitors and VIP. For those without decreased diffusion capacity, but with MHM, we surmise the future of VIP therapy alone appears to be bright in correcting the underlying transcriptomic findings in PCS patients.

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