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Systemic Inflammation and Control of Breathing Patterns in Humans with History of COVID-19 Infection

A Thesis submitted in partial satisfaction
of the requirements for the degree of

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in

Biomedical Sciences

by

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ABSTRACT OF THE THESIS

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by

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Dr. Erica Heinrich, Chairperson

The emergence of SARS-CoV-2 resulted in millions of hospitalizations and 6.9 million deaths to-date. Many individuals who recover from COVID-19 report prolonged dyspnea, with this symptom persisting for months following recovery. Furthermore, data suggests COVID-19 has been linked to systemic and neuronal inflammation which may have downstream impacts on the neural control of breathing. As such, we hypothesized that individuals recovered from COVID-19 may exhibit changes in their ventilatory chemosensitivity to CO_2 and/or O_2 and that these changes may be linked to higher levels of systemic inflammation. To test this hypothesis, we measured baseline ventilatory patterns and chemoreflex sensitivity using a modified rebreathing technique in individuals recovered from COVID-19 (n = 77), as well as an age and sex-matched control group (n = 41). Peripheral blood samples were also collected for inflammatory

profiling. Recovered participants demonstrated lower ventilatory responses to hypercapnia, particularly under a combined hypoxic stimulus ($p = 0.032$). Furthermore, higher levels of plasma IL-1β was associated with higher hypoxic ventilatory responses among the recovered group ($R = 0.44$, $p = 0.004$), highlighting a potential link between acute systemic inflammation and depressed ventilatory chemoreflex sensitivity. When separated by time-post-recovery, we observed a decreased ventilatory recruitment threshold (VRT) beginning at 4 months post-recovery ($p = 0.019$) that returned to baseline after one-year post-recovery. A decrease in sensitivity to CO_2 was also noted immediately after recovery with no return to baseline observed within the two-year tested time frame ($p = 0.023$). Overall, this data indicates that (1) COVID-19 may have impacts on the neural control of breathing, and (2) systemic inflammation may play a role in modulating ventilatory chemoreflex sensitivity. These findings may have implications for the pathology of long-COVID symptoms, including sleep disturbance and prolonged dyspnea.

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INTRODUCTION

The Emergence of COVID-19

In late 2019 and early 2020, the world faced the emergence of a new virus – SaRS-CoV-2, more commonly known as the Coronavirus or COVID-19. The World Health Organization (WHO) classified the spread of COVID-19 as a global pandemic in March of 2020, and since it reached the United States in late January 2020, there have been more than 6.9 million deaths and millions of hospitalizations caused by COVID-19 (Coronavirus Disease (COVID-19) Pandemic, 2023). The virus first emerged in Wuhan, China in December of 2019 and was originally thought to be a type of pneumonia of unknown origin. One month later, it was officially termed "severe acute respiratory syndrome coronavirus 2", or SaRS-CoV-2. It spread rapidly and COVID-19 infection caused several concerning symptoms.

As a result, researchers around the world began studying its pathology. One early study followed 38 confirmed COVID-19 cases and shared each patients' symptoms, severity, and hospitalizations (Spiteri et al., 2020). 35 of these cases were hospitalized with one case resulting in death and four cases requiring mechanical ventilation. The symptoms reported by each person were textbook symptoms of acute respiratory infection. Those who experienced more severe symptoms reported shortness of breath or difficulty breathing, referred to as dyspnea (Spiteri et al., 2020). These early reported symptoms were consistent with a respiratory infection since COVID-19 produced a pro-inflammatory cytokine storm, which is associated with conditions such as pneumonia and

acute respiratory distress syndrome (ARDS) (Maiese et al., 2021). Further studies on the respiratory symptoms of SaRS-CoV-2 reported impaired lung function in individuals with moderate to severe cases (Van Willigen et al., 2023). Another study examined lung function immediately after infection and three months post-infection in recovered patients to determine the acute and long-term impacts of infection on respiratory function in patients with severe versus moderate cases. Of the severe cases, 20% demonstrated a nonsignificant trend for impaired lung function at 3 months post-recovery, while all others' resting breathing patterns had returned to normal. Those who still exhibited breathing abnormalities 3 months post recovery were often afflicted with comorbidities, such as obesity, or experienced mechanical ventilation, sedation, or medication as a result of their COVID-19 infection (Stockley et al., 2021). Both moderate and severe cohorts showed evidence of pulmonary restriction in 55.3% of patients from both cohorts combined and had an increased pulmonary diffusions capacity (measured as the transfer coefficient for carbon monoxide, K_{CO}), with 78.1% of these cases having an increased K_{CO}, with a more significant increase in the severe group (Stockley et al., 2021). While these studies provided much needed information about the acute and long-term impacts of COVID-19 on the lungs and resting breathing patterns, data is missing regarding the possible link between infection and the neural reflex control of breathing. Since current COVID-19 studies show decreased lung function during, and immediately following, infection, we can speculate that there may be long-term changes in some breathing parameters following COVID-19 infection.

Lung Physiology and Alveolar Gas Exchange

The neural reflexes that control breathing are dependent on oxygen (O_2) and carbon dioxide (CO_2) partial pressures (P) within the body. O_2 and CO_2 are exchanged through the blood-gas interface of the pulmonary capillary system. This gas exchange surface has a surface area of 50 to 100 square meters due to the presence of millions of small circular structures called alveoli. Respiratory gas exchange of O_2 and CO_2 occurs across alveolar-capillary membranes (West & Luks, 2016). Since alveolar gas pressures are directly dependent on the rate of alveolar ventilation, the ventilation rate is the primary driving force for maintaining a homeostatic range of arterial partial pressure of oxygen (Pa_{O2}) and carbon dioxide (Pa_{CO2}). As these partial pressures fluctuate from their normal levels, 75 to 100 mmHg for arterial P_{O2} (Ortiz-Prado et al., 2019) and 35 to 45 mmHg for arterial P_{CO2} at sea level (Messina & Patrick, 2022), ventilatory rates change rapidly to compensate for non-homeostatic conditions. Arterial P_{CO2} is tightly regulated because increases in P_{CO2} decrease the body's normal pH (~7.35-7.45) due to the presence of excess hydrogen (H+) ions that are released when CO_2 combines with water in the blood (Hopkins et al., 2022). Because respiratory rate is dependent on P_{CO2} within arterial blood, CO_2 must be continuously exhaled to maintain this equilibrium.

Neural Control of Breathing

Chemoreceptors are groups of cells which sense and respond to the chemical composition of blood (figure 1). Peripheral chemoreceptors detect and respond to both arterial P_{O2} and P_{CO2}, while the central chemoreceptor detects and responds to arterial P_{CO2} through its influence on cerebral tissue pH (Nattie & Li, 2012). Respiratory chemoreceptors are found centrally in the brain stem and peripherally in the aortic and carotid bodies. Upon detecting CO_2 and O_2 partial pressures in arterial blood, signals are sent to the medulla, the respiratory control center of the brain, via glossopharyngeal or vagus nerves. Inspiratory neurons, located at the dorsal region of the medulla, will increase their firing rate when increased CO_2 or decreased O_2 levels are detected (Nattie & Li, 2012), modulating the respiratory pattern. The diaphragm and intercostal muscles receive input from the medulla via phrenic nerve efferents and modulate respiration by increasing or decreasing the rate at which they contract and relax (Jensen et al., 2020).

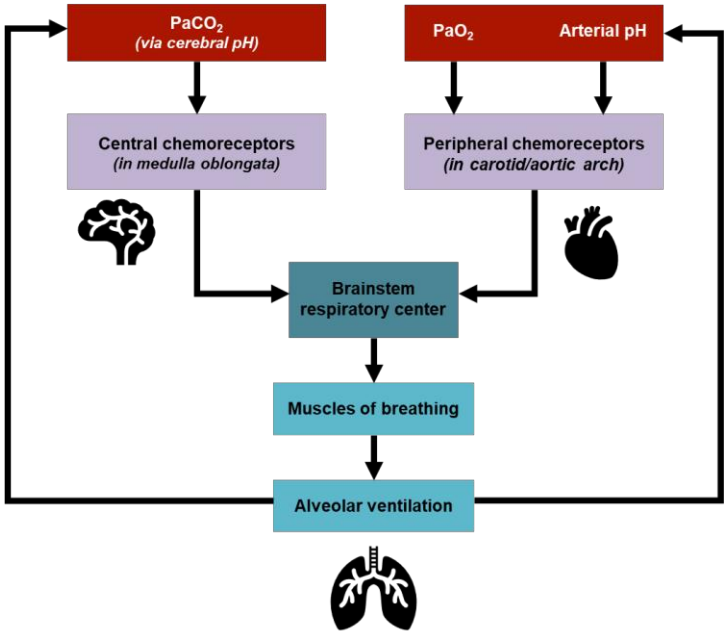

Figure 1. Chemoreceptors. Peripheral and central chemoreceptors detect arterial O_2 and CO_2, sending information to the brainstem and modulating breathing accordingly.

The respiratory chemoreflexes are only one of three neural mechanisms that help remove metabolically produced CO_2 via the lungs. Homeostasis of CO_2 in the body is also driven by neural processes called central command and somatic afferent feedback (Guyenet & Bayliss, 2015). Furthermore, muscle afferents are activated during physical exercise and contribute to hyperpnea to preemptively prevent both the buildup of excess CO_2 and decrease in pH due to lactate, H+, and K+ ion production in the muscles. Central command refers to structures in the brain that are responsible for locomotion, such as the hypothalamus. Furthermore, when a body is in motion, specifically during physical exercise, feedback generated by a feed-forward mechanism in the locomotive control region of the brain works alongside the same motor pathways that drive respiration (Guyenet & Bayliss, 2015).

During hypercapnia, the respiratory rate will increase to quickly remove H^+ ions to compensate for the increase in P_{CO2} (Nattie & Li, 2012). The P_{CO2} at which the respiratory rate begins to rapidly increases is known as the ventilatory recruitment threshold (VRT). The hypoxic ventilatory response (HVR), however, is more plastic and may lead to an increase or a decrease in ventilation in the presence of decreased arterial O_2 saturation. The depressed Pa_{O2} causes the central chemoreceptors to "activate", rapidly firing stimuli to the central nervous system (CNS) and respiratory control center in the brain to determine the body's response to hypoxia (Pamenter & Powell, 2016). Those with a lower HVR may have the same firing rate from the central chemoreceptors as those with a higher HVR but tend to have a less sensitive response to hypoxia. This is due

to the exhaustive overuse of the lungs and respiratory muscles and is usually found in people with chronic respiratory diseases or those who live at higher elevation.

Hypoxia and Neuroinflammation

Acute tissue hypoxia results in inflammatory signaling. This adaptive response evolved in humans to protect tissues during infection or injury, which typically are accompanied by hypoxia as a result of impaired blood flow, edema, and cellular recruitment (Pham et al., 2021). To protect affected tissues, inflammatory signaling is responsible for recruiting immune cells to the affected area and promoting tissue healing and angiogenesis (Pham et al., 2021). Long-term hypoxia exposure, whether through injury, infection, high-altitude exposure, or chronic respiratory illnesses, can result in maladaptive chronic inflammation, leading to development of downstream diseases promoted by chronic systemic inflammation (Pham et al., 2021).

Previous work indicates that inflammation affects the neural control of breathing. From a systemic perspective, several hypoxia-related diseases, with characteristic impairments in the neural control of breathing reflexes, have been linked to systemic inflammation. These include obstructive sleep apnea (OSA), chronic obstructive pulmonary disease (COPD), asthma, and sudden infant death syndrome (SIDS) (Peña-Ortega, 2019).

SIDS occurs in infants under one year of age. Prenatal exposure to cigarette smoke, alcohol, prone sleeping, or side sleeping increases the risk of SIDS. Infants are

more likely to re-inhale expired gases when not sleeping supine, resulting in hypoxic or hypercapnic (increased P_{CO2}) states. Prenatal tobacco smoke exposure causes a depressed ventilatory response to hypoxia and hypercapnia, where ventilation does not increase as rapidly as it should to return the arterial blood gas tensions and pH to homeostasis (Moon et al., 2016). There may also be genetic underpinnings to SIDS development, as some studies show links between SIDS prevalence and polymorphisms or mutations in several inflammation-related genes such as the *CXCL8* and *interleukin 13* (*IL13*) genes (Ferrante et al., 2010).

Additionally, COPD is another pulmonary disease that often results from chronic exposure to cigarette smoke or other particulate matter and chemical irritants such as agricultural dust. There are many comorbidities that can predispose patients to COPD, such as cardiovascular, respiratory, psychiatric, and endocrinology-related pathologies (Recio Iglesias et al., 2020). COPD is associated with chronic bronchitis and abnormal breathing patterns. Furthermore, patients with COPD exhibit obstructive ventilatory patterns which can lead to chronic respiratory failure with concomitant hypoxemia (Raherison & Girodet, 2009). Patients with severe COPD experience more asthma-like symptoms and commonly experience comorbid OSA. When COPD and OSA occur together, both can be associated with intermittent hypoxemia and hypopneas, leading to a more drastic deficit of oxygen saturation at nighttime (Czerwaty et al., 2022; Owens et al., 2017). The hypoxic stress resulting from these pathologies produces systemic inflammation, with elevated systemic inflammatory markers upon waking in the morning, which can exacerbate upper airway inflammation, reduced airway muscle function, or

mucosal inflammation in nasal passages (Owens et al., 2017). Patients with OSA also exhibit snoring at night due to a narrowed airway space during sleep from an elongated soft palate, increased tongue size, and decreased positioning of the floating hyoid bone (Patil et al., 2007). This airway obstruction, commonly associated with a metabolic disorder or obesity, results in temporary cessation of breathing while asleep as well as daytime sleepiness and fatigue. Reduced respiratory drive in OSA patients can exacerbate the duration and severity of airway obstructions since insufficient inspiratory effort is produced to overcome the obstruction, and decreased efferent signals to the upper airway muscles allow the airway to close more easily.

In addition to potential links between systemic inflammation and breathing control, new evidence suggests that neuroinflammation, which is mediated by glial cells in the central nervous system (CNS), also plays a key role in the plasticity of the neural control of breathing (Peña-Ortega, 2019). In the presence of neuroinflammation, inflammatory mediators work alongside chemosensory signals to change breathing patterns and modulate the strength of respiratory reflexes, resulting in an abnormal respiratory rhythm, and influencing responses to both hypoxia and hypercapnia (Peña-Ortega, 2019).

Studies in rodent models as well as clinical populations show that inflammation likely plays a role in modulating ventilatory responses to hypercapnia and hypoxia. For example, Bavis et al., (2020) found that rats exposed to chronic hypoxia from birth developed a blunting of the HVR (Bavis et al., 2020). To determine why this happens, it's important to note that in rats, the carotid body is a major producer of the

inflammatory cytokines interleukin-6 (IL-6), interleukin-1beta (IL-1β), and tumor necrosis factor alpha (TNF-α) (Porzionato et al., 2013). Further studies in rats indicate that increased expression of these cytokines is linked to blunted HVRs (Bavis et al., 2020; Porzionato et al., 2013). Consequently, when chronic hypoxia is prevalent and the body adapts to exhibiting a lower HVR for energy conservation purposes to avoid the exhaustive use of the lungs and respiratory muscles, systemic inflammation will likely persist, increasing expression of inflammatory cytokines such as IL-6, IL-1β, and TNF-α, which leads to association with a blunted HVR.

Additionally, Popa et al. (2011) determined that when given ibuprofen, rats that were acutely exposed to hypoxia had a decreased HVR and decreased expression of IL-6 and IL-1β, but no significant change in HVR for those chronically exposed to hypoxia (Popa et al., 2011). Coincidentally, this phenomenon was also observed in humans, where individuals brought up to high altitude from sea level living had blunted HVRs when given ibuprofen every 4 hours (Basaran et al., 2016). This data clearly demonstrates that broad spectrum anti-inflammatory treatment, such as ibuprofen and potentially other NSAIDS, can prevent ventilatory acclimatization (plasticity in HVR). This indicates that inflammatory signaling plays a key role in ventilatory chemoreflexes.

Furthermore, IL-6 expression in humans is often associated with the development of acute mountain sickness, where hypoxic conditions are prevalent (Pham et al., 2021). Knowing this and correlating the fact that peripheral chemoreceptors located on the carotid bodies detect arterial O_2 levels and activate under hypoxic conditions, we can deduce that expression of some inflammatory cytokines are likely linked to blunted

HVRs in humans. Considering the cytokine storm that occurs during SaRS-CoV-2 viral infiltration in the body, we can form a hypothesis regarding the link between hypoxia-induced inflammatory status and how the virus potentially changes the ventilatory response to hypoxia.

Autopsy Findings and Neuropathology

Maiese et al., (2020) conducted a systemic review pertaining to COVID-19 from the initial outbreak until June 2020 where 341 cases were studied to determine common occurrences in autopsy findings of those who died from COVID-19 (Maiese et al., 2021). One major deformity that was noted from autopsies was the presence of diffuse alveolar damage (DAD) within the lungs, along with microthrombi in pulmonary vessels, DVT (deep vein thrombosis), and pulmonary embolisms (Maiese et al., 2021). Liver tissues and myocardium did not exhibit consistent pathologies between autopsies, but all individuals exhibited some form of alveolar or pulmonary vessel deformity, often associated with DAD, fibrin congregations, and inflammation within the vessels and alveoli (Maiese et al., 2021; Tian et al., 2020). Furthermore, fever, dyspnea, headache, and anosmia in many COVID-19 patients posed a concern for central nervous system involvement. Indeed, autopsy findings reported viral particles from the novel coronavirus in both endothelial cells and within the frontal lobe (Maiese et al., 2021; Paniz-Mondolfi et al., 2020).

Concordantly, another study reviewed 18 autopsies and looked at brain tissue biopsies obtained from the frontal lobe and olfactory nerves. Quantified nucleocapsid proteins from SaRS-CoV-2 showed evidence of the protein infiltrating the brain (Solomon et al., 2020). Further autopsy results of the brain used microscopic techniques to view clustering of microglia, indicating microglial activation within the brainstem, which is also observed during ventilatory acclimatization to hypoxia. Autopsy results also showed T-cell infiltration in some individuals, but not consistent enough and not with a lack of comorbidities to determine if the findings were solely due to SaRS-CoV-2 infection (Mukerji & Solomon, 2021). However, olfactory system findings were consistent with inflammation, microglial activation, and viral particle infiltration between autopsied individuals, indicating that the virus can enter the brainstem, and therefore the respiratory centers, via the olfactory system (Deigendesch et al., 2020; Mukerji & Solomon, 2021).

Newer research demonstrates that viral infections by SARS-CoV-2 affect the vagus nerve, resulting in inflammatory cell infiltration (Woo et al., 2023). With previous evidence showing increased inflammatory responses in the brainstems of autopsied individuals with COVID-19 (Radke et al., 2023), researchers analyzed vagal nuclei in the brain stem to determine inflammatory status and discovered that cytokine expression and inflammatory cell presence were in line with previous evidence of viral brain infiltration, showing an increase in $CD8^+$ T-cells and HLA-DR^+ monocytes (Woo et al., 2023). Inflammation in the vagal nerve causes a disruption to the autonomic nervous system, which controls involuntary physiological processes such as breathing, heart rate, blood

pressure, and food digestion (Waxenbaum et al., 2023). More autopsies of 27 patients who died from COVID-19 analyzed vagal nerves and compared them against 5 individuals who did not have COVID-19 when they died. The findings suggested a significant increase in expression of genes that regulate antiviral responses, indicating that brain infiltration of the COVID-19 virus is directly tied to vagal nerve inflammation caused by the virus' RNA infecting vagal nuclei (Woo et al., 2023).

Conclusively, brain autopsies show evidence of viral infiltration via the vagus nerve into the brain stem, including brain regions involved in regulating breathing patterns, affecting the autonomic nervous system. Therefore, there is a possibility that changed breathing patterns during COVID-19 infection are due to the likelihood that the virus damaged these brain regions or otherwise impacted the overall neural control of breathing circuit (figure 2).

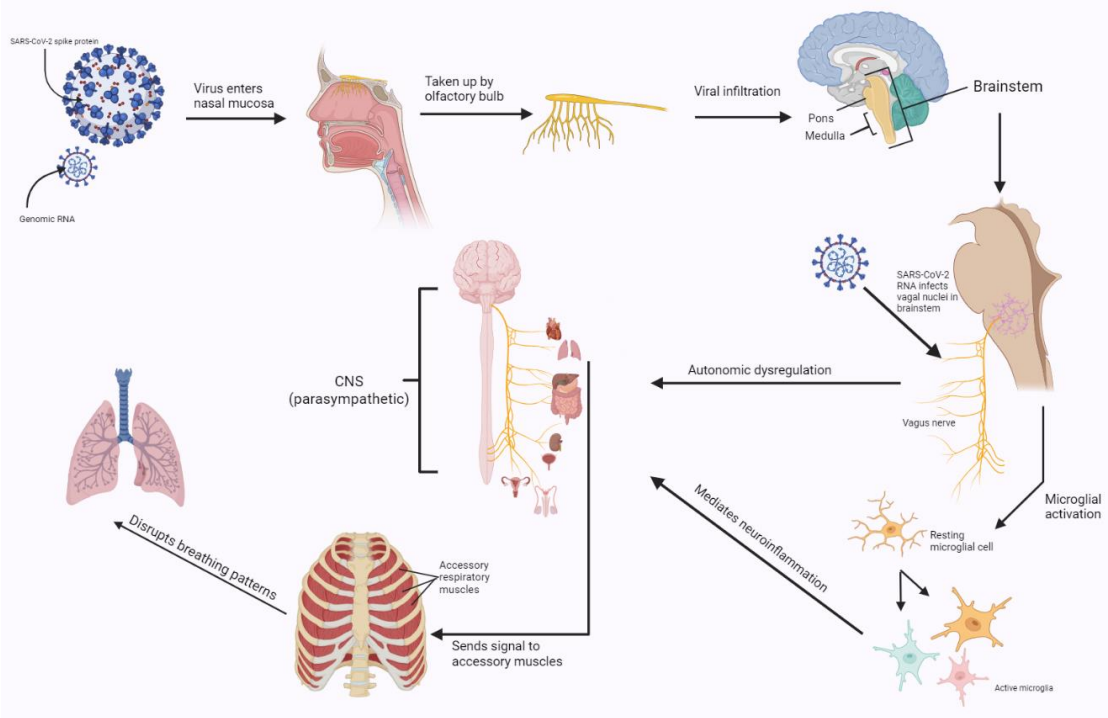

Figure 2. SARS-CoV-2 in brainstem. Figure showcases viral infiltration of the brain and respiratory control centers of the brainstem. One way virus particles are taken up is by the olfactory bulb where they are transported to the brainstem, infecting vagal nuclei. The virus can also be taken up from the lungs via vagal afferents innervating the pulmonary system, potentially moving from periphery to the brain stem via the vagus nerve infiltrating vagal nuclei with its RNA. This leads to autonomic dysfunction and microglial activation and potentially causes peripheral inflammation in the CNS, signaling the respiratory muscles to dysregulate breathing patterns.

COVID-19 and its Effects on Control of Breathing Parameters

Respiratory related problems are the most common symptoms reported around the world in COVID-19 infected individuals. Hospitals report exhausting all ventilatory equipment, such as mechanical ventilators and continuous positive airway pressure (CPAP) machines (Islam et al., 2020). Upon hospitalization, most patients had decreased oxygen saturation levels, some reaching as low as 60% while normal levels should be

above 95% (Islam et al., 2020). This oxygen hungry state led to reports of dyspnea and severe respiratory distress, where oxygen therapies, such as nasal cannula on 5-6L oxygen or hyperbaric chambers for hypoxemic patients were initiated (Islam et al., 2020). However, while dyspnea was common, contradictory reports of "happy hypoxia", or "silent hypoxemia" were also noteworthy. In these cases, patients presented with severe hypoxemia with no shortness of breath, potentially supporting a link between COVID-19 and blunting of respiratory drive. In addition to these acute breathing difficulties, COVID-19 is also associated with the development of new impairments in sleep quality and sleep disordered breathing was significantly more common in COVID-19 patients (Perger et al., 2021). With many people reporting difficulty breathing with the onset of COVID-19 and information from studies showing evidence of pro-inflammatory cytokine storms (Maiese et al., 2021), we sought address the gaps in our knowledge regarding the impact of COVID-19 on the neural control of breathing.

In summary, based on the available data discussed above, I hypothesize that:

(1) Higher levels of systemic inflammation in recovered patients will be associated with a long-term change in the hypoxic ventilatory response.
(2) We will observe a decrease in the hypercapnic ventilatory response and ventilatory recruitment thresholds in people who have recovered from COVID-19 as opposed to those who have never contracted the virus.

These hypotheses are based on the following rigorous preliminary evidence:

- COVID-19 significantly impairs lung function and is associated with significant systemic inflammation.
- Systemic inflammation has been linked to modified ventilatory chemoreflex sensitivity.
- COVID-19 has been linked to sleep disturbance and sleep disordered breathing.
- Autopsy evidence indicates potential activity of SARS-CoV-2 in the brainstem respiratory centers via direct viral activity via entry through the olfactory bulb, or via vagal afferents.

To test this hypothesis, I measured ventilatory chemoreflexes in recovered patients and in participants that have never had COVID-19 (control group). I also obtained blood samples from each participant and determined the expression levels of various inflammatory cytokines. Correlations between levels of inflammatory marker expression and ventilatory chemoreflex sensitivity, specifically the HVR, were then quantified to determine if systemic inflammation is associated with control of breathing reflexes. When possible, within-subject paired parameters were analyzed for both ventilatory chemoreflex tests and blood samples for any controlled participants that returned after COVID-19 infection.

METHODS

Participant Demographics

We recruited 118 participants between April of 2022 and May of 2023. Of the 118 participants, 41 reported never having COVID-19 (control group) and 77 reported confirmed positive cases of COVID-19 (recovered group). Of the recovered participants, five were previous control group participants that came back for the study again after COVID-19 infection, thus making it possible to investigate the within-subject changes in ventilatory control before and after COVID-19 infection. Each participant self-reported their ethnicity for this study with some reporting more than one ethnicity (figure 3). Recruitment was performed via word of mouth, posting flyers around UCR campus and local clinical sites, and social media posts.

Inclusion criteria included being at least 18 years of age. Participants with current severe cardiac or pulmonary illness were excluded or conducted limited testing within safe limits. One participant with a history of lung cancer and partial lobectomy completed only an abbreviated version of the chemoreflex test and did not participate in the hypoxia phase. Exclusion criteria included confirmed or suspected active COVID-19 infection. Participants who were currently pregnant were also excluded from the chemoreflex test portion of the study due to links between hypoxia exposure and development of preeclampsia (Tong & Giussani, 2019).

Participants were asked not to consume caffeine on the morning prior to the study and to abstain from taking any anti-inflammatory medication, corticosteroids, or other

medications that could potentially interfere with control of breathing measures and inflammatory marker expression (Peña-Ortega, 2019). If participants were not able to stop taking the referenced medications, their data was excluded. Of note, participants were not required to fast prior to their study appointments since these samples were also compared to ICU patient samples, for which it was not possible to require fasting.

Consent procedures were performed in the participant's native language with study personnel fluent in the language (English or Spanish). All participants were provided with a copy of the consent form prior to their appointment and were informed of the purpose of the study, including all risks and benefits. After all information was provided, both written and verbal consent were required to move forward with the study.

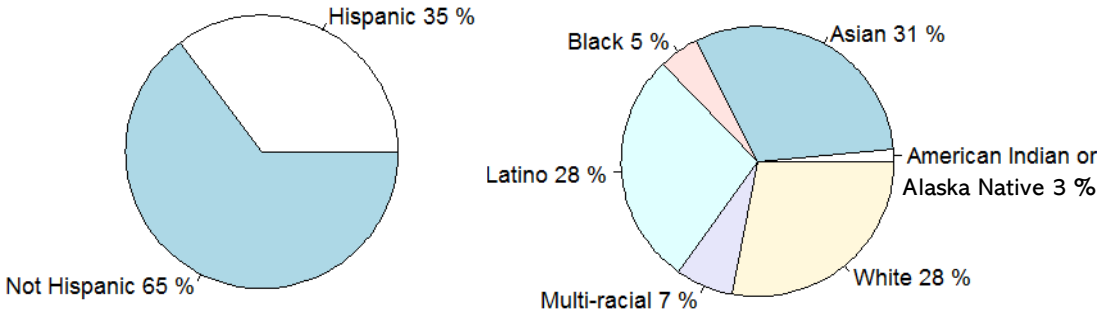

Figure 3. Participant race and ethnicity. Demographics provided here are self-reported. Some participants indicated two ethnicities and those who indicated 3 or more are categorized under multi-racial.

Study Design

Each participant had their blood pressure taken using a stethoscope and manual sphygmomanometer, body temperature was collected from the forehead using an infrared thermometer, and height and weight were recorded. Participants then completed a series of questionnaires regarding their past medical history, demographics, and long-COVID symptoms. Peripheral venous blood samples were then collected via standard venipuncture procedures by a certified phlebotomist. Samples were preferentially collected from the median cubital vein, or the hand if necessary. The participant then completed a spirometry test to measure baseline lung function, followed by ventilatory chemoreflex measures. The complete study appointment lasted approximately two hours.

Blood Sample Processing

Approximately 25 mL of blood was collected in vacutainer tubes containing EDTA (20 mL, PaxGene RNA stabilization reagent (2.5 mL), or no additive (2.5 mL)). After being stored at room temperature for no more than 4 hours, blood samples were processed and separated for plasma and white blood cells (WBCs) for protein analysis. To separate blood plasma from WBCs, the samples were centrifuged at 2500g for ten minutes. After separation, plasma used for inflammatory cytokine assays was stored at -20 °C for short term storage and at -80 °C for long term storage.

LEGENDPlex Assays

A large subset of plasma samples were chosen at random (control: N = 41; recovered: N = 72) from the larger sample set for cytokine biomarker analyses, matched for age, sex, and BMI, and measured via LEGENDplex protocols. The Human Anti-Virus Response Panel (13-plex; BioLegend, San Diego, CA, US), which consists of cytokines IL-1β, IL-6, CXCL8 (IL-8), IL-10, IL-12p70, IFN-α2, IFN-β, IFN-λ1 (IL-29), IFN-λ2/3 (IL-28a/b), IFN-γ, TNF-α, CXCL10 (IP-10), and GM-CSF, and Human Vascular Inflammation Panel 1-TC (13-plex; BioLegend, San Diego, CA, US), which consists of cytokines Myoglobin, Calprotectin (MRP8/14), Lipocalin A (NGAL), C-Reactive Protein (CRP), MMP-2, Osteopontin (OPN), Myeloperoxidase (MPO), Serum Amyloid A (SAA), IGFBP-4, ICAM-1 (CD54), VCAM-1 (CD106), MMP-9, and Cystatin C, were used for this study, following manufacturer protocols. Each sample was treated with Triton X diluted with assay buffer at a 1:2 ratio for the anti-virus response panel and 1:100 for the vascular inflammation panel. Samples were then tested in duplicates. Upon completion, each plate was run through the Novocyte flow cytometer to determine cytokine concentrations.

Pulmonary Function Testing

Each participant was required to do a pulmonary functions test before initiating the ventilatory chemoreflex portion of the study. A breathing apparatus, consisting of a mouthpiece, respiratory air filter, and a flow meter (AD Instruments). The participant was

then fitted with a nose clip to ensure no air escaped through nasal airways and was instructed to take a deep breath and quickly exhale all air in their lungs for 6 seconds as fast as they could. After repeating this test three times, the pulmonary functions testing was complete, and each participant's volume and flow data was recorded, measured as the forced expired volume in 1 second (FEV_1) and the forced vital capacity (FVC). The obtained values determined whether or not there was significant airflow obstruction or volume restriction, and if the participant was safe to move forward with the study. Participants with an FEV_1/FVC of >80% were allowed to participate in the ventilatory chemoreflex testing.

Ventilatory Chemoreflex Testing

Ventilatory chemoreflex measures were conducted using the modified Duffin rebreathing technique (Duffin, 2007). Oxygen and carbon dioxide gas analyzers, flow meters, and pulse oximeters were calibrated following manufacturer instructions within 1 hour prior to testing. Participants were seated in a chair in a semi recumbent position with their legs uncrossed. They were then fitted with a pulse oximeter and a 3-lead electrocardiogram (ECG) to monitor their vitals throughout the study (figure 4). If participants were wearing nail polish, they were asked to remove the nail polish if O_2 saturation was <95%. For the ECG, participants were asked to clean off their skin of any debris or excess body hair for 3-lead electrode placement.

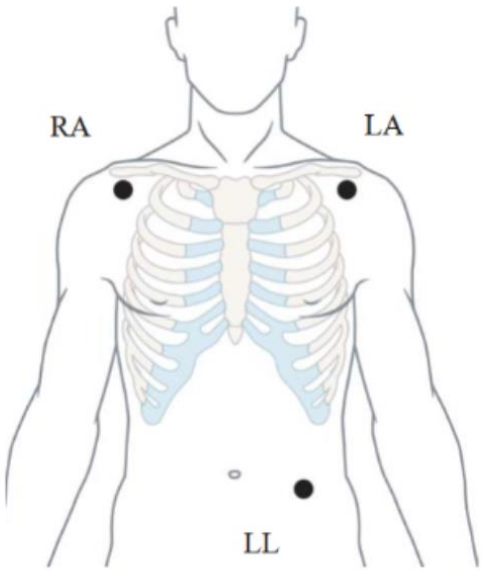

Figure 4. **Three-Lead Electrode Placement for ECG**. A white lead was placed over the right subclavicular region, denoted RA. A black lead was placed over the left subclavicular region, denoted LA. Lastly, a red lead was placed over the left lower abdominal region, denoted LL.

Each participant was fitted with a breathing apparatus that consisted of a respiratory filter with separately attached mouthpiece (Hans Rudolph), a flow meter (AD Instruments, ML 1000), a two-way valve fitted with O_2 and CO_2 gas analyzers (VacuMed, model #17625, Ventura, CA; VacuMed, model #17630, Ventura, CA) to maintain accurate gas concentrations, and a rebreathing bag (figure 5). At the base of the rebreather bag, a line for an O_2 concentrator (DeVilbiss) was attached. During the resting portions of the chemoreflex testing, the two-way valve was set to allow room air breathing. During the chemoreflex test period, the valve was switched to allow rebreathing from the rebreathing bag which allowed end-tidal P_{CO2} to slowly increase over a period of 5-10 minutes while O_2 concentrations could be controlled at a constant level via the tubing attached to the bottom of the rebreathing bag. Nose clips were placed

on each participant to ensure air was not being inhaled or exhaled through the nasal passageways.

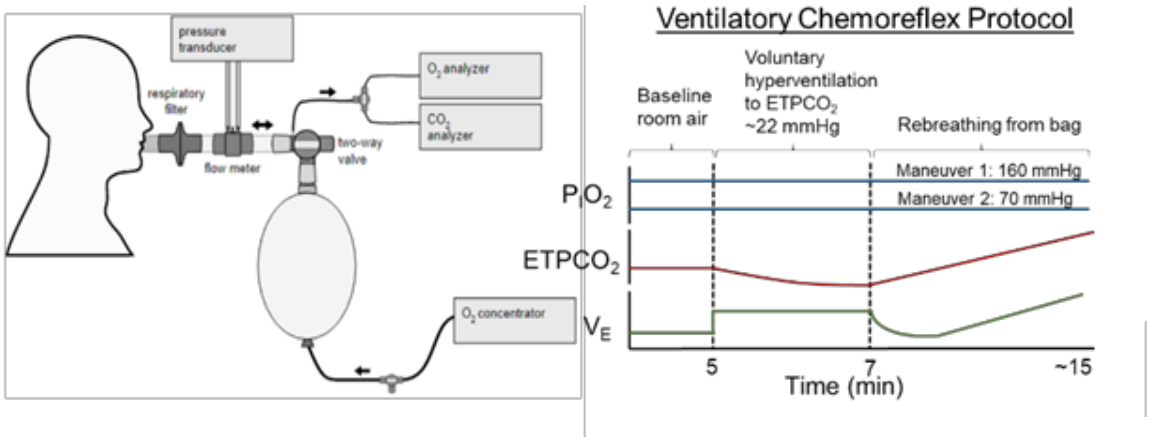

Figure 5. Chemoreflex Breathing Apparatus Set-up. The ventilatory chemoreflex protocol required a breathing apparatus to be suspended from the air and fitted to each participant. Mouth pieces were reusable and sanitized between each test subject. Apparatus was set up in a way that the participant could remove themselves from it if they experienced any discomfort during the test.

During testing, participants were first instructed to relax and breathe normally for five minutes. Throughout this time, the participants were breathing room air and were asked to refrain from talking, moving, or looking at their cell phones or other devices. Vital signs and end-tidal P_{CO2} (ETP_{CO2}) were closely monitored during the resting portion of the test. At the conclusion of the five-minute rest phase, the participants were asked to voluntarily hyperventilate by inhaling deeply and exhaling out all air from their lungs, while avoiding panting. The hyperventilation phase lasted for approximately 2 minutes, or until the ETP_{CO2} reached 22 mmHg. The purpose of this phase is to reduce the ETP_{CO2} below the VRT to ensure this parameter is detected during the test. This phase also

ensures that when the participant begins breathing from the rebreathing bag, their alveolar gas equilibrates with the gas pressures in the bag. This equilibration is detected by a plateau in ETP_{CO2} shortly after the onset of rebreathing.

Immediately after the hyperventilation phase, the participants were switched from breathing room air to breathing from a 6L rebreathing bag and asked to relax and breathe normally. Participants stayed on the rebreathing bag for several minutes while ETP_{CO2} was allowed to slowly increase over time from their starting value to 60 mmHg. During this time, PO_2 levels in the bag were maintained at a constant level. The test was repeated twice, first with a hyperoxic gas mixture which maintained an inspired oxygen concentration of 30%, then with a hypoxic gas mixture which maintained an $ETPO_2$ level of 50 mmHg (PIO2 approximately 70 mmHg, allowing average desaturation to approximately 80-85%). Between these two tests, participants rested and breathed room air for 15 minutes to allow them to return to baseline. Tests were terminated if ETP_{CO2} reached 60 mmHg, SpO_2 reached or dropped below 70%, total ventilation reached 100 L/min, or if the participant pulled themselves off.

Data Collection

During the test, analog output from each data source (gas analyzers, flow meter, pulse oximeter, ECG) was collected by a PowerLab data acquisition system (AD Instruments) which converted the data to a digital signal that was sent to a PC for collection in LabChart 8 software (AD Instruments). Here, fifteen channels worked in

tandem to measure breathing parameters. These channels included the EKG channel, which was monitored to ensure there was no irregular heart rhythm throughout the test, and the SpO_2 channel, which monitored the participants oxygen saturation. The flow channel, oxygen percent channel, and carbon dioxide percent channels all correlated to the amount of air inhaled/exhaled and concentration of the associated gases that were present in the air the participants were breathing. Partial pressure of oxygen, partial pressure of carbon dioxide, and spirometry volume were also measured throughout the protocol. The integral of the flow channel was used to record inspiratory volumes. All volumes were converted to BTPS units. Figure 6 provides an example of a raw data trace.

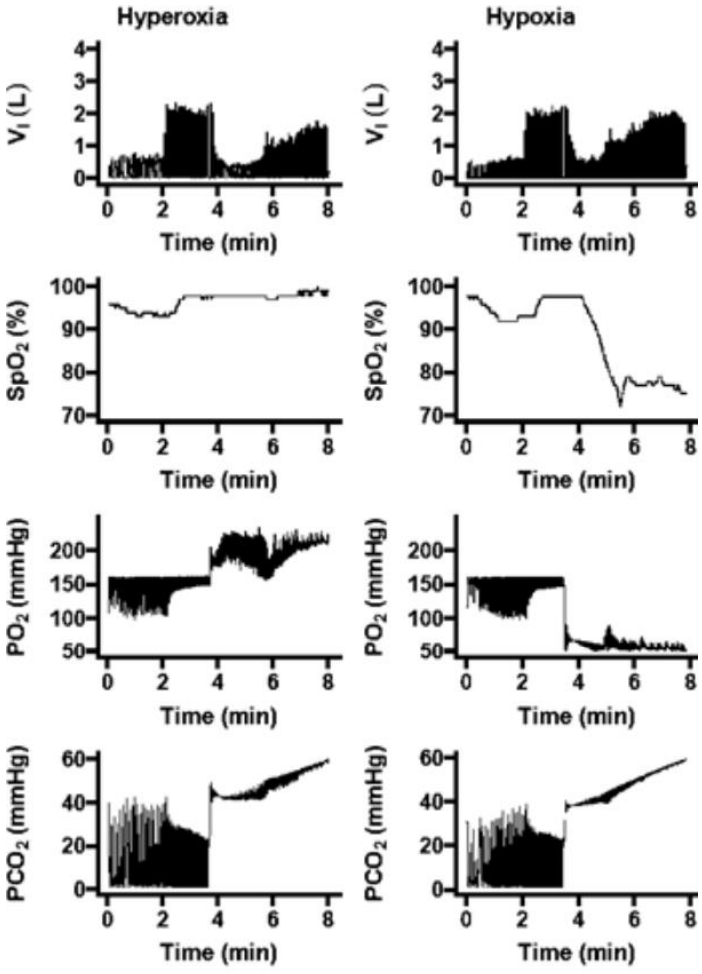

Figure 6. Hyperoxia vs. Hypoxia. Immediately after hyperventilation, test conditions were initiated and visualized in LabChart. The above channels were closely monitored to ensure test conditions were in the correct parameters and participants were performing the ventilatory chemoreflex testing within safe limits.

The test data from each participant was edited in LabChart to include one minute of pre-test data and data from the duration of the test-phase of the chemoreflex test. Calibration data and hyperventilation phases were removed. Files were converted to .txt files for statistical analysis in Rstudio.

Chemoreflex Data Analysis

Chemoreflex data was pre-processed in LabChart 8 and analyzed in Rstudio. Each participant's VRT under each test condition was determined by fitting 15 estimates within the *mcp* package (Lindeløv, 2020). The results of the *mcp* bootstrapping function were plotted within Rstudio to visually check for accuracy of the VRT estimate (figure 7). The HCVR slope was determined by plotting the output data, creating the "hockey stick" figure seen in the VRT images in figure 7. The numerical "m2", or slope of the second, steeper, segment of the line provided the HCVR. Each measurement was recorded in Excel (Microsoft) for further calculations, including the hypoxic ventilatory response (HVR) of each participant.

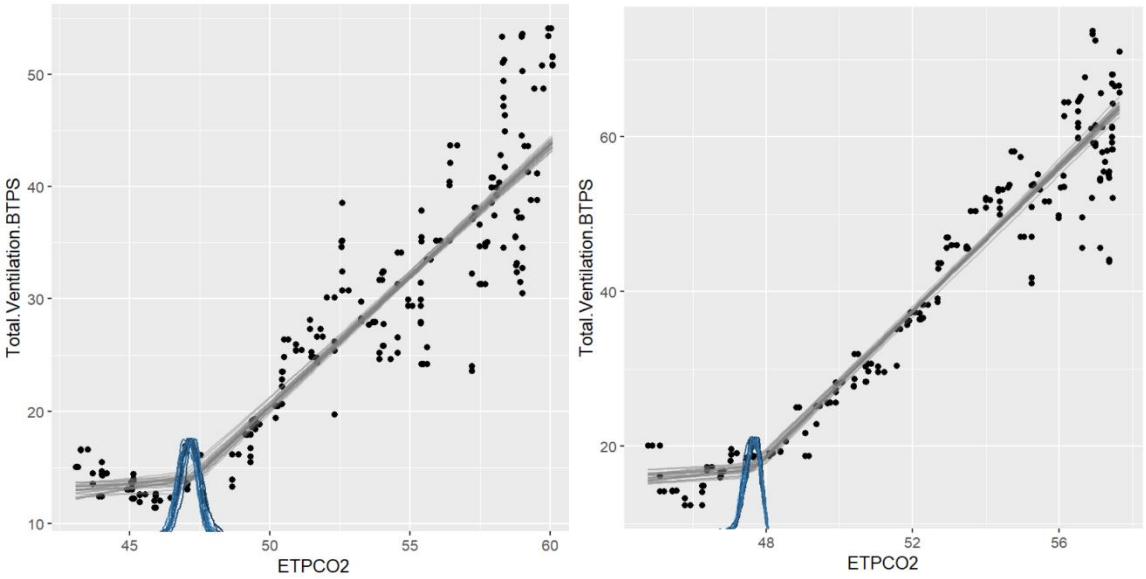

Figure 7. VRT plots. Obtained from the same participant, the hyperoxia VRT plot (left) and hypoxia VRT plot (right) show the relationship between total ventilation in L/min and $ETPCO_2$, measured in mmHg. The point at which the first- and second-lines meet is the participants VRT in each test condition.

After obtaining the VRT and HCVR slope values in Rstudio, we were able to determine the hypoxic ventilatory response (HVR) from each participant. To do this, we recorded the SpO_2 in at four given ETP_{CO2} levels (45 mmHg, 50 mmHg, 55 mmHg, and hyperoxic VRT + 3 mmHg) from the hypoxic and hyperoxic conditions. We then used the equation for the HCVR response to calculate the ventilation rate (V_E) at each ETP_{CO2} level under each oxygen condition. The HVR was then calculated as:

$$HVR = \frac{\dot{V}_{E_{hypoxia}} - \dot{V}_{E_{hyperoxia}}}{SpO_{2_{hyperoxia}} - SpO_{2_{hypoxia}}}$$

For example, at a given 55 mmHg ETP_{CO2}, one recovered participant had an SpO_2 of 98.81% in the hyperoxic test and an SpO_2 of 83.01% in the hypoxic test. By taking the difference between the two, we have $\Delta SpO_{2\cdot 55} = 15.8\%$. Next, we calculated the *y* in

$y=mx+b$ of the second, steeper line in the VRT plot from figure 6, with m being the slope of line two, x being one of the four previously mentioned $ETPCO_2$, and b corresponding to the y-intercept of the HCVR fit line, provided by the *mcp* package. The difference between each value of ventilation from the two test conditions was then recorded as. For example, for the same recovered participant, the calculated V_E at 55 mmHg ETP_{CO2} in the hyperoxic test was 27.245 L/min and 44.219 L/min in the hypoxic test. By taking the difference between the two, we have $\Delta V_{E\cdot55}$ = 16.974 L/min. Thus, to determine the HVR, we divided $\Delta V_{E\cdot55}$ by $\Delta SpO_{2\cdot55}$. For example, the HVR for the recovered participant at ETP_{CO2} 55 mmHg is 16.974 L/min / 15.8 % SpO_2 = 1.074 L/min/%SpO_2.

LEGENDPlex Analysis

All samples from control participants (N=41) and 72 out of 77 samples from recovered participants were included in the cytokine analysis. Analysis of cytokine expression data was performed via LEGENDplex™ Data Analysis Software by BioLegend® using manufacturer's protocols. After uploading all samples to the software, manual gating was used to include an analyte that was originally not gated for. Four recovered participants and one control participant were excluded from the final results for the human anti-viral response panel due to mis-plating samples, and no participants were excluded from the final results in the vascular inflammatory panel. The resulting concentrations were then downloaded for statistical analysis in RStudio.

Statistical Analysis

To determine if there were significant effects of group (control versus recovered) on each cytokine expression level, a general linear model was performed with age, sex, and BMI as covariates (Cytokine level ~ Group + Sex + Age + BMI). A Spearman correlation was also performed to determine positive or negative relationships between cytokine expression and HVR. Study group cohorts and test phases were analyzed via ANOVA (detailed in results section) to determine statistical significance.

RESULTS

Participant Demographics

Of the 118 participants recruited, 69 were female, 47 were male, and 2 did not report biological sex. Age, weight, height, systolic blood pressure (SBP), and diastolic blood pressure (DBP) were averaged for the control and recovered cohorts and presented in Table 1.

	Female (n=69)		Male (n=47)		Unknown sex (n=2)
	C (n=21)	R (n=48)	C (n=20)	R (n=27)	R (n=2)
Age (years)					
Mean ± SD	30.7 ± 10.8	28.6 ± 12.3	26.2 ± 5.1	29.0 ± 11.0	34.0
Median	26.0	24.5	26.0	26.0	n/a
Weight (lbs)					
Mean ± SD	158.3 ± 37.4	152.3 ± 39.6	200.8 ± 59.0	193.5 ± 55.4	230.0 ± 1.4
Median	145.0	141.0	182.0	183.5	230.0
Height (cm)					
Mean ± SD	162.3 ± 4.8	161.2 ± 6.9	177.5 ± 7.4	175.96 ± 8.59	180.8 ± 4.6
Median	161.5	161.3	176.0	176.8	180.8
BMI					
Mean ± SD	27.0 ± 5.2	26.2 ± 6.4	28.7 ± 7.1	27.8 ± 6.8	32.0 ± 1.4
Median	25.6	26.0	26.8	26.0	32.0
SBP (mmHg)					
Mean ± SD	120.5 ± 12.0	76.0 ± 8.4	127.8 ± 10.4	77.1 ± 13.5	123 ± 5.7
Median	118.0	118.0	126.5	125.0	122.0
DBP (mmHg)					
Mean ± SD	119.3 ± 11.9	73.1 ± 12.5	132.5 ± 21.7	81.6 ± 13.5	76.3 ± 11.6
Median	75.0	74.5	75.0	79.0	76.0

Table 1. Participant characteristics. Physiological measures are reported separated by group and biological sex. Weight is reported in pounds (lbs), height is reported in centimeters (cm), and blood pressure is reported in mmHg. N/A is reported in median age under column "other" as both participants were the same age. No statistical significance was found between sex, group, and vitals (nonsignificant results via chi-squared tests in Rstudio).

Men and women were distributed evenly across control and recovered groups ($p = 0.89$). Age and BMI were not significant among groups (age: $p = 0.772$, BMI men: $p = 0.422$, and BMI women: $p = 0.471$). 37 control participants in the control group reported being vaccinated against COVID-19 with 2 not vaccinated and 2 not indicating vaccine status. Of the recovered group, 75 participants indicated vaccination against COVID-19; however, whether the vaccine was obtained before or after infection is unknown. Two recovered participants reported an unvaccinated status.

Participants exhibited a range of side effects in response to the chemoreflex procedure with the most common side effect being lightheadedness. Other side effects either reported or observed were headaches, dizziness, anxiety, and temporarily blurred vision. All participants reported feeling normal again within a few minutes of completing the ventilatory chemoreflex test. Following blood draw, participants did not report any side effects but were made aware of the possibility of bruising and advised to keep gauze and bandage in place for at least 20 minutes to avoid bleeding at the site.

Inflammatory Marker Analysis

To determine if there were significant effects of group (control versus recovered) on each cytokine, a general linear model was performed with age, sex, and BMI as covariates. A change in expression levels between control and recovered cohorts was found statistically significant for IL-10, Cystatin C, IGFBP-4, OPN, MMP-2, NGAL, and Myoglobin. Furthermore, an effect of sex indicated significance for IGFBP-4 and IL-8,

and an effect of BMI indicated significance for MRP8/14, NGAL, CRP, ICAM-1, VCAM-1, and MMP-9. There were no significant values returned for an effect of age alone. Additionally, family-wise Bonferroni p-value adjustments were performed ($p<0.0038$ being considered statistically significant). Despite the familywise adjustment, significant findings were still prevalent (Table 2). There were highly significant effects of group on vascular inflammatory cytokines (n=6; 46.15%) as opposed to the human anti-inflammatory cytokines (n=1; 7.69%), with a total of thirteen cytokines per panel.

Inflammatory markers	p-values			
	Group	Age	Sex	BMI
Vascular Inflammation Panel				
Myoglobin	**0.0427***	0.307	0.101	0.876
MRP8/14	0.101	0.275	0.150	**0.00073***, ł**
NGAL	**0.00706****	0.669	0.092	**0.0057****
CRP	0.161	0.939	0.204	**1.29 x 10^{-5}***, ł**
MMP-2	**0.00073***, ł**	0.675	0.639	0.948
OPN	**0.00377**, ł**	0.938	0.289	0.704
MPO	0.089	0.550	0.789	0.785
SAA	0.965	0.109	0.521	0.096
IGFBP-4	**0.0102***	0.892	**0.028***	0.221
ICAM-1	0.327	0.547	0.444	**0.0044****
VCAM-1	0.153	0.127	0.153	**0.0032**, ł**
MMP-9	0.427	0.528	0.490	**0.0057****
Cystatin C	**0.000549***, ł**	0.813	0.948	0.123
Human Anti-Inflammatory Panel				
IL-1β	0.467	0.287	0.249	0.135
IL-6	0.174	0.332	0.547	0.688
TNF-α	0.141	0.583	0.544	0.102
IP-10	0.119	0.114	0.410	0.996
IFN-λ1	0.535	0.208	0.461	0.710
IL-8	0.368	0.121	**0.032***	0.794
IL-12p70	0.162	0.947	0.104	0.423
IFN-α2	0.286	0.396	0.399	0.544
IFN-λ2/3	0.068	0.969	0.830	0.676
GM-CSF	0.410	0.504	0.756	0.349
IFN-β	0.303	0.158	0.158	0.078
IL-10	**0.0499***	0.932	0.827	0.624
IFN-γ	0.620	0.418	0.415	0.190

Table 2. Results of general linear models. Table demonstrates significant effects of group, age, sex, and BMI on plasma cytokine expression levels. *Indicates significance at the $p<0.05$ level, and ł indicates significance after family-wise Bonferroni adjustment.

Inflammatory Markers: Time-Point-Separation Analysis

Inflammatory markers that demonstrated significant differences across healthy and recovered participants were further analyzed to determine if time post recovery impacted expression levels. The recovered cohort was split into categories based on the amount of time passed since they last tested positive for COVID-19 (0 to 3, 4 to 6, 7 to 11, 12 to 24, or 24+ months). Here, a multivariate plot was created, showing significant p-values and the relationship between the control group (C) and time-point separation recovered categories (figure 8). A student's t-test comparing the categories to each other was used to determine significance. Contrary to our expectations, each cytokine evaluated showed a pattern for reduced expression as a function of time post recovery, compared to the control group, with some factors returning to baseline levels following 2 years post recovery. Most markers demonstrated the most significant decreases compared to the control group at 7-11 months post recovery.

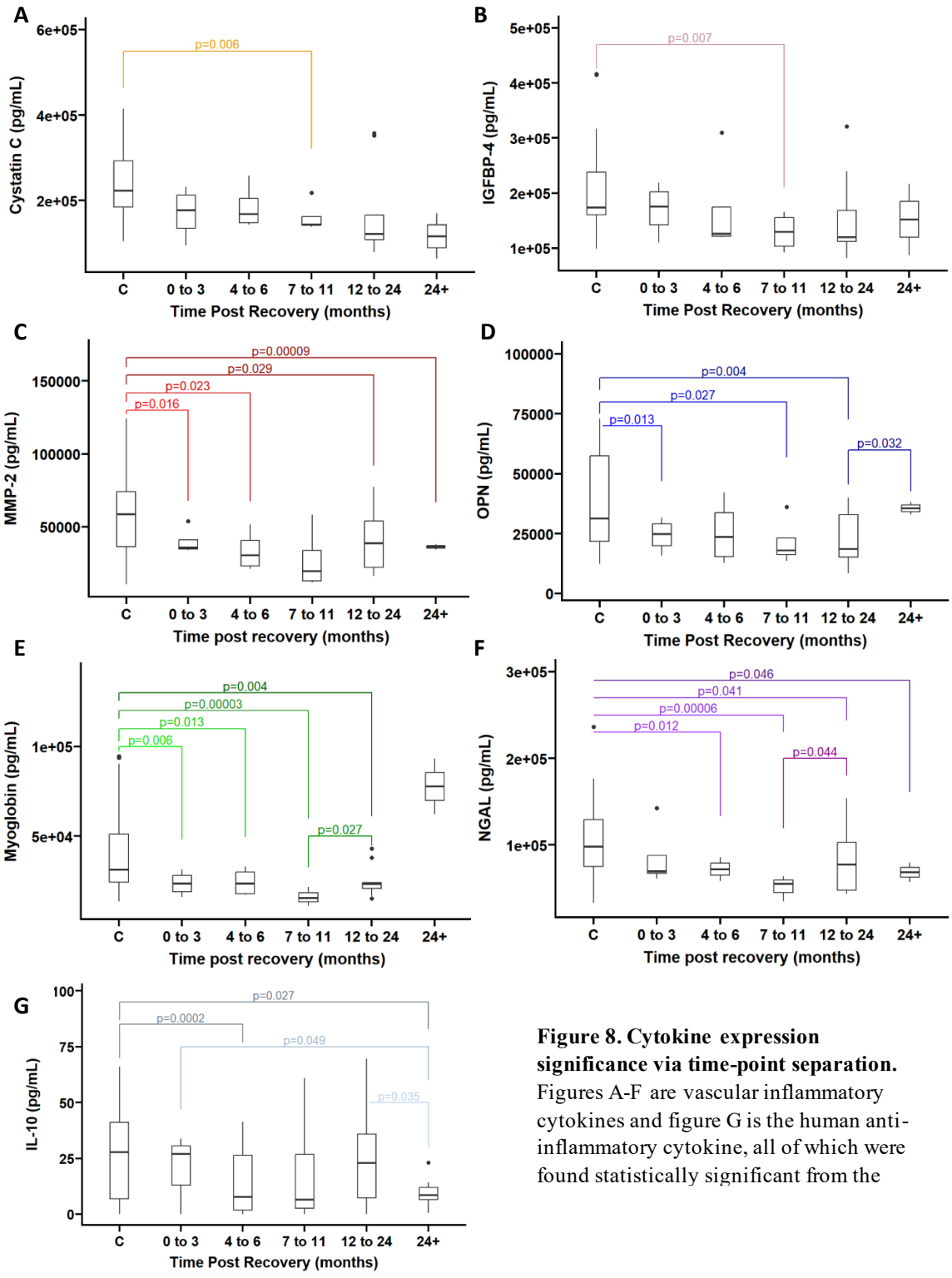

Figure 8. Cytokine expression significance via time-point separation. Figures A-F are vascular inflammatory cytokines and figure G is the human anti-inflammatory cytokine, all of which were found statistically significant from the

Chemoreflex Test: Pulmonary Functions Data

To determine if lung function was significantly impacted after recovery from COVID-19, participants completed standard spirometry testing. There was no significant difference in the forced vital capacity (FVC) or forced expired volume in 1 second (FEV_1) between recovered and control groups (figure 9). This data indicates a return to normal lung volume and airflow rate after infection.

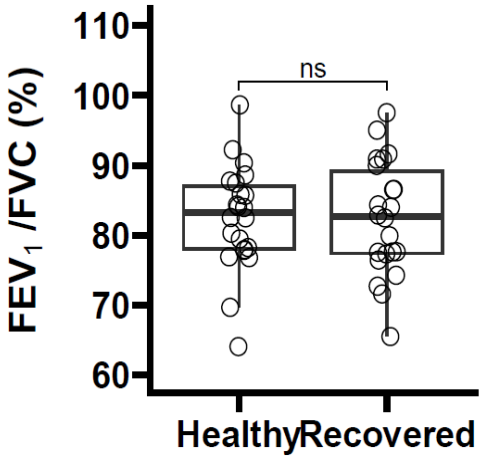

Figure 9. Spirometry. Statistical analysis used for resting values were done with a t-test.

Chemoreflex Test: Statistical Analysis

Resting values obtained from the ventilatory chemoreflex test included resting $ETPCO_2$, SpO_2, tidal volume, breathing frequency, and resting ventilation. No statistically significant differences in resting breathing patterns were found between participant study groups (figure 10).

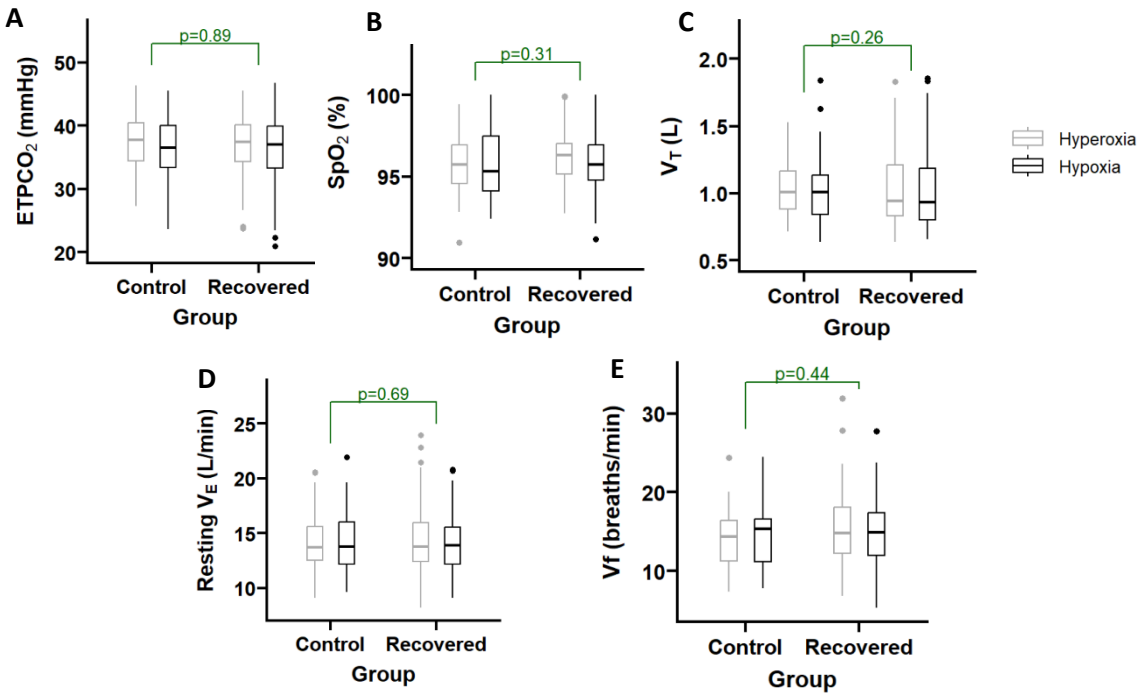

Figure 10. Resting Figures. Statistical analysis used for resting values were done with ANOVA. **A.** End-tidal carbon dioxide. **B**. Average resting oxygen saturation between groups and test. **C.** Average tidal volume in liters. **C.** Resting ventilation in liters per minute and **D.** breathing frequency measured in breaths per minute.

VRT and HCVR

To determine if COVID-19 infection significantly impacted ventilatory reflex control, we conducted chemoreflex testing using a Duffin modified rebreathing technique. A two-way ANOVA revealed a significant main effect of group on VRT and HCVR (figure 11). Post-hoc pairwise t-tests were performed to determine if these differences were driven by changes in the hyperoxic or hypoxic HCVR reflex. A significant effect was found in the HCVR (p=0.032) between groups in the hypoxic test phase, where the recovered group had a lower average HCVR than the control group.

Significance for VRT, however, was found between the hyperoxic phase of the control group and hypoxic phase of the recovered group. However, the VRT showed an overall decreasing trend from control to recovered in same-test phases.

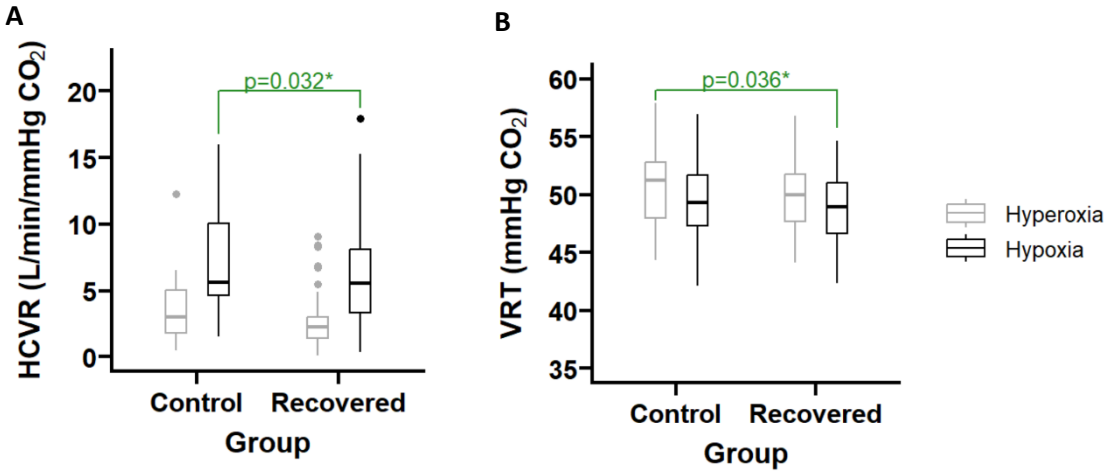

Figure 11. Test Figures. A. The HCVR figure shows the quantified ventilatory response to carbon dioxide within the body in both control and recovered cohorts. **B.** The VRT figure shows the average $ETPCO_2$ at which ventilation begins to rapidly increase to compensate for the rising CO_2 and decreased pH in both control and recovered cohorts.

Because of the observable trend towards an overall decreased HCVR and VRT in both study cohorts, we performed a more in-depth analysis and separated the recovered group into time frames based on when they were tested after recovering from COVID-19 infection (table 3). We identified a significant decrease in the VRT (p=0.019) from control to 7-11 months post recovery, which matched the timeframe of peak changes in inflammatory cytokine expression. Furthermore, a statistically significant decrease was also noted in the HCVR (p=0.023) from control to 2+ years post recovery (figure 12).

	n = x		VRT	HCVR
0-3 months	n = 8	Hyperoxia	51.889 ± 1.752	**2.709 ± 2.426**
		Hypoxia	50.526 ± 2.366	8.210 ± 4.007
4-6 months	n = 9	Hyperoxia	**49.558 ± 2.267**	**2.503 ± 1.645**
		Hypoxia	**48.965 ± 1.930**	**6.482 ± 1.645**
7-11 months	n = 8	Hyperoxia	**48.798 ± 2.447**	**1.758 ± 0.825**
		Hypoxia	**47.286 ± 2.181**	**6.105 ± 2.938**
1-2 years	n = 10	Hyperoxia	**50.314 ± 3.042**	**3.111 ± 1.698**
		Hypoxia	**48.764 ± 3.757**	**7.214 ± 4.893**
2+ years	n = 3	Hyperoxia	53.620 ± 2.538	5.778 ± 3.947
		Hypoxia	51.199 ± 0	**4.688 ± 3.183**
Controls	n = 37	Hyperoxia	*50.374 ± 3.599*	*3.704 ± 2.312*
		Hypoxia	*49.833 ± 3.074*	*8.046 ± 5.732*

Table 3. Time-post recovery for VRT and HCVR. Values in bold are lower than the control group. Here we can observe a trend towards decreasing values in both VRT and HCVR until about 1 year post recovery, when VRT and HCVR start to return to pre-infection levels.

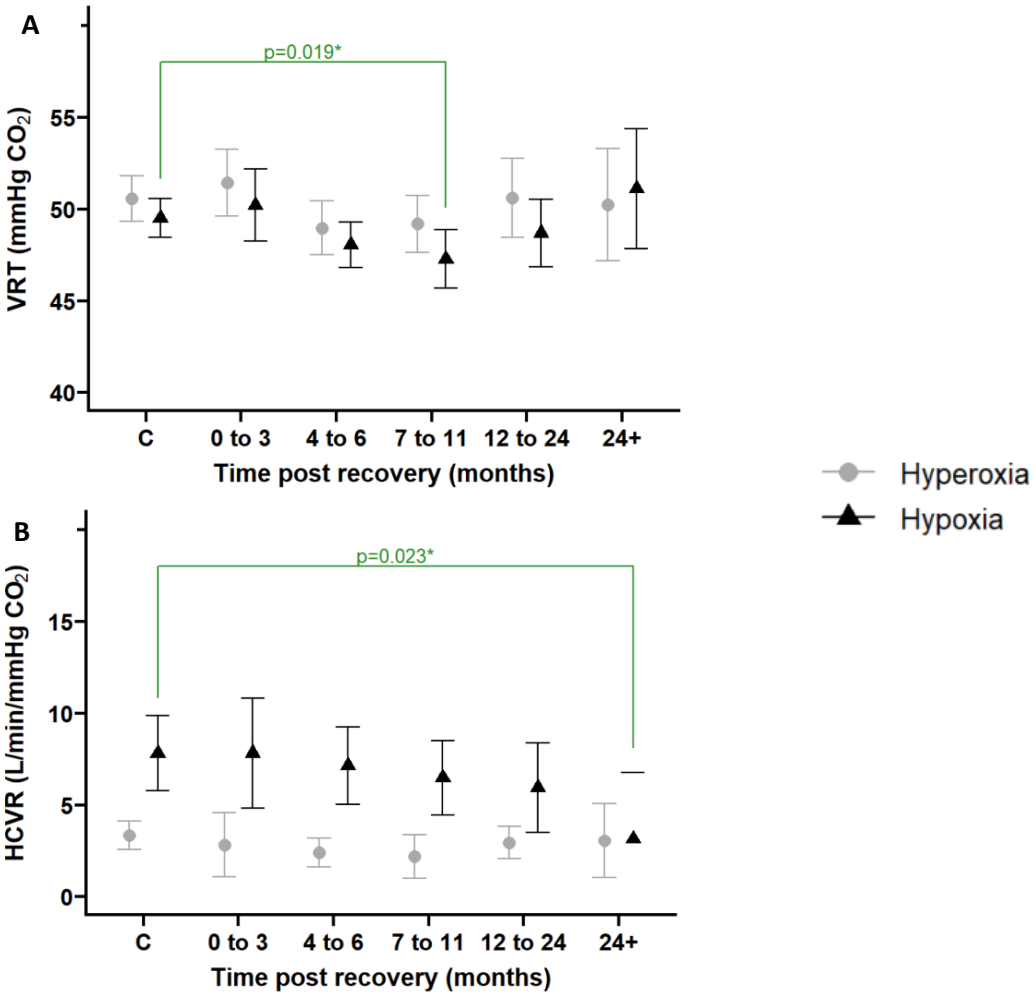

Figure 12. Time-post recovery for VRT and HCVR. While there is an observable downward trend until 1 year post recovery in both hypoxic and hyperoxic test phases, a significant difference was noted in the hypoxic test phase for both VRT and HCVR.

Hypoxic Ventilatory Response

The hypoxic ventilatory response (HVR) was measured at four different $ETPCO_2$ values. Each measurement indicated no significant difference between control and recovered groups (figure 13).

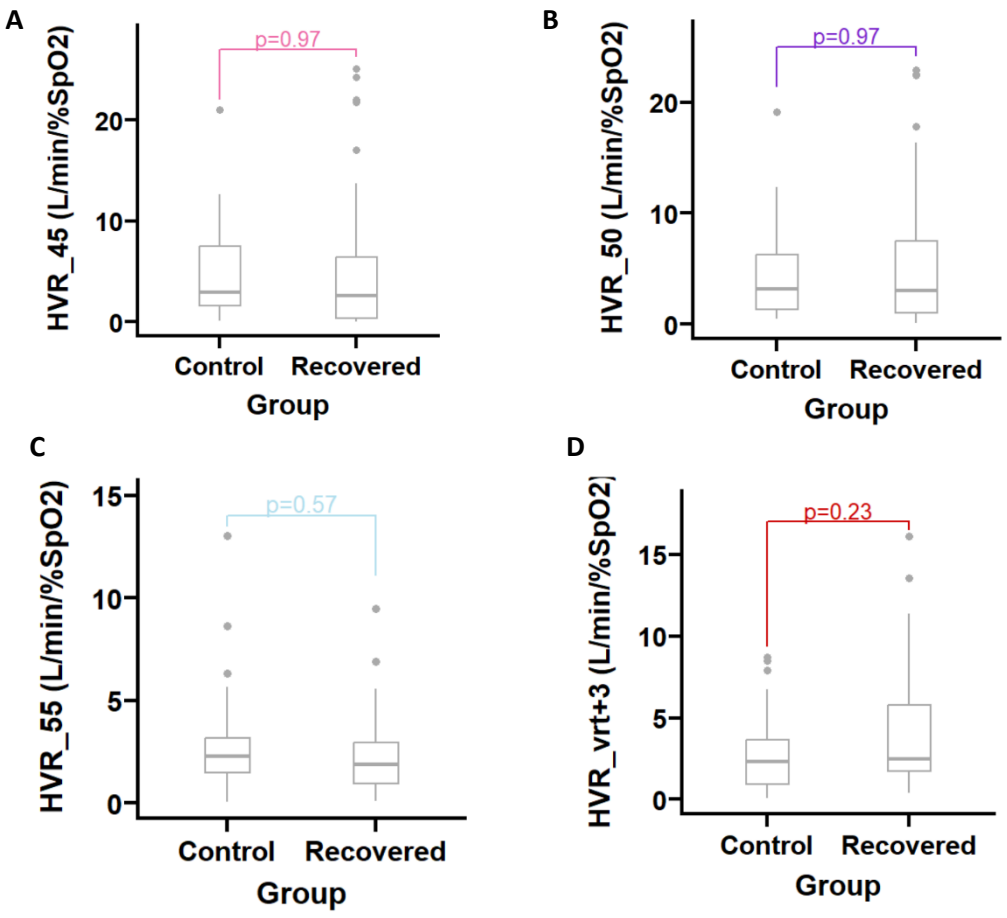

Figure 13. HVR plots. Plots show average HVR obtained at a given $ETPCO_2$ (mmHg) concentration. **A**. Measured at 45 mmHg, **B**. measured at 55 mmHg, **C**. measured at 50 mmHg, and **D**. measured at VRT+3 (obtained at three numbers above each participant VRT).

Despite non-significance, we see a trend towards an increased HVR for many participants in the recovered group. To further evaluate this, we performed an additional analysis with the same time post recovery separation as annotated in figure 12 above. Using unpaired students t-tests to compare each recovered timepoint group to the control group, we determined significant changes at different time points in HVR 50, HVR 55, and HVR VRT+3, shown in figure 14.

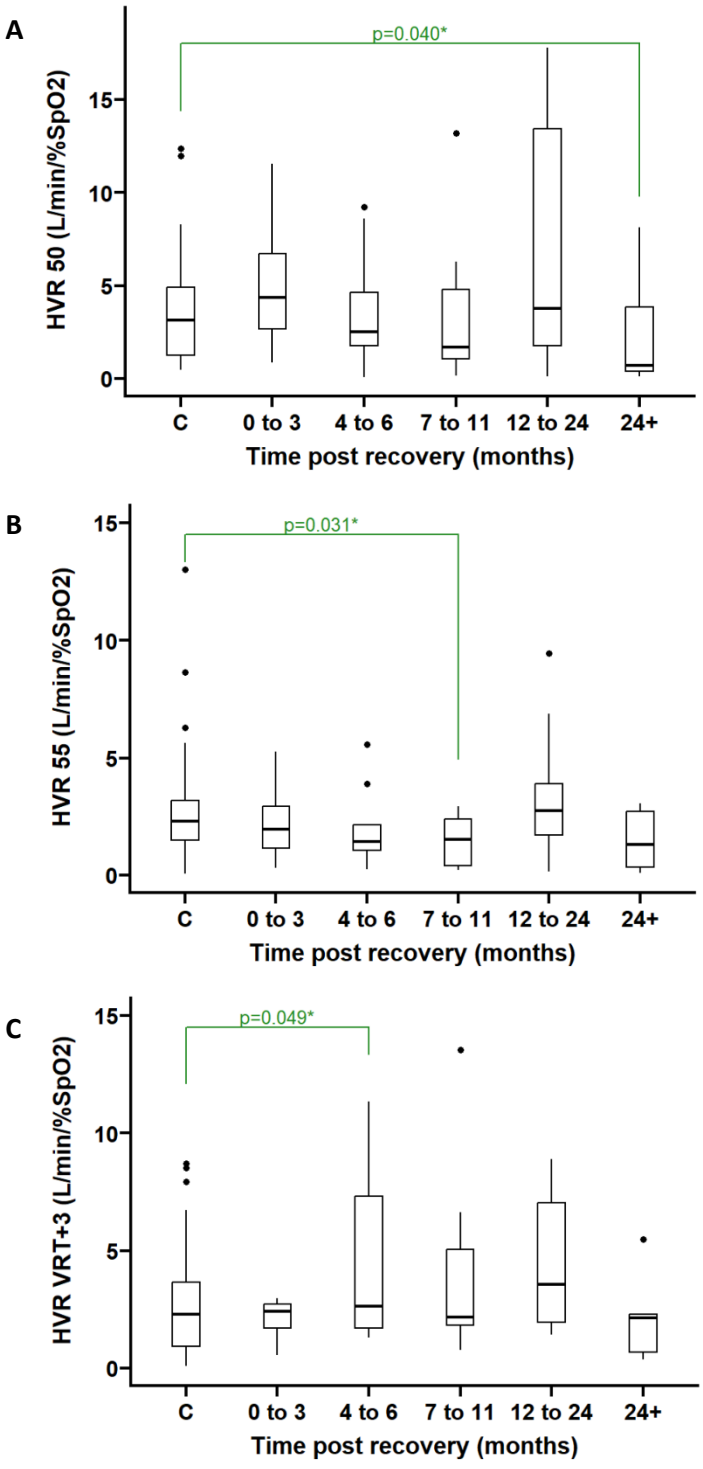

Figure 14. HVR plots with time-post recovery. Significant changes in HVRs are prevalent at specific time points after recovery from COVID-19.

Cytokine Expression and HVR

Once both HVR and inflammatory marker data were obtained from each participant, we performed spearman rank correlation analyses to determine if there was any correlation between systemic cytokine expression and HVR sensitivity within the two study cohorts. For HVRs collected at VRT+3, we found 9 statistically significant correlations from either control or recovered groups, indicated in figure 15.

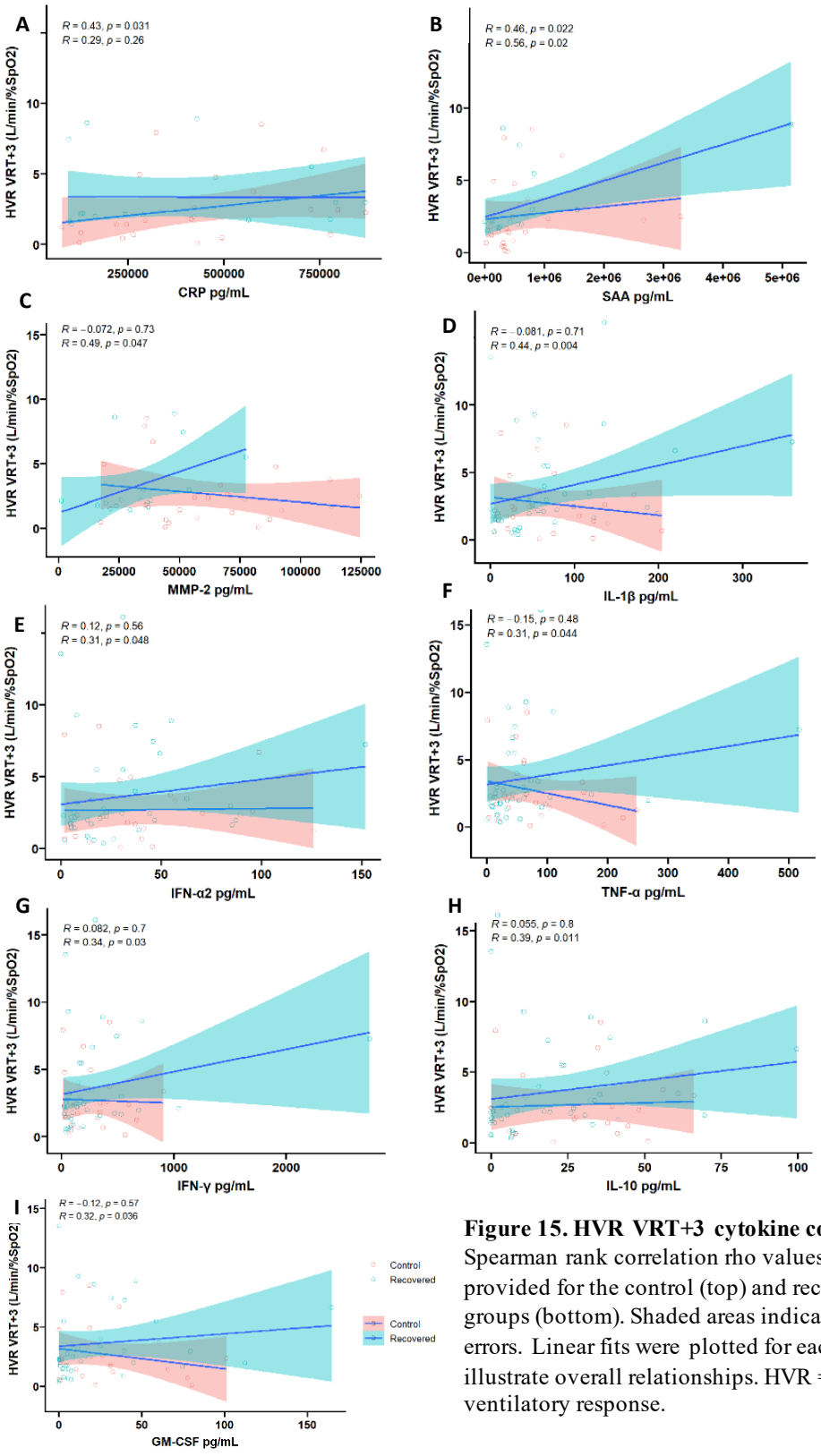

Figure 15. HVR VRT+3 cytokine correlation. Spearman rank correlation rho values are provided for the control (top) and recovered groups (bottom). Shaded areas indicate standard errors. Linear fits were plotted for each group to illustrate overall relationships. HVR = hypoxic ventilatory response.

For HVRs collected at $ETPCO_2$ of 55mmHg, we found 3 statistically significant correlations between HVR 55 and cytokine expression, shown in figure 16.

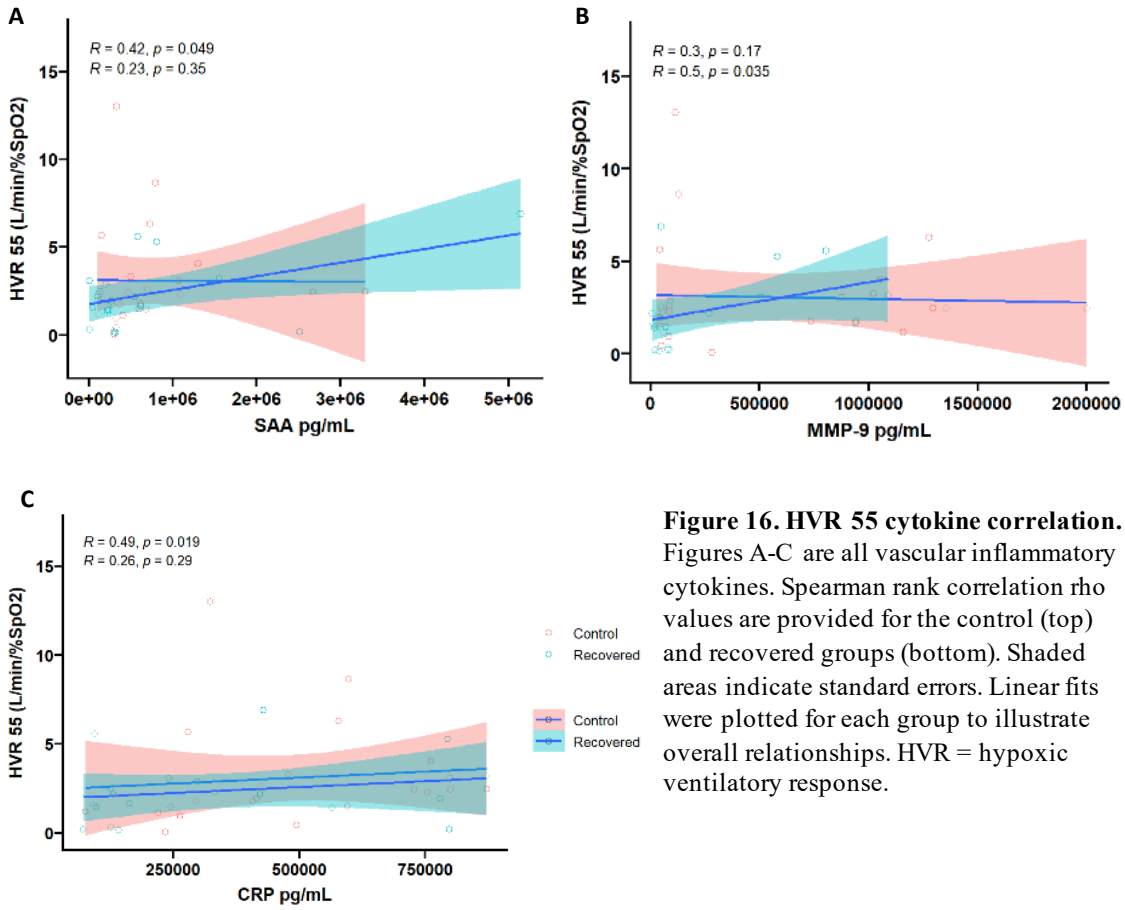

Figure 16. HVR 55 cytokine correlation. Figures A-C are all vascular inflammatory cytokines. Spearman rank correlation rho values are provided for the control (top) and recovered groups (bottom). Shaded areas indicate standard errors. Linear fits were plotted for each group to illustrate overall relationships. HVR = hypoxic ventilatory response.

HVRs collected at $ETPCO_2$ of 50mmHg, we found 6 statistically significant correlations between HVR 50 and cytokine expression, shown in figure 17.

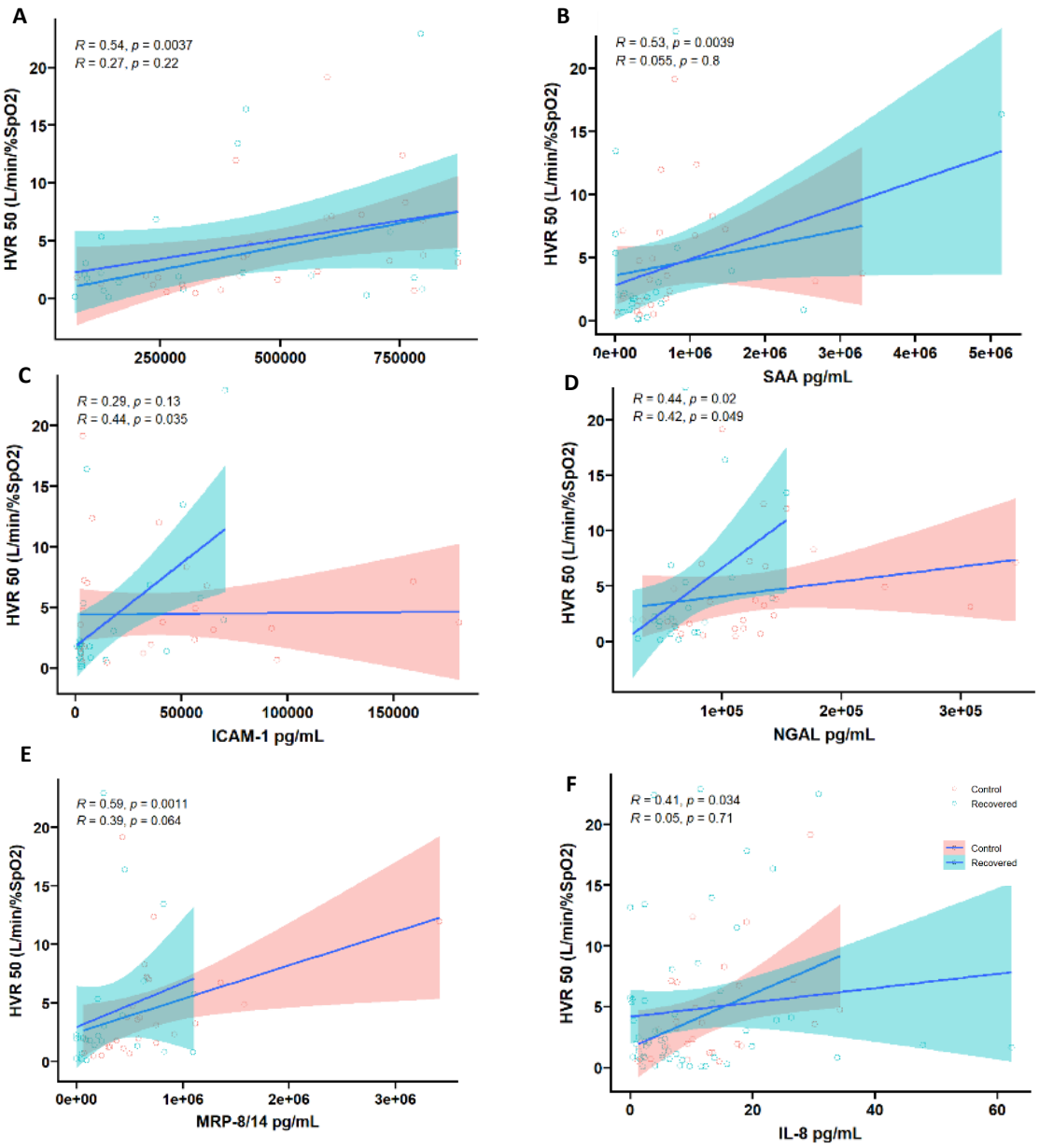

Figure 17. HVR 50 cytokine correlation. Figures A-E are vascular inflammatory cytokines and figure F is a human anti-viral cytokine. Spearman rank correlation rho values are provided for the control (top) and recovered groups (bottom). Shaded areas indicate standard errors. Linear fits were plotted for each group to illustrate overall relationships. HVR = hypoxic ventilatory response.

For HVRs collected at $ETPCO_2$ of 45 mmHg, we found 9 statistically significant correlations between HVR 45 and cytokine expression, shown in figure 18.

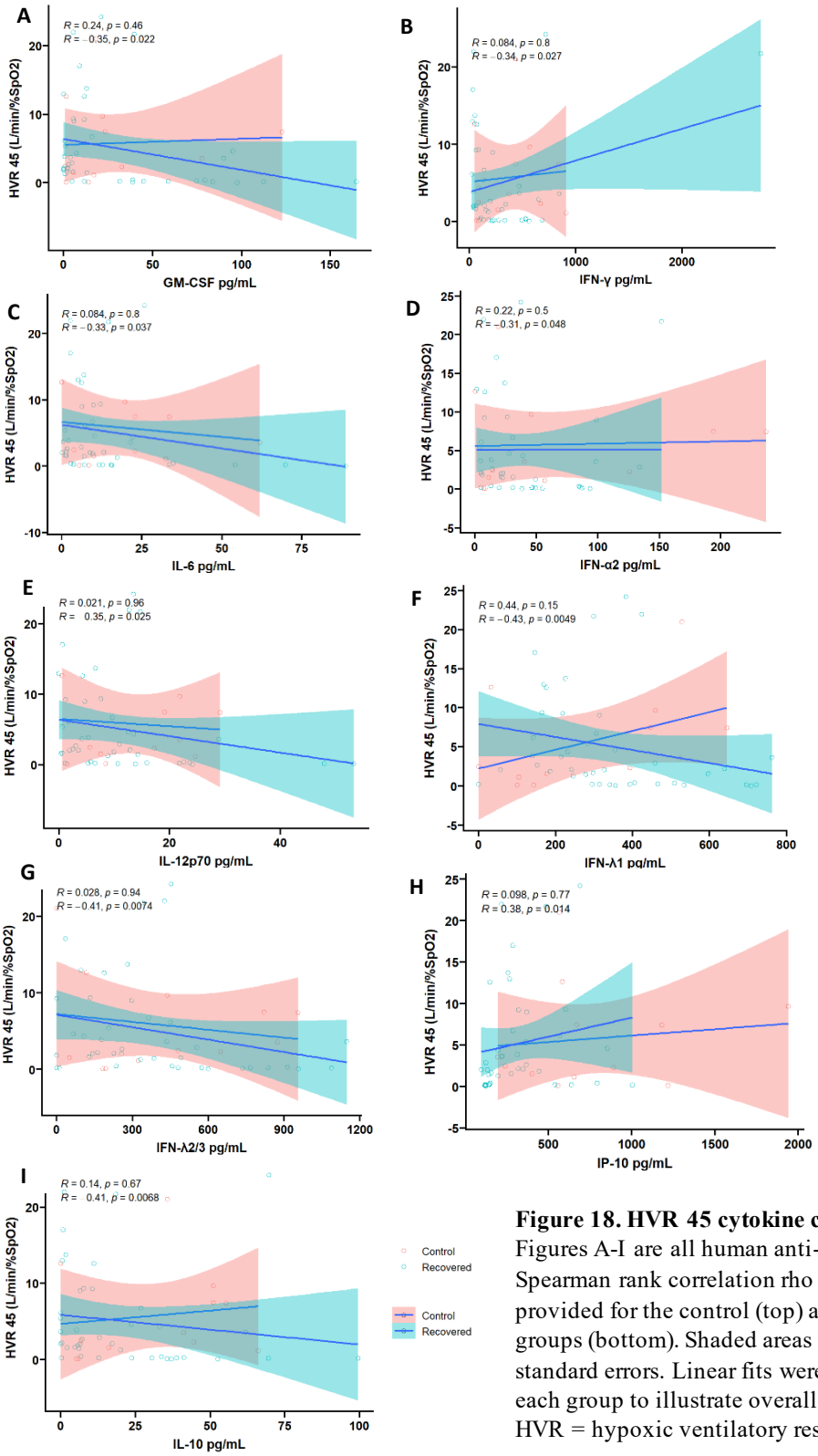

Figure 18. HVR 45 cytokine correlation. Figures A-I are all human anti-viral cytokines. Spearman rank correlation rho values are provided for the control (top) and recovered groups (bottom). Shaded areas indicate standard errors. Linear fits were plotted for each group to illustrate overall relationships. HVR = hypoxic ventilatory response.

Overall, many inflammatory markers were positively associated with HVR, regardless of measurement point. This is particularly true in the recovered group as some inflammatory markers trended a negative association with HVR in the control group.

Within Subject Findings

From the control study cohort, five participants returned after COVID-19 infection to participate in the study as a recovered participant. Because of this, analysis of between-subject findings was performed. Four out of five of the participants were found to have a decreased CO_2 reflex, represented by a decreased hyperoxic HCVR from control to recovered. Participant 2, however, had the opposite effect, accompanied by an increased hypoxic VRT from control to recovered.

	Group	Test	VRT	HCVR	HVR VRT+3
Participant 1	Control	Hyperoxia	48.064	12.290	0.208
		Hypoxia	47.307	10.985	
	Recovered	Hyperoxia	48.050	4.938	6.751
		Hypoxia	47.377	7.538	
Participant 2	Control	Hyperoxia	51.886	5.989	1.487
		Hypoxia	48.085	7.453	
	Recovered	Hyperoxia	54.363	8.320	1.487
		Hypoxia	49.036	10.271	
Participant 3	Control	Hyperoxia	53.017	1.051	3.312
		Hypoxia	51.707	15.401	
	Recovered	Hyperoxia	52.289	0.736	5.405
		Hypoxia	53.693	15.284	
Participant 4	Control	Hyperoxia	50.009	4.692	0.882
		Hypoxia	47.860	6.422	
	Recovered	Hyperoxia	50.588	1.784	7.581
		Hypoxia	50.684	8.777	
Participant 5	Control	Hyperoxia	52.270	3.042	5.412
		Hypoxia	51.709	10.039	
	Recovered	Hyperoxia	50.726	2.764	3.232
		Hypoxia	49.720	10.609	

Table 4. Within subject ventilatory parameters. Values are representative of differences in control of breathing between control and recovered same participants. Participants 1 and 4 were tested again 4-6 months post recovery and the remaining participants 0-3 months post recovery.

Furthermore, we performed analyses to determine if there was an overall change in inflammatory cytokine expression before and after COVID-19 infection. Unfortunately, we did not have enough power to determine significance (n=3; participants 2, 3, and 5 from table 4); however, we did observe trends in changed levels of cytokine expression in each of the three participants. The changes observed included decreased expression of myoglobin, NGAL, OPN, and MMP-2 and increased expression of IGFBP-4 and IL-10 after 0-3 months recovery from COVID-19.

DISCUSSION

Inflammatory Biomarkers of long-COVID

The primary aim of this study was to investigate the role of systemic inflammation in long-COVID, a greatly misunderstood and long-lasting impact of COVID-19 infection. Furthermore, we investigated the impact of COVID-19 on the neural control of breathing and potential links between inflammatory biomarkers and altered breathing responses. Our findings that COVID-19 significantly impacted the expression of vascular inflammatory cytokines, even following many months after recovery, supports previous findings of vascular damage and inflammation during acute infection (Shabani et al., 2023). However, unexpectedly, we found that many of these vascular inflammation markers showed decreased expression levels in the plasma. This may represent a rebound effect in which expression of these vascular inflammation mediators becomes blunted following recovery in response to significant overexpression during the acute injury phase. Indeed, during recovery, upregulation of anti-inflammatory and antioxidant pathways may contribute to this result. Nonetheless, decreased expression of many of these markers may also be a sign of pathology, as decreased expression of many of our significant cytokines (Cystatin C, IGFBP-4, MMP-2, OPN, Myoglobin, NGAL/Lipocalin, and IL-10) has been linked to other comorbidities and viral or bacterial-based diseases (Flonta et al., 2009; Salgado et al., 2013; Shirakabe et al., 2010; Singh et al., 2014; Torres et al., 2023; Wei et al., 2020).

Decreased expression of Cystatin C is linked to cardiovascular disease, especially prevalent in patients with atherosclerosis and coronary artery disease. Its decreased presence has also been noted in viral infection, leading to reduced resistance against a virus (Salgado et al., 2013). Because its decreased expression is a significant finding in our recovered participant cohort, this may be indicative of long-lasting cardiac or other vasculature related symptoms within the human body. Interestingly, we also observed decreased levels of IGFBP-4, MMP-2, OPN, Myoglobin, NGAL/Lipocalin, and IL-10 – each of which have an association with each other or with vasculature.

The observed decrease in IGFBP-4 after infection may be beneficial to long-term recovery due to increasing levels of expression being associated with higher rates of pulmonary arterial hypertension (PAH) and decreased survival rates from PAH (Torres et al., 2023). Similarly, increased levels of matrix metalloproteinases, or MMPs, (Shirakabe et al., 2010) and OPN (Singh et al., 2014) have an association with acute heart failure (AHF), myocardial dysfunction, and are indicative of lower survival rates to AHF and other cardiac-related events (Shirakabe et al., 2010; Singh et al., 2014), potentially meaning that the decreased expression of MMP-2 and OPN we observed in long-term recovery is correlated with a better recovery outcome. Interestingly, decreased expression of myoglobin is not indicative of disease; however, myoglobin is expressed by heart or skeletal myocytes and act as an oxygen reservoir (Flonta et al., 2009), which may pertain to a role in changed breathing parameters, especially in response to hypoxia.

The expression of NGAL/Lipocalin and IL-10 tend to go hand-in-hand since IL-10 stimulates the production of NGAL (Jung et al., 2012). Previous studies on COPD

patients analyzed the role that these two cytokines had concomitantly and determined that expression levels of both increased in the presence of acute exacerbated COPD and decreased in a study cohort of individuals with no known respiratory health detriments (Wei et al., 2020). Interestingly, expression of both NGAL and IL-10 within our study went hand-in-hand, however we observed a decreased expression in the face of a respiratory disease. It may be possible that this decreased expression, which trends towards normal expression levels over time, may be a result of a rebound effect, where expression was likely increased during infection and quickly dropped off upon recovery.

While we observed a trend towards "back-to-normal" levels of cytokine expression ("normal" correlating to levels of expression for the control group), we still did not observe a full recovery by the time individuals were 2+ years post-recovery, meaning these long-COVID effects may last at least three or more years.

Changed Control of Breathing Parameters

Results from our ventilatory chemoreflex protocol showed several trends towards altered control of breathing parameters following recovery from COVID-19 (Figure 12). Specifically, we observed decreases in the ventilatory recruitment threshold (VRT) and hypercapnic ventilatory response (HCVR) several months after recovery. The functional significance of these changes is that for a given increase in arterial P_{CO2}, there is a lower increase in ventilation. However, this may be compensated for by a reduction in the VRT. In other words, the slope of the ventilatory response to CO_2 becomes blunted, but the y-

intercept of the response increases. The reduction in VRT may be a compensatory response to a reduced HCVR to maintain a normal ventilation rate at a given arterial P_{CO2}. This may explain why baseline ventilation patterns were not different across healthy and recovered groups. Instead, changes in CO_2 sensitivity may predispose patients for disordered breathing under specific stress situations in which P_{CO2} is modified, such as in exercise and sleep apnea syndromes.

As expected, we observed a decreased VRT and increased HCVR during co-stimulus with hypoxia. This effect was expected because this co-stimulus of hypoxia and hypercapnia activates both the central chemoreceptors and peripheral chemoreceptors in tandem.

When examining differences in chemoreflex parameters across study cohorts without timepoint separation, we observed a minor, but statistically significant decrease in HCVR under hypoxia co-stimulus between control and recovered cohorts. We observed the same trend, although nonsignificant, within the same parameters for the VRT. In each test phase, the recovered group had a decrease in both HCVR and VRT. However, to come to a more definitive conclusion, it was necessary for us to perform a time-post-recovery separation in each breathing parameter, where we found the VRT and HCVR followed a similar trend as the cytokine expression annotated above. Both exhibited decreased values immediately after recovery that continued to decrease until approximately one-year post-recovery, then began to return to normal. Unfortunately, we did not observe a full "return-to-normal" for the HCVR and HVR, indicating that our

breathing response to both hypoxia and increasing CO_2 levels is impacted and less sensitive than before COVID-19 infection, with no known recovery time frame.

When separated by time-post-recovery, we also observed a significant effect of COVID-19 recovery on the HVR collected at three of the four ETP_{CO2} concentrations. No significant effect was detected at 45mmHg $ETPCO_2$; however, this is unsurprising as many participants did not achieve their VRT by 45mmHg which is necessary to get an accurate HVR measurement. The significant effect found at VRT+3 may hold the most legitimacy as it ensures that every participant has reached their VRT by the time HVR was recorded.

Recovery from COVID-19 and long-COVID

Previous findings in rodent-based studies indicate that we may see a decreased HVR with increased inflammatory cytokine expression. However, we observed the opposite effect in many of the inflammatory cytokines post-recovery. In the presence of long-COVID, many pro-inflammatory cytokines, such as IL-6, IL-1β, TNF-α, and IFN-γ, showed positive correlations with increased HVRs. Interestingly, these cytokines are known to be upregulated in adaptive immunity, or rather acute inflammation that is often a result of illness or injury, whereas decreased HVR with increased inflammatory cytokine expression is associated with chronic diseases, such as COPD or emphysema (Pham et al., 2021; Walmsley et al., 2014). Because of this, it makes sense that we see

more expression of many of these cytokines linked to increased HVR in the recovered group than in the control group.

Limitations

A limitation of this study includes not knowing all participants past medical history, making it difficult to derive a definitive conclusion about systemic inflammation and its link to long-COVID. Additionally, another limitation to this study is the lack of follow-up data. While trends and significance were observed in many of our results, having data from each participant at multiple time points post recovery would strengthen our results and conclusion, providing more in-depth analysis of within subject findings.

Future Directions

This study provided a framework for future respiratory-related research and experiments in the face of emerging diseases. In terms of COVID-19, we may use the findings from this study to discover new experimental aims, such as analyzing these trends by following a set of participants over a long period of time and testing them multiple times at certain post-recovery time points to ensure these findings hold true within each person. Furthermore, the significant impact of vascular inflammatory cytokines opens up a plethora of possibilities related to cardiac and vasculature related studies pertaining to COVID-19. Determining more ways that COVID-19 affects the

cardiovascular system may provide more links to the altered neural control of breathing parameters and tie-in to its relationship with COVID-19-induced-systemic inflammation.

CONCLUSION

This study successfully identified a potential role of systemic inflammation after recovery of COVID-19 as well as some effects of long-COVID. Furthermore, it determined that there exists a strong impact of COVID-19 on the vasculature of the human body with no full recovery yet prevalent. There also exists a changed ventilatory response to both hypoxia and increasing levels of arterial CO_2, providing evidence that the effects of COVID-19 include an increased sensitivity to both oxygen-limiting environments and CO_2 sensitivity that last long after recovery. Moreover, the proinflammatory cytokine storm associated with COVID-19 quickly decreases after recovery; however, some inflammatory cytokines still had increased expression with higher HVRs, further showing the body's increased sensitivity to hypoxia after recovery from COVID-19.

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