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Advancing Clinical Practices and Patient Outcomes Through Computational Analyses in Medicine: A Focus on SARS-CoV-2 Epidemiology, COVID-19, and Long-COVID Neuropathogenesis

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ABSTRACT

This dissertation comprehensively explores Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and its implications. In particular, SARS-COV-2 effects on Coronavirus Disease 2019 (COVID-19), disease severity, long-COVID pathogenesis, neurological sequelae (i.e., post-acute sequelae SARS-CoV-2 infection [PASC]), and patient health outcomes are explored. A deep focus is on investigating specific SARS-CoV-2 variants that are the more prevalent and cause worsened COVID-19 and long-COVID disease severity, along with examining SARS-CoV-2 effects on latent herpesvirus (HHV) reactivation. In addition, we investigate the impact of the COVID-19 pandemic on the quality of life of patients undergoing treatment for gliomas.

Investigation of SARS-CoV-2 through wastewater-based-epidemiology (WBE) in the Reno-Sparks metropolitan region provided a broad overview of the status of COVID-19 and the circulating SARS-CoV-2 variants and pathogens in the region. We investigated COVID-19 patients by analyzing nasopharyngeal (NP) swab specimens using Next-Generation RNA Seq and analyzed cellular and molecular characteristics, gene disturbances, and cellular pathways. We correlated these findings with important patient factors (*e.g.,* comorbidities, age, gender, vaccination/booster status, symptoms, and disease progression timelines).

We identified SARS-CoV-2 variants during primary infection, which resulted in worsened severity of COVID-19 infection and patient outcomes. Notably, we found underlying human herpes viruses (HHVs) in some nasopharyngeal specimens, prompting further investigation into the molecular mechanisms involved in SARS-CoV-2-induced reactivation of latent HHVs. This line of inquiry yielded insights into the potential

interplay between HHVs, SARS-CoV-2 variants, and patient outcomes. These analyses supplied insights to improve patient and public health outcomes for COVID-19 patients and those with underlying HHVs. Our literature investigation highlighted the molecular underpinnings involved in the role of SARS-CoV-2 infection, COVID-19 disease, and its effect on underlying HHVs.

In the context of glioma patients, we explored the impact of the COVID-19 pandemic on their treatment outcomes and quality of life. We analyzed preliminary data from a cohort of patients from the University of Utah (U of U) Department of Neurosurgery (Salt Lake City, UT) enrolled in a clinical trial for glioma treatment; some underwent surgery and completed study questionnaires before and during the pandemic. Notably, poor prognosis in gliomas may result from certain pandemic-influenced factors and, thus, can be targeted to improve the treatment of gliomas and clinical outcomes. Furthermore, we investigated the molecular determinants and mechanisms involved in long-COVID neuropathogenesis. We report preliminary cohort analyses, including clinical variables and SARS-CoV-2 variants at COVID-19 primary or repeat infection and their association with severity. We also describe the future directions of two studies, evaluating serum proteins and functional-MRI (fMRI) changes in long-COVID patients with neurological symptoms and evaluating glioma patients' quality of life concerning specific tumor biomarkers and imaging data.

Published abstracts and manuscripts are appended at the end of the dissertation. These underscore the application of these computational analyses to yield improvement in patient health outcomes. Our findings highlight the implications of infectious diseases on our health to improve clinical practices and patient outcomes.

DEDICATION

To the love of my life, Maliha Masud, and our four-legged companions Babloo, Nugget, Miso, Sesame, and Kobe – my beacons in the toughest of days and darkest of nights – you are all the grace that has shaped my life for the better. Jaan, you have been the unwavering strength that has picked me up and held me tight against even the most treacherous tides of challenges that would pull me under the water as soon as I got near the surface. Your outpouring of love, countless sacrifices, patience with me, and whispers of encouragement in those moments of doubt would often allow stones of hardship to become stepping stones for me – *probably because you know I still can't swim at 25.* Every day, I humbly admire the woman you are – your strength, the calm in your soothing voice, the way you chase after your dreams, your creativity and artwork, and your ability to solve every single one of my problems effortlessly. The soul-deep connection that intertwines our lives has transformed the fabric of my life – you've single-handedly weaved my strands of impossible into possible.

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"The only way we'll get freedom for ourselves is to identify ourselves with every oppressed people in the world." – Malcolm X

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CHAPTER 1

1. INTRODUCTION

1.1 Background*

The expanding field of computational biology resides at the crossroads of biology, public health, and computing, a discipline that leverages various and complex data analyses to help solve complex real-world biological problems – largely focusing on improving human health, clinical outcomes, and the healthcare system. One of society's most demanding diseases to date, the Coronavirus Disease 2019 (COVID-19) pandemic, caused by the novel Betacoronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has established its dominance and persistence worldwide since early 2020.[1-5] As we approach our fourth year since the start of the global pandemic, healthcare and research communities remain challenged – longer and more demanding working hours, coupled with global financial considerations (*e.g.*, inflation), have left many overworked and burnt out.[6-11] The field of computational analyses in the sciences and healthcare is particularly relevant in improving human health, clinical outcomes, and healthcare systems. Recent years have demanded flexibility across all sectors;

***Abbreviations**

2-HG, 2-Hydroxyglutarate; AA, Anaplastic Astrocytoma; AAV, Adeno-Associated Virus; ACE2-R, Angiotensin-Converting Enzyme 2 Receptor; ADA, Americans with Disabilities Act; β, beta; BBB, Blood-Brain Barrier; BEI, Biodefense and Emerging Infections; BSL, Biosafety Level; CDC, Centers for Disease Control and Prevention; CNS, Central Nervous System; DCE-MRI, Dynamic Contrast-Enhanced Magnetic Resonance Imaging; DSC-MRI, Dynamic Susceptibility Contrast Magnetic Resonance Imaging; DMEM, Dulbecco's Modified Eagle Medium; DNA, Deoxyribonucleic Acid; dsDNA, Double-Stranded DNA; DWI/ADC, Diffusion-Weighted Imaging/Apparent Diffusion Coefficient; EBV, Epstein-Barr Virus; EMEM, Eagle's Minimum Essential Medium; FBS, Fetal Bovine Serum; GBM, Glioblastoma; HIF1α, Hypoxia Inducible Factor 1 alpha; HBMEC, Human Brain Microvascular Endothelial Cells; HR, Hazard Ratio; IDH1, Isocitrate Dehydrogenase 1; IL, Interleukin; IFA, Immunofluorescence Assay; KPS, Karnofsky Performance Status; LGG, Low-Grade Glioma; MOI, Multiplicity of Infection; NRP-1, Neuropilin-1; NGS, Next-Generation Sequencing; NIH, National Institutes of Health; NP, Nasopharyngeal; PASC, Post-Acute Sequelae of SARS-CoV-2 Infection; PDGFRA, Platelet-Derived Growth Factor Receptor Alpha; QoL, Quality of Life; RNA, Ribonucleic Acid; RPMI, Roswell Park Memorial Institute; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; TNF-α, Tumor Necrosis Factor-Alpha; TMPRSS2, Transmembrane Serine Protease 2; WBE, Wastewater-Based Epidemiology

fortunately, these increased efforts and expansions of current ideologies have led to rapid modern-day advancements in research, healthcare, and medicine. The World Health Organization (WHO) has reported over 772 million confirmed positive COVID-19 cases worldwide and at least 6.98 million deaths (as of December 2023).[12,13] Emerging and continuously advancing technologies (*e.g.*, genomic sequencing, metagenomics, and wastewater-based epidemiology [WBE]) have fostered tremendous strides in understanding SARS-CoV-2 and subvariant dynamics.

We have seen the SARS-CoV-2 virus undergo countless respective mutations in various viral proteins, particularly the spike protein(s).[14-20] This mutation has allowed the virus to produce numerous sub-variants, some that become predominant in a community, city, state, or country in just a matter of days in some cases.[14,16,17,21-23] Several mutants caused worsened severity – the underlying pathogenesis, virulence factors, interactions with other viruses and bacteria, our immune system, and especially patient management guidelines are still being investigated and continue to be improved upon.[24-26]

The COVID-19 pandemic has uncovered the need for integrating computational analyses in various facets of biology, medicine, public and human health, and clinical medicine. Using these computational tools to analyze and interpret extensive and complex biological datasets allows us to gain valuable insights into the behavior of viruses like SARS-CoV-2, their impact on human health, and the effectiveness of various interventions.[27,28] Computational biology is a discipline that applies data analysis and algorithmic techniques to understand and solve complex biological problems.[29,30] This approach can create a theoretical framework that allows us to process and interpret large

and complex datasets, providing insights into the behavior of SARS-CoV-2 and its impact on patient health outcomes.

This dissertation aims to address gaps in our knowledge by leveraging computational biology to analyze large-scale biological and patient-derived datasets, thereby providing critical insights into the virus's behavior and its implications for public health and clinical practices to tackle one of the most pressing health challenges of our time. Here, we sought to investigate the prevalence of SARS-CoV-2 variants in the Reno-Sparks metropolitan region using WBE, the impact of these specific SARS-CoV-2 variants and other underlying comorbidities and patient variables that correspond to worsened disease outcomes of COVID-19. There are reports on the potential role of underlying herpesviruses (HHVs) co-infection in modulating clinical symptoms and outcomes in COVID-19 patients.[31-47] Furthermore, the Hypoxia Inducible Factor (HIF1α) gene has been known to play a crucial role in cellular responses to hypoxia, and its overexpression has been associated with poor prognoses in various diseases and cancers, including HHVs and gliomas.[48-55] This association prompted the assessment of the impact of COVID-19 and the quality of life (QoL) of glioma clinical trial patients.

The significance of this research lies in its potential to contribute to our understanding of SARS-CoV-2, particularly its presence in the wastewater to its impact on patient health outcomes, and the role of underlying factors (*e.g.*, co-infections or reactivation of latent viruses) on disease pathogenesis, molecular pathways on patient health outcomes. This research could inform public health strategies, contribute to developing interventions to improve patient health, and provide a robust analytical

framework that can be applied to other viral diseases. The insights gained may have far-reaching implications on public health, medicine, healthcare economics, clinical practices and guidelines, and the broader scientific community.

1.1.1 Computational Analyses in Medicine and the Sciences

Computational analyses and approaches have been utilized to assess various aspects of patient outcomes, quality of life, and improvement in medicine and the sciences, including the potential for computational modeling to predict patient outcomes.[56,57] These analyses can provide insights into the quality of life (QoL) in patients;[58] moreover, in the context of clinical trials for cancer patients, the association between progression-free survival and QoL has been investigated using these analyses, highlighting the relevance of patient-reported outcomes and impact of surgical procedures on QoL endpoints.[59-63] The delivery of genomic medicine has also emphasized the role of computational approaches in enhancing genomic literacy in modern medicine practices towards advancing genomic medicine and personalized medicine to improve patient outcomes in various healthcare fields.[63,64] Computational analyses have also assisted in evaluating healthcare practices and adherence to clinical standards.[65]

The development of vaccines has benefitted from computational models (*e.g.,* influenza vaccines), which integrate data sources from protein structural modeling to advanced phylodynamic modeling.[66] These analyses have been used to simulate the

spread of diseases, such as COVID-19, which allows for estimating vaccine rollout and effectiveness.[67,68] Furthermore, computational vaccine design (*i.e.,* computational vaccinology) encompasses epitope mapping, antigen selection, and immunogen design using computational tools to assist in the rapid development of novel vaccines.[69] Moreover, computational simulation tools and structure-based vaccine design offer alternative possibilities to traditional empirical vaccine development.[70] Furthermore, computational modeling has been employed to prioritize COVID-19 vaccine strategies based on age, serostatus, and vaccine efficacy, and this impact has been evaluated using computational models to estimate the number of averted COVID-19 cases due to vaccination.[71,72] In addition to vaccine development and effectiveness, computational modeling has been applied to understand vaccine hesitancy among healthcare professionals and the general population, providing valuable information for predicting vaccination intentions and guiding interventions to address hesitation (*e.g.,* assessment of public perception and sentiment towards vaccines on social media platforms).[73,74]

The use of computational analyses and techniques has rapidly advanced in the fight against COVID-19, with applications ranging from taxonomic classification of COVID-19 genomes to predicting mortality and severity risk assessment of patients.[75] These technologies have also benefitted various aspects of COVID-19 management (*e.g.,* drug repositioning, disease susceptibility, and survival prediction of severe COVID-19 patients).[75,76] By applying computational techniques to clinical data, the prediction of mortality among COVID-19 patients has provided early implications to addressable variables to reduce mortality and improve outcomes, demonstrating the use case potential

of these analyses in producing valuable prediction models.[76] The utility of deep learning models for clinical prediction of COVID-19 suggests that combinatory use of computational algorithms and electronic health records can provide more accurate mortality risk prediction for COVID-19.[77-79] Development of a clinical decision support system based on computational models for severity risk assessment and triage of COVID-19 patients at hospital admission have demonstrated various practical applications of these analyses in improving patient management and resource allocation strategies.[78] Rapid and accurate classification of whole COVID-19 genomes has showcased the potential significance of these analyses in pathogen classification and identification.[14,17,80,81] Computational analyses have allowed for the development of end-to-end applications for predictive identification and modeling of biomarkers for COVID-19, highlighting the potential for risk assessment and identification of infected individuals.[80] Hybrid computational approaches have also been developed to predict COVID-19, offering alternative models for outbreak prediction (*e.g.,* predicting COVID-19 survival rates and patient discharge times based on clinical data).[82-84]

By applying computational power analyses to clinical and laboratory data, improvement in the performance of COVID-19 prediction can be seen, as these models are able to leverage more diverse data sources.[84,85] A comparative analysis of computational models and traditional computing models for predicting COVID-19 outbreaks highlighted the potential significance and improvement of these newer applications to traditional epidemiological models.[85,86] Predictors of COVID-19 in-hospital mortality using a set of computational techniques showcased this potential and

significance of clinical outcome prediction and assisting in clinical decisions for specific disease states (*e.g.*, acute respiratory failure in COVID-19 patients)[86-88] Other examples include prognostic models for predicting mortality among confirmed COVID-19 patients which demonstrate the practical application of ML in mortality risk prediction and decision-making support.[88,89] When combined with classic epidemiological methods, computational analyses can improve predictive modeling by analyzing clinical information in electronic health records of COVID-19 patients.[89-91] These studies have led to integrating these analyses into electronic health records (*e.g.*, calculating real-time risk scores for COVID-19 outcomes), allowing healthcare providers to address novel and complex health questions seen due to the pandemic.[91,92]

There is a broad diversity in applications of computational analyses (*e.g.,* machine learning, deep learning, artificial intelligence, prediction modeling, forecasting, *etc.*) in medicine. The use of these analyses to analyze data and their results can be used from preoperative assessments to cancer clinical trials and even applied to real-time personalized medicine applications. Computational immunology has also become critical for understanding the emergent properties of cells and whole tissues, advancing the field of immunotherapeutic discovery and development.[93] Computational analyses have played a pivotal role in COVID-19; thus, we can improve patient outcomes and public health across various domains worldwide by integrating them into existing and future medical and scientific applications.

1.2 Review of The Literature

1.2.1 Betacoronaviruses; SARS-CoV-2 Replication and Life Cycle

Betacoronaviruses are a large family of viruses known to cause disease in mammals, including humans, encompassing Human coronavirus (HCoV), Middle East Respiratory Syndrome (MERS-CoV), and SARS-CoV, which include more variants (*e.g.*, HCoV-NL63, HCoV-OC43, HCoV-HKU1, and MERS-CoV).[94] Typically, these cause respiratory symptoms ranging in severity from a common cold to MERS and SARS (*i.e.,* severe respiratory disease).[95-97] The recently discovered betacoronavirus, SARS-CoV-2, originated in Wuhan, China on December 2019 and is the causative pathogen responsible for the COVID-19 pandemic, which had spread from a localized outbreak to a global pandemic by March 2020, resulting in widespread uncertainty, panic, and fear, resulting in millions of confirmed cases and deaths worldwide.[98-100] Alongside SARS-CoV-2's rapid transmissibility and spread, COVID-19 also demonstrated wide variability in clinical symptom manifestations ranging from asymptomatic, mild, moderate, severe, and critical cases where infected individuals experienced a constellation of symptoms (mainly severe respiratory distress, organ damage), with many millions of people, very unfortunately, deceased from COVID-19.[12]

SARS-CoV-2 has four protein types (spike proteins, membrane proteins, envelop proteins, and nucleoproteins), which have been utilized to detect this virus in an array of sample types.[101] Genome sequence of SARS-CoV-2 suggests that it stemmed from bat or pangolin-origin coronaviruses.[102] SARS-CoV-2 has high infectivity in humans, attributed to the strong affinity of its spike (S) protein to angiotensin-converting enzyme 2 (ACE-

2).[103] Notably, antibody response after vaccination with inactivated SARS-CoV-2 is broad and durable in individuals with a history of SARS-CoV infection.[104] Breast milk has also been found to inhibit the infectivity of SARS-CoV-2.[105] RNA interference has been proposed as a potential treatment against SARS-CoV-2.[106]

Epidemiological analysis has been conducted to understand the spread of the virus in different populations.[107] Monitoring the spread of these variants became a significant focus of public health efforts and initiated new avenues of research into the behavior of these variants. We began investigating different variables to try and understand the underlying cellular and molecular processes behind the wide spectrum of disease severity presentations in COVID-19. Some factors (*e.g.*, age, gender, and underlying health conditions) were identified as potential contributors to disease severity. Underlying latent infection with viruses (*e.g.*, HHVs) is a significant factor when it comes to SARS-CoV-2 and COVID-19 disease severity and may even have larger implications for post-acute sequelae of SARS-CoV-2 infection (PASC), commonly referred to as long-COVID. The rapid spread and diverse symptom range of SARS-CoV-2 have posed significant challenges to public health efforts and instigated new lines of research into the virus's behavior and its variants.

The SARS-CoV-2 viral life cycle is a complex process involving various stages and interactions with host cells.[108] The virus enters target host cells by binding the viral spike glycoprotein to angiotensin-converting enzyme 2 (ACE-2) on the cell surface.[109] Post-entry, the SARS-CoV-2 N protein binds to the viral RNA genome and begins regulating viral replication, transcription, genome packaging, and modulation of host cell

processes.[110] SARS-CoV-2 utilizes multiple mechanisms to invade host cells; these host cell entry inhibitors are being assessed as potential therapeutic strategies against COVID-19.[111]

Studies suggest that calcium is essential for viral entry, gene replication, and virion maturation and release, although specific evidence for SARS-CoV-2 is limited.[112] The SARS-CoV-2 viral RNA associates with different RNA-binding host proteins at each stage of its life cycle.[113] Moreover, m6A-marked RNA and hRNPA1 interaction have been shown to regulate the early translation to replication.[114] Replication of genomic and subgenomic RNAs is essential for the stability and infectivity of SARS-CoV-2.[115] This stability is greatly influenced by the broad genetic variability in host cell entry factor expression, which has clinical implications for infectivity and pathogenesis.[17,116-118] Clinically, viral RNA load dynamics in infected children have been observed to peak around days 2-3 of illness.[119] SARS-CoV-2 may enter host cells through interactions with beta-adrenergic receptors, in which case the PI3K/AKT pathway has been implicated in its entry and replication process.[120,121]

SARS-CoV-2 can shed in respiratory droplets from infected individuals, which can then be transmitted to others through close contact or inhalation. The virus can also be detected in wastewater samples, providing a means to monitor the prevalence and spread of the virus within communities. Wastewater-based epidemiology (WBE) has emerged as a promising tool for monitoring the spread of SARS-CoV-2, as it offers a cost-effective and non-invasive method to detect the presence of the virus.[17-19,21,117,122-127] Coupled with advances in genomic sequencing, WBE has created new opportunities to

detect and monitor the presence and spread of different SARS-CoV-2 variants at the community level.

1.2.2 COVID-19 Disease Severity and Hospital Load

The impact of SARS-CoV-2 variants on hospitals and hospital load has been a topic of interest in the literature, with some studies showing that certain variants, such as the delta variant, have been associated with an increased risk of hospital admissions due to pathogenicity of infection compared to previous variants.[109] The severity of COVID-19 caused by different variants has also been investigated, with some studies suggesting that certain variants may be associated with more severe disease outcomes.[109] Understanding the impact of variants on hospital load and disease severity is crucial for healthcare systems to manage and allocate resources effectively during a pandemic.

The COVID-19 pandemic has led to a decrease in interhospital patient transfers and an increase in the length of hospital stays, resulting in rapidly increasing demand for healthcare in hospitals and intensive care units (ICUs) worldwide.[128-130] The impact of the pandemic on emergency department visits and patient safety has also been substantial.[130,131] Various healthcare resources have been affected, with studies showing that the healthcare system will soon be overwhelmed, emphasizing the necessity of establishing multi-level medical systems and strengthening primary and tertiary medical care systems to reduce the pressure on high-level hospitals during public health emergencies.[132] Outcomes for patients admitted for non-COVID issues have also been

affected.[133-135] The length of hospital stay for severe COVID-19 patients experience significantly longer hospital stays.[136-138] Furthermore, in-hospital factors (*e.g.,* nutrition service) have been found to be correlated with these effects, indicating that patient nutrition greatly affects their health improvement and length of stay in the hospital.[139] Interhospital transfers driven by physical capacity and staff shortages during the pandemic are performed safely without clinically relevant changes in vital signs.[140-142]

The severity of COVID-19 has been extensively studied in the literature.[4,143-145] Certain factors (*e.g.*, age, gender, and underlying health conditions) have been identified as potential contributors to disease severity.[109] Older individuals and those with pre-existing health conditions are at higher risk of developing severe symptoms. Additionally, recent studies have suggested that co-infections or reactivation of latent viruses (*e.g.*, human herpesviruses [HHVs]) may also influence disease severity in COVID-19 patients.[35,39,146-150] Understanding the factors contributing to disease severity is crucial for effectively managing and treating recurring COVID-19.

1.2.3 Human Herpesviruses: Life Cycle and Disease Severity

Human herpesviruses (HHVs) are known for establishing persistent infections in the human population.[151-158] There are eight known human herpesviruses, including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and human herpesvirus 8 (HHV-8).[159-164] These viruses

have coevolved with humans over millions of years and have developed sophisticated immune evasion strategies, allowing them to persist in the host for life.[162] HHVs infect and establish chronic infections in over 90% of the population.[159]

In healthy individuals, these latent HHVs do not cause serious problems as these people can resolve these reactivations through their innate and adaptive immune responses.[159] Individuals with weakened immune systems may experience more frequent and severe reactivations, often in response to stimuli such as fever, UV light exposure, or hypoxia.[31,146,147,165-172] Co-infection of viruses may trigger increased reactivation of HHVs, which is significant in SARS-CoV-2 infection.[31,146,147,171] Understanding the replication and life cycle of the virus, disease severity factors, virus shedding, and the implications of long-COVID are crucial for effective management and treatment of COVID-19.[171] Additionally, the potential overlap between SARS-CoV-2 infection and reactivation of HHVs highlights the need further to investigate the roles of HHVs in disease severity.

HHVs have complex replication and life cycles. After primary infection, these viruses establish latency in specific cell types, persisting in quiescence.[160] During latency, the viral genome is maintained as an episome, and only a limited number of viral genes are expressed.[160] Reactivation from latency can occur in response to various stimuli, producing infectious virions and spreading the virus to new host cells.[160] The replication and gene expression of HHVs are tightly regulated, involving interactions with host cellular factors and modulation of the host immune response.[160]

HHVs can have a significant impact on hospitals and hospital load. In immunocompromised individuals, HHV infections can lead to prolonged hospital stays, increased healthcare costs, and higher mortality rates.[39,45,147-149,172-178] The emergence of HHV variants (*e.g.*, drug-resistant strains) can further complicate the management of HHV infections in healthcare settings.[172,179] Understanding the prevalence and impact of HHV variants is crucial for effective infection control and treatment strategies in hospitals. Understanding the cellular and molecular underpinnings surrounding HHVs and SARS-CoV-2 and further investigating the relationship between SARS-CoV-2 antigens' effects on HHVs will lead to effective patient management and treatment options. Indeed, further research is still needed to unravel the complex interactions between HHVs and the host immune system – in time, the goal would be to develop targeted therapies and prevent HHV- and SARS-CoV-2-associated severe and long-term disease.

1.2.4 Association of SARS-CoV-2 and the Brain

SARS-CoV-2 has been associated with various neurological manifestations in both adults and infants.[180-200] Investigating the impact of viral infection on neural-stem and -progenitor cells and its implications for brain growth is crucial for human health.[200-203] The exact mechanisms underlying SARS-CoV-2 neonatal infant neuropathy are not fully understood. Still, several potential pathways have been proposed.[204,205] One proposed mechanism is viral spike protein binding to the ACE2 receptor (ACE-2R), leading to reduced ACE2 function and increased levels of angiotensin 2 (Ang II).[180,189,195]

This increase in Ang II may result in local vasoconstriction, leading to reduced blood flow and potential nerve damage.[180,189,195] SARS-CoV-2 may directly invade the central nervous system (CNS) through hematogenous and neuronal routes and neurological immune responses.[185] The invasion of the CNS by SARS-CoV-2 may contribute to the neurological symptoms observed in COVID-19 patients, including infants.[185] Further research is needed to elucidate the specific mechanisms underlying SARS-CoV-2-neuropathy, including studies investigating the role of ACE2 and Ang II in developing neuropathy and the pathways through which SARS-CoV-2 invades the CNS.

In addition to understanding the underlying mechanisms, future research should focus on the clinical manifestations and outcomes of SARS-CoV-2-neuropathogenesis. Longitudinal studies can provide valuable insights into the condition's natural history, including the progression of symptoms and potential long-term effects on neurodevelopment. These studies should also investigate the efficacy of different treatment approaches (*e.g.*, chemotherapy, psychiatric meds, steroids, *etc.*).[184] Exploring the potential risk factors is important; for instance, age has been identified as a potential factor associated with developing neuropathy in COVID-19 patients.[182,184] Understanding more of these risk factors can help identify higher-risk populations early and inform preventive strategies. Currently, there is limited evidence on the optimal management strategies for neuropathy associated with COVID-19. Investigating the efficacy of different treatment options (*e.g.*, antiviral drugs, vasodilators, and immunomodulatory therapies) can also provide valuable insights into the potential therapeutic approaches.[191] Because SARS-CoV-2-neuropathy is so complex, with multiple potential mechanisms

and clinical manifestations, investigation of the underlying mechanisms, risk factors, big data, and experimental models can provide valuable insights into the condition's natural history and inform clinical management strategies.

Developing effective treatments for SARS-CoV-2 neonatal infant neuropathy is also crucial. Investigating the efficacy of antiviral drugs, immunomodulatory therapies, and neuroprotective agents can provide potential therapeutic options for managing and preventing neurological complications in affected infants.[190,196,200-204,206-208] Future research on SARS-CoV-2 neonatal infant neuropathy should focus on understanding the mechanisms of viral entry and CNS invasion, comparing the clinical manifestations and outcomes with Zika virus, identifying risk factors and biomarkers, elucidating the pathogenesis at the molecular level, and developing effective therapeutic approaches. This comprehensive approach will contribute to a better understanding of the neuropathogenic effects of SARS-CoV-2 and inform strategies for the prevention, diagnosis, and treatment of neonatal neuropathy.

1.2.5 Viruses and the Blood-Brain Barrier

The mechanisms by which pathogens, including SARS-CoV-2, cross the blood-brain barrier (BBB) are central to understanding their pathogenicity in the CNS. However, certain pathogens have developed mechanisms to cross the BBB and invade the brain, leading to various neurological diseases and complications. The ability of SARS-CoV-2, pathogens, and other metabolites to cross the blood-brain barrier (BBB)

has been a subject of research for some time.[209-219] SARS-CoV-2 can disrupt the BBB and enter the brain.[220] The interactions between SARS-CoV-2 and the BBB are multifactorial, involving the virus crossing the BBB, infecting brain endothelial cells, and altering BBB functions.[221] Though the exact mechanisms by which SARS-CoV-2 crosses the BBB are still under investigation, it is believed to involve several receptors and signaling cascades. SARS-CoV-2 can induce neuroinflammation and neuronal injury once in the brain. Additional viruses and other pathogens have also been shown to cross the BBB (*e.g.*, *Japanese encephalitis virus, Herpes simplex virus type 1*[HSV-1], *Neisseria meningitides* and *Streptococcus suis*, *Toxoplasma gondii, Streptococcus agalactiae,* and *Haemophilus influenzae*, *etc.*) and cause acute encephalitis.[219,222-226]

These pathogens utilize various mechanisms and virulence factors to breach the BBB (*e.g.*, alterations in tight junction proteins, disruption of the basal lamina, actin cytoskeleton disruption, endocytosis, and activation of host proteins).[227,228] The BBB is a physical barrier as much as a part of the neurovascular unit. There is cross-talk between different brain components and the BBB for optimal brain and body function, and disruption to this process can lead to inflammation, pleocytosis, blood-brain barrier disruption, and neuronal injury.[229,230] The exact mechanisms by which pathogens enter the CNS and cause these effects are still being investigated.

Understanding how pathogens cross the BBB is crucial. Various approaches have been explored, including nanoparticles, monoclonal antibodies, and viral components as transporters for drug delivery to the brain.[231,232] Additionally, early research on the permeation of CNS-active drugs through the BBB has provided insights into the

possibility of enhancing drug delivery to the brain.[231,233] Further research is needed to determine how SARS-CoV-2 modulates the BBB and its potential targets in the CNS. These findings may help us understand the neurological symptoms and complications (*i.e.,* sequelae) associated with COVID-19 and long-COVID. Some individuals experience these neurological sequelae after recovering from acute SARS-CoV-2 infection when their effects can persist for weeks or months.[234] They may include various symptoms (*e.g.*, fatigue, shortness of breath, cognitive difficulties, and organ damage).[235] The implications of long-COVID are still being actively investigated.

1.2.6 CNS Insights: Pathologies in long-COVID

Grasping the CNS pathologies linked to long-COVID, which involve alterations in brain structure and function, is crucial for devising effective treatments.[236,237] Furthermore, functional connectivity can distinguish long-COVID from non-COVID and correlate these changes with the severity of clinical outcomes.[238] Moreover, fMRI studies can also be used for structural and functional connectivity analysis as a treatment response-monitoring tool for long-COVID patients.[239] fMRI also serves as a valuable tool to establish baseline neurosensory dysfunction (resting-state [RS-fMRI]).[240,241]

Early studies using fMRI to evaluate these brain changes have found long-COVID patients to have a greater reduction in grey matter thickness and tissue contrast in certain brain regions compared to their non-long-COVID counterparts, greater changes in markers of tissue damage in brain regions that are functionally connected to the primary

olfactory cortex, as well as a greater reduction in global brain size.[242,243] fMRI functional connectivity analysis has identified significant regulatory connections between the self-monitoring brain system and clinical neuropsychological phenotypes in long-COVID (*i.e.,* loss of cognitive awareness).[244,245]

SH-SY5Y neuroblastoma cells show the capability of SARS-CoV-2 infection through a noncanonical mechanism involving spike-neuropilin-1 (NRP-1) interaction.[246,247] Furthermore, SH-SY5Y cells were investigated for mRNA expression levels of NRP-1 and ACE2, as well as transmembrane serine protease 2 (TMPRSS2), a cell surface protein involved in spike protein priming following ACE2-R binding.[247] However, whether ACE2, TMPRSS2, or NRP-1 are involved in S1-induced long-COVID pathogenesis is still unknown and warrants investigation. Collectively, these studies suggest that there may be a relationship between the SARS-CoV-2 S1/spike protein and the development of long-COVID symptoms, highlighting the need for further research to understand the underlying mechanisms. The significance of circulating S1 in the serum is notwithstanding precursory SARS-CoV-2 infection or the necessary co-factors associated (*e.g.*, viral entry, replication, *etc.).*[248,249]

Some HHVs can cross the blood-brain barrier, which can lead to neurological complications and diseases, such as encephalitis and meningitis.[159,250,251] The mechanisms by which HHVs cross the blood-brain barrier are not fully understood and require further investigation. Understanding the ability of HHVs to invade the central nervous system is important for developing targeted therapies and prevention strategies for HHV-associated neurological diseases. Recent studies have suggested a potential link

between HHV reactivation and the development of long-COVID symptoms, mainly contributing to the persistence of symptoms and organ damage).[159] HHVs infect and establish lifelong latency in humans, which is the foundation of this hypothesis – that underlying HHVs may contribute to developing and maintaining long-COVID/PASC following COVID-19 infection due to certain SARS-CoV-2 variants, other underlying variables and comorbidities, and severe COVID-19 disease.

Long-COVID is now recognized as an ADA-recognized disability, which highlights the urgent need to identify the underlying mechanisms driving this condition.[252] By identifying the molecular markers involved in spike/S1-induced long-COVID symptoms, new research could pave the way for developing novel interventions and treatments to improve patient outcomes and QoL. Two underlying mechanisms may be at play: host immune system dysregulation, resultant neuroinflammation, and direct invasion of the central nervous system (CNS) by the SARS-CoV-2 virus or its components (*e.g.,* S1 spike protein).

1.2.7 Pathways and Inflammatory Biomarkers

Uncovering pivotal cellular and molecular changes during long-COVID sheds light on the underlying mechanisms of PASC/long-COVID. The kynurenine (kyn) pathway plays a significant role in cellular energy production, mediating immune responses and neuroinflammation through metabolites.[253] Serum kyn pathway metabolites are correlated with the severity of COVID-19, suggesting that they could

serve as useful biomarkers for acute, long, and post-COVID-19 diagnostics.[254] In addition, diabetes is a significant predictor of morbidity and mortality in COVID-19 patients.[255] There are several theories as to how diabetes and SARS-CoV-2 are associated with influencing symptom severity and mortality. Elevated levels of inflammation, coagulation, and immune response impairment in people with diabetes could aggravate viral infection and symptoms;[256] however, evidence supports direct interactions between viral and diabetic mechanisms within cells.[257-260] As endogenous ACE2 regulates glucose uptake and insulin sensitivity, virus-mediated dysregulation of insulin signaling could further aggravate symptoms in people with diabetes.[261] It becomes imperative to evaluate the interactions between the viral (ACE2-initiated) and diabetic (insulin) pathways and identify signaling molecules common to these pathways.

The kyn pathway may contribute to chronic fatigue syndrome (CFS), which has common abnormalities in long-COVID.[262,263] The pathophysiology underlying long-COVID is complex and multifactorial. Several factors contribute to its development, including residual damage or viral proteins from acute infection, persistent immune system activation, and underlying comorbidities.[264-268] There is a posited potential role of persistent circulating SARS-CoV-2 spike/S1 proteins in contributing to neurological symptomology, a critical area of research as neurological complications are more prevalent in long-COVID and cause long-lasting impacts to patients.[265,269-272] SARS-CoV-2 spike protein consists of two separate polypeptides (S1 & S2), of which S1 binds to ACE2-receptor, producing cleavage (via host furin-like proteases) and subsequent circulation of S1 into the serum.[268,273,274] These conditions present as a constellation of

symptoms that bear resemblance to CFS, including chronic malaise, diffuse myalgia, depressive symptoms, cognition challenges, and non-restorative sleep.[275-304] Both conditions are associated with prolonged and profound fatigue, and some post-acute COVID-19 patients have reported symptoms similar to those experienced by individuals with CFS.[276-281,285,286,297,298,303] Furthermore, studies have identified chronic fatigue as the most common symptom of long-COVID, further implicating the overlap in mechanisms and presentation of disease.[286,299] The similarities between the two conditions have suggested that long-COVID may overlap for treatment and prevention strategies.[283,287] The symptoms of long-COVID, such as chronic fatigue, have also been compared to those experienced by patients with myalgic encephalomyelitis (ME), a condition often triggered by infection and immune activation.[277,278,280,281,285,286,297,298,303] The potential involvement of autoimmune dysfunction syndromes, such as fibromyalgia, in these disorders further highlights the similarities between these conditions.[282] Furthermore, CFS is highly prevalent among recovered COVID-19 patients.[286] These similarities have prompted discussions on the need for specialized multidisciplinary support for patients with long-COVID, similar to the support provided for individuals with CFS and ME.[288] Studies have reported the shared cellular and molecular signatures between the two conditions, indicating common underlying mechanisms.[294,301]

Recent studies have identified levels of the polypeptide that makes up the SARS-CoV-2 spike protein (S1) and immune markers (*e.g.*, IL-6, IL-1β, and IL-18) to be of particular interest associated with long-COVID, which may lead to an identification of underlying mechanisms and novel treatment avenues for long-COVID.[268,273,305] The

presence of increased systemic inflammatory biomarkers (*e.g.,* cytokines) among individuals with severe acute COVID-19 has been reported previously.[306] Moreover, anti-inflammatory agents (*e.g.*, corticosteroids & IL-6-blocking agents) are associated with positive outcomes in patients hospitalized with COVID-19. Long-lasting systemic inflammation is present in people with more severe forms of long-COVID, which provides biological plausibility for the neurological impairments in people with persistent symptoms after acute COVID-19.[19,209,265,273,306,307] Understanding the relationship between inflammatory biomarkers and these complications is crucial for developing effective diagnostic and treatment strategies.

Recent studies have identified potential mechanisms underlying the development of long-COVID symptoms. It is understood that spike/S1 binding to ACE-2, one of its receptors, is critical for SARS-CoV-2 pathogenicity and transmissibility;[308,309] however, there are also other potential receptors for spike protein entry (Transmembrane Serine protease 2 [TMPRSS2] & Natriuretic Peptide Receptor 1 [NPR1]).[247] Circulating S1 is produced following binding with ACE2, undergoing conformational change, and cleaved into circulation.[274] This process is facilitated by furin proteases (cleavage) and increases the amount of spike binding to ACE2.[310] Johnson et al. have shown that loss of the furin cleavage site on the SARS-CoV-2 virus attenuates viral pathogenesis *in vivo*.[310] Paiardi et al. also showed this attenuation effect using heparin to prevent furin-mediated spike cleavage.[274] Moreover, histamine was shown to potentiate spike entry into endothelial cells, though this mechanism is likely histamine and histamine receptor signaling-

dependent.[311] These interactions highlight the tight regulation of the spike/S1/ACE-2 binding domain and their effects on overall viral pathogenesis.

Swank *et al.* analyzed plasma samples from a cohort of previously infected COVID-19 patients, including those diagnosed with post-acute sequelae of SARS-CoV-2 infection (PASC, or long-COVID), and found the concentration of SARS-CoV-2 antigens (S1-spike, full-length spike glycoprotein, and nucleocapsid) were detectable in approximately 65% of the PASC patients several months after SARS-CoV-2 infection, However, S1 was quantified to a lesser degree than full-length spike and nucleocapsid, while S1 and nucleocapsid were prominent in hospitalized and severe cases.[268] Additionally, Patterson *et al.* found that non-classical monocytes, a subset of monocytes involved in inflammation, were significantly elevated in patients with PASC up to 15 months after initial infection compared to healthy controls.[273] This study also detected the presence of the S1 protein in non-classical monocytes from severe COVID-19 patients and PASC patients, indicating that these cells may be a source (or potential indicator) of inflammation in long-COVID.[273] Recent data suggests that other receptors may also be able to bind to S1 in other organs (*e.g.,* liver, kidneys, intestines, *etc.*), further highlighting the importance of investigating the role of circulating S1 in the multi-organ etiology of long-COVID.[269] Long-COVID patients experience persistent symptoms that significantly impair their QoL (*e.g.*, brain fog, fatigue, headaches, insomnia, memory issues, episodic disorders, cerebrovascular events, sensory and motor deficits, and mental health changes).[234,264,312-316]

Kyn pathway metabolites are correlated with the severity of COVID-19, suggesting that they could serve as useful biomarkers for acute, long- and post-COVID-19 diagnostics.[254] Similarly, the kyn pathway may contribute to CFS,[262,263] which has common abnormalities with long-COVID. These studies suggest the kyn pathway may lead to CFS and long-COVID pathogenesis. High levels of inflammation, coagulation, immune response impairment, *etc.*, in individuals with pre-existing comorbidities (*e.g.*, diabetes) could aggravate viral infection and symptoms.[256] Since endogenous ACE-2 regulates glucose uptake and insulin sensitivity, virus-mediated dysregulation of insulin signaling could further aggravate long-COVID symptoms in individuals with certain pre-existing comorbidities (*e.g.*, HTN, Metabolic Syndrome).[261]

1.2.8 COVID-19 Pandemic and Cancer Patient Quality of Life Outcomes

The COVID-19 pandemic has profoundly impacted healthcare services globally, including surgical Neuro-Oncology. Surgical Neuro-Oncology, being a crucial component of hospital services and a high consumer of intensive care unit (ICU) resources, faced significant strategic shifts during the pandemic (*e.g.*, ventilators, personal protective equipment, and medical staff) and disrupted the delivery of surgical neuro-oncology services, which led to detrimental outcomes of postponing surgeries for cancers such as glioblastoma (GBM), the most common and lethal primary brain tumor.[317] [317] Studies have been focusing on various aspects of the pandemic on clinical outcomes, mortality rates, treatment decisions, and the influence of active oncologic treatment for these patient types, which has revealed higher rates of death in this population.[318] Other studies

have highlighted the impact of active oncologic treatment on clinical outcomes in cancer patients hospitalized for COVID-19.[319] While limited, these studies underscore the vulnerability of cancer patients to severe outcomes from COVID-19. By identifying higher relative risk patients for severe outcomes and death, especially in patients with cancer, a significant impact can be made in their outcomes compared to those without.[320] Consistent with the understanding that cancer patients are uniquely vulnerable and that severe COVID-19 is due to immunosuppression from cancer and cancer therapy, it is an area of research that is needed, especially as we continue to see disruptions in routine clinical care.[321] One group identified discontinuation and changes in cancer treatments during the COVID-19 pandemic in about 10 % of cases, highlighting the challenges faced in maintaining cancer care during the pandemic.[322] Concerns regarding the potential adverse outcomes if treatment pathways for cancer patients do not improve post-pandemic.[323] Moreover, the influence of the pandemic on treatment schedules and provider decision-making has been a subject of investigation, with one group reporting delays in lung cancer treatment for about 9 % of patients during the pandemic, likely due to the complexities in managing cancer care in the context of COVID-19 and respiratory involvement of SARS-CoV-2.[324,325]

Gliomas are primary brain tumors that encompass various subtypes, including low-grade astrocytoma and oligodendroglioma (LGG, WHO grade II), anaplastic astrocytoma and anaplastic oligodendroglioma (AA, AO, WHO grade III), and glioblastoma (GBM, WHO grade IV).[326] Among these, GBM is the most common malignant brain tumor, with a median survival of approximately 14 months.[326 317]

Gliomas exhibit significant heterogeneity and are involved in various molecular pathways and tumor invasion (*e.g.,* EGFR, IDH1, 1p19q, MGMT, PTEN, MIB-1 proliferation index, ATRX, p53, BRAF, and IDH2 mutations).[326] Glioma patients are particularly vulnerable due to their relative immunocompromised status from previous radiation and chemotherapy treatments; moreover, the postoperative mortality rate is higher in patients with cancer than those with benign diseases.[317] Amid the pandemic, the benefit of urgent surgery to resect gliomas must be balanced with the risk of exposing patients and staff to COVID-19 infection.[317] This balance created a challenging issue for healthcare providers and patients worldwide.

The Karnofsky Performance Status (KPS) is a standardized tool that evaluates a patient's capacity to perform everyday tasks and activities.[327] It scores patients on a scale of 0 to 100, with 100 representing perfect health and 0 indicating death. It is a comprehensive measure of a patient's overall health status, considering physical and psychological well-being. It has been extensively validated in different patient populations and is widely used in clinical trials and observational studies. Strengths of the KPS include its simplicity and broad applicability to various diseases and health conditions; however, it is a subjective measure and can vary depending on who makes the assessment (*e.g.*, a physician, a nurse, or the patient). It also lacks sensitivity to slight changes in a patient's condition. It may not accurately reflect the patient's QoL, focusing more on physical functioning and less on psychological or social well-being.

The MDASI (MD Anderson Symptom Inventory) assessment is a multi-symptom patient-reported outcome measure that assesses the severity of multiple symptoms

common to cancer patients and the interference of these symptoms with daily functioning.[328-330] The MDASI includes thirteen core items measuring symptom severity and six measuring symptom interference with daily activities. Strengths of the MDASI include its focus on symptoms relevant to cancer patients (*e.g.*, the inclusion of symptom severity, interference items, and its good psychometric properties). Limitations of the MDASI include its reliance on patient self-report, which can be influenced by factors such as mood, attentiveness, and recall biases. They may also not capture all symptoms relevant to a specific patient or tumor type.

The FACT (Functional Assessment of Cancer Therapy) assessment is a comprehensive set of QoL questionnaires specifically designed for use with cancer patients.[331] It measures multidimensional aspects of QoL, including physical, social/family, emotional, and functional well-being. Strengths of the FACT include its multifaceted approach to QoL assessment, ease of use, and extensive validation in various cancer populations. It also includes cancer-specific modules relevant to specific types of cancer or treatments *(e.g.,* GBM). The limitations of the FACT include its length, which may be burdensome for some patients, and its reliance on patient self-reporting. Given the strengths and weaknesses of each QoL measure, using them in combination can provide a comprehensive and robust assessment of patient QoL. The KPS provides an overall measure of patient functioning, the MDASI focuses on symptom severity and interference, and the FACT comprehensively assesses various aspects of QoL – they provide a holistic view of the patient's health status and QoL.

The COVID-19 pandemic has profoundly impacted healthcare services, particularly in surgical Neuro-Oncology. The disruption of surgical neuro-oncology services and the challenges faced by healthcare providers and patients worldwide have highlighted the need for prompt and strict regulations to ensure uninterrupted oncological surgical service during challenging times. We have seen significant changes in planned elective and emergent surgical procedures and appointment-based health services due to decreased hospital capacity and strained healthcare professionals focused on fighting the pandemic.[332] Though we have seen an expansion of telemedicine and other technologies in delivering healthcare services, undoubtedly, any interruption to the oncological surgical services has significant and detrimental effects on patients [333] The pandemic has also placed an unprecedented strain on the healthcare system, affecting the continuity of health service delivery, including the reallocation of medical personnel to treat COVID-19 patients, which disrupted healthcare services in other ways and may have contributed to reduced healthcare utilization.[334,335] Thus, analyzing the effect of the pandemic on their QoL is essential for making regulatory and clinical changes aimed at improving overall QoL, ensuring that certain key factors contributing to worsening QoL are not missed.

The lessons learned from this health crisis are valuable for healthcare workers worldwide, especially neurosurgeons and neuro-oncologists. This dissertation contributes to these efforts by analyzing comprehensive assessment measures (KPS, MDASI, and FACT), which can provide valuable insights into the impact of the pandemic on the QoL of glioma patients and may also contribute to improvements in the healthcare system across all services.

1.3 Overview of Dissertation

This dissertation encapsulates a series of computational analyses investigating viral diseases, particularly SARS-CoV-2. It illustrates how harnessing computational results can advance our disease containment and treatment strategies, from exploring viral diseases to demonstrating the application of these computational results to enhance disease treatment strategies and improve patient health outcomes. We aligned findings from clinical wastewater-based viral analyses with nasopharyngeal swab analyses to detect emerging SARS-CoV-2 variants. We correlated clinical wastewater-based viral analyses with nasopharyngeal (NP) swab analyses to detect the emergence of SARS-CoV-2 variants. These analyses were also associated with demographic and symptomology information. Computational analyses were applied to investigate potential links between underlying herpesvirus infections in patients and their role in the modulation of COVID-19 clinical symptoms. The work performed in these studies is the first to tie together how computational analyses can be used in a small urban metropolitan region to advance public health during pandemics.

1.3.1 Chapter 2

Chapter 2 was published in *OneHealth*.[17] We identified the presence of pathogens within wastewater via WBE methods, which has been an effective method for monitoring shifts in viral prevalence in a community. WBE also aids in the prompt detection of emerging and circulating viral variants as well as the ability to monitor mutations, which

aids in facilitating rapid responses to viral outbreaks. Studying SARS-CoV-2 variants in specific geographic regions can yield crucial insights into the emergence and prevalence of new variants within communities and provide insight into viral spread mechanisms.

We conducted a year-long study from November 2021 to November 2022, collecting weekly wastewater samples from the Reno-Sparks metropolitan region. This analysis enabled us to assess the prevalence of SARS-CoV-2 and other respiratory viruses across different seasons. Through genome sequencing of the viral RNA, we found that wastewater samples could accurately quantify the SARS-CoV-2 genomic copies and allow the detection of specific variant frequencies and variant identifiers (*e.g.*, lineage). We concluded wastewater-based monitoring to be an efficient approach for the early identification of environmental variants and monitoring community spread of SARS-CoV-2, bolstering the importance of WBE initiatives worldwide as a primary or supplemental tool, depending on resources, for clinical respiratory virus testing in the public health response. Unlike other seasonal respiratory viruses, we also identified the continuous presence of SARS-CoV-2, demonstrating its broad genetic diversity and enduring ability to infect susceptible hosts. Additionally, we detected antimicrobial resistance genes (AMR) using the wastewater samples, suggesting WBE's additional value and viability in detecting and monitoring community AMRs.

This work sets the stage for the evaluation of clinical COVID-19 samples from patients at our local hospital (Renown Regional Medical Center, RRMC) and the Nevada State Public Health Laboratory (Reno, NV, USA) for the investigation of SARS-CoV-2 variants, disease severity, and evaluation of lag between wastewater- and clinical-based

identification of new and emerging variants, especially those on the World Health Organization (WHO) list as variants of concern and interest.

1.3.2 Chapter 3

Building on the foundation laid by wastewater surveillance, Chapter 3 presents a comprehensive analysis of clinical samples. In this chapter, we establish a correlation between viral signatures found in wastewater and those detected in nasopharyngeal (NP) swabs, focusing specifically on the emergence and spread of SARS-CoV-2 variants. We investigated a large cohort of 1,760 patients for correlations between health information and their accompanying genetic data from NP swab specimens. These confirmed COVID-19 cases, ranging from pediatrics (< 17 years old), adults (18 - 65), and elderly (>65), were seen by RRMC physicians between March 2022 and January 2023.

Our goal was to understand whether: a) there are specific SARS-CoV-2 variants that are associated with worsening manifestations of COVID-19 symptomology, b) whether associations were dependent on different age groups or vaccinations, and c)the presence of a secondary respiratory infection (*e.g.*, Influenza, RSV, *etc.*) caused more severe COVID-19 symptomology. These findings showed that despite the unlimited amount of risk factors and variables involved in altering COVID-19, the SARS-CoV-2 variant at primary infection and underlying clinical comorbidities can affect patient outcomes in COVID-19. These initial outcomes may have implications for long-COVID pathogenesis.

1.3.3 Chapter 4

Chapter 4 extends our inquiry into the interaction between SARS-CoV-2 and human herpesviruses (HHVs). Considering the widespread prevalence of HHVs and their potential impact on COVID-19 severity, this chapter explores the intersection of viral pathogenesis and host response. As our lab has a longstanding history of studying HHVs, we took insights gained from our analyses in Chapter 3 and began our investigation into whether SARS-CoV-2 variants interact with and reactivate HHVs.

Through a retrospective cohort study of 85 patients, we aimed to discover whether SARS-CoV-2 infection triggers HHV reactivation, potentially increasing COVID-19 severity. The key question was whether underlying (latent) HHV infection affects COVID-19 symptom severity and if this effect is present in an *in vitro* setting. Since most people have chronic (latent) infections with HHVs *(i.e.,* underlying, latent, non-active, co-infection with HHVs), we analyzed the roles and impacts of HHVs on SARS-CoV-2 and clinical COVID-19 disease symptomology. The severity and variability in symptoms of COVID-19 come from multiple factors *(e.g.,* viral-induced cellular damage, inherent genetic differences, varying immune response, *etc.*). Understanding the biological impact of HHVs will provide clues to the underlying mechanisms that control the varying disease severity and may also lead to the identification and development of combinatorial therapy.

We utilized RT-qPCR and RNA-Seq metagenomics analysis to detect SARS-CoV-2 and HHV infections that could be detected in blood samples, categorizing patients based on the SARS-CoV-2 variant. RNA-Seq metagenomics analysis of these samples

confirmed the presence of SARS-CoV-2 and significant levels of other respiratory viruses and bacteria in some samples. By mapping the transcriptome, we identified significantly increased levels of lytic HHV genes (EBV & KSHV) in patients with severe and critical illnesses, indicating the role of SARS-CoV-2 in reversing epigenetic silencing of HHV viral DNA for escape from latency. Further, the analysis of the cellular genes via RNA-Seq revealed 234 differentially expressed genes in HHV-positive COVID-19 patients, which were associated with a wide range of biological processes and molecular functions. We also confirmed this link between HHV reactivation and SARS-CoV-2 infection through *in vitro* validation assays. SARS-CoV-2 variants were used to infect HHV cell lines (EBV- and KSHV) to evaluate for SARS-CoV-2-infected HHVs-Reactivation (*i.e.,* post-infection latent to lytic HHVs progression). Collectively, these findings suggest that SARS-CoV-2 can alter HHVs and that HHV reactivation may elevate the severity of COVID-19 symptoms in patients with underlying latent HHV infection.

1.3.4 Chapter 5

With insights into viral interactions and patient outcomes, Chapter 5 shifts focus to the long-term effects of COVID-19. Here, we examine the phenomenon of long-COVID, its neuropathogenic aspects, and the resultant implications for patient care and QoL. We have collected a database with patient health variables of COVID-19 and long-COVID patients from Renown Regional Medical Center (RRMC) in northern Nevada (Reno, NV, USA). The preliminary long-COVID patient cohort was investigated to

understand the neuropathogenesis of long-COVID to identify insights into SARS-CoV-2 variants and underlying patient variables on COVID-19 disease severity and long-COVID outcomes. Initial outcomes and future directions of this future study are included in this chapter, as well as a literature review, including background and significance surrounding the study, which involves using fMRI in evaluating long-COVID patients and understanding the neuropathogenesis of long-COVID.

Additional key variables (*e.g.,* age, gender, date of infection, SARS-CoV-2 variant, vaccination and booster status, clinical symptoms, disease severity, *etc.*) were evaluated and correlated with the findings. The long-COVID diagnosis was confirmed by a doctor based on the reported symptoms and recorded into the patient chart with the ICD-10 code. To compare against our long-COVID cohort, the same variables are obtained from COVID-19 patients without long-COVID symptoms (controls), matched for age, gender, and ethnicity, along with the date of sample collection (to normalize for the circulating variants), and symptomatology of the first exposure of SARS-CoV-2 will be obtained for correlative studies.

We aimed to determine a few key findings, such as the frequencies of SARS-CoV-2 variants causing long-COVID (*i.e.,* whether any variants have led to significantly more long-COVID diagnoses), the contribution of symptomatology of the first exposure to long-COVID, any specific co-morbid factor(s) contributing significantly to long-COVID disease states, whether vaccination reduces the symptoms of COVID-19 and progression or disease severity in long-COVID.

1.3.5 Chapter 6

Chapter 6 narrows the lens to a vulnerable patient population – those with gliomas. The chapter assesses the pandemic's impact on treatment protocols, patient outcomes, and overall QoL, providing a poignant illustration of COVID-19's ripple effects through specialized healthcare services. Patients undergoing treatment for gliomas are particularly vulnerable due to their relative immunocompromised status from previous radiation and chemotherapy. Amid the pandemic, the benefit of urgent surgery to resect gliomas needed to be balanced with the risk of exposing patients and staff to COVID-19 infection. Undoubtedly, in an already otherwise difficult circumstance, this balance created a challenging issue for healthcare providers and patients worldwide.

We examined a small cohort of clinical trial patients from the University of Utah Department of Neurosurgery (Salt Lake City, Utah, USA), some of whom underwent surgery and completed study questionnaires before the pandemic started in March 2020, some who were operated on before COVID began but did the questionnaires during the pandemic, and those who both underwent surgery and completed study questionnaires during the pandemic. The questionnaires were administered pre-operatively and at 1, 3, 6, and 12 months post-operatively. Additionally, a Karnofsky Performance Status (KPS) score was also recorded, thus allowing for the evaluation of all three metrics (KPS score, Functional Assessment of Cancer Therapy [FACT], and MD Anderson Symptom Inventory [MDASI]) concerning the overall QoL evaluation for these patients.

1.3.6 Chapter 7

The dissertation concludes with Chapter 7, which synthesizes the research findings and discusses their implications. This chapter also outlines potential future directions from this work, underlining the importance of interdisciplinary approaches in pandemic preparedness and response. The data and experimental methods, taken together, showcase the strengths of combining disciplines from various public health, biological, and medical fields, along with the help of modern-day computational power and robust analyses, in investigating novel research questions and identifying significant results that can have powerful impacts on the lives of those suffering from a novel disease such as COVID-19. We have made tremendous advances over the last two decades and even more in the last three years since the pandemic; now more than ever, we are prepared for any future viral outbreaks that may occur, whether on a small, community-based level or even worldwide.

We investigated SARS-CoV-2 through computational analyses, focusing on its presence in wastewater and impact on patient health outcomes. Despite significant advancements in our understanding of SARS-CoV-2, gaps remain in our knowledge about its behavior and effects on human health. The global COVID-19 pandemic has presented unprecedented challenges to public health, economies, and societies worldwide. While this has presented many challenges, it has also provided unique opportunities for innovation in computational biology. As we strive to understand, manage, and overcome this pandemic, computational analyses have proven invaluable in studying the virus, predicting its spread, and identifying potential treatments.

The findings presented fill some of these gaps by using computational analyses to link significant findings to patient health outcomes. These findings have far-reaching implications for public health and clinical practices for all humans. They could inform strategies for monitoring the spread of infectious pathogens and disease, provide insights into the factors that influence worsened patient outcomes, and guide the development of new interventions to improve human health.

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CHAPTER 2

2. SIGNIFICANCE OF WASTEWATER SURVEILLANCE IN DETECTING THE PREVALENCE OF SARS-COV-2 VARIANTS AND OTHER RESPIRATORY VIRUSES IN THE COMMUNITY – A MULTI-SITE EVALUATION

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2.1 Abstract

Detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral genome in wastewater has proven useful for tracking virus prevalence trends within the community. The surveillance also provides precise and early detection of new and circulating variants, which aids in response to viral outbreaks. Site-specific monitoring of SARS-CoV-2 variants provides valuable information on the prevalence of new or emerging variants in the community. We sequenced the genomic RNA of viruses present in the wastewater samples. We also analyzed the prevalence of SARS-CoV-2 variants and other respiratory viruses for one year to account for seasonal variations. The samples were collected weekly from the Reno-Sparks metropolitan area between November 2021 and November 2022. Samples were analyzed to detect the levels of SARS-CoV-2 genomic copies and variants identification. This study confirmed that wastewater monitoring of SARS-CoV-2 variants can be used for community surveillance and early detection of circulating variants and supports wastewater-based epidemiology (WBE) as a complement to clinical respiratory virus testing as a healthcare response effort. Our study showed the persistence of the SARS-CoV-2 virus throughout the year compared to a seasonal presence of other respiratory viruses, implicating SARS-CoV-2's broad genetic diversity and strength to persist and infect susceptible hosts. Through secondary analysis, we further identified antimicrobial resistance (AMR) genes in the same wastewater samples and found WBE to be a feasible tool for community AMR detection and monitoring.

2.1.1 Highlights

- WBE better represents virus prevalence under declining testing and clinical reporting rates.
- SARS-CoV-2 virus was present throughout the year compared to other respiratory viral pathogens.
- WBE can provide insights into co-circulating respiratory viruses, causing flu-like symptoms.
- WBE-AMR gene monitoring is feasible and provides valuable insight into regional AMRs.
- Our workflow enables relative proportion estimation of different variants in any pooled samples.

2.1.2 Keywords

WBE, Diagnostics, RVP, Variants, Coinfection, AMRs

2.2 Introduction*

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) virus, responsible for the Coronavirus Disease of the 2019 (COVID-19) pandemic, continues to be prevalent globally. In the United States of America (USA), case numbers are approaching 100 million, with a death rate that has surpassed 1.09 million persons as of 12/05/2022.[1] Infectious diseases are a significant threat to global and public health, with many drivers causing increased spread and transmission.[2] Following CDC direction, many clinical and public health laboratories, academic institutions, and private sectors have contributed significant efforts to monitor virus evolution through next-generation sequencing (NGS) technologies; these have supported tracing efforts and assisted in research on transmission dynamics and host response.[3] However, alternative and complementary technologies are also necessary for this effort as barriers to the accessibility of sequencing technologies exist (*i.e.*, rising costs and sizeable necessary sequencing volumes).[4,5] Furthermore, leveraging the SARS-CoV-2 monitoring to include co-occurring pathogens in the community, including other respiratory viruses that cause

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***Abbreviations**

AMR, Antimicrobial Resistance; cDNA, Complementary DNA; CDC, Centers for Disease Control; Ct, Cycle Threshold; COVID-19, Coronavirus Disease of 2019; DNA, Deoxyribonucleic Acid; DNase I, Deoxyribonuclease 1; EPH3, Elute, Prime, Fragment High Concentration Mix; gc, Genome Copies; HCoV-OC43, Human Coronavirus-OC43; ID, Identification; KDa, Kilodaltons; LoD, Limit of Detection; MD, Maryland; NCBI, The National Center for Biotechnology Information; NGS, Next-Generation Sequencing; NT, Nucleotide; NV, Nevada; PCR, Polymerase Chain Reaction; PMMoV, Pepper Mild Mottle Virus; PE, Paired-End; QC, Quality Control; RNA, Ribonucleic Acid; RNO, Reno-Tahoe International Airport; rPM, Reads Per Million; RSV, Respiratory Syncytial Virus; RSWRF, Reno-Stead Water Reclamation Facility; RT-qPCR, Reverse Transcription and Quantitative Polymerase Chain Reaction; RVP, Respiratory Viral Pathogens; RVOP, Respiratory Virus Oligo Panel; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; SD, Standard Deviation; SE, Single-End; STMWRF, Sparks Truckee Meadows Water Reclamation Facility; TMWRF, Truckee Meadows Water Reclamation Facility; VOC, Variants of Concern; VOI, Variants of Interest; VBM, Variants Being Monitored; WBE, Wastewater-Based Epidemiology; WHO, World Health Organization; WRF, Water Reclamation Facilities

similar symptoms, is critical to accurately determining community health and disease prevalence.

SARS-CoV-2 viral particles are present in the feces of virus carriers and subsequently shed into sewage systems and downstream water reclamation facilities (WRFs).[6,7] Thus, wastewater-based epidemiology (WBE) within these sewage networks and WRFs can provide valuable insight into virus prevalence and variant evolution in sewershed.[8,9] Moreover, WBE shows a low risk of virus infectivity to personnel, although caution should still be used.[10,11] Wastewater samples allow for the anonymous quantification of SARS-CoV-2 viral RNA from individuals in a defined sewershed, representing the overall spatial and temporal prevalence of SARS-CoV-2 in a community at a given time point.[12-14] WBE has been shown to detect circulating respiratory viruses and variants of concern (VOCs) earlier, which can appear days to weeks before clinical cases are seen in healthcare settings (*e.g.*, detection of B.1.1.519 preceding the Omicron wave).[15-17] It has also been shown that WBE of SARS-CoV-2 strongly correlates with clinically diagnosed case numbers;[12] further, the WBE methodology is not dependent on external factors (*e.g.*, testing and reporting rates, reporting lag, and reporting biases).[12,18,19]

In this study, we captured and enriched SARS-CoV-2 in the wastewater, reported site-specific sequencing and variant analysis, and described the implications of these data. Further, we performed a respiratory viral pathogens (RVP) panel analysis for other respiratory disease-causing viruses in these samples. The use of widespread antimicrobial agents during the pandemic could potentially trigger higher antimicrobial resistance

(AMR) occurrences in the community. Thus, to contribute additional insights into community health risks due to other pathogens, this study correlated the presence of the AMR genes in wastewater with SARS-CoV-2 and other respiratory viruses. The goal was that analyzing SARS-CoV-2, other RVPs, and AMRs in the wastewater may allow for increased knowledge and clarity of impending infection waves, which may provide insight into the prevalence and evolution of respiratory viruses in the community and inform public health decision-making.

2.3 Materials and Methods

2.3.1 Sample and Study Sites

This study was conducted in the Reno-Sparks metropolitan area in Nevada (NV), USA, between November 2021 and November 2022. Seven local sub-sewershed and influent (pre-treatment) sites from three water reclamation facilities (WRF) were included in the study, representing wastewater collected from about 0.4 million residents. The WRFs ranged from the southern Truckee Meadows region to the northern Reno-Stead region and included the Truckee Meadows WRF (TMWRF), the South Truckee Meadows WRF (STMWRF), and the Reno-Stead WRF (RSWRF). The Truckee Meadows WRF (TMWRF) represents sewershed catchments from both Reno and Sparks, NV, serving over 205,000 and 115,000 residents, respectively, and has an approximate 121,000 m^3/day flow rate (~30 million gallons/day), accounting for about 80 % of Washoe County's wastewater. The South Truckee Meadows WRF (STMWRF) serves over 52,000 residents and has an approximate 96,000 m^3/day flow rate (~2.5 million gallons/day). The Reno-Stead WRF (RSWRF) serves over 18,000 residents and has an approximate 64,000 m^3/day flow rate (~1.6 million gallons/day). In addition to these WRFs, we also analyzed influent from two hotel-casinos to represent Travel-Influenced Sites and combined influent from three sewer sub-catchments with inflow exclusively from residential housing areas (approximately 500 residential units each) to represent Sub-Neighborhoods (Sparks, NV). Two elementary schools, one in Reno and one in Sparks, NV, were also included to represent Elementary Schools in the region.

2.3.2 SARS-CoV-2 Specimen Collection and Quantification in Wastewater

The methodology for virus enrichment and quantification in wastewater was performed as described in our previous study.[20] Briefly, 1 liter (L) of untreated wastewater was obtained after preliminary treatment from facilities between 9:00 a.m. and noon and transported directly to the laboratory on ice. Samples were kept at 4 °C until further treatment. Samples were centrifuged at 3000×g for 15 min, and the resulting supernatants were sequentially filtered through 1.5, 0.8, and 0.45 μm sterile membrane filters to remove debris and large particles. The resulting supernatant was used to concentrate the viruses. The virus concentration was performed via ultrafiltration using 100 KDa Amicon® Ultra-15 Centrifugal Filter Cartridge Units (Millipore Sigma, St. Louis, MO, USA). Depending on the wastewater virus concentration levels, we processed 60 mL of samples (*i.e.*, to concentrate the viruses to a detectable level). After ultrafiltration, a cartridge of ~ 500 μL of the concentrate was collected and stored at −80 °C for downstream analysis (unless analyzed that day).

The total RNA from the concentrated samples was extracted using an AllPrep PowerViral DNA/RNA kit, following the protocol provided (QIAGEN, Inc., Germantown, MD, USA). Reverse transcription and quantitative polymerase chain reactions (RT-qPCR) were completed using the CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Per US-CDC recommendations, N1 and N2 primers and probes were used for the RT-qPCR assay.[21] The RT-qPCR was conducted by SARS-CoV-2 RT-qPCR Kits for wastewater (Promega, Madison, WI, USA) according to the kit instruction manual. Briefly, each reaction contained 10 μL GoTaq® wastewater

Probe qPCR MasterMix (x 2), 1 µL N1 and N2, respectively, and PMMoV Primer/Probe/IAC Mix (x 20), 0.2 µL GoScript® Enzyme Mix (x 50), and 5 µL of the total genomic RNA template, into a total 20 µL solution. The RT-qPCR reaction was then conducted according to the following protocol: RT at 45 °C for 15 min, RT inactivation and GoTaq® activation at 95 °C for 2 min, followed by 40 cycles of 15-second denaturation at 95 °C, 60-second annealing/extension. The plate was read after each cycle.

The RT-qPCR data were analyzed using the CFX Manager Software (BioRad, Hercules, CA, USA). The default algorithm in the CFX Manager Software determined the cycle threshold (Ct) values. Each run contained positive and non-template controls. Extraction RNA blanks were included monthly for field and RNA. Calibration curves (range 0 to 5-log) were generated with tenfold serial dilutions of SARS-CoV-2 positive control (IDT, Coralville, IA, USA) in the range from 200,000 to 2 genome copies (gc)/µL. Correlation coefficients (R2) > 0.99 were obtained for all calibration curves, with 90 % to 100 % amplification efficiencies. Each qPCR assay RNA elution had a limit of detection (LoD) of > 4 gc/µL, showing more than 50 % positive signal, with Ct values of the lowest-dilution positive control.

For the endogenous biomarker target, pepper mild mottle virus (PMMoV) was used for concentration method validation (*i.e.*, samples positive for PMMoV but negative for SARS-CoV-2 were considered under LoD for SARS-CoV-2 virus; if both were negative, we reprocessed the samples for confirmation). For the recovery control, human coronavirus-OC43 (HCoV-OC43) was used to evaluate the recovery rate in the

wastewater due to its similar envelope structure. The SARS-CoV-2 recovery efficiency was conducted as described previously by Gharoon et al.[22]

2.3.3 Library Preparation and Sequencing

Sequencing libraries for genotyping the SARS-CoV-2 virus in wastewater were prepared using one of two methods. Wastewater samples for variant analysis (all WRFs and sewershed) collected from November 2021 to July 2022 were processed as previously described by our group in Hartley et al.[23] Briefly, RNA was linearly amplified into dsDNA, sheared, and ligated to Illumina-compatible sequencing adapters with the QIAGEN QIAseq FX Single Cell RNA Library Kit. These PCR amplicons were sequenced as 2 x 151. These libraries were then enriched for SARS-CoV-2 sequences with an Arbor Biosciences library enrichment kit and SARS-CoV-2 specific enrichment probes. These libraries were sequenced as 2 x 60. These libraries were sequenced as paired reads with the NextSeq 2000 P2 100-cycle sequencing kit. Due to decreasing coverages (described in *Limitations*) as the study period progressed, wastewater samples collected between August 2022 and November 2022 (and select samples acquired before August 2022) were processed with the QIAGEN QIAseq DIRECT SARS-CoV-2 kit according to manufacturer instructions and sequenced as paired reads with a NextSeq 2000 P1 300 cycle sequencing kit. Depending on kit availability, RVP samples were sequenced as 2 x 151 or 2 x 60.

For the identification of respiratory pathogens in the wastewater, RNA was extracted from samples with Ct values (range 35 – 37), as previously described.[12] Briefly, samples were treated with DNase I (QIAGEN, Inc., Germantown, MD, USA) for 30 minutes at room temperature before concentrating through RNeasy Minlute spin columns (QIAGEN, Inc., Germantown, MD, USA). Once concentrated, samples were converted into Illumina-compatible sequencing libraries using the Respiratory Pathogen ID/AMR Enrichment Panel kit or the Respiratory Virus Oligo Panel (RVOP) according to the manufacturer's protocols (Illumina, Inc.). Briefly, RNA was first denatured using the Elute, Prime, Fragment High Concentration Mix (EPH3) for 5 minutes at 65 ◦C. Hexamer-primed RNA fragments were then reverse-transcribed to produce first-strand complementary DNA (cDNA). Second-strand synthesis was performed to complete the cDNA. AMPure XP beads were used to clean up the cDNA for tagmentation. Following the tagment step, premixed Index 1 (i7) and Index 2 (i5) adapters (Illumina, Inc.) were added to the sample and subjected to 16 PCR cycles to amplify the tagged cDNA and incorporate the adapters. Samples were again cleaned with AMPure XP beads. The cDNA was then normalized and consolidated into one-plex samples using undiluted libraries for overnight hybridization at 58 ◦C. Enrichment of SARS-CoV-2 and RVPs was done via the Respiratory Pathogen ID/AMR Enrichment or RVOP Enrichment Oligos (labeled with biotin), captured with streptavidin-coated beads and washed. The resulting enrichment pools were quantified and normalized using High Sensitivity D1000 ScreenTape® and TapeStation Analysis Software 3.2. Sequencing was performed using an Illumina NextSeq mid-output (2 x 75) or NextSeq 2000 P2100 cycle (2 x 50). The generated FASTQs from the sequencing reaction were subjected to the detection of RVP signatures.

2.3.4 Bioinformatics and Data Analysis

Single-end (SE) or paired-end (PE) FASTQ files generated from the Illumina sequencing were analyzed using a custom bioinformatics pipeline publicly available on GitHub. Details about the pipeline and setup can be obtained from its GitHub page (https://github.com/Nevada-Bioinformatics-Center/snakemake_freyja_covidwastewater). The pipeline starts by trimming the reads using fastp to remove poor-quality bases and adapter contamination (https://github.com/OpenGene/fastp). Then, the reads are classified using Kraken2 using its standard taxonomic database (https://github.com/DerrickWood/kraken2) to assess the quality and content of organisms sequenced in the wastewater sample. The trimmed reads are mapped to the Wuhan-Hu-1 reference (MN908947.3) using minimap2 (https://github.com/lh3/minimap2), and the resulting BAM files are assessed for quality control (QC) using Qualimap (http://qualimap.conesalab.org/). A combined QC report of all samples, including fastp trimming, Kraken2 classification, and Qualimap, is then generated by MultiQC (https://github.com/ewels/MultiQC). Freyja (https://github.com/andersen-lab/Freyja) runs on each sample to recover the relative lineage abundances from the mixed SARS-CoV-2 samples using the mapped BAM files. Freyja identifies the total coverage per sample and the abundances for each type of variant derived from the UshER global phylogenetic tree. Lastly, an aggregated report is generated, which includes viral concentration data and the relative abundances of each SARS-CoV-2 variant. This report is then used to visualize and compare the data across different sites over time and to assess the correlations

between viral concentration and the relative abundances of different SARS-CoV-2 variants.

2.3.5 Detection of RVPs and AMR Genes

We used an open-source IDseq pipeline (v3.7, https://czid.org/) to analyze the presence of RVPs and AMR genes.[24] Briefly, the pipeline performs subtractive alignment of the human genome (NCBI GRC h38) using STAR (v2.5.3),[25] followed by quality filtering with subsequent removal of cloning vectors and phiX phage using Bowtie2 (v2.3.4).[24] The identities of the remaining microbial reads are then queried against the NCBI nucleotide (NT) database using GSNAP-L in the final steps of the IDseq pipeline.[24,26] After background correction and filtering, retained taxonomic alignments in each sample were aggregated at the genus level and sorted by abundance, independently for each sample, measured in NT reads per million (NT-rPM).

2.4 Results

2.4.1 Overview

In this study, we aimed to determine the presence and concentrations of SARS-CoV-2 in wastewater samples collected from the Reno-Sparks metropolitan area and understand how the concentration of SARS-CoV-2 in wastewater correlates with any emerging variants of the SARS-COV-2 virus. We also conducted metagenomic analyses to detect other respiratory viruses and genes associated with AMR in the samples. We collected 175 wastewater samples from three wastewater treatment plants (WRFs) and seven regional sites in the Reno-Sparks metropolitan area between November 2021 and November 2022. The recovery rate of the viral genomic RNA from the wastewater matrix was about 23 %. The concentration of SARS-CoV-2 gene copies ranged between 4 gc/µL (the LoD), 8.48×10^5 gc/L (N1 gene), and 3.32×10^6 gc/L (N2 gene). We detected viral genomes with Ct values as low as 38.54 (about 1.35×10^4 gc/L).

2.4.2 SARS-CoV-2 Concentrations in Wastewater

Measuring the concentration of SARS-CoV-2 in wastewater can provide insights into the prevalence of COVID-19 in a community and help predict future outbreaks. By analyzing the concentration of SARS-CoV-2 in wastewater samples collected over 12 months, we could track changes in the virus concentration of COVID-19 in the area. Longitudinal analysis of genomic RNA copies of the SARS-CoV-2 nucleocapsid genes, N1 and N2, are represented for TMWRF (**Figure 1** [logarithmic gc]**)** and all other sites

(**Supplementary Figure 1** [linear gc/L]). At TMWRF, there were lower relative concentrations of N1 (log 3.3 ± 0.20) and N2 (log 3.67 ± 0.27) in the wastewater from November 2021 until early December 2021 (Delta, pre-Omicron period) (mean ± standard deviation [SD]).

We found that the SARS-CoV-2 N1 and N2 concentrations in the wastewater varied over time. During the Omicron wave (December 2021-mid January 2022), the concentration rose sharply and returned to early December levels. A similar pattern was observed during the Stealth Omicron wave (April-early August 2022). However, in the later months of the study (October-November 2022), we saw an increase in the N1 and N2 concentrations of SARS-CoV-2 in the wastewater. This pattern was also seen at other WRFs and sewershed. (**Supplemental Figures 1A-E**).

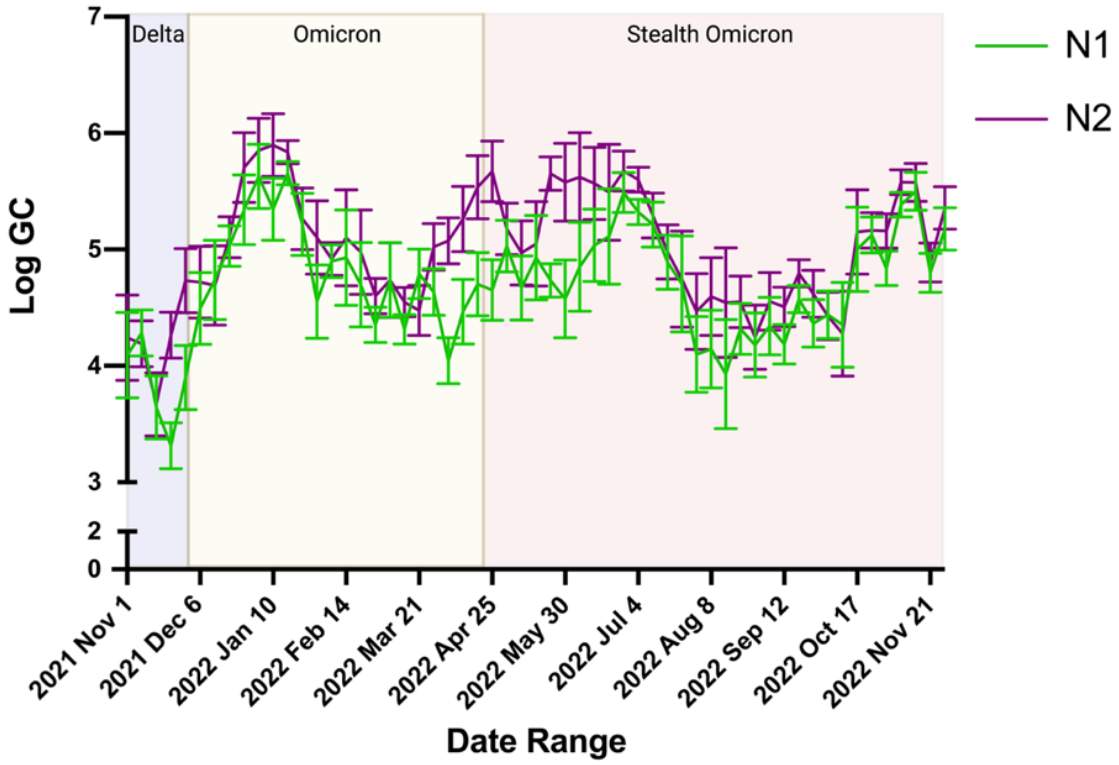


Figure 1. Longitudinal Analysis of Genomic RNA of SARS-CoV-2 Nucleocapsid Genes, N1 and N2. Influent was collected from November 2021 to November 2022. RT-qPCR results for N1 (green) and N2 (purple) are plotted over time. Weekly mean logarithmic N1 and N2 GC are plotted with standard deviation error bars.

2.4.3 Detection of SARS-CoV-2 Variants in TMWRF Sewershed

We performed SARS-CoV-2 variant analysis at TMWRF using the updated Freyja variant profiling classification analysis to classify the variants (snakemake pipeline) (**Figure 2**). By chronologically depicting the variant data, we visualized SARS-CoV-2 evolutionary inflection points (*i.e.*, the presence of a variant [occurring for short – or long-term] preceding the generation of a new variant(s) that then proceed to become predominant). These inflection points were determined to be December 6th, 2021 (beginning of the Omicron wave) with B.1.1.529, and April 8th, 2022 (beginning of the Stealth Omicron wave), with BA.2.12.1. Thus, following these variant introductions, predominant variants during each respective wave were identified as described below. Further, these inflections can also be visualized where significant N1 and N2 (log gc) rises preceded the Omicron and Stealth Omicron waves, respectively (**Figure 1**).

During the Omicron wave (December 2021 through January 2022), the variants with greater than 75 % prevalence were BA.1, BA.1.1.16, BA.1.1.13, BA.1.1.12, BA.1.1.18, BA.2, and BA.2.3 (**Figure 2**). Other variants, including BA.3 and its subvariants, were detected during this period but were not predominant (*e.g.*, BA.3 was only found in a few weekly samples at a prevalence of less than 25 %). The introduction

of the BA.2.12.1 variant led to a new Stealth Omicron wave (April 2022 through October 2022). The predominant variants during this wave (greater than 75 % prevalence) were BA.2.12.1, BA.2.3.2, BA.2.12, BA.2.11, BA.5, and BA.5.10. BA.5 subvariants were the most predominant, but their prevalence decreased in October 2022 (**Figure 2**). BA.4 and its subvariants were detected during the Stealth Omicron wave but were not predominant. As plotted, we show respective subvariants with at least two weeks of detection (*i.e.*, variants detected once were reclassified to their parent BA.X variant group). In our most recent TMWRF influent samples, we detected BQ (BQ.1 [24.88 %] and BQ.1.1 [0.66 %]), BF (mainly BF.7 and subvariants [96.3%]), and BE (BE.1.1.1 [29.68 %]) variants. BQ.1 and BE.1.1.1 have established a combined prevalence of 54.31 %, surpassing the declining BA.5 variants (41.52 %). The prevalence of BQ.1, BE.1.1.1, and BA 5.2.1 variants have increased in recent samplings from the TMWRF sewershed (**Figure 2**); however, these variants were sparsely detected in the other studied collection sites (**Supplemental Figure 2**).

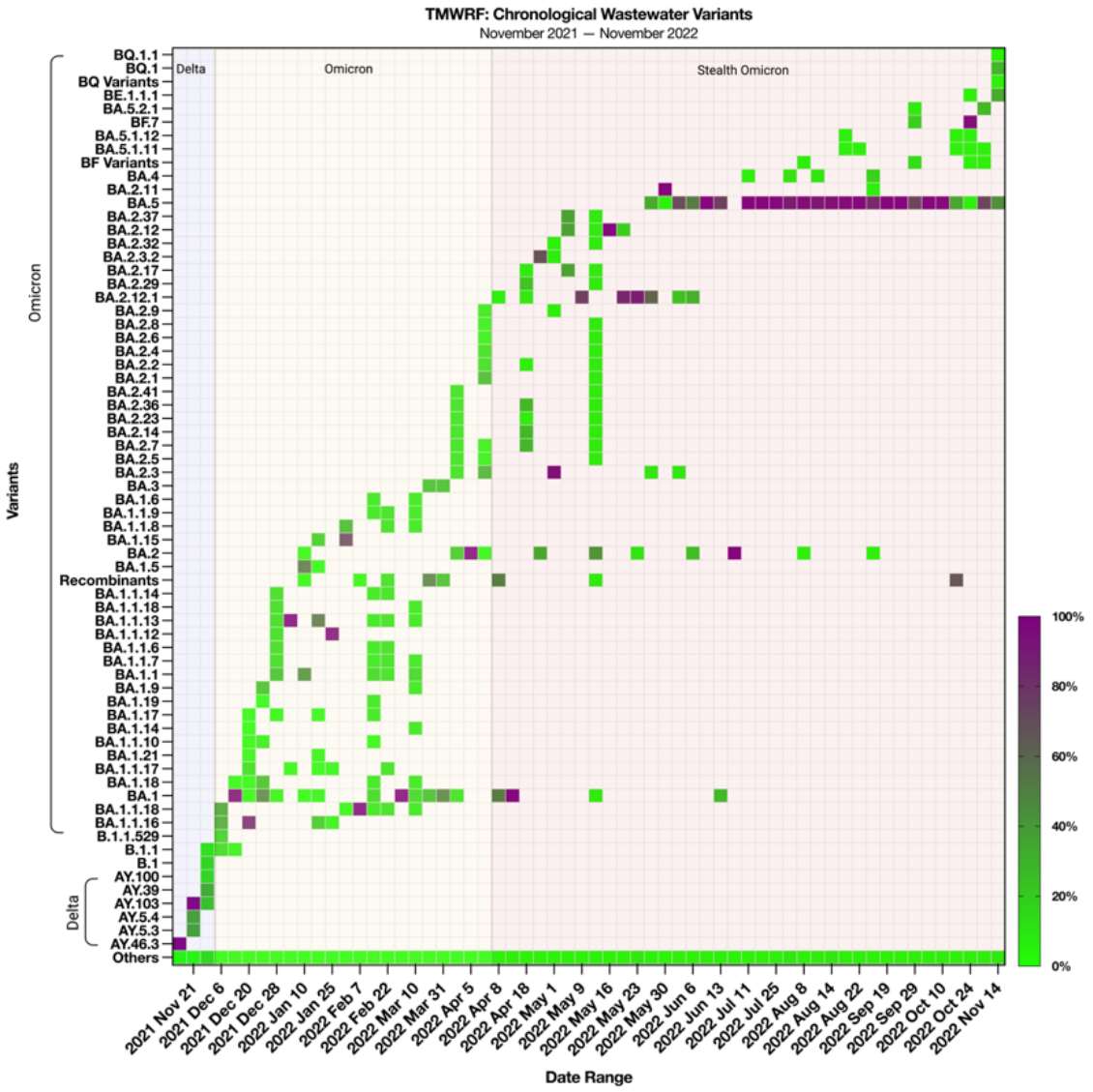


Figure 2. Chronological Detection of SARS-CoV-2 Variants in TMWRF Sewershed. Influent was collected from November 2021 to November 2022. The relative proportion of variants was determined by Freyja, a SARS-CoV-2 variants analysis pipeline (https://github.com/andersen-lab/Freyja). B.1.1.529 (Omicron) was first detected in the wastewater sample collected on December 6th, 2021 – before the first clinical identification of BA.2.12.1 (Omicron) in Washoe County on April 8th, 2022.

*TMWRF geographic location is represented in **Figure 4.**

2.4.4 Detection of RVPs in TMWRF Sewershed

In addition to analyzing the SARS-CoV-2 variants, we also performed metagenomic analysis to identify other co-occurring human respiratory disease-causing pathogens, which allowed us to assess the prevalence of multiple respiratory viruses in the community (**Figure 3**). The results of the metagenomic analysis showed that the prevalence of SARS-CoV-2 was consistently high throughout the study period, while the prevalence of other viruses had a seasonal pattern. For example, the prevalence of human Mastadenovirus increased during the early winter months, while Paraechovirus and Parvovirus (NIH-CQV) had a higher prevalence during the summer months. Low levels of enteric viruses, such as Mastadenovirus (F) and Enterovirus sp. (B and C), were detected throughout the year as expected. Other human respiratory disease-causing viruses that were detected in the metagenomic analysis included Paraechovirus (A), Mastadenovirus sp. (A, B, C, D, E, and G), Influenza (A and C), Rhinovirus sp. (A, B, and C), human Polyomavirus (1), HMO Astrovirus (A), and human Bocavirus.

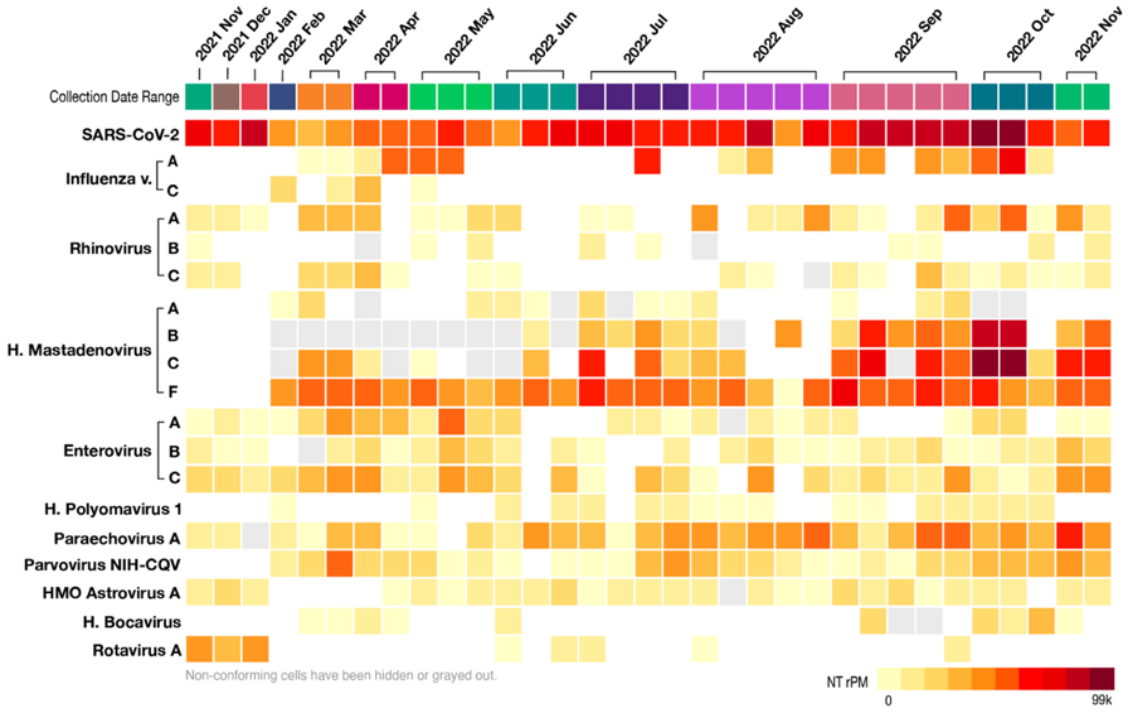


Figure 3. RVP Metagenomic Analysis of TMWRF Sewershed. Influent was collected from November 2021 to November 2022. The relative proportion of SARS-CoV-2 and other RVPs was determined by sequencing genomic RNA using an RVP kit (Illumina). The sequences were analyzed through the Pathogen Detection tool of CZiD.org. A heatmap was generated based on the total nucleotide reads per million (NT-rPM) of each pathogen detected relative to their overall abundances.

2.4.5 Detection of AMR Genes in TMWRF Sewershed

To investigate the presence of AMR genes in the TMWRF region, we conducted a secondary analysis to identify genes that confer resistance to various antibiotic classes (**Table 1**). We detected AMR genes that confer resistance to macrolide, beta-lactamase, tetracycline, sulfonamide, glycopeptide, fluoroquinolone, trimethoprim, aminoglycoside, phenicol, and colistin antibiotics. The AMR gene coverage ranged from 2.34 % to 94.28

%, and AMR gene reads (per million) ranged from 0.01 to 1911.99 RPM. Our samples' most predominant AMR genes belonged to the macrolide and tetracycline antibiotic classes, followed by beta-lactamase and aminoglycoside. The sulfonamide antibiotic class had two AMR genes detected at a greater than 90 % prevalence. The glycopeptide, fluoroquinolone, trimethoprim, phenicol, and colistin antibiotic classes detected fewer AMR genes, with 1 or 2 dominant AMR genes in the respective antibiotic class (40-77 % prevalence).

Table 1. Antimicrobial Resistance Gene Analysis from Wastewater Influent

Antibiotic Class	Gene	Sample Counts	Avg. Coverage of AMR Gene (%)	Avg. rPM of AMR Gene
Macrolide	**MphE**	**31**	**89.43**	**530.71**
	MsrE	**31**	**88.66**	**1193.98**
	MefA/ Mel	**31**	**66.34**	**105.50**
	MsrD	31	53.02	0.94
	ErmF	**30**	**83.78**	**382.78**
	ErmG	**30**	**79.10**	**92.04**
	Mef(B)	29	38.23	0.52
	ErmB	**28**	**70.91**	**163.25**
	LnuC	**28**	**58.07**	**71.07**
Beta-lactamase	**OXA-7**	**31**	**73.61**	**94.27**
	OCA-2	**31**	**62.42**	**204.32**
	TEM-1D	**31**	**67.07**	**104.44**
	CfxA	**30**	**70.58**	**1.11**
	OXA-211	27	49.24	99.91
	OXA-5	22	27.93	1.86
	CARB-5/ BlaRTG-2	19	33.71	12.59
	CEPH	15	23.85	1.77
Tetracycline	TetC	31	36.30	0.52
	TetQ	**30**	**94.28**	**1483.71**
	TetX	**30**	**86.70**	**974.86**
	TetW	**30**	**81.13**	**1911.99**

	TetO	**30**	**76.17**	**566.78**
	TetM	30	38.20	65.54
	TetB-P	30	40.45	0.83
	TetA-P	30	38.90	0.60
	Tet-32	29	38.42	126.82
	Tet-39	29	49.00	0.61
	Tet-44	**29**	**53.15**	**1.16**
	Tet-40	26	27.79	1.62
Sulfonamide	**SulI**	**30**	**64.05**	**173.47**
	SulII	**28**	**52.85**	**77.65**
Glycopeptide	VanA-G	16	20.97	0.08
	VanRD	1	13.59	0.14
	VanTC	2	3.33	0.05
	VanSA	1	2.25	0.10
	VanYD	1	8.15	0.05
	VanRF	1	6.03	0.02
Fluroquinolone	QnrVC5	13	48.67	21.10
	OqxB	24	6.14	0.23
	QnrVC1	8	20.09	5.52
	QnrD	8	38.02	22.66
Trimethoprim	DfrA5	13	46.30	21.41
	DfrA16	8	32.75	3.65
	DfrA3	6	23.79	0.81
	DfrA1/ Dfr1	6	39.30	6.84
Aminoglycoside	Aph3-III	30	49.13	116.08
	ANT(6)-Ib	**27**	**54.03**	**67.68**
	ANT(6)-Ia	24	42.64	43.85
	Aac3-I	20	33.91	17.87
	APH(3")-Ia/ AphD2/ AphE	15	20.76	0.14
Phenicol	FloR	3	5.79	0.06
	Cmr	2	9.86	0.03
	CmlB1	2	2.77	0.04
	CatA2	1	17.13	0.03
	CatB	1	4.09	0.11

Colistin	MCR-4.1/ MCR-4	2	3.88	0.03
	MCR-1.1/ MCR-1	1	2.34	0.01

*****AMR genes with >50 % average coverage are bolded

2.4.6 Detection of SARS-CoV-2 Variants in RSWRF, STMWRF, and Sub-Sewershed

We analyzed the prevalence of SARS-CoV-2 variants at all the tested sites to understand the distribution and evolution of the virus in the community. The results showed that all the sites had a similar prevalence of variants, with slight variations in the order of appearance (**Figure 4**). BA.5 was the predominant variant at TMWRF from April 8th, 2022 (inflection point for the Stealth Omicron wave). However, our sample collection at the non-TMWRF sewershed began on May 3rd, 2022. RSWRF showed an increasing prevalence of BA.5 (range 43-99 %) over the collection period, with a single sample with a high prevalence of BA.2.12.1 (33.64 %) on July 13th, 2022. The first overall detection of BF and respective subvariants occurred on August 3rd, 2022, at RSWRF (4.32 %), with subsequent detections at STMWRF on October 3rd, 10th, 24th, and November 7th (0.35 %, 0.44 %, 0.44 %, 65.81 %, respectively), Travel-Influenced sites on August 9th and 10th, 2022 (10.76 %, 55.45 %) and Elementary Schools on October 19th, 2022 (74 %). There were no detections of BF variants at Sub-Neighborhoods. BG and respective subvariants were detected in the effluent collected from the Elementary Schools on May 3rd, 2022 (97.4 %), followed by Sub-Neighborhoods on June 14th, 2022 (0.16 %), and at STMWRF on June 20th, 2022 (0.65 %). There were no detections of the BG variants at TMWRF. There was only one low trace detection of BE and respective

subvariants in STMWRF on August 29th, 2022; however, there has since been no detection of BE at any non-TMWRF site (as of October 24th, 2022). BQ and respective subvariants were detected as early as October 31st, 2022, in STMWRF, confirming the introduction of this new variant.

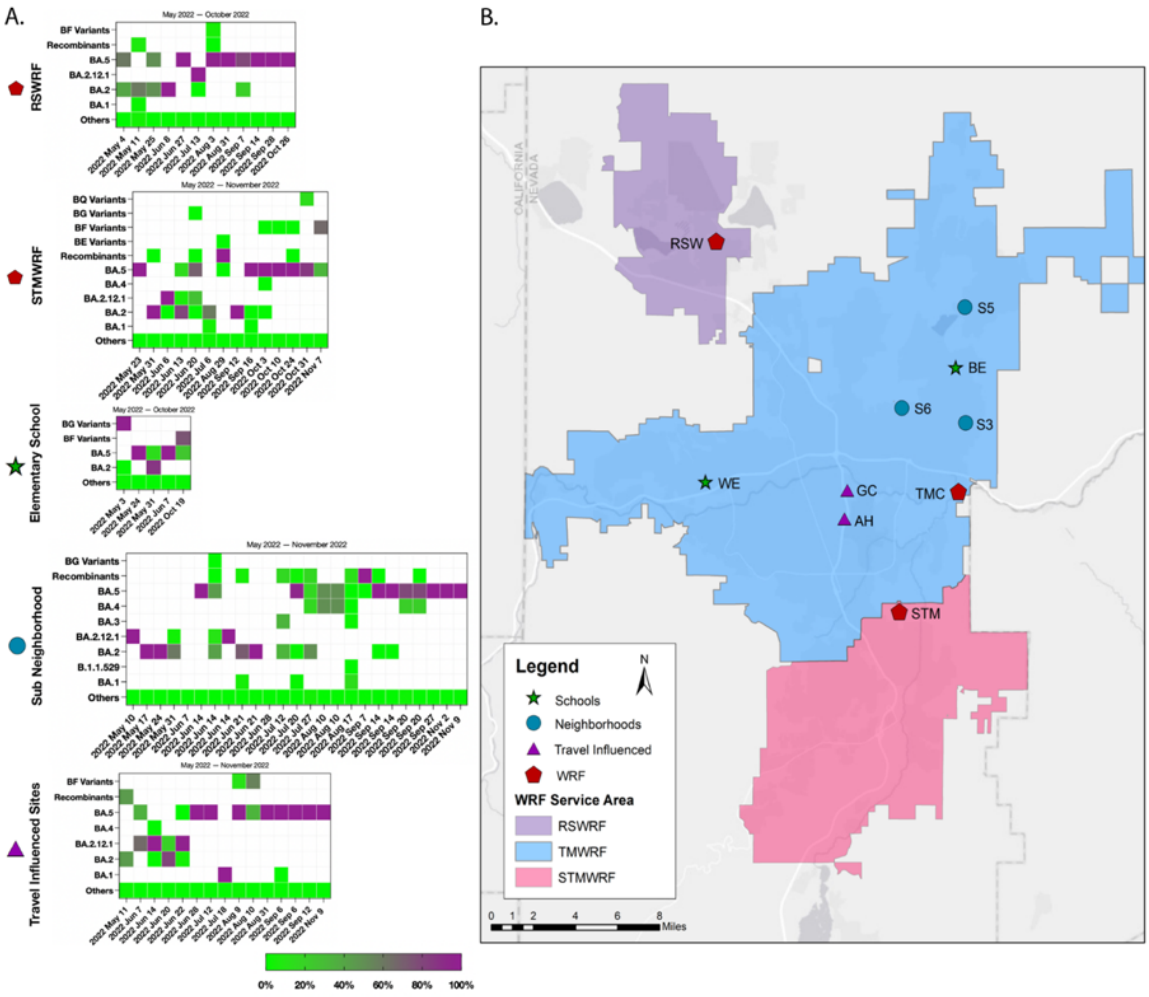


Figure 4. Detection of SARS-CoV-2 Variants in STMWRF, RSWRF, and Other Sub-sewershed. Influent was collected from May 2022 – October 2022 at two WRFs and three sewershed (A). A map of the Reno-Sparks metropolitan area depicting WRFs, sewershed locations, and servicing areas is shown (B). Collective occurrences of the main variants of SARS-CoV-2 Delta and Omicron (*e.g.*, AY.X, BA.1.X, BA.2.X, BA.3.X, BA.4.X, BA.5.X, BE.X, BF.X, BG.X, and BQ.X) were calculated. The relative

proportion of variants was determined using Freyja (https://github.com/andersen-lab/Freyja). BA.2.12.1 was excluded from summation to depict the significance of detection.

*TMWRF variant data is shown in **Figure 2.**

2.5 Discussion

WBE for SARS-CoV-2 analysis is a recent global endeavor to mitigate low testing frequencies and reporting information to gather more comprehensive data on the current status of COVID-19 in a community.[20,27-30] WBE has effectively detected key variants responsible for the evolution of SARS-CoV-2 infectivity and pathogenicity.[13,15,20,31] In this study, we analyzed multiple WRFs and local collection sites in the Reno-Sparks metropolitan area using RT-qPCR to quantify the presence of SARS-CoV-2 and its variants. To contribute additional insight to the literature, the present study sought to independently assess regional WRFs and local collection sites to quantify SARS-CoV-2 and respective variants via frequent sample collection and an up-to-date variant classification bioinformatics analysis. Furthermore, our study is strengthened through secondary analysis and identification of other respiratory disease-causing viruses and AMR genes not included in other studies. Our findings provide valuable information on the COVID-19 status in the Reno-Sparks area and demonstrate the usefulness of WBE in detecting circulating viruses. Our data showed detections of new variants. We noted a recent rise in viral genome concentrations not reflected in the number of clinically diagnosed cases, indicating a discrepancy between testing and reporting at the community level.

Our RT-qPCR data on wastewater's N1 and N2 genes show early signs of all new variants (**Figure 1**) – continual monitoring for variants allowed for early and specific detection of new VOIs and VOCs. WBE is particularly useful in times of low testing frequencies and can provide comprehensive, up-to-date data on the COVID-19 status of a

community, including individuals who are not being tested. This is particularly valuable in rural areas where access to testing may be limited. Previous studies have shown that SARS-CoV-2 cases tend to spike in the summer and winter months, leading to the emergence of new variants; moreover, based on the current pattern seen in our data, this could be driving the rise in BE, BF, and BQ variants.[32,33] We also noted a recent rise in viral genome concentrations; however, we do not see the same trend in clinically diagnosed cases, alluding to a discourse between testing and reporting at a community level. Thus, WBE of SARS-CoV-2 is once again proving to be an effective tool during times of low testing frequencies and reporting, and further, can be used as a more effective and inclusive (*i.e.*, acquisition of virus prevalence amongst testing and non-testing persons) diagnostic in rural settings to provide public health and city officials of up to date community data, especially as the process becomes increasingly more streamlined and broadly accepted.[34,35]

2.5.1 Variants and Genomic Surveillance

There are nationally recognized entities that exist to provide updated information on variants of interest (VOIs), variants of concern (VOCs), and variants being monitored (VBMs) (*e.g.*, Centers for Disease Control [CDC], World Health Organization [WHO]).[36,37] Current CDC guidelines report pre-Omicron variants as VBMs (*e.g.*, Alpha, Delta, Wuhan strains). One variant is reported as a VOC (B.1.1.529); however, no VOIs are currently established.[37] During the beginning of this study (November 2021), almost all the signatures detected were of the Delta (AY) variant, which was replaced following

the introduction of the B.1.1.529 Omicron variant (**Figure 1**). We have not re-detected Delta signatures since their disappearance in the wastewater (**Figure 2**). This was a similar WBE result seen by Galani et al. in Greece (*e.g.*, the Delta variant being replaced by B.1.1.519, signifying the start of the Omicron wave).[38] The B.1.1.529 (Omicron) variant was first detected in our study on December 6th, 2021, which preceded the Omicron wave by one week and consisted of predominantly BA.1, BA.1.1.18, BA.1.1.12, BA.1.1.13, and BA.2 variants.

Preceding the Stealth Omicron wave of BA.2 and respective subvariants, we saw the highest prevalence of BA.2.12.1, which we then hypothesized to be the predominant variant in all our study sites to precede BA.5 in high concentrations (**Figure 2**). This followed early data reporting that US sequences submitted to GISAID accounted for 26 % of BA.2.12.1 and that BA.4 and BA5 comprised more than 90 % of the genomes sequenced in South Africa.[39] Furthermore, BA.2.12.1 has been observed to overcome immunity from earlier Omicron infections, suggesting that it may cause re-infections and increase infectivity.[40,41] In our TMWRF samples, which represent the most extensive collection area, BA.2.12.1 was detected seven weeks before the first sample containing BA.5. Although sample collection dates at non-TMWRF sites are limited, we did see BA.2.12.1 as the predominant variant at two other sites (Sub-Neighborhood and Travel-Influenced Sites) preceding BA.5; however, BA.2.12.1 was predominant alongside BA.5 in two other sites (STMWRF, RSWRF) (**Figure 4**). In contrast to B.1.1.529, which had a brief prevalence preceding the BA.2 Omicron wave, BA.2.12.1 had a longer time course before BA.5.

The detection of BF and respective subvariants in our sub-sewershed sites on August 3rd, 2022, is noteworthy (**Figure 4 and Supplemental Figure 2**). Despite the early detection and rapid drop of these BF and respective subvariants, these variants have the potential to become the predominant strain in the region, as evidenced by the increasing prevalence observed in our largest site (TMWRF): August 8th, 2022, with BF.8 (0.8 %), followed by BF.7 (15 %) on September 29th, and BF.7 (96.35 %) on October 24th, 2022. The last TMWRF sample with the BF variant was on October 31st, 2022, with BF.4 (0.44 %). Other sub-sewershed sites also showed detections of BF variants, with RSWRF detecting BF.3.1 (4.03 %) and BF.1 (0.21 %) on August 3rd, 2022, and Travel-Influenced sites detecting BF (10.76 %) and BF.2, BF.4, and BF.18 (26.14 % each, respectively) on August 9th, 2022. Elementary schools also detected BF.12, BF.7, and BF.28 (24.5 % each, respectively) on October 19th, 2022, where these BF subvariants were more predominant than BA.5. These data collectively indicate that BF may also become the predominant variant in the region, especially when considering rising abundance at TMWRF.

Our TMWRF data suggests that BQ.1 and BE.1.1.1 are beginning to establish strong predominance in the region, except for STMWRF, which continued to show a higher prevalence of BA.5.2 and BA.5.2.1 subvariants in recent samples (October 24th – November 7th, 2022). The higher prevalence of BQ.1 is following the CDC COVID-19 variant foresight and prevalence tracker, which uses a NOWCAST model to estimate and predict proportions of circulating variants based on recently circulating variant proportions, is predicting that BQ.1, BQ.1.1, and BF.7 variants to become the

predominant future variants in the region including NV (Region 9).[42] Our data indicate that these emerging variants with rising prevalence are consistent with data on major variants circulating in the U.S., but that smaller sub-regions may be slower to pick up this detection (*e.g.*, BQ variant); thus, large, central wastewater collection is key to the early detection of emerging predominant variants in a defined region. The variant dynamics seen in our study are generally consistent with the trends observed nationally, reinforcing the significance of wastewater surveillance in capturing the regional prevalence of SARS-CoV-2 variants.[3,42]

2.5.2 RVP and AMR Analysis

This WBE analysis consistently detected elevated levels of SARS-CoV-2 throughout the year compared to other respiratory viruses (*e.g.*, Influenza), which varied seasonally (**Figure 3**). This may suggest that SARS-CoV-2 was constantly present in the community but caused fewer clinical cases in the summer months. Further, regional health data from 2021-2022 in Washoe County (Reno, NV) showed that Influenza A (93.8 %) was more prominent during the same study period in hospitalized patients; however, Influenza C (6.2 %) was not identified, whereas Influenza B was prevalent, although in low amounts.[43] Of these hospitalized patients, only 35.6 % were vaccinated with the seasonal flu vaccine. Interestingly, other regions in the US did report circulating Influenza B, suggesting that Influenza B and C's viral patterns are consistent with seasonal and regional patterns and may be influenced by factors such as vaccination. Moreover, in our study, the detection of SARS-CoV-2 sub-variants and the seasonal

patterns of other respiratory viruses closely align with the CDC's reports on the persistence of SARS-CoV-2 and the seasonal fluctuations of respiratory viruses such as adenoviruses and Influenza.[44] This further supports the utility of wastewater surveillance as a reliable tool for monitoring the prevalence of respiratory pathogens in communities.

Cold temperatures are known risk factors for respiratory infections, particularly in the winter, when they can cause irritation and inflammation of the airways, making it easier for respiratory viruses to enter and infect the body (*e.g.*, irritation and inflammation of the airways, making it easier for respiratory viruses to enter and infect the body).[45,46] Furthermore, studies have found that respiratory infections are higher during the winter months as people are more likely to be exposed to cold temperatures (*e.g.*, the rate of Influenza infections in the US was significantly higher at colder temperatures and climates compared to warmer temperatures and climates).[47,48] Another study associated lower temperatures with higher rates of SARS-CoV-2 infection.[32] WBE, paired with secondary analyses for respiratory disease-causing viruses, provides a comprehensive assessment of the total microbial burden in a community and can inform healthcare responses.[49,50]

The introduction of antibiotics has played a crucial role in treating diseases globally. Still, the simultaneous delivery of these antibiotics has allowed microbes to develop antibiotic resistance, reducing their therapeutic effect in humans and leading to difficulties in managing many infectious diseases.[51] The rise in AMR (and associated AMR genes) is responsible for over 700,000 worldwide deaths yearly.[52] Brumfield et al. performed secondary metagenomic, metatranscriptomic, and targeted SARS-CoV-2

analyses on wastewater samples as cases were rising and found the presence of other pathogens and important spike mutations, as well as potential coinfection insights and AMR genes.[7] Similarly, our study identified significant macrolide and beta-lactamase AMR genes.

A recent study investigated the levels of beta-lactam antibiotics via the MecA gene by comparing wastewater samples from hospitals and other healthcare facilities to other sources and a higher prevalence of the MecA gene in hospitals and healthcare facilities.[53] In our study, the most abundant beta-lactamase AMR genes were MphE and MsrE (89.43 % and 88.66 %, respectively), suggesting that the increased use of antibiotics may be related to secondary bacterial infections associated with SARS-CoV-2 and may have implications for changes in the human gut microbiome.[54,55] While we found high levels of AMRs for many antibiotic classes, we also identified antibiotics with lower levels of detected resistance, implicating potential therapeutic effects against antibiotic-resistant pathogens.

2.5.3 Study Limitations

WBE provides valuable insight into regional virus prevalence and can provide data into circulating viral variants and antimicrobial resistance genes. However, it does not provide specific information on persons infected, and tracing efforts are difficult as wastewater samples are pooled at WRF sites (unless a specific sub-sewershed is used). WBE is a new research approach to the public health and medical field, and a few ethical

concerns must be considered. For example, there is the potential for discrimination or stigmatization of individuals or communities based on the results of the research, which may lead to social and economic consequences leading to undermined trust in research and public health efforts (*e.g.*, if there is a high abundance of a particular RVP in a community, they may be targeted and blamed). Thus, anonymizing or aggregating data is important, as well as developing and following strict protocols for handling and sharing sensitive information.

Furthermore, although the more significant sample regional area site (TMWRF) provides more generalizable data, there were slight differences between the smaller sub-sewershed, which indicates a decrease in the degree of generalizability provided by more extensive sample-area data. We also noted a gradual worsening in coverages in our variant analysis as the study period progressed, which required us to switch our amplification method (Qiagen). This could be partly due to the original enrichment oligos not working well due to evolution in the viral sequence, quality issues with the wastewater samples themselves, or degradation of reagents. Lastly, our sample collection, processing, and sequencing methodology were continuously analyzed and updated throughout the study period, which may contribute to some sample variation between early and recent data.

2.6 Conclusion

Overall, our findings highlight the values of WBE for monitoring the presence and dynamics of SARS-CoV-2 in an urban setting, employing multiple fields and expertise in the process (*e.g.*, virology, bacteriology, epidemiology, public health research, and surveillance). We emphasize that WBE can be an essential tool for monitoring the transmission of pathogens between humans, animals, and the environment, providing valuable insights into the community prevalence of SARS-CoV-2 variants and other respiratory viruses and AMRs.

2.6.1 Utility of Wastewater-Acquired Variant Data and Monitoring Strategies

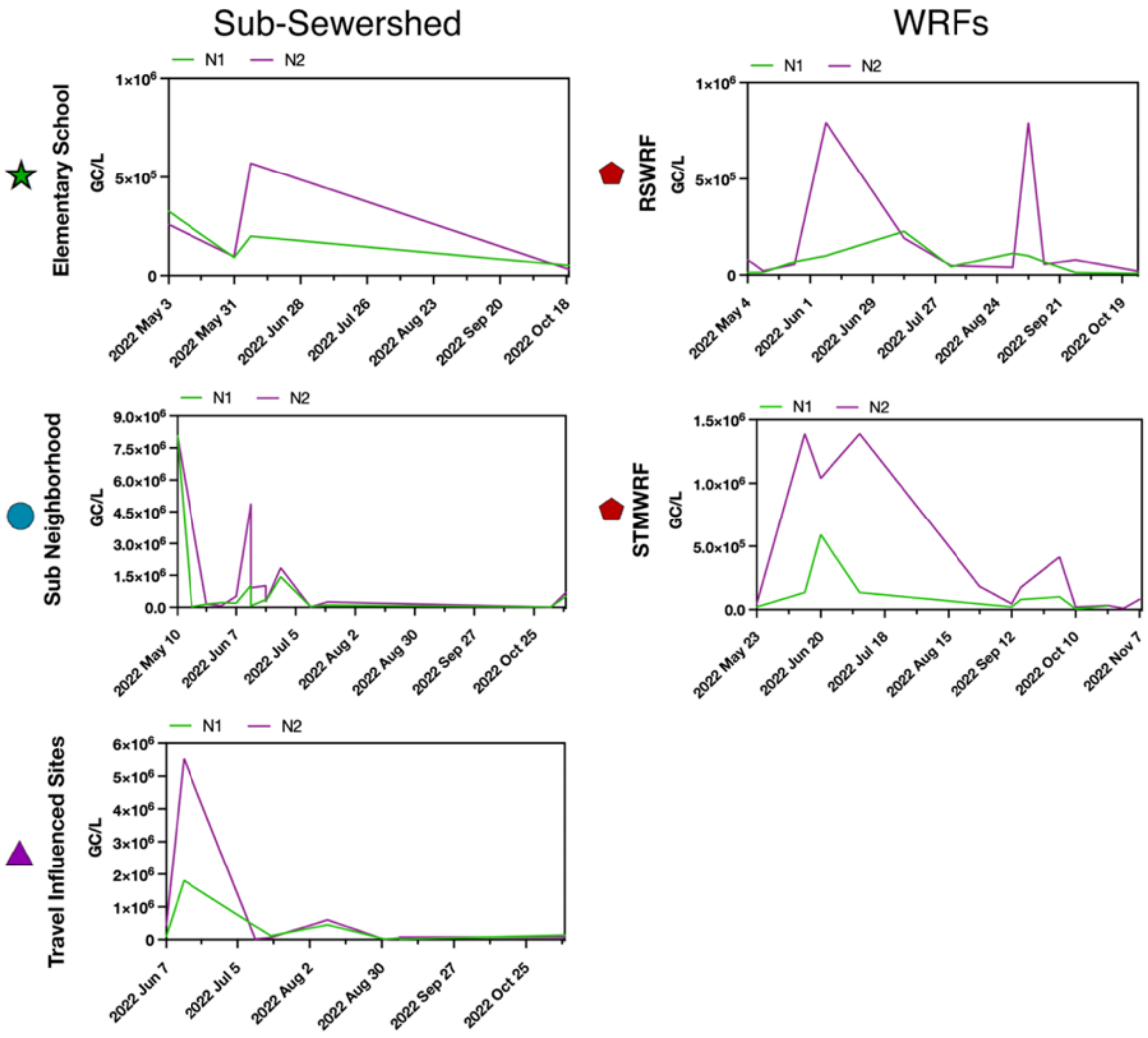
By collecting samples from multiple sites, we were able to identify and correlate regional differences in virus prevalence, detected the emergence of new variants that preceded the inflections of the two dominant Omicron waves, and showed that near-proximity regional differences in virus prevalence exist (*i.e.*, different variants are more prevalent in certain areas). The CDC-NOWCAST model's prediction of the BQ.1, BQ.1.1, and BF.7 variants becoming the predominant future variants in the region aligns with our findings, further supporting the effectiveness of WBE in detecting and tracking the spread of SARS-CoV-2 and its variants.[42] Moreover, this method does not rely on the requirement for persons to self-report or depend on testing infrastructure, as our samples are community-pooled wastewater, which provides better insight into community virus

prevalence and supports WBE as a sufficient and acceptable alternative to clinical-based testing surveillance.

Our analysis also identified the presence of other RVPs and AMR genes, providing insight into the dynamics of SARS-CoV-2 co-occurring viruses, which can provide valuable information for healthcare teams. We observed a wave-like pattern of occurrence for respiratory disease-causing viruses aside from SARS-CoV-2 and Mastadenovirus F, as well as identifying dominant AMR genes (*e.g.*, MphE and MsrE). Additionally, our analysis revealed the seasonality of certain viruses (*e.g.*, Influenza), with SARS-CoV-2 and Mastadenovirus variant levels (B, C, and F, specifically) remaining consistently high throughout the year. In contrast, other viruses showed higher prevalence only during specific seasons.

2.7 Appendix

2.7.1 Supplementary Material



Supplemental Figure 1. Longitudinal Analysis of Genomic RNA of SARS-CoV-2 Nucleocapsid Genes, N1 and N2, at Sub-Sewershed. Influent was collected from May 2022 -November 2022. RT-qPCR results for N1 (green) and N2 (purple) are plotted over time and subdivided by respective sewershed or WRF collection sites.

Supplemental Figure 2. Expanded Detection of SARS-CoV-2 Variants in WRFs and Sub-Sewershed. Influent collected from November 2021 to November 2022. Collective occurrences of SARS-CoV-2 Delta and Omicron (*e.g.*, AY.X, BA.1.X, BA.2.X, BA.3.X, BA.4.X, BA.5.X, BE.X, BF.X, BG.X, and BQ.X) were calculated. Relative variant proportions were determined using Freyja (https://github.com/andersen-lab/Freyja).

2.7.2 Data Availability

The data for the study are submitted as BioSamples to NCBI Genbank under BioProject ID PRJNA772783 (https://www.ncbi.nlm.nih.gov/bioproject/772783).

2.7.3 Acknowledgments

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2.7.4 Conflicts of Interest

The authors declare no conflicts.

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CHAPTER 3

3. INVESTIGATING SARS-COV-2 VARIANTS IN WASTEWATER AND CLINICAL SAMPLES: AN ANALYSIS OF VARIANT PREDOMINANCE AND VIRULENCE ON COVID-19 DISEASE SEVERITY

3.1 Abstract

The transmission and prevalence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and its variants have been observed to exhibit seasonal trends. This study aims to evaluate the correlation between specific SARS-CoV-2 variants detected in wastewater and clinical samples, the lead and lag times in their identification, and the mean time taken for variant predominance. It also investigates the association and factors of specific SARS-CoV-2 variants that contribute to the severity of COVID-19 disease. We collected and analyzed wastewater samples in the Reno-Sparks metropolitan area (Reno, NV, USA) over 16 months (November 2021 to March 2023). A separate clinical cohort of 1,748 patients seen at Renown Regional Medical Center (RRMC, Reno, NV, USA) over 19 months (April 2022 and November 2023) was analyzed to investigate the association of key SARS-CoV-2 variants, patient health variables, and disease severity. Our findings suggest that certain SARS-CoV-2 variants took more time to predominance (days until > 50 % prevalence of total circulating variants) compared to Delta and early-Omicron sub-variants, which may indicate decreasing virulence and infectiousness of the SARS-CoV-2 virus. Additionally, our study identified key variants associated with heightened disease severity and certain key underlying factors that may be involved (*e.g.*, underlying herpesvirus [HHVs] infections) in worsened pathogenesis. This work underscores the utility of analyzing combined clinical and wastewater specimens for surveillance of disease, tracking variant dynamics of SARS-CoV-2, and tracking other pathogens and co-infections to inform public health strategies and improve patient health outcomes.

3.1.1 Highlights

- Correlation between wastewater (WBE) and clinical (NP Swab) SARS-CoV-2 variants.
- Extended lag times and mean days-to-predominance (> 50 %) for late SARS-CoV-2 variants.
- Specific SARS-CoV-2 variants were identified as significantly contributing to COVID-19 disease severity.
- Wastewater and clinical surveillance provide complementary insights into the dynamics of SARS-CoV-2 infections in a population.
- Climatic, environmental, and population flux may influence the prevalence and dynamics of SARS-CoV-2 variants.
- WBE epidemiology effectively captures the regional variation of SARS-CoV-2 strains and can recommend public health responses.

3.1.2 Keywords

SARS-CoV-2, Variants, Wastewater-Based Epidemiology, WBE, Public Health, Wastewater Surveillance, Clinical Surveillance, Disease Severity

3.2 Introduction*

The COVID-19 pandemic, caused by SARS-CoV-2, continues to pose significant global health challenges. As of November 2023, there have been over 770 million confirmed cases and nearly 7 million deaths worldwide.[1] Global vaccination has certainly limited the spread of SARS-CoV-2, yet the emergence and spread of novel SARS-CoV-2 variants has continued.[2-6] These variants (*e.g.*, Alpha, Delta, Omicron; B.1.1.529, BA.2.12.1, BA.5, BF.7, BQ.1, *etc*.) have emerged and reign predominant in certain regions, likely due to mutations that cause increased infectivity, and in some cases, represent potential for immune escape.[1,2,7,8] The continual evolution of the virus underscores the need for comprehensive surveillance and a nuanced understanding of these emerging variants in relation to disease severity, vaccine efficacy, and public health measures.[9]

We sought to approach this question from multiple angles to address gaps in our understanding of the different effects of specific SARS-CoV-2 variants and determine whether they have a role in worsened COVID-19 pathogenesis. i) Next-generation

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*Abbreviations

AAV, Adeno-Associated Virus; ACE2-R, Angiotensin-Converting Enzyme 2 Receptor; ADA, Americans With Disabilities Act; B, Beta; BBB, Blood-Brain Barrier; BEI, Biodefense And Emerging Infections; BSL, Biosafety Level; CDC, Centers For Disease Control And Prevention; CNS, Central Nervous System; DMEM, Dulbecco's Modified Eagle Medium; DNA, Deoxyribonucleic Acid; dsDNA, Double-Stranded DNA; EMEM, Eagle's Minimum Essential Medium; FBS, Fetal Bovine Serum; HCoV, Human Coronavirus; HBMEC, Human Brain Microvascular Endothelial Cells; HR, Hazard Ratio; IL, Interleukin; IFA, Immunofluorescence Assay; MOI, Multiplicity of Infection; NGS, Next-Generation Sequencing; NIH, National Institutes Of Health; NP, Nasopharyngeal; PASC, Post-Acute Sequelae Of SARS-Cov-2 Infection; RNA, Ribonucleic Acid; RPMI, Roswell Park Memorial Institute; RT-qPCR, Reverse Transcription Quantitative Polymerase Chain Reaction; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; TMWRF, Truckee Meadows Water Reclamation Facility; TNF- α, Tumor Necrosis Factor-Alpha; VOC, Variant of Concern

sequencing (NGS) of nasopharyngeal (NP)-swab specimens to identify the prevalence of circulating SARS-CoV-2 variants (Pango lineage, NextStrain Clade, and World Health Organization [WHO] Label designations). ii) Analyses of a biorepository of COVID-19 patient electronic medical records (EMR) (*e.g.,* patient health variables; age, gender, ethnicity, comorbidities, vaccination status, symptoms, comorbidities, *etc.*). iii) Wastewater-based epidemiology (WBE) sample metagenomic analyses for circulating pathogens and NGS for SARS-CoV-2 variant identification.[10] We aimed to correlate SARS-CoV-2 variants detected to evaluate lead and lag times in variant identification between these two sources of data, analyze the chronology of variants, and determine associations between SARS-CoV-2 variants and certain patient variables on COVID-19 disease severity. We collected and analyzed weekly wastewater influent (i.e., untreated wastewater) collected from Truckee Meadows Water Reclamation Facility (TMWRF) from the Reno-Sparks metropolitan area (Nevada, USA) over 16 months (November 2021 to March 2023). Patient NP swabs were collected from Renown Regional Medical Center (RRMC, Reno, NV, USA) between April 2022 and November 2023.

Genomic analysis of wastewater and NP swab specimens provided insights into the region's clinical prevalence of circulating SARS-CoV-2 variants. Focusing on the correlation between variants identified through the WBE and NP swab surveillance methods, our analyses provided insights into the prevalent clades and variants, temporal patterns, and their relationships with clinical variables and disease severity. By leveraging the wastewater data, we also propose its use as a potential leading indicator as it may help draw earlier insights into the detection and shifts of certain variants of

concern (V\OCs).[2] The wastewater and clinical data analyses reveal underlying connections that reflect broad trends in variant distribution and mutations. The findings contribute valuable information to the ongoing efforts to monitor and manage the COVID-19 pandemic.

3.3 Materials and Methods

3.3.1 Data Collection Sources

Our study drew from two primary data sources. Our first data set was drawn and updated from wastewater samples obtained through our ongoing WBE efforts in partnership with the Department of Civil & Environmental Engineering (University of Nevada, Reno, Reno, NV, USA). Wastewater sampling provides a community-level perspective on the prevalence of SARS-CoV-2. It can act as an early warning system for the emergence of new variants.[10] We obtained and processed new samples from TMWRF to add data to our previous study, accounting for November 2022 and March 2023 (November 2021 to March 2023 total). The variant detection and analysis methodologies and detailed descriptions of the sample collection, SARS-CoV-2 detection, variant analysis, and climatic data were consistent with those in our previous study (Chapter 2).[10] Briefly, wastewater influent samples (*i.e.*, untreated wastewater) were collected from TMWRF weekly and were analyzed for the presence of viral and bacterial pathogens. The TMWRF represents about 325,000 residents within the Reno-Sparks Metropolitan Area and has an approximate daily flow rate of 121,000 m^3 (~30 million gallons/day), accounting for roughly 80 % of Washoe County's wastewater collection.

The second data source consisted of 1,748 NP swab specimens sequenced from individuals tested at RRMC (Reno, NV, USA) from April 2022 to November 2023 (**Table 1**). This cohort comprised of in- and out-patients hospitalized or presenting to RRMC for COVID-19 with varying disease severities. Some of these individuals also initially presented with flu-like symptoms and were later confirmed to have positive

influenza A infection in addition to COVID-19 (*i.e.*, co-infection). The NP swabs were collected using a standardized protocol to ensure the consistency and reliability of the samples. All protected health data (PHI) of the patients in our cohort were anonymized and securely stored to maintain confidentiality. PHI was obtained through chart review (*e.g.*, clinical variables) to acquire additional patient variables for detailed analyses of the relationship between certain SARS-CoV-2 variants and disease severity. Disease severity was classified for COVID-19 patients per the CDC COVID-19 treatment guidelines (*i.e.,* asymptomatic, mild, moderate, severe, and critical illness categories).[11]

3.3.2 Specimen Library Preparation and Sequencing

Sequencing libraries for genotyping SARS-CoV-2 in wastewater and NP Swabs were prepared as previously described by our group.[12,13] Briefly, a combination of Next-Generation Sequencing (NGS), library preparation, and bioinformatics analyses. RNA was linearly amplified into dsDNA, sheared, and ligated to Illumina-compatible sequencing adapters with the QIAGEN QIAseq FX Single Cell RNA Library Kit. These PCR amplicons were sequenced as 2 x 151. These libraries were then enriched for SARS-CoV-2 sequences with an Arbor Biosciences library enrichment kit and SARS-CoV-2 specific enrichment probes. These libraries were sequenced as 2 x 60. These libraries were sequenced as paired reads with the NextSeq 2000 P2 100-cycle sequencing kit. Notably, decreasing coverages were noted in wastewater samples obtained between August 2022 and November 2022 (and select samples acquired before August 2022); thus, these were processed with the QIAGEN QIAseq DIRECT SARS-CoV-2 kit

according to manufacturer instructions and sequenced as paired reads with a NextSeq 2000 P1 300 cycle sequencing kit (described in *Limitations*). Depending on kit availability, RVP samples were sequenced as 2 x 151 or 2 x 60 for the identified samples as previously described.[14]

To identify other pathogens, samples were treated with DNase I (QIAGEN, Inc., Germantown, MD, USA) for 30 minutes (RT), concentrated (RNeasy Minlute spin columns; QIAGEN, Inc., Germantown, MD, USA), and converted into Illumina-compatible sequencing libraries according to the manufacturer's protocols (Illumina, Inc.). RNA was first denatured (Elute, Prime, Fragment High Concentration Mix [EPH3]; 5 min, 65 °C). Hexamer-primed RNA fragments were reverse-transcribed to produce first-strand complementary DNA (cDNA), and second-strand synthesis was performed. AMPure XP beads were used to clean up the cDNA. Tagmentation was performed, followed by PCR amplification (16 x cycles) and incorporation of adapters (Index 1 [i7] and Index 2 [i5]; Illumina, Inc.). Samples were cleaned (AMPure XP beads), and cDNA was normalized into one-plex samples (overnight hybridization at 58 °C). Enrichment pools were quantified and normalized (High Sensitivity D1000 ScreenTape® and TapeStation Analysis Software v3.2). Sequencing was performed using an Illumina NextSeq mid-output (2 x 75) or NextSeq 2000 P2100 cycle (2 x 50), and generated FASTQs were subjected to secondary NGS analyses.

Total RNA from NP Swabs was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), and a direct-zol RNA extraction kit (Zymo Research) was used according to the manufacturer's recommendations. Relative quantification of viral genomic copies was

performed (TaqPath) using N1 primer probes RT-qPCR assay (Thermo Fisher Scientific, Waltham, MA, USA). To quantify SARS-CoV-2 viral copies in the virus preparation, 5 µL of extracted total RNA was evaluated against a standard curve (tenfold serial dilutions of Wuhan-Hu-1 genomic RNA (BEI Resources, catalog #NR-52727; 2.0×10^8 genome equivalents/mL).

3.3.3 Bioinformatics and Data Analysis

We utilized our publicly available specialized bioinformatics pipeline to analyze the Illumina sequencing generated single-end (SE) or paired-end (PE) FASTQ files. A GitHub page of the pipeline provides information regarding its setup and other details (https://github.com/Nevada-Bioinformatics-Center/snakemake_freyja_covidwastewater). Briefly, The pipeline starts by trimming the reads using fastp to remove poor-quality bases and adapter contamination, followed by read classification using Kraken2 with its standard taxonomic database to assess the quality and content of organisms sequenced in the samples (OpenGene, https://github.com/OpenGene/fastp; kraken2, https://github.com/DerrickWood/kraken2). The trimmed reads are then mapped to the Wuhan-Hu-1 (MN908947.3) reference using minimap2, and the resulting BAM files are assessed for quality control (minimap2, https://github.com/lh3/minimap2; Qualimap, http://qualimap.conesalab.org/). A combined QC report of all samples is then generated (MultiQC, https://github.com/ewels/MultiQC). Freyja runs on each sample to recover the relative lineage abundances from the mixed SARS-CoV-2 samples using the mapped BAM files (https://github.com/andersen-lab/Freyja). An aggregated report is generated,

which includes viral concentration data and the relative abundances of each SARS-CoV-2 variant. This report is then used to visualize and compare the data across different sites over time and to assess the correlations between viral concentration and the relative abundances of different SARS-CoV-2 variants.

The presence of pathogens was analyzed using an open-source IDseq pipeline (v3.7, https://czid.org/).[15] Briefly, the pipeline performs subtractive alignment of the human genome (NCBI GRC h38; STAR v2.5.3).[16] Quality filtering and subsequent removal of cloning vectors and phiX phage are then performed (Bowtie2, v2.3.4).[15] The identities of microbial reads are then queried and compared against the NCBI nucleotide (NT) database (GSNAP-L).[15,17] Following background correction and filtering, taxonomic alignments per sample are aggregated and sorted by abundance, measured in NT reads per million (NT-rPM).

Additional statistical analyses were performed using Prism 10.0 software (GraphPad Inc., San Diego, CA, USA), and *p*-values were calculated using 2-way ANOVA. A *p-value* of $p < 0.05$ was considered significant (*), and a $p < 0.01$ was considered highly significant (**). Descriptive statistics were computed for the data, summarizing key metrics. The datasets underwent further cleaning (*e.g.*, correcting errors, removing duplicates, and standardization). The Wastewater data included an additional column for abundance, treated as a weight variable. A time-series analysis was performed to investigate the date of first appearance at 5 % prevalence and the number of days the top 5 variants took from 5 % to 50 % prevalence. The distribution of clades between wastewater and County data was compared, focusing on the top 5 clades.

3.3.4 Ethics Approval

Deidentified human specimens (NP swabs) were used to extract viral RNA. All the experiments were done under the guidelines set forth by the University of Nevada, Reno Institutional Review Board (IRB) and under federal regulations set forth under federal Health Insurance Portability and Accountability Act (HIPAA) law.

3.4 Results

3.4.1 COVID-19 NP Swab Cohort

A total of 1,686 participants were included in our analysis (**Table 1**). The distribution across age categories included 228 pediatric (0-18 years), 1,162 adults (19-64 years), and 358 elderly (≥ 65 years) individuals. The average age was 8.3 years for pediatrics (standard deviation [SD] = 5.2), 40.04 years for adults (SD = 13.4), and 74.5 years for the elderly (SD = 6.8). Regarding gender, females constituted 42 % (n = 95) of pediatrics, 62 % (n = 725) of adults, and 57 % (n = 204) of the elderly. Males were 58 % (n = 133) of pediatrics, 38 % (n = 437) of adults, and 43 % (n =154) of elderly cases.

Table 1. COVID-19 Cohort Demographics: RRMC Biorepository Cohort

	Age Categories					
Total (n = 1,748)	**Pediatric (0 - 18, n = 228)**		**Adult (19 - 64, n = 1,162)**		**Elderly (≥ 65, n = 358)**	
Age, years (mean, SD)	8.3	5.2	40.04	13.4	74.5	6.8
Sex						
Female (n, %)	95	42 %	725	62 %	204	57%
Male (n, %)	133	58 %	437	38 %	154	43%
Comorbidities						
Respiratory (n, %)	17	8.25 %	173	17.64 %	46	19.01 %
Neurological (n, %)	14	6.80 %	173	17.64 %	32	13.22 %
Gastrointestinal (n, %)	13	6.31 %	173	17.64 %	63	26.03 %
Reproductive (n, %)	20	9.71 %	61	6.22 %	6	2.48 %
Metabolic (n, %)	5	2.43 %	337	34.35 %	148	61.16 %
Cardiovascular (n, %)	5	2.43 %	189	19.27 %	132	54.55 %
Autoimmune (n, %)	2	0.97 %	39	3.98 %	20	8.26 %
Neoplastic (n, %)	0	0.00 %	33	3.36 %	46	19.01 %
Renal (n, %)	1	0.49 %	44	4.49 %	26	10.74 %
Disease Severity						
Asymptomatic (n, %)	13	6.31 %	95	9.68 %	25	10.33 %

Mild illness (n, %)	179	86.89 %	774	78.90 %	167	69.01 %
Moderate illness (n, %)	12	5.83 %	133	13.56 %	39	16.12 %
Severe illness (n, %)	1	0.49 %	7	0.71 %	8	3.31 %
Critical illness (n, %)	1	0.49 %	3	0.31 %	2	0.83 %
Deceased (n, %)	1	0.49 %	0	0.00 %	1	0.41 %

Comorbidities were observed across the cohort with Respiratory conditions in 17 pediatric cases (8.25%), 173 adults (17.64%), and 46 elderly (19.01%); Neurological issues were present in 14 pediatrics (6.80%), 173 adults (17.64%), and 32 elderly (13.22%); Gastrointestinal problems were found in 13 pediatrics (6.31%), 173 adults (17.64%), and 63 elderly (26.03%); Reproductive health issues were reported in 20 pediatrics (9.71%), 61 adults (6.22%), and 6 elderly (2.48%); Metabolic disorders were seen in 5 pediatric cases (2.43%), 337 adults (34.35%), and 148 elderly (61.16%); Cardiovascular conditions were identified in 5 pediatrics (2.43%), 189 adults (19.27%), and 132 elderly (54.55%); Autoimmune diseases were recorded in 2 pediatric cases (0.97%), 39 adults (3.98%), and 20 elderly (8.26%); Neoplastic conditions were found in 33 adult cases (3.36%) and 46 elderly individuals (19.01%); Renal pathologies were observed in 1 pediatric case (0.49%), 44 adult cases (4.49%), and 26 elderly (10.74%).

There were 13 pediatric cases (6.31%) with asymptomatic symptoms as well as 95 adults (9.68%) and 25 elderly (10.33%); Mild severity was the most common with 179 pediatric cases (86.89%), 774 adults (78.90%), and 167 elderly (69.01%); Moderate severe was seen in 12 pediatric cases (5.83%), 133 adults (13.56%), and 39 elderly (16.12%); Severe disease was least common with 1 pediatric case (0.49%), 7 adult cases (0.71%), and 8 elderly cases (3.31%); Critical disease was seen in 1 pediatric case

(0.49%), 3 adults (0.31%), and 2 elderly (0.83%). There were 2 deceased cases in our cohort (1 pediatric [0.49 %] and 1 elderly [0.41%]).

The top 10 most prevalent SARS-CoV-2 variants were identified from the cohort through NGS analysis (**Figure 1**). The respective proportions of the variants include BA.2.12.1 (21.2 %), BA.2 (13.2 %), BA.5.5 (10.7 %), BA.5.2.1 (9.3 %), BA.5 (6.1 %), XBB 1.5 (5.2 %), BQ.1.1 (4.4 %), BA.5.1 (4 %), and BA.5.2 (3.6 %). Undetermined variants (25.1 %) constituted those not accounted for in the top 10 most frequent. These were noted and used to compare these prevalent variant frequencies with the WBE data.

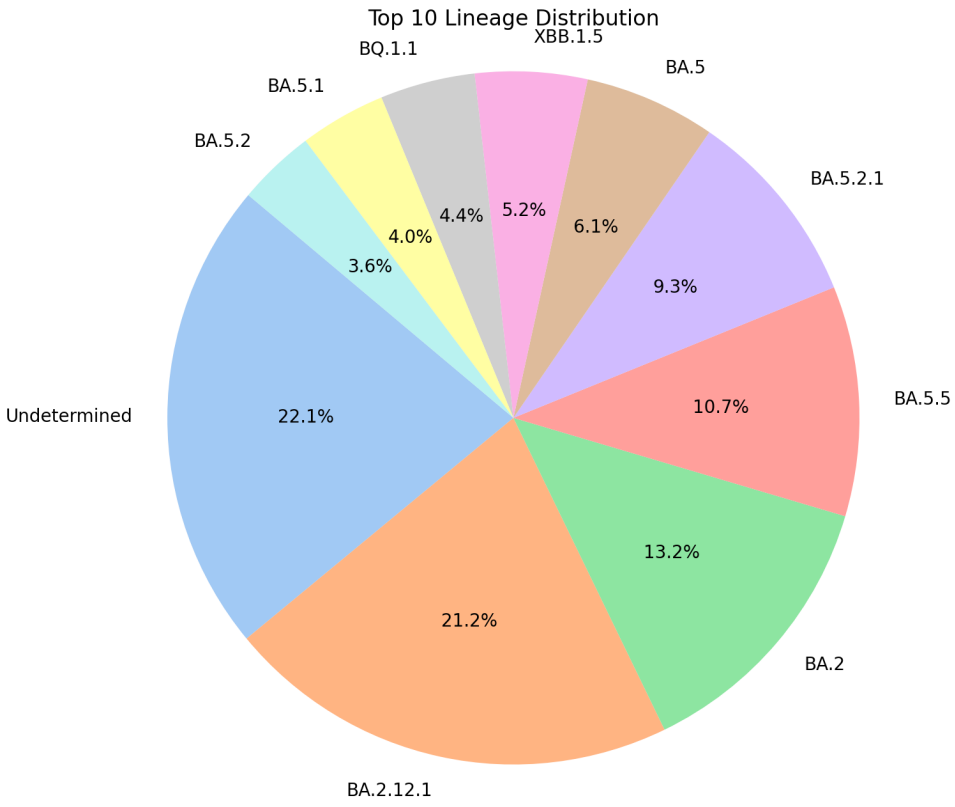

Figure 1. Top 10 SARS-CoV-2 Variants from NP Swabs. The percentages indicate the proportion of each lineage of the top 10. Undetermined variants are those not in the top 10 most frequent.

3.4.2 WBE SARS-CoV-2 Analysis: Update

We obtained and re-processed new influent samples from TMWRF to add data to our prior study between November 2022 and March 2023.[10] The analysis of the SARS-CoV-2 variants found in the wastewater revealed new variants, as well as recent variants with significant (>50 %) abundances (*e.g.,* XBB.1.5.X) (**Figure 2**).

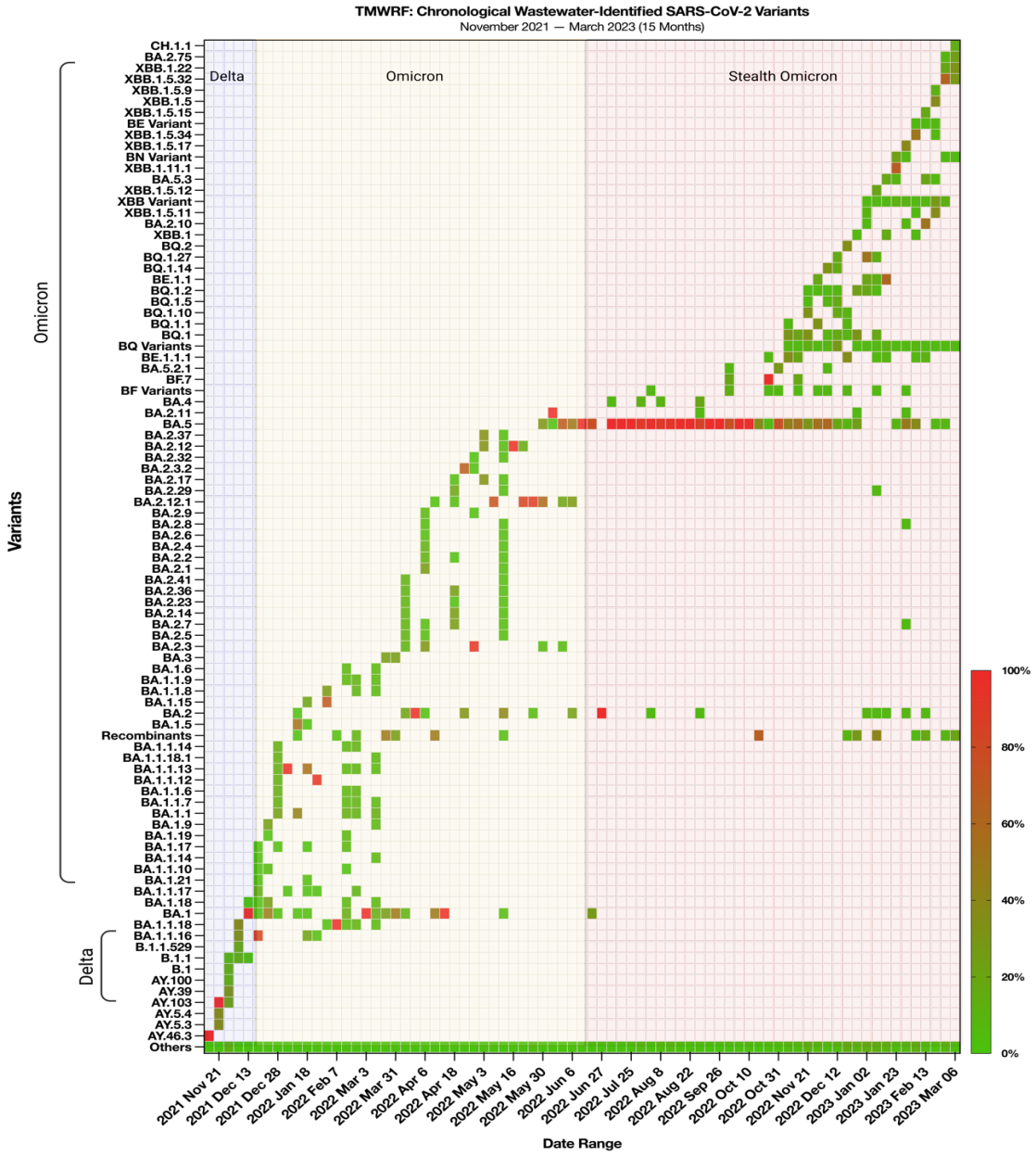

Figure 2. Chronological Detection of SARS-CoV-2 Variants in TMWRF Sewershed. Influents were collected from November 2021 to March 2023. The relative proportion of variants was determined by Freyja, a SARS-CoV-2 variants analysis pipeline (https://github.com/andersen-lab/Freyja). *TMWRF geographic location is represented in Chapter 2**, Figure 4.**

3.4.3 Correlation of Variants with Disease Severity

Data used to rate COVID-19 severity were obtained from patients' charts and subsequently computed into 5 categories rated as follows: Asymptomatic (0), Mild (1), Moderate (2), Severe (3), and Critical (4) (**Figure 4**). Our analysis showed a significant association between specific SARS-CoV-2 variants and disease severity. The variants most prevalent in critical COVID-19 were BA.2.75, XBB, BA.2.12.1, BA.4, BA.5, BXX.1.5, BQ.1.11, BA.2.29, BA.2.21, and BQ.1.13.1 (**Figure 4A**). These variants were associated with a higher risk of severe disease, particularly in elderly patients and those with pre-existing underlying conditions.

In terms of the distribution of disease by patients' age, asymptomatic disease severity was seen in 6.31 % (n = 13) of pediatrics, 9.68 % (n = 95) in adults, and 10.33 % (n = 25) in the elderly (**Figure 4B**). Mild disease severity was seen in 86.89 % (n = 179) of pediatrics, 78.90 % (n = 774) of adults, and 69.01 % (n = 167) of the elderly. Moderate disease severity was experienced in 5.83% (n = 12) of pediatrics, 13.56 % (n = 133) of adults, and 16.12 % (n = 39) in the elderly. Severe disease severity was reported in 0.49 % (n = 1) of pediatrics, 0.71% (n = 7) of adults, and 3.31 % (n = 8) in the elderly. Critical illness was reported in 0.49 % (n = 1) of pediatrics, 0.31 % (n = 3) of adults, and 0.83 %

(n = 2) in the elderly. Mortality occurred in 1 pediatric case (0.49 %) and 1 elderly case (0.41 %).

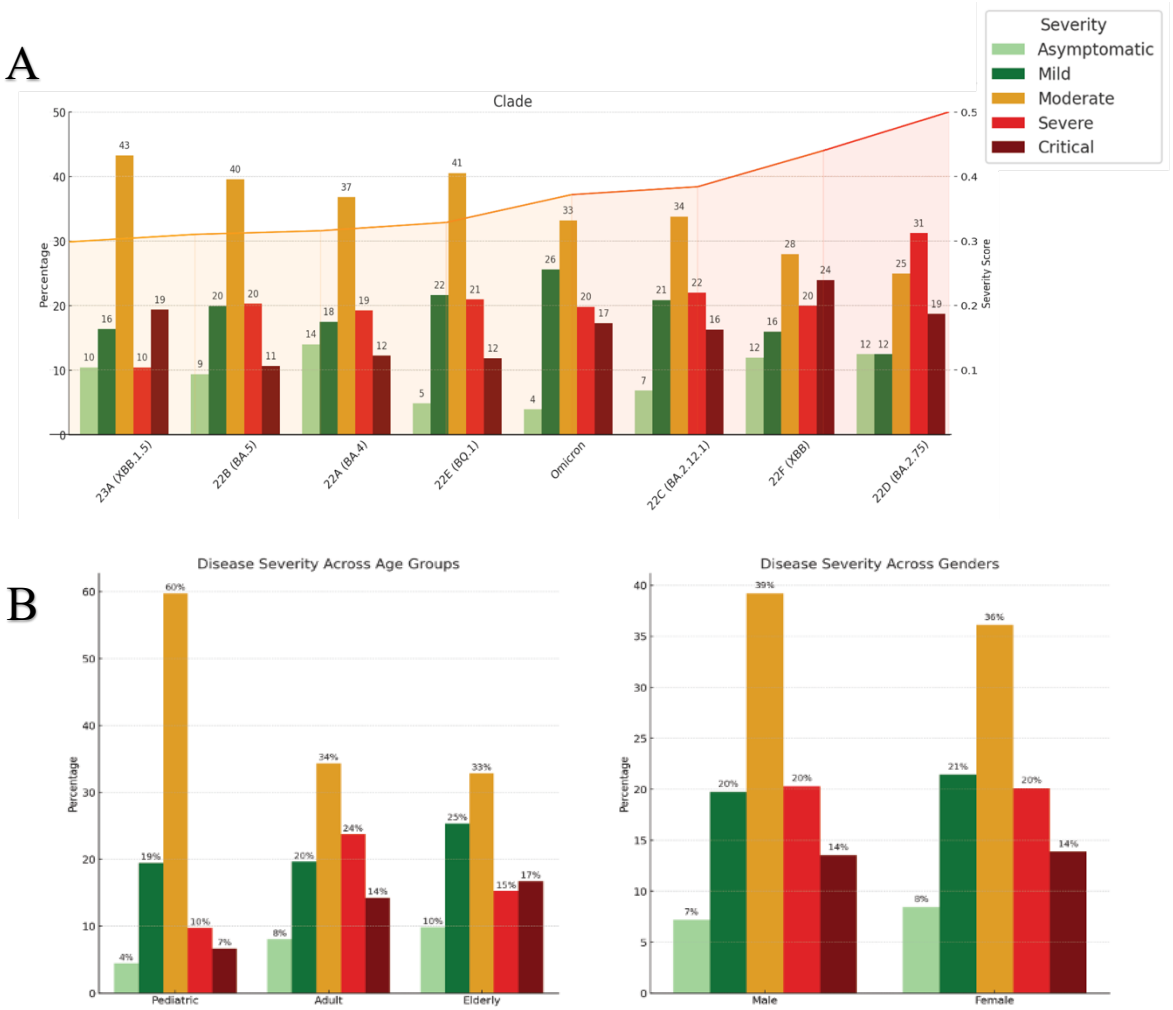

Figure 4: Association Between SARS-CoV-2 Variants Worsening Overall Disease Severity. Graph A shows the risk of severe disease associated with the most common SARS-CoV-2 variants arranged in the order of mean disease severity. The severity line indicates hazard ratio (HR), with increasing values indicating an increased overall disease severity. COVID-19 severity by age was also plotted (B). The cohort was divided into 3 separate age categories (pediatric, < 18 years; adult, 18-65; elderly, 65+). *Disease severity was calculated based on symptom profiles (qualitative) in patient charts and computed into 5 categories for quantification: Asymptomatic, 0; Mild, 1; Moderate, 2; Severe, 3; and Critical, 4.

3.4.4 Association of Comorbidities and Severity

Regarding the individual preexisting comorbidities seen in our cohort, respiratory issues were present in 8.25 % (n = 17) of pediatric participants, 17.64 % (n = 173) of adults, and 19.01 % (n = 46) of elderly participants. Neurological conditions were found in 6.80% (n = 14) of pediatric participants, 17.64 % (n = 173) of adults, and 13.22 % (n = 32) of the elderly. Gastrointestinal problems were present in 6.31 % (n = 13) of the pediatric group, 17.64 % (n = 173) of adults, and 26.03 % (n = 63) of elderly individuals. Reproductive issues affected 9.71 % (n = 20) of pediatric participants, 6.22 % (n = 61) of adults, and 2.48 % (n = 6) of the elderly. Metabolic conditions were found in 2.43 % (n = 5) of pediatric participants, 34.35 % (n = 337) of adults, and 61.16 % (n = 148) of the elderly. Cardiovascular issues affected 2.43 % (n = 5) of pediatric participants, 19.27 % (n = 189) of adults, and 54.55 % (n = 132) of the elderly. Autoimmune conditions were present in 0.97 % (n = 2) of the pediatric group, 3.98 % (n = 39) of adults, and 8.26 % (n = 20) of elderly individuals. Neoplastic conditions were found in 0.00 % (n = 0) of pediatric participants, 3.36 % (n = 33) of adults, and 19.01 % (n = 46) of the elderly. Renal issues affected 0.49 % (n = 1) of pediatric participants, 4.49 % (n = 44) of adults, and 10.74 % (n = 26) of the elderly. In this study, there was about a two-fold increase in the frequency of severe disease between zero and two or more comorbidities (**Figure 5**)

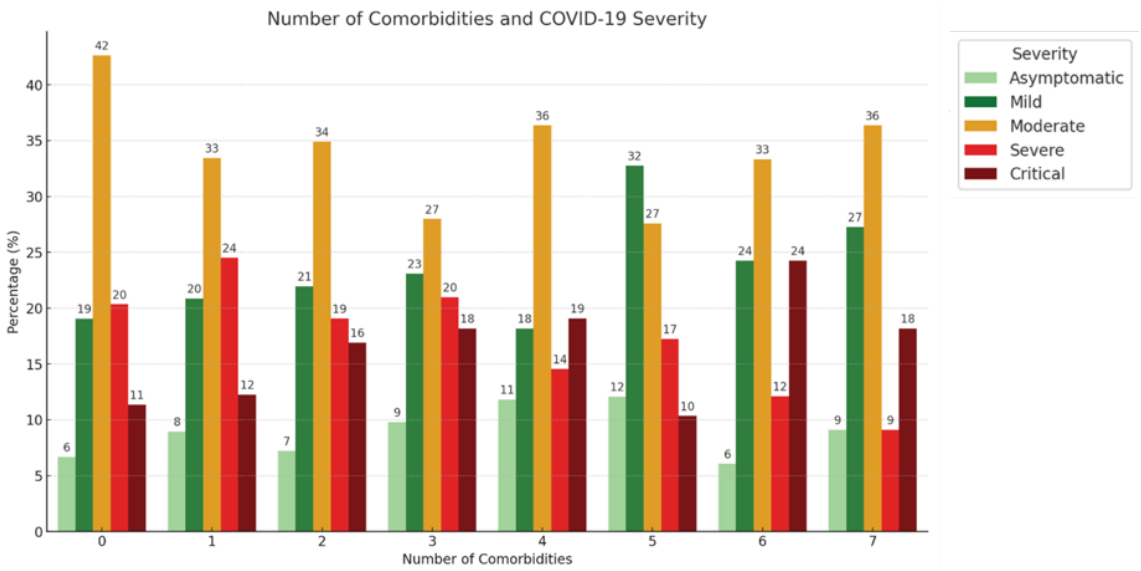

Figure 5. Association of the Number of Comorbidities and Disease Severity. The number of increasing comorbidity counts are in ascending order. COVID-19 disease severity is represented based on the category of severity per comorbidity count. Total case counts (n) are indicated on top of each disease severity per comorbidity.

*Disease severity was calculated based on symptoms (qualitative) and computed into 5 categories for quantification: Asymptomatic, 0; Mild, 1; Moderate, 2; Severe, 3; and Critical, 4.

3.5 Discussion

3.5.1 Understanding WBE Findings

Our group has been tracking SARS-CoV-2 variants in the community by sequencing COVID-19-positive NP swabs obtained locally, as well as assessing samples obtained via wastewater-based epidemiology (WBE) studies in partnership with Environmental Science and Engineering colleagues Pagilla et al.[10,12,13,18-21] Through these efforts, our group has made several significant discoveries, including the identification of the first case of SARS-CoV-2 acute re-infection in a healthy adult, WBE-based earlier detection of circulating SARS-CoV-2 variants (compared to clinical case identification),[10] and identification of significant SARS-CoV-2 viral copies year-round (compared to other respiratory viruses).[10,19,22-25] However, the prevalence and dynamics of all SARS-CoV-2 variants remained to be elucidated. WBE has already emerged as a powerful surveillance tool for tracking the spread of SARS-CoV-2 in the community, offering the advantage of representing a large population regardless of individual testing rates.

The findings in this present study underscore the importance of considering other factors (*e.g.*, population immunity, social behavior, and public health interventions) in understanding the dynamics of SARS-CoV-2 transmission and variant distribution. These insights are valuable for public health authorities in designing interventions and strategies to control the continued spread of SARS-CoV-2. However, it is important to note that the impact of new variables on SARS-CoV-2 dynamics is still warranted to fully understand the complex interplay between these factors in shaping the continuing course of the

COVID-19 pandemic. Our study provides critical insights into the relationship between specific SARS-CoV-2 variants and disease severity.

The WBE findings offer a macroscopic vantage point of the pandemic's progression and provide a preemptive glimpse into variant trends that may not yet be evident in clinical settings. The lag times observed suggest that WBE could serve as a cornerstone of early pandemic response, offering vital lead time for healthcare systems to brace for potential surges in specific variants. We found a significant correlation between variant prevalence in wastewater and clinical cases, suggesting that increases in variant prevalence in wastewater usually precede clinical cases with that viral variant. These findings have important implications for public health strategies. Early detection of emerging variants through WBE could allow for more proactive measures to control the spread of these variants. Furthermore, understanding the association between specific variants and disease severity could inform treatment strategies and vaccine rollouts.

3.5.2 Understanding Data from Clinical Specimens

The clinical data paint a more granular picture, allowing us to discern patterns and associations at the individual level. The distinct association between certain SARS-CoV-2 variants and increased disease severity reinforces the need for continued genomic surveillance and personalized medicine approaches in managing COVID-19. Our findings indicate that certain variants are significantly associated with disease severity, especially among elderly patients with pre-existing conditions. This pattern suggests that

these variants may have evolved to become more virulent; moreover, these results may suggest that these cardiovascular patient populations are particularly vulnerable to severe disease. Our study elucidates the complex relationship between SARS-CoV-2 variants and COVID-19 severity. The significant associations between specific variants and increased severity, especially in the elderly and those with specific and increased comorbidities, should be noted. Since our WBE data effectively identified emerging variants, its utility can be used to aid in public health surveillance and early detection and action programs.

Certain underlying conditions, such as cardiovascular abnormalities and obesity, have already been reported to increase the risk of severe illness in pediatric COVID-19 patients.[26] Understanding the different factors associated with disease severity in pediatric COVID-19 patients is crucial for providing appropriate care and developing preventive strategies.[26,27] Further research is needed to explore the long-term effects of COVID-19 in children and assess vaccination's effectiveness in preventing severe illness and long-term complications.[28] In the early stages of the pandemic, children were thought to be at low risk for severe COVID-19, but it has become evident that children can also experience severe illness and death following infection.[26] COVID-19 in pediatric populations can lead to severe and critical symptoms, including pneumonia, acute respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC), critical inflammatory disease, and multi-organ failure.[26] Several SARS-CoV-2 variants have been associated with an increased risk of severe illness in children.[29]

NGS of coronaviruses SARS-CoV-2-infected patients allows for the identification of mutations in critical genes.[29] These sequencing techniques have been used to identify key mutations in viral spike glycoprotein (S) genes and variations in human genes such as ACE-2R, TLR-3, and RNA-dependent RNA polymerase (RdRp).[29-31] Unique mutations in the RNA-dependent (RdRp) gene of SARS-CoV-2 highlight the importance of genomic surveillance in tracking the evolution of the virus.[29] Several non-genetic factors have been associated with severe illness in pediatric COVID-19 patients. A study conducted in the United States found that age over 12 years, obesity, type 1 diabetes, and cardiac and circulatory congenital anomalies were associated with a higher risk of hospitalization.[32] Another study reported that strokes and comorbidities were risk factors for mortality in pediatric COVID-19 patients.[33] It is crucial to identify these risk factors to better understand disease severity and provide appropriate care for pediatric patients. This computational technology plays a crucial role in understanding the genetic variations of the virus and their potential impact on disease severity.[30,31]

The evidence of increased virulence in certain variants, particularly among elderly patients with certain underlying conditions, resonates with the global narrative of COVID-19's impact on vulnerable populations.[34] This has been a focal point of concern for health authorities worldwide, underscoring the necessity for prioritizing these populations in vaccination rollouts and booster campaigns. Recognizing severe pediatric cases has prompted a re-evaluation of risk profiles and has reinforced the importance of inclusive vaccination programs that protect all age groups.[35] Our findings contribute to a more nuanced understanding of pediatric risk and the urgent need for tailored clinical

management and preventive strategies for children. Genomic analyses of wastewater have been instrumental in detecting the entry of described lineages or Variants of Concern (VOCs) into populations, characterizing new outbreaks, and aiding in viral strain tracking.[36] This has prompted a re-evaluation of risk profiles and reinforced the importance of inclusive vaccination programs that protect all age groups.[37]

Moreover, our findings contribute to growing evidence suggesting that personalized medicine should be central to future pandemic responses, allowing for tailored strategies that consider individual genetic and health profiles. Exploration of non-genetic factors such as age, obesity, and existing comorbidities in disease severity provides valuable epidemiological data that can improve public health strategies globally.[38] By understanding these factors, health systems can better allocate resources, prioritize high-risk groups, and implement effective interventions to mitigate the impact of COVID-19. These findings reinforce the importance of continued research in the face of an evolving pathogen and demonstrate the critical role of data sharing and collaboration across borders.[38] Informing international databases, contributing to a centralized repository of genomic information that can expedite the development of vaccines and therapeutics. This genomic surveillance is critical for understanding the current pandemic and preparing for future zoonotic spillovers.[34] As we move forward, it is essential that the global scientific community continues to leverage computational technologies to understand the genetic underpinnings of SARS-CoV-2 and its implications for disease severity, vaccine development, and public health policymaking.

3.5.3 Insights into Vaccination/Booster Effect

While our study did not directly measure vaccine efficacy, the observed trends in disease severity across different variants offer indirect insights into vaccine performance. As newer variants emerge, real-world vaccine effectiveness studies become even more crucial for guiding booster shot formulations and immunization schedules. Following the second dose of mRNA vaccines, such as the Pfizer (BNT162b2) and Moderna (mRNA-1273) vaccines, efficacy rates of the Pfizer and Moderna vaccines of 100 % and 93.3 % have been reported in adolescents and children, respectively.[26,39,40] Systemic events, such as injection site pain, fatigue, and headache, were more frequently reported after the second dose of an mRNA vaccine. Still, adverse events were generally mild and moderate in severity and short-lived.[26,39,40] The current data suggest that approximately 68 % of children with COVID-19 have mild or no symptomatic presentation, 8 % present with severe symptoms, and 4 % require Intensive Care Unit (ICU) care.[26] The most common symptoms in children with COVID-19 include fever, cough, and nasal symptoms.[41] Additional symptoms may include diarrhea, headache, rhinorrhea, sore throat, and vomiting.[41] Children with congenital heart disease and chronic lung conditions may also be at higher risk of severe illness.[26,42] It is essential to consider these underlying conditions (*e.g.*, cardiovascular abnormalities, obesity, diabetes mellitus type 1, and diseases of white blood cells) when assessing the risk of severe illness in pediatric COVID-19 patients.

3.5.4 Limitations

The limitations of our study highlight the inherent challenges of pandemic research, where the rapid evolution of pathogens and changing public health landscapes can outpace data collection and analysis. Future studies will benefit from more dynamic data collection methods and real-time analytics to keep pace with the ever-evolving nature of the SARS-CoV-2 virus. Further, our study was confined to a specific geographic region, potentially limiting the generalization of our findings. Second, our reliance on retrospective patient data and only weekly wastewater samples may introduce limitations; thus, the data may only partially capture chronological events. Additionally, the rapidly evolving nature of the SARS-CoV-2 virus and the changing pandemic may mismatch with early correlations and findings. Both clinical situations and therapeutic guidelines have also changed since data collection for this study began. It is important to acknowledge that the study's retrospective nature limits the ability to establish causality. The study's sample size may also impact the generalization of the findings. Further research is needed to validate and expand upon these findings.

3.5.5 Future Work

Future research initiatives focusing on extending our surveillance to a wider geographic area, incorporating real-time data analytics, and exploring the long-term effects of COVID-19 are needed. Interdisciplinary collaborations will be essential for developing comprehensive models incorporating social, environmental, and biological

factors to predict and manage future pandemics. Future studies could incorporate additional variables (*e.g.,* air quality, geographic region, state funding*, etc.)*, which could also influence the transmission dynamics of SARS-CoV-2. Comprehensive datasets involving multiple geographical locations would be beneficial in validating and extending our findings. Integrating WBE data with other epidemiological and public health data can also provide a more holistic understanding of the social factors influencing the spread of SARS-CoV-2. Applying advanced machine learning techniques may help identify complex patterns and interactions between certain variables and SARS-CoV-2 dynamics and severity. Whether there is a significant link between certain factors and post-COVID (*i.e.*, PASC/long-COVID) remains to be investigated.

3.6 Conclusion

Wastewater data was a leading indicator for changes in viral variants observed in Washoe County. These findings have substantial implications for public health strategies, particularly for high-risk individuals, and may influence future vaccine development.[43] The far-reaching implications allow for developing and improving public health strategies, particularly for individuals at high risk. For instance, it was initially believed that children had a low risk of experiencing severe COVID-19 symptoms; however, it has now been observed, in our results as well as other studies, that children can also experience severe illness and death following infection.[26] Computational approaches aid understanding of COVID-19 and can guide the development of preventive strategies. Further computational research is likely to shed light on long-term COVID/PASC.

Our findings underscore the utility of WBE as a surveillance tool for capturing SARS-CoV-2 evolution and transmission, which can guide public health response strategies.[43] This study advocates for integrating WBE in standard public health surveillance, highlighting its potential in guiding preemptive measures and resource allocation. The findings also prompt a re-evaluation of clinical management strategies, as the associations between variants and severity could influence treatment pathways. As we advance, computational biology and data analytics will remain at the forefront of pandemic research, shaping our responses to future global health challenges. The findings from this study support and encourage monitoring SARS-CoV-2 variants on a large scale to allow for early detection and alerting of potential shifts in pathogens to alert developments in health outcomes.

3.7 Appendix

3.7.1 Data Availability Statement

The wastewater data are submitted as BioSamples to NCBI Genbank under BioProject ID PRJNA772783 (https://www.ncbi.nlm.nih.gov/bioproject/772783). Specific patient datasets generated and analyzed during the current study are available upon reasonable request and governed as protected health information (PHI) following federal Health Insurance Portability and Accountability Act (HIPAA) regulations.

3.7.2 Acknowledgments

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3.7.3 Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 4

4. REACTIVATION OF LATENT HERPESVIRUSES BY SARS-COV-2 INCREASES COVID-19 SYMPTOMS – A COHORT STUDY

4.1 Abstract

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the causative agent of Coronavirus Disease 2019 (COVID-19), a respiratory illness that has caused a global pandemic. The spectrum of symptoms observed in SARS-CoV-2 infected individuals ranges from no symptoms to critical illness and death. There remains debate as to whether the latently persisting viruses (*e.g.,* human herpesviruses [HHVs]) impact COVID-19 disease severity. We sought to determine whether SARS-CoV-2-infection triggered the reactivation of HHVs, which may have contributed to increased symptom severity in COVID-19 infection, addressing the question through a retrospective cohort study. We analyzed nasopharyngeal swabs collected from 85 Renown Regional Medical Center (Reno, NV) patients from June 2020 through June 2022. RT-qPCR and RNA-Seq analyses assessed SARS-CoV-2 and HHV infections, respectively. The predominant SARS-CoV-2 variant was used as the feature to classify the COVID-19 patient cohorts: Wuhan (Wuhan-Hu-1), Delta (B.1.617.2), and Omicron (BA.1). RNA-Seq metagenomic analyses showed SARS-CoV-2, as expected. Some specimens also showed significant frequencies of other respiratory viruses (*e.g., Influenza A*) and bacteria (*e.g., Klebsiella pneumoniae*). Analysis of the key HHV genes by transcriptome mapping revealed elevated levels of viral BZLF1 lytic genes in severe/critically ill patients. Analysis of the cellular genes through RNA-Seq identified 234 differentially expressed genes in HHV-positive COVID-19 patients. These upregulated genes were associated with multiple functions, including regulation of immune responses, cell adhesion, response to hypoxia, RNA metabolism, and processing, RNA splicing and translation, DNA repair, and

replication. Furthermore, the SARS-CoV-2 *in vitro* reactivation of key immediate-early lytic genes using EBV- and KSHV-harboring cells supports a link between SARS-CoV-2 infection and HHV reactivation *in vivo*. Collectively, these results indicate that reactivation of HHVs could increase disease severity during COVID-19.

4.1.1 Keywords

HHVs, SCoV2, PASC, ARDS, NGS, RNA-Seq, Long-COVID, Lytic Reactivation

4.2 Introduction[1]

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent for the coronavirus disease 2019 (COVID-19) pandemic, has resulted in a massive health crisis, responsible for more than 6.9 million global deaths and an infection prevalence surpassing 766 million people (as of Aug 20th, 2023).[1] The clinical manifestations of COVID-19 infection are highly variable, ranging from asymptomatic cases to severe respiratory failure (*i.e.,* acute respiratory distress syndrome, ARDS) and even death, particularly in vulnerable populations such as infants, the elderly, and those with pre-existing comorbidities.[2,3] Despite the widespread impact of COVID-19, the root causes of the extensive clinical variability remain largely unclear. However, numerous clinical reports indicate a correlation between worsened COVID-19 severity and the presence of underlying conditions (*e.g.,* diabetes, hypertension, heart failure, asthma, cystic fibrosis, cardiovascular disease, obesity).[4] Moreover, chronic viral illness or infection (*e.g.,* herpesviruses [HHVs]) have recently been identified as a risk factor for severe illness in COVID-19.

HHVs are a group of eight viruses with a large, linear, double-stranded DNA genome, which are ubiquitous in the global human population.[5,6] These viruses have a

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[1] **Abbreviations**
ARDS, Acute Respiratory Distress Syndrome; COVID-19, Coronavirus Disease of 2019; DE, Differentially Expressed; dsDNA, Double-Stranded DNA; EBV or HHV-4, Epstein–Barr Virus; GO, Gene Ontology; HCMV or HHV-5, Cytomegalovirus; HIF-1α, Hypoxia-Inducible Transcription Factor-1α; HHV, Human Herpesvirus; HSV-1, Herpes Simplex Virus Type 1; HSV-2, Herpes Simplex Virus Type 2; KEGG, Kyoto Encyclopedia of Genes and Genomes; KSHV or HHV-8, Kaposi's Sarcoma-Associated Herpesvirus; log FC, Logarithmic Fold Change; NGS, Next Generation Sequencing; NP, Nasopharyngeal Swab; PFUs, Plaque-Forming Units; QC, Quality Control; RT-qPCR, Reverse Transcription-Quantitative Polymerase Chain Reaction; RVP, Respiratory Viral Panel; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; VZV or HHV-3, Varicella-Zoster Virus.

biphasic life cycle, alternating between latent (silent) and lytic (reactivation) phases. While HHVs can persist in the host in their latent phase, various stressors (*e.g.,* immunosuppression, inflammatory disorders, and other pathogenic viral infections) can trigger viral reactivation, resulting in elevated expression of viral lytic genes, amplification of viral DNA, production, and release of mature progeny virions, and death of the infected cells.[7,8] Severe COVID-19-associated ARDS is characterized by disrupted lung alveolar cells, altered permeability to cause pulmonary edema, and reduced blood oxygen saturation (SpO2 < 90 %, hypoxia),[9,10] The consequential lung hypoxia triggers the production of hypoxia-inducible transcription factor-1α (HIF-1α). This HIF-1α activation, in turn, activates the release of pro-inflammatory cytokines (*e.g.,* IL-6, TNF-α, and IL-1β), leading to a cytokine storm.[11,12] The HIF-1α-driven cytokine storm has been identified as a key for lytic reactivation of HHVs in infected patients.[13,14]

Recent clinical reports suggest that the hypoxia-driven cytokine storm may play a role in the lytic reactivation of HHVs in COVID-19 patients regardless of vaccination status.[15-17] However, the impact of reactivated HHVs on the severity of COVID-19 disease remains largely unknown. To address this critical gap in our understanding, we made a retrospective cohort study using a clinically characterized, large, and diverse COVID-19 patient cohort to determine rates of nasopharyngeal coinfection with SARS-CoV-2 and HHVs, correlating coinfections with the severity of COVID-19. We also tested in *vitro* cell culture models to determine whether coinfection with different SARS-CoV-2 variants (Wuhan [Wuhan-Hu-1], Delta [B.1.617.2], and Omicron [BA.1]) could trigger HHV reactivation. Identifying the underlying factors contributing to the extensive

clinical variability observed in COVID-19 patients is crucial, particularly for vulnerable populations. Our study aims to advance our understanding and inform the development of more effective diagnostics and targeted treatments for patients suffering from severe COVID-19 and long-COVID with underlying comorbidities such as chronic viral illness (*e.g.,* HHVs).

We sought to approach this question from multiple angles: i) Using a large (~1,700 patient entries) retrospective cohort study with associated biorepository of next-generation sequenced (NGS) nasopharyngeal (NP)-Swab specimens and associated identified SARS-CoV-2 variant information (Pango lineage, NextStrain Clade, and World Health Organization [WHO] Label),[18] along with electronic medical record (EMR) sourced patient health information (PHI) (*e.g.,* age, gender, ethnicity, comorbidities, vaccination status, symptoms, comorbidities, *etc.*) and notably HHV-positive infection, along with corresponding HHV-negative matched-controls; we also performed ii) *in vitro* Biosafety Level (BSL)-3 cell culture experiments using HHV cell lines (BC-1, KSHV & AGSiZ, EBV) as reactivation models challenged with different SARS-CoV-2 variants (*e.g.,* [Wuhan, Wuhan-Hu-1], [Delta, B.1.617.2], and [Omicron, BA.1]).

Our overall goal was to investigate whether HHVs reactivation is variant-dependent and whether the reactivation alters key cellular pathways that may lead to worsened clinical disease severity. By identifying these underlying factors in patients, particularly those with underlying comorbidities such as chronic viral illness, specifically latent HHV infection, we can inform the development of more effective diagnostics and targeted treatments for preventing severe COVID-19 symptomology. Our study aims to

identify key targets and pathways causing worse COVID-19 symptomology – ultimately advancing our understanding of the complex relationship between SARS-CoV-2-dependant latent-HHV reactivation and subsequent COVID-19 severity.

4.3 Materials and Methods

4.3.1 Nasopharyngeal Swab Specimen Demographics

The NP swab specimens (deidentified) were collected from Renown Regional Medical Center (RRMC, Reno, NV) between June 2020 and June 2022. Briefly, we performed RNA extraction and diagnostic evaluation to identify the SARS-CoV-2 strain (Wuhan [Wuhan-Hu-1], Delta [B.1.617.2], and Omicron [BA.1]). We then performed library sequencing preparation for next-generation sequencing (NGS), differential gene expression, and metagenomic analysis on the specimens. Associated clinical health information was obtained and deposited into our biorepository for analysis of symptomology and underlying comorbidities.

4.3.2 RNA Extraction and RT-qPCR

Viral genomic RNA was detected through reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from mock- and SARS-CoV-2-treated Vero E6 cell supernatants using Trizol reagent (Invitrogen, Carlsbad, CA, USA). According to the manufacturer's recommendations, RNA was extracted from the samples using a direct-zol RNA extraction kit (Zymo Research). Relative quantification of viral genomic copies was performed using TaqPath and N1 primer probes via an RT-qPCR assay (Thermo Fisher Scientific, Waltham, MA, USA) using a 5 μL aliquot of the extracted total RNA. A standard curve was generated using tenfold serial dilutions of

Wuhan-Hu-1 genomic RNA (BEI Resources, catalog #NR-52727; 2.0×10^8 genome equivalents/mL) to quantify SARS-CoV-2 viral copies in the virus preparations.

4.3.3 SARS-CoV-2 Library Preparation and Next-Generation Sequencing

Sequencing libraries for genotyping SARS-CoV-2 virus were prepared as previously described by our group in Hartley et al.[19] Briefly, this process involved converting RNA to double-stranded DNA (dsDNA), shearing the dsDNA into small pieces, and attaching Illumina-compatible sequencing adapters to the pieces using the QIAGEN QIAseq FX Single Cell RNA Library Kit. The resulting PCR amplicons were sequenced using the NextSeq 2000 P2 100-cycle kit in pairs (2 x 151). The libraries were enriched for SARS-CoV-2 sequences using the Arbor Biosciences library enrichment kit and specific enrichment probes and sequenced in pairs (2 x 60). The libraries were sequenced as paired reads with the NextSeq 2000 P2 100-cycle sequencing kit.

4.3.4 Metagenomic Analysis

The presence of co-occurring respiratory pathogens was identified using the open-source IDseq pipeline (czid.org, v3.7) to analyze the respiratory viral panel (RVP).[20] All samples were included in the metagenomic analysis due to high-quality control (QC) pass percentages for the viral genome. Briefly, this pipeline aligns the human genome (NCBI

GRC h38) using STAR (v2.5.3),[21] performs quality filtering. This process removes cloning vectors and phiX phage using Bowtie2 (v2.3.4),[20] and queries the remaining microbial reads against the NCBI nucleotide database using GSNAP-L.[20,22] After background correction and filtering, the retained taxonomic alignments for each sample are aggregated at the genus level, sorted by abundance (measured in nucleotide reads per million [NT-rPM]), and analyzed independently for each sample.

4.3.5 Differential Gene Expression and Pathway Analysis

To evaluate the changes in gene expression from the NP Swab specimens of our COVID-19 patient cohort, we used the Illumina BaseSpace DRAGEN Suite to align the RNA-Seq reads to a reference human genome (Hg38) and quantified the gene expression levels. After quality control (QC) check and verification of appropriate sample cohorts, we identified the top 60 differentially expressed genes between the SARS-CoV-2 sample groups (SARS-CoV-2-positive HHVs-present *vs.* SARS-CoV-2-positive HHVs-absent) (**Table 2**). We used appropriate covariates, such as batch effects and technical replicates, to control for unwanted variation.

We used iPathwayGuide (Advaita Inc. Ann Arbor, MI, USA) to identify enriched pathways for the differentially expressed genes.[23] Briefly, the data are analyzed in the context of pathways obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database,[23] gene ontologies from the Gene Ontology (GO) Consortium

database,[24-26] miRNAs from the miRBase (MIRBASE Version: 22.1,10/18) and TARGETSCAN (Targetscan version: Mouse: 8.0, Human: 8.0) databases,[27] regulatory network relations from BioGRID: Biological General Repository for Interaction Datasets (v4.4.203),[28] and chemicals/drugs/toxicants from the Comparative Toxicogenomics Database.[29] Thresholds in this study included a *p-value* of 0.05 as significant, along with a minimum log fold change of at least 0.4 (absolute). These analyses provide insight into the biological processes that are affected by changes in gene expression and may suggest potential therapeutic targets or pathways that could be targeted to modulate the disease process.[23]

4.3.6 EBV- and KSHV-Harboring Cell Lines

Vero E6 cells were purchased from ATCC and maintained in DMEM medium supplemented with 10 % fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA, USA), 2 mM L-glutamine, 25 U/mL penicillin, and 25 µg/mL streptomycin. The cells were grown at 37 °C and 5 % CO_2 in a humidified chamber. Other HHV-harboring cell lines (EBV-GFP, AGSiZ cells, BC-1, and TRExBCBL-1) were maintained in DMEM and RPMI medium, respectively, supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 25 U/mL penicillin, and 25 µg/mL streptomycin. The TRExBCBL-1 cells were maintained with 20 µg/mL of additional hygromycin. SARS-CoV-2 strains, namely Wuhan-Hu-1, B.1.1.7, and B.1.617, were obtained from BEI Resources and propagated in Vero E6 cells. The Vero E6 cell monolayer was infected with the virus for

2 hours at 37 °C. After 2 hours, the unattached virus was removed by washing and adding fresh medium. After 3 days, the supernatant containing the virus was harvested following cell debris removal by centrifugation, and the virus was aliquoted and stored at −80 °C until further use. The viral copies in the harvested supernatant were quantified by RT-qPCR using a standard curve and by plaque assay. All the assays were performed under BSL-3 containment.

4.3.7 SARS-CoV-2 Infection of EBV- and KSHV-Harboring Cell Lines

Vero E6 cells were seeded in a 12-well plate (100,000 cells/well) with a thin cert (pore size 1 μm, Greiner bio-one) in 500 μL of medium for 2 hours. The cells were then infected with mock- and SARS-CoV-2 virus for 2 hours, after which the unattached virus was removed by washing with 1X PBS and the addition of fresh medium. The infected cells on the thin cert were transferred to another 12-well plate containing HHV cells (100,000 cells/well). The infected cells were incubated at 37 ° C with 5 % CO_2 for 72 hours. After 3 days of incubation, mock- and virus-infected HHV cells were washed with 1 X PBS and subjected to total RNA extraction and RT-qPCR to analyze SARS-CoV-2 (N1 gene) levels and lytic HHVs genes.

4.3.8 Viral and Cellular Gene Profiles

To evaluate the changes in gene expression from our in-vitro assay, we used the Illumina BaseSpace (Illumina, Inc.) DRAGEN Suite to align the RNA-Seq reads to the respective viral reference genomes (KSHV and EBV) and quantify the gene expression levels.[30] After quality control (QC) check and verification of appropriate sample cohorts, we identified the top 25 differentially expressed genes between the SARS-CoV-2 sample groups (SARS-CoV-2-positive HHVs-present *vs.* SARS-CoV-2-positive HHVs-absent). We used appropriate covariates, such as batch effects and technical replicates, to control for unwanted variation.

4.3.9 Statistical Analysis

The data presented are the average of 3 independent experiments, and the error bars represent the standard deviation across these independent experiments. Statistical analyses were performed using Prism 10.0 software (GraphPad Inc., San Diego, CA, USA), and *p*-values were calculated using 2-way ANOVA. A *p-value* of $p < 0.05$ was considered significant (*), and a *p-value of* $p < 0.01$ was considered highly significant (**).

4.4 Results

4.4.1 NP Swab Patient Cohort

In this study, 85 patients were evaluated, consisting of NP swabs from 72 (84.7 %) confirmed COVID-19-positive patients, with varying disease outcomes and SARS-CoV-2 variants, and 13 (15.3 %) healthy controls (**Table 1**).

Table 1. Demographic Information for COVID-19 Patient Cohort

	n = 85		
Variables	***COVID-19 Patients***		***Healthy Controls***
Total cases, n (% total cases)	*72 (84.7)*		*13 (15.3)*
SARS-CoV-2 status			
Wuhan	*5 (6.9)*		*-*
Delta	*5 (6.9)*		*-*
Omicron	*62 (79.1)*		*-*
Clinical Severity		***Variants***	
Asymptomatic	*8 (11.1)*	*BA.2.12.1 (50), BA.2 (12.5), BA.2.12 (12.5)*	*-*
Mild	*18 (25)*	*BA.2 (16.7), BA.2.12.1 (16.7), BA.2.23 (2), BA.2.3 (11.1), BA.2.9 (11.1)*	*-*
Severe	*20 (27.8)*	*BA.2 (10), BA.1.1 (5), BA.2.3 (5), BA.2.9 (5), BA.2.12.1 (5), BA.2.23 (5)*	*-*
Critical	*2 (2.8)*	*BA.2.12.1 (1)*	*-*
Unknown	*14 (19.4)*	*Unassigned*	*-*
Gender			
Male	*34 (43.1)*		*5 (38.5)*
Female	*34 (43.1)*		*8 (61.5)*
Unknown	*4 (5.5)*		*0 (0)*

Age (Male)			
Pediatric, ≤18	*9 (12.5)*		*0 (0)*
Adult, 19-64	*45 (62.5)*		*11 (84.6)*
Elderly, 65+	*12 (16.7)*		*2 (15.4)*
Unknown	*6 (8.3)*		*0 (0)*
HHV-Containing Specimens	**Age**	**Gender**	**Variant (Ct value)**
W1	48	M	Wuhan-Hu-1
D1	21	M	B.1.617.2
O17	18	F	BA.2 (22.774)
O19	N/A	N/A	N/A
O23	72	F	BA.2.9 (18.757)
O34	17	M	BA.2.10 (16.405)
O38	48	F	BA.2
O39	49	F	BA.2.12.1 (16.689)
O46	46	F	BA.2.12.1 (18.922)
O51	32	F	BA.2
O55	72	F	BA.2 (18.515)

The samples were composed of 39 males (45.9 %), 42 females (49.4 %), and 4 with unknown gender (5.5 %). Additionally, the age distribution of the samples was as follows: 9 patients younger than 18 years old (10.6 %), 56 adults (age 19 to 64, 65.9 %), and 14 elderly (age 65 and older, 16.5 %). Specimens with identified SARS-CoV-2 variants included Wuhan (5, 6.9 %), Delta (5, 6.9 %), and Omicron (5, 79.1 %). COVID-19 symptom severity information was available for a subset of the samples, with 8 asymptomatic (11.1 %), 18 mild (25 %), 20 severe (27.8 %), 2 critical (2.8 %), and 14 unknown severity classifications (19.4 %). Additionally, due to low mapping percentages to the Hg38 Human reference genome, only a subset of the samples were included for differential gene and cellular pathways analysis.

4.4.2 Characterizing HHV Status and Metagenomic Analysis in COVID-19 NP Swabs

Since NP swab samples are widely used for clinical and laboratory diagnosis of both HHVs and SARS-CoV-2, we aimed to investigate the presence of HHVs, SARS-CoV-2, and other viruses and bacteria in the NP swab samples by performing metagenomic analysis (**Figure 1**). A total of 85 clinical NP swab samples were included in the metagenomic analysis, which consisted of negative controls (COVID-19-negative) samples, Wuhan-, Delta-, and Omicron-infected specimens. Further, some specimens in the Omicron group had accompanying symptomology data: 8 Asymptomatic (12.9 %), 18 Mild (29 %), 20 Severe (29 %), 2 Critical (2.9 %), and 14 Undefined (20.3 %). Of the COVID-19-positive samples, all had SARS-CoV-2 present, with some samples containing significantly high viral copies (NT rPM approaching 913,000) (**Figure 1A**). Of the COVID-19-positive group, 11 were found to have the presence of one HHV (13 %), with 2 samples found to have 2 HHVs present (2.4 %) (**Figure 1B**).

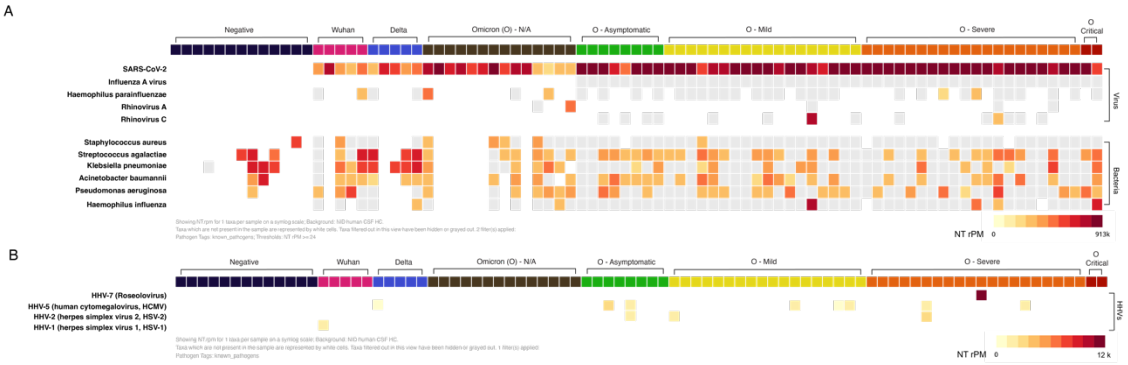


Figure 1. COVID-19 NP Swab Metagenomic Analysis of Viral Pathogens in SARS-CoV-2 Positive Patients. Patients are categorized based on SARS-CoV-2 status: 13 SARS-CoV-2 Negative; 58 SARS-CoV-2 Positive [5 Wuhan (Wuhan-Hu-1), 5 Delta

(B.1.617.2), and 48 Omicron (BA.1)]. The presence and relative abundance (NT rPM) of pathogens (**A**) and HHVs (**B**) are analyzed per sample. NT rPM range, 0 – 913k.

4.4.3 SARS-CoV-2 Variant Analysis in HHV-Reactivation COVID-19 Patients

11 SARS-CoV-2-positive HHV-containing samples were analyzed for SARS-CoV-2 variant classification via PCR (Ct value obtained) or provided by the Nevada State Health Lab. Among the analyzed samples, the Ct value ranged from 16.405 to 22.774. Of the 11 samples, 2 (18.2 %) had no Ct value. The remaining 9 samples had a median Ct value of 18.757, indicating a moderate viral load. Of the 9 samples with available Ct values, 2 (22.2 %) were asymptomatic, 3 (33.3 %) had mild symptoms, and 2 (22.2 %) had severe symptoms. The disease severity of 2 (22.2 %) samples was not reported. Of the identified variants, BA.2.10 had the lowest Ct value (16.405), while BA.2 had the highest Ct value (22.774). Among the 9 samples with Ct values available, BA.2.10 had the lowest median Ct value (16.405), indicating high viral load, while BA.2 had the highest median Ct value (18.757), indicating moderate viral load.

4.4.4 Identification of Differentially Expressed Genes in COVID-19 NP Swabs

In our cohort, we identified 234 differentially expressed (DE) genes were identified out of 9,692 genes with measured expression. Only the top 60 up- and down-regulated DE genes are reported in **Table 1**, while all analyzed differentially expressed

genes are reported in **Supplementary Table 1**. Among the downregulated genes, CRIP1, NOP2, ZBTB40, and RNU6-1 had the lowest log fold change (LogFC) values of -10.00, indicating the most significant differences in expression levels. Meanwhile, among the upregulated genes, DHX9, IRF8, and SRP14 had the highest LogFC values of 10.00, respectively. In addition, we identified several genes with moderate differential expression levels. For instance, DGKG and RPL11 were downregulated with LogFC values of -8.52 and -8.26, respectively, while AQP9 and RSC1A1 were upregulated with LogFC values of 7.58 and 7.41, respectively.

Table 2. Top 60 Differentially Expressed Human Genes in Samples Differing in Detectable HHV Coinfections. SARS-CoV-2 comparison sample groups include SARS-CoV-2-positive, HHVs-present (n = 7) NP Swabs *vs.* SARS-CoV-2-positive, HHVs-absent NP Swab specimens (n = 22). *n = 29 Full list of differentially expressed genes in **Supplemental Table 1.**

DOWNREGULATED GENES			**UPREGULATED GENES**		
GENE	**LogFC**	***p-value***	**Gene**	**LogFC**	***p-value***
CRIP1	-10.00	**0.00**	**DHX9**	10.00	**0.00**
NOP2	-10.00	**0.00**	**IRF8**	10.00	**0.00**
ZBTB40	-10.00	**0.00**	**SRP14**	10.00	**0.00**
RNU6-1	-10.00	**0.00**	**CIR1**	10.00	**0.00**
ZFYVE1	-10.00	**0.00**	**CLDND1**	10.00	**0.00**
AZIN2	-10.00	**0.00**	**GRAMD1B**	10.00	**0.00**
ARMCX2	-9.57	**0.00**	**CFAP97**	10.00	**0.00**
CD320	-9.39	**0.00**	**PARP12**	10.00	**0.00**
TPPP3	-9.21	**0.00**	**PYM1**	10.00	**0.00**
LINC02591	-9.18	**0.00**	**OVCA2**	10.00	**0.00**
RNU6-738P	-9.04	**0.00**	**SPTSSA**	10.00	**0.00**
DGCR5	-8.97	**0.00**	**ADGRF1**	10.00	**0.00**
RNU6-11P	-8.91	**0.00**	**GLDN**	10.00	**0.00**
RNU6-7	-8.74	**0.00**	**CYTH2**	9.52	**0.00**
OR10G8	-8.66	**0.00**	**STX16**	8.62	**0.00**

LINC00698	-8.66	**0.00**	**ESRP1**	7.65	**0.00**
DGKG	-8.52	**0.00**	**AQP9**	7.58	**0.01**
RPL11	-8.26	**0.00**	**RSC1A1**	7.41	**0.00**
EMC10	-7.70	**0.00**	**MCEMP1**	7.13	**0.02**
RNASE4	-7.57	**0.01**	**COL6A3**	7.10	**0.00**
UBL4A	-7.52	**0.00**	**LYZ**	6.98	**0.00**
TUBGCP3	-7.37	**0.01**	**MSN**	6.41	**0.00**
RNU6-25P	-7.28	**0.01**	**RPL26P28**	6.27	**0.03**
LINC01354	-7.28	**0.01**	**TBC1D5**	6.19	**0.01**
ZMYND10	-7.20	**0.00**	**KRT6A**	6.12	**0.01**
HSPA1L	-7.14	**0.02**	**NFKBIZ**	5.87	**0.00**
LINC02318	-7.11	**0.02**	**CDC42SE2**	5.83	**0.00**
AKR1B1	-6.93	**0.01**	**CCDC6**	5.80	**0.00**
FGFR1OP	-6.92	**0.02**	**NR4A2**	5.58	**0.03**
EYA1	-6.89	**0.00**	**PCMTD1**	5.56	**0.02**

* 56 specimens were excluded from this analysis due to poor alignment to the hg38 reference genome and/or poor FASTQ QC check percentages.

Upregulated genes that were found to be associated with biological processes and molecular functions included regulation of immune response (*e.g.,* IRF8, IFITM3), response to hypoxia (*e.g.,* EGLN1, BNIP3L), and viral processes (*e.g.,* OASL, IFIT1) (**Figure 2**). Many downregulated genes were involved in regulating RNA metabolism and processing (*e.g.,* DHX9, NOP2), while others were involved in regulating DNA replication and repair (*e.g.,* MSH2, PCNA). In addition, other downregulated genes were involved in cell adhesion (*e.g.,* CLDND1, COL6A3) and regulation of apoptotic processes (*e.g.,* TNSF10, GADD45A). Some genes were also associated with GO terms, including "response to virus" (*e.g.,* IFIT1, OASL), "inflammatory response" (*e.g.,* S100A8, S100A9), and "response to oxidative stress" (*e.g.,* GCLM, TXNRD1).

Downregulated genes that were found to be involved in biological processes and molecular functions included regulation of RNA metabolism and processing (*e.g.,* DHX9, NOP2), regulation of apoptotic processes (*e.g.,* BIRC3, BNIP3), and response to oxidative stress (*e.g.,* HMOX1, SLC7A11). Additionally, other downregulated genes were involved in DNA replication and repair (*e.g.,* MSH2, PCNA), cell adhesion (*e.g.,* CLDND1, COL6A3), and regulation of immune response (*e.g.,* NFKBIZ, TNFRSF10D). These downregulated genes were also associated with a range of GO terms, including "response to virus" (*e.g.,* IFITM1, MX1), "inflammatory response" (*e.g.,* S100A8, S100A9), and "response to hypoxia" (*e.g.,* EGLN3, HIF3A).

4.4.5 Enrichment Analysis of Cellular Pathways and GO Terms

There were 5 pathways, 587 GO terms, 66 upstream gene regulators, 90 upstream chemical regulators, and 33 diseases found to be significantly impacted or enriched. The top 25 GO terms in the Biological Processes, Cellular Components, and Molecular Functions are shown (**Figure 2)**. The top 5 pathways identified by the KEGG pathway analysis were found to be related to various infectious diseases and immune responses. The most significant pathway was the "pathogenic Escherichia coli infection" pathway (*p-value* = 0.018, FDR = 0.763), which involves bacterial adhesion and colonization of host cells, host cell signal transduction, and the host immune response. The "Kaposi sarcoma-associated herpesvirus infection" pathway (*p-value* = 0.029, FDR = 0.763) was also identified as significant and is known to modulate host signaling pathways to promote viral replication and persistence. Other identified pathways include the "C-type

lectin receptor signaling pathway" (*p-value* = 0.021, FDR = 0.763), which is involved in pathogen recognition and activation of the immune response, "Yersinia infection" (*p-value* = 0.021, FDR = 0.763), which involves bacterial adhesion, colonization, and evasion of the host immune response, and "Fructose and mannose metabolism" (*p-value* = 0.032, FDR = 0.763), which is a metabolic pathway involved in the breakdown and utilization of fructose and mannose in cells and may reflect altered metabolic needs of cells and tissues during viral infection.

The upstream regulator analysis revealed several potential regulators that may be associated with the observed gene expression changes in severe COVID-19 patients. The top-ranked predicted activated regulator was peroxisome proliferator-activated receptor gamma (PPARG, DTA score of 5, *p-value* of 0.00012), indicating a strong association with the observed gene expression changes. GNA12 and AQP3 were also identified as predicted activated regulators (DTA scores of 5 and 2, *p-values* of 0.059 and 0.013, respectively). Other predicted activated regulators identified include cold-inducible RNA-binding protein (CIRBP) and DNA damage-binding protein 2 (DDB2) (DTA score of 2, *p-value* of 0.372). These upstream regulators are known to be involved in a variety of biological processes, including lipid metabolism (PPARG), cell signaling (GNA12), and water transport (AQP3).

The analysis of ranked inhibited upstream regulators revealed SIRT6, MAP3K7, BNIP3L, GLS, and HHEX as the top five regulators. The top-ranked inhibited regulators included Sirtuin 6 (SIRT6) and mitogen-activated protein kinase-kinase-kinase 7 (MAP3K7) (DTA score of 2, *p-value* of 0.014 and 0.748, respectively). BNIP3L,

glutaminase (GLS), and hematopoietically expressed homeobox (HHEX) were also identified as potential inhibited regulators (DT score of 1, *p-value* of 0.027 and 0.748, respectively). These upstream regulators are known to be involved in a variety of biological processes, including cellular metabolism (GLS), autophagy (BNIP3L), and transcriptional regulation (HHEX).

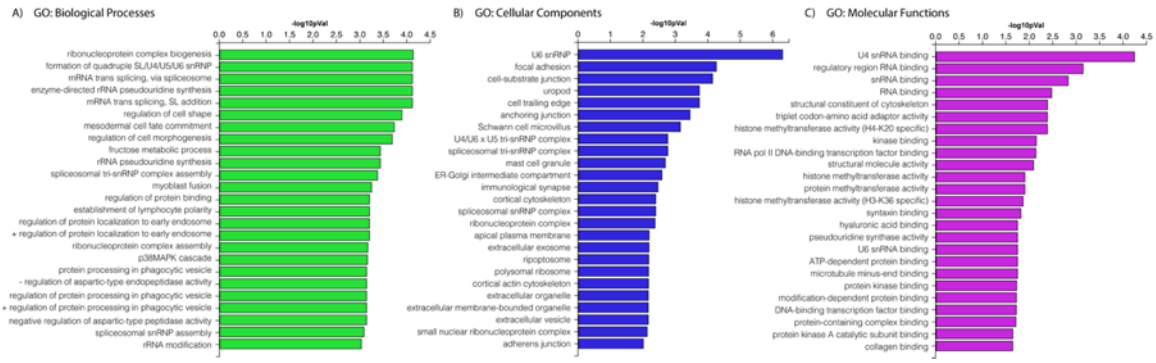


Figure 2. Gene Ontology Analysis of Differentially Expressed Genes in Samples Differing in Detectable HHV Coinfections. Top 25 GO terms related to enriched A) Biological Processes (green), B) Cellular Components (blue), and C) Molecular Functions (magenta). A -log10 scale denotes (*p-value*) respective to each GO term. SARS-CoV-2 comparison sample groups include SARS-CoV-2-positive, HHVs-present (n = 7) NP Swabs *vs.* SARS-CoV-2-positive, HHVs-absent NP Swab specimens (n = 22).

4.4.6 In vitro Evaluation of SARS-CoV-2 Variants on HHV Reactivation

To further confirm that an association between HHVs and SARS-CoV-2 affects infection severity, we tested their interaction using an *in vitro* cell culture model (**Figure 3**). For this, we co-cultured mock-, Wuhan-hu-1, B.1.1.7, and B.1.617 infected Vero E6 cells with EBV- (EBV-GFP, and AGSiZ cells), and KSHV- (BC-1, and TrexBCBL-1)

harboring cells for 3 days using a trans-well thin cert (**Figure 3A**). EBV-GFP and AGSiZ cell lines are EBV-infected oral epithelial cells, the TrexBCBL-1 cell line, a B-cell lymphoma is infected with KSHV, and the BC-1 cell line, derived from a body cavity-based B-cell lymphoma, is dually infected with EBV and KSHV. We and others have demonstrated that HHVs can establish latent infection in these lymphocytes and oral epithelial cells. Significant amounts of the SARS-CoV-2 viral genome copies were detected through RT-qPCR in the SARS-CoV-2 infected HHVs, compared to mock-infected controls (**Figure 3B**). This proved the EBV- and KSHV-cell lines to be permissive to SARS-CoV-2 infection. Additionally, elevated levels of EBV (BZLF1 and GP350) and KSHV (RTA and ORF59) early lytic genes, quantified by RT-qPCR using specific primers, confirmed the presence of reactivated HHVs in the SARS-CoV-2 infected cells, compared to uninfected controls. Interestingly, compared to B.1.1.7-infected cells, the relative lytic gene expression was higher for both Wuhan-hu-1 and B.1.617-infected HHV cells.

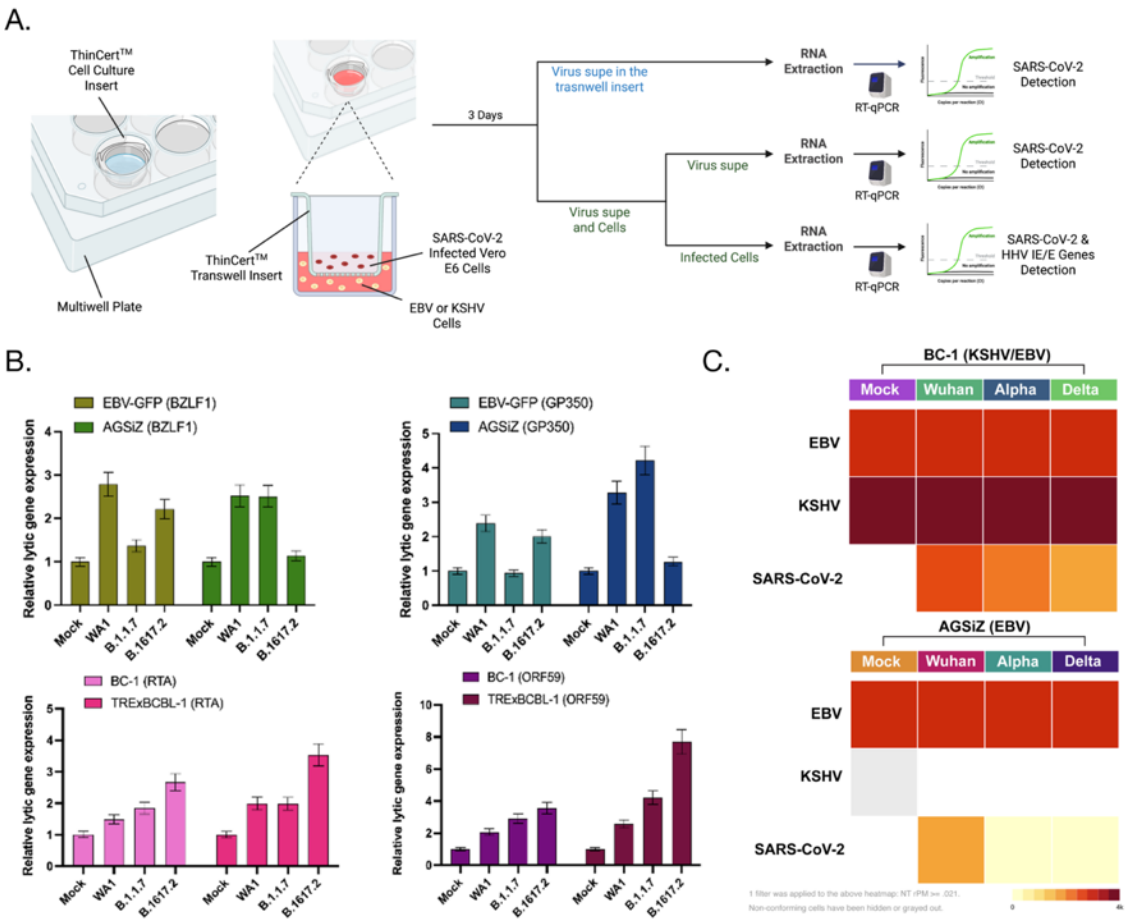


Figure 3. *In vitro* Reactivation of HHVs following SARS-CoV-2 Coinfection. A) Schematic diagram of SARS-CoV-2-infected Vero E6 cells co-cultured with EBV (AGSiZ)- and KSHV (BC-1)-harboring cells. Virus supernatant from infected Vero E6 and EBV- and KSHV cells were subjected to RNA extraction and RT-qPCR to detect SARS-CoV-2 (N1 gene). B) HHV-4 (EBV) and HHV-8 (KSHV) viral lytic gene signatures *in vitro* infected cell culture model. RNA from SARS-CoV-2 infected cells were subjected to SARS-CoV-2 (N1 gene) and HHV early lytic gene detection using RT-qPCR and post-RNA extraction. C) In the *in vitro* cell culture model post-SARS-CoV-2 infection, viral genome copies and cell line respective HHVs are represented as nucleotide reads per million (NT-rPM). RNA Seq FASTQ metagenomic analysis (CZiD.org) for SARS-CoV-2 and HH*Vs.*

To identify differentially expressed viral genes in the in vitro cell culture model, we analyzed the RNA-Seq data of SARS-CoV-2-infected EBV- and KSHV-harboring cells (**Figure 4**). To determine the differentially regulated herpes viral transcripts, RNA isolated from the infected cells was also subjected to total RNA-Seq at the Nevada Genomics Center using NextSeq2000. The obtained sequences were mapped to HHV transcriptome reference (KSHV & EBV) using CLC Genomics Workbench. Differential expression of KSHV (**Figure 4C**) genes is represented as a heatmap. ORF50, ORF48, ORF8, ORF75, ORF64, vIRF-1, and vIRF-2 were found to be upregulated in the cells infected with all the SARS-CoV-2 variants studied. Upregulation of these genes is essential for lytic reactivation and the production of virions. In addition, the Differential expression of EBV (**Figure 4A**) genes is represented as a heatmap. Elevated levels of BNLF2a and BLLF1, early lytic genes, were found in SARS-CoV-2-infected EBV cell lines. The upregulation of these viral genes confirms the presence of reactivated HHVs in SARS-CoV-2-infected cells and suggests worsened SARS-CoV-2 infection severity. Differential expression of common human cellular genes in all infected samples revealed increased TIGIT, RELN, SRRM5, FLIP1, and DBIL5P levels (**Figures 4B and 4D**).

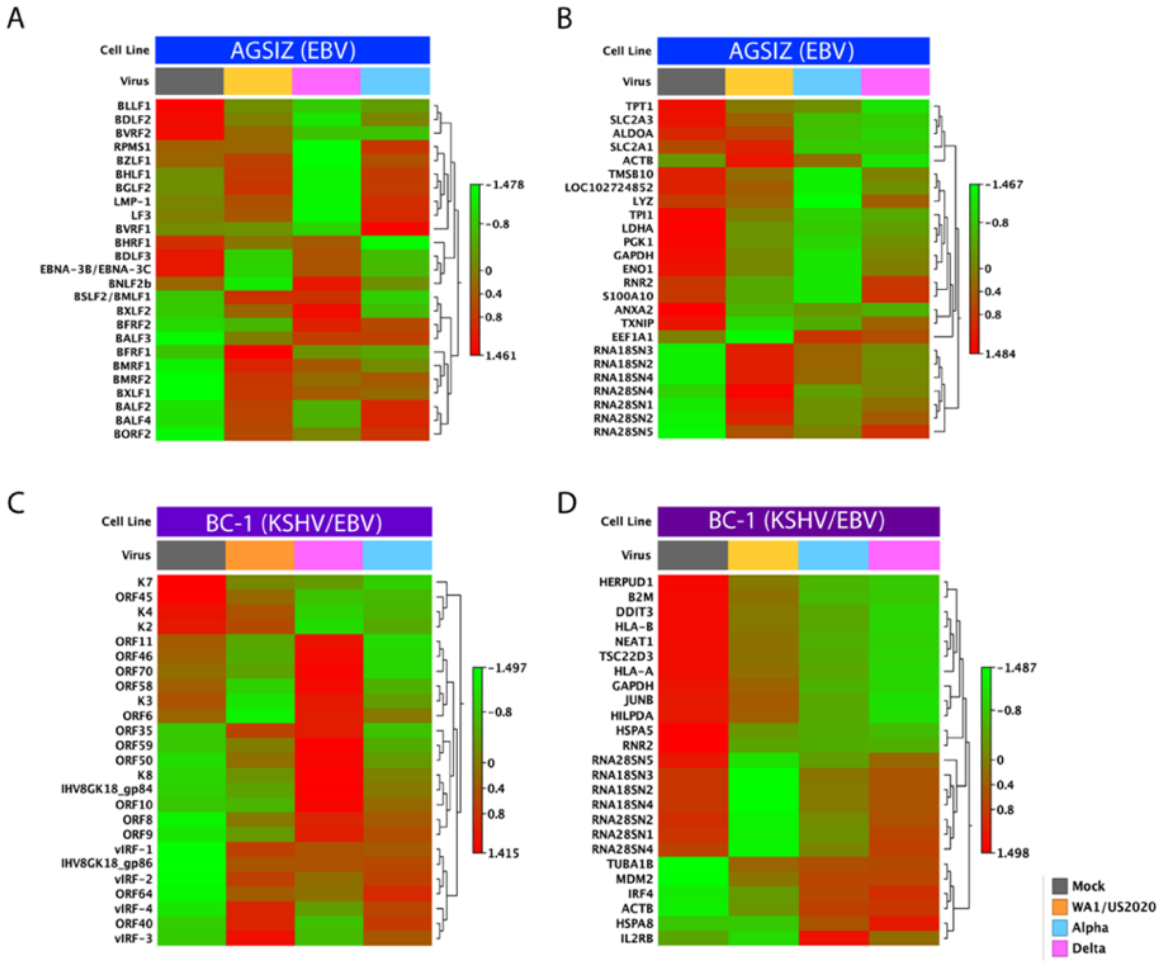


Figure. 4: Viral and Cellular Differential Gene Expression Analysis. Heatmap of Top 25* HHV-Viral (**A & C**) and -Cellular (**B & D**) Differentially Expressed Genes. EBV-harboring cell line (AGSiZ) infected with Mock (gray) and SARS-CoV-2 variants [WA-1 (Wuhan, orange), B.1 (Alpha, blue), and B.1.1.519 (Delta, pink)]. **A**) Perturbed EBV and **B**) Human Cellular genes shown. Identified genes commonly upregulated due to infection with all variants include BNLF2a and BLLF1. Identified genes are commonly downregulated due to infection with all variants, including BALF5. KSHV-harboring cell line (BC-1) infected with Mock (gray) and SARS-CoV-2 variants [WA-1 (Wuhan, orange), B.1 (Alpha, blue), and B.1.1.519 (Delta, pink)]; Perturbed KSHV **C**) and **D**) Human Cellular genes shown. Identified genes commonly upregulated due to infection with all variants include ORF48, ORF8, ORF75, ORF64, vIRF-2, and vIRF-1. Identified genes commonly downregulated due to infection with all variants include K4, K7,

ORF17.5, ORF17, ORF27, ORF57, ORF75. *Absolute FC > 1.2, $p < 0.05$. Venn diagrams of shared genes between SARS-CoV-2 infected variants *vs.* Mock are shown in **Supplemental Figure** 1 and **Supplemental Table 1.**

4.4.7 SARS-CoV-2 – HHVs Reactivation: Cellular Pathways Analysis

The RNA-Seq analysis identified several genes and associated pathways (**Figure 5**). These genes, their associated up- and down-regulated identification, and associated measured non-significant and non-measured genes are described, including their associated interactions (*e.g.,* activation, binding, catalysis, expression, inhibition) and interaction effects (activation, inhibition, or unknown). Genes associated with herpes simplex virus type 1 (HHV-1) infection (**Figure 5A**), Epstein-Barr virus (HHV-4) infection (**Figure 5B**), Human cytomegalovirus (HHV-5) infection (**Figure 5C**), Kaposi sarcoma Herpesvirus (HHV-8) infection (**Figure 5D**), COVID-19 infection (**Figure 5E**), Cytokine-cytokine receptor interaction (**Figure 5F**), and T-Cell receptor signaling (**Figure 5G**) are depicted as connected gene networks.

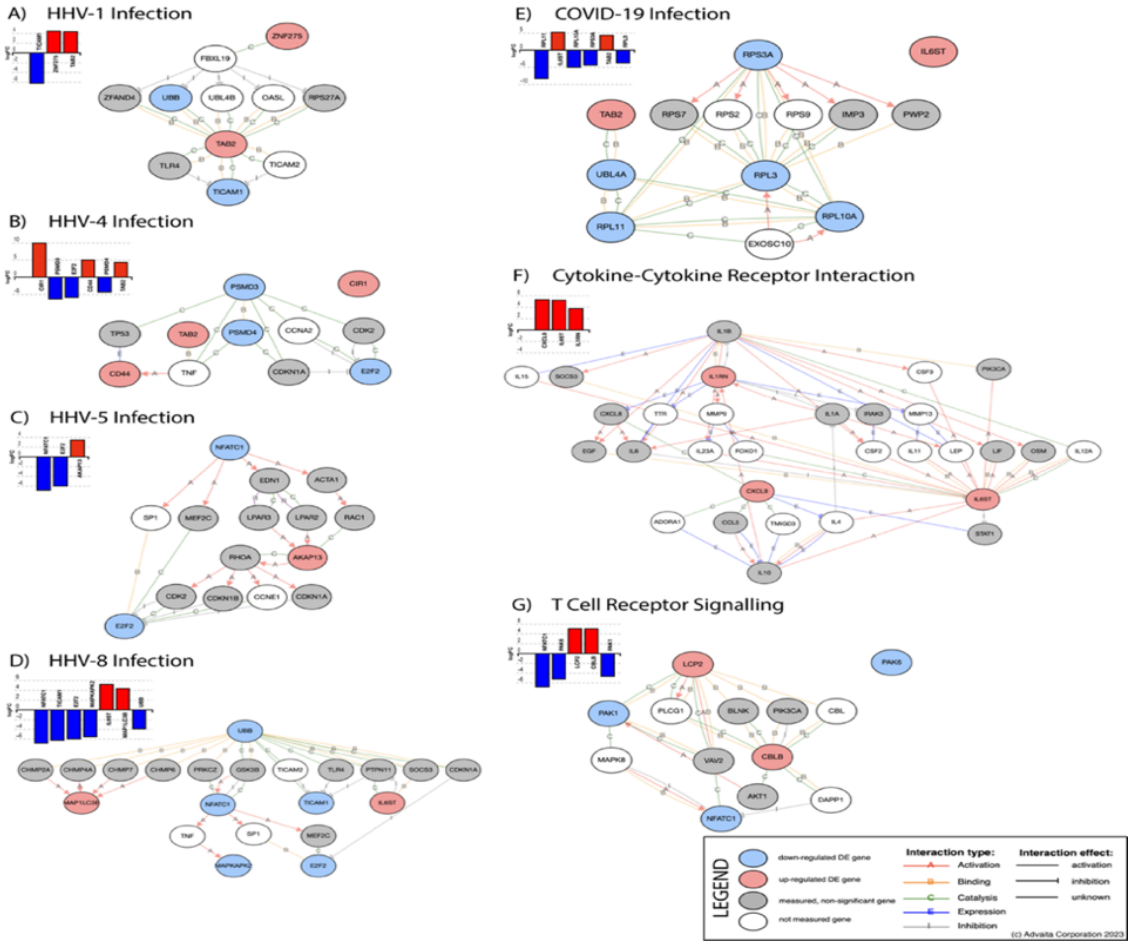


Figure 5. Pathways Analysis of Differentially Expressed Genes: Network Analysis of Key Regulators. Chosen pathways included those relevant to HHVs: A) HHV-1 Infection, B) HHV-4 Infection, C) HHV-5 Infection, D) HHV-8 Infection; SARS-CoV-2, E) COVID-19 Infection; and the Immune System, F) Cytokine-Cytokine Receptor Interaction, G) T Cell Receptor Signaling. The volcano plot depicting genes with an Absolute FC of > 1.2 is shown in **Supplemental Figure 2.**

4.5 Discussion

4.5.1 HHVs and SARS-CoV-2 Disease Pathophysiology and Symptomology

HHVs are infectious DNA viruses with a linear, large, double-stranded DNA genome that can cause an array of diseases in humans (*e.g.,* chickenpox, shingles, and mononucleosis).[5,6,31] There are currently eight known HHVs: herpes simplex virus types 1 (HSV-1) and 2 (HSV-2); Varicella-zoster virus (VZV or HHV-3); Epstein–Barr virus (EBV or HHV-4); Cytomegalovirus (HCMV or HHV-5); HHV-6; HHV-7; and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8).[31] HHVs are extremely common, with most people infected with at least one HHV in their lifetime. Once an HHV has infected a host, it remains in the body for life, reactivating under certain situations (*e.g.,* compromised immune system, infection with a pathologic virus, *etc.*). Viral lytic reactivation promotes elevated expression of viral lytic genes, amplification of viral DNA, production, and release of mature progeny virions. The absence of virus particles in patient serum has been reported, implicating the use of serum for diagnostics.[32,33] Further, in SARS-CoV-2 viremia patients, virus particles are associated with increased disease severity and may even predict clinical outcomes.[34] Thus, although we attempted to analyze severe- and critical-diseased COVID-19 patients to identify SARS-CoV-2 and HHVs, perhaps inclusion and comparison of patient PBMCs with significant enough disease or viremia is warranted.

Studies have demonstrated an association between HHV reactivation and SARS-CoV-2 infection.[35-37] HHVs can be reactivated by various stimuli, including fever, stress,

hypoxia, and coinfection with other viruses.[38,39] SARS-CoV-2 infection and replication in the lungs have been shown to cause cell death and the secretion of mucous/proteinaceous substances into the alveoli, leading to impaired breathing.[40-42] The damaged alveoli can further cause hypoxia in infected individuals.[9,10,43-46] Recent reports of clinical cases suggest that hypoxia-cytokine storm may be a potential cause of lytic reactivation of HHVs in COVID-19 patients and vaccinated patients (*i.e.,* by creating a favorable environment for HHV reactivation). However, more research is needed to understand the exact mechanisms behind this potential relationship and to determine the clinical implications of HHV reactivation in COVID-19. Further, HHVs, particularly HCMV and EBV, can establish latent infections in various cells in the body, including lymphocytes, nasopharyngeal cells, and oral epithelial cells,[47] and can also lead to the production of cytokines,[48-50] which has been observed in COVID-19 patients and the patient cohort of this study.[51] Some studies have highlighted the role of HHV reactivation due to SARS-CoV-2 coinfection in severe COVID-19 cases.[15,16,52]

A recent study reported that HHV reactivation was more common in patients with COVID-19 compared to controls, with HHV-6 and HHV-7 being the most detected viruses.[53] Another study reported similar findings, with HHV-6 and HHV-7 being the most detected HHVs in patients with COVID-19.[53] One case study reported the use of metagenomic analyses to investigate HHV reactivation (HHV-1) in a patient with severe COVID-19 and that HHV reactivation may be associated with a longer hospital stay and higher risk of death.[54] The reactivation of HHV-6 due to the mRNA vaccine was reported by Kolansky et al.[55] Moreover, a single-center study on pediatric COVID-19 infections

found that HHV-6 infection was present in one-third of patients with febrile seizures, implicating the significant effect of HHVs in the context of COVID-19 and neurological sequelae.[56]

4.5.2 Future Directions

Not much is known about the role of reactivated HHVs in the aggravated COVID-19 disease; thus, there is an urgent need to increase our understanding of HHVs coinfection with SARS-CoV-2, proceeding HHVs reactivation and associated clinical symptomology, including morbidity and mortality. Long-COVID Syndrome is a new area of focus for research, and it is important to understand the underlying etiology of this prolonged illness. Also known as post-COVID syndrome or long-term effects of COVID-19, long-COVID refers to the ongoing or long-lasting symptoms that can occur in some people following infection with SARS-CoV-2, the virus that causes COVID-19. These symptoms can persist for weeks or months after the initial infection and can range from mild to severe. The use of non-invasive sampling techniques such as NP swabs offers a better snapshot into what's going on in the patient's lung parenchyma and can be used to evaluate for long-COVID. Further, the use of metagenomic analysis to assess the presence of herpesviruses and SARS-CoV-2 from patient NP swabs may provide new insights into the role of HHV and viral reactivation in the pathogenesis of long-COVID. It is thought that the cytokine storm and inflammation that can occur in severe cases of COVID-19 may create a favorable environment for HHV reactivation. It is possible that

the reactivation of HHVs may contribute to the development or persistence of long COVID symptoms. However, more research is needed to understand the exact mechanisms behind this potential relationship and to determine the clinical implications of HHV reactivation in long COVID.

4.5.3 Study Limitations

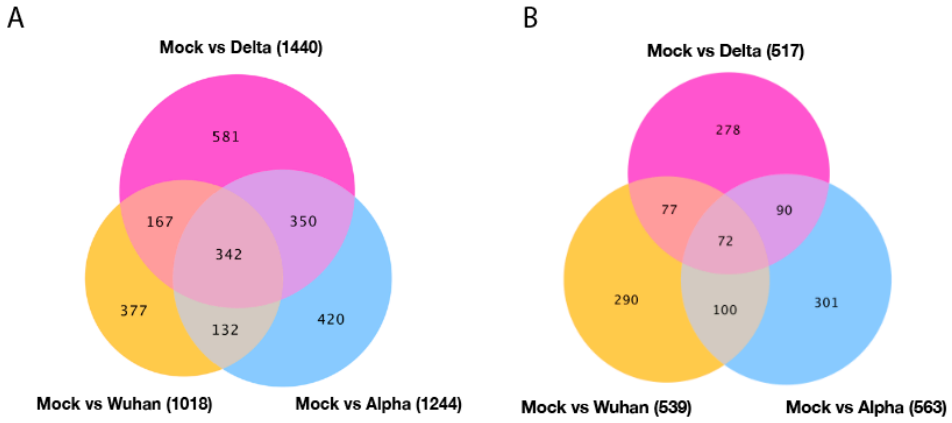
Our study was limited by the fact that we did not have COVID-19 patient PBMC samples. PBMCs are not valid reservoirs for HHVs; however, in severe COVID and viremia patients, the presence of HHVs can be used to evaluate clinical characteristics – which would have allowed for analysis. Furthermore, only some of our NP swab samples could be used for RNA-Seq differential expression analysis because the (non-selected) samples contained low mapping percentages to the human reference genome (Hg38). Thus, our differential gene expression and cellular pathway analysis results are limited by low sample size. Luckily, for metagenomic analysis, since the reference genome excludes the human genome, we were able to conclude metagenomic results for both sample groups. Additionally, while our study accounted for a broad array of microbes, we did not assess for the presence or interaction of HHVS with other specific pathogens, which may be important to consider when evaluating patient outcomes and the potential role of HHVs reactivation in COVID-19 and long-COVID.

4.6 Conclusion

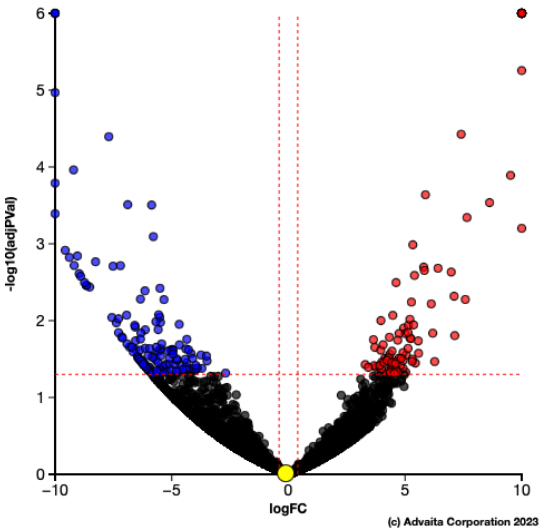
We investigated the potential role of SARS-CoV-2 in triggering the reactivation of HHVs in patients with COVID-19 and the potential effects of this reactivation on the severity of COVID-19 symptoms. Additionally, we used an *in vitro* cell culture model to study the mechanisms behind this potential relationship at a molecular level. We noted a relationship between SARS-CoV-2 and HHVs as well as the potential sequela of reactivation during COVID-19. Reactivation of HHVs during COVID-19 infection may contribute to increased severity of symptoms. Identifying key immediate-early lytic genes in severe COVID-19 patients and the increased prevalence of HCMV and HSV viruses in these patients suggests that HHVs play a role in COVID-19 disease progression. Further research elucidating the mechanisms of interaction between the SARS-CoV-2 and HHVs is needed, which may contribute to novel targets and pathways that may contribute to potential therapeutic opportunities that may arise from targeting them. By better understanding the role of HHVs in COVID-19 pathogenesis, we may be able to develop more effective treatment strategies for patients with pre-existing HHV infections.

4.7 Appendix

4.7.1 Supplementary Material



Supplemental Figure 1. Venn Diagrams of Shared Differentially Expressed Human Cellular Genes Between Infected SARS-CoV-2 variants *vs.* Mock.* EBV-harboring cell line (AGSiZ) infected with Mock and SARS-CoV-2 variants [WA-1 (Wuhan, orange), B.1 (Alpha, blue), and B.1.1.519 (Delta, pink)]. **A**) Shared perturbed EBV and **B**) Human Cellular genes are shown. Absolute FC > 1.2, $p < 0.05$. *Heatmap of Top 25* HHV-Viral and -Cellular Differentially Expressed Genes are shown in **Figure 4,** and a full list of genes is included in **Supplemental Table 1.**



Supplemental Figure 2. Volcano Plot of RNA Seq Differentially Expressed Genes. EBV-harboring cell line (AGSiZ) infected with Mock and SARS-CoV-2 variants [WA-1 (Wuhan), B.1 (Alpha), and B.1.1.519 (Delta)]. Non-significant differentially expressed genes are shown below the threshold in black. Log fold-change for upregulated (red) and downregulated (blue) gene products is shown. Absolute FC > 1.2, $p < 0.05$.

Supplementary Table 1. All Differentially Expressed Genes in SARS-CoV-2-*positive* HHVs-present NP Swab Specimens (n = 7). The comparison group included SARS-CoV-2-positive HHVs-*absent* NP Swab specimens (n = 22). *Total n = 29

Downregulated Genes			**Upregulated Genes**		
GENE	**Log FC**	***p-value***	**GENE**	**Log FC**	***p-value***
CRIP1	-10	0.0001	**DHX9**	10	0.0001
NOP2	-10	0.0001	**IRF8**	10	0.0001
ZBTB40	-10	0.0001	**SRP14**	10	0.0001
RNU6-1	-10	0.0001	**CIR1**	10	0.0001
ZFYVE1	-10	0.0001	**CLDND1**	10	0.0001
AZIN2	-10	0.0001	**GRAMD1B**	10	0.0001
ARMCX2	-9.57	0.0001	**CFAP97**	10	0.0001
CD320	-9.39	0.0001	**PARP12**	10	0.0001
TPPP3	-9.21	0.0001	**PYM1**	10	0.0001
LINC02591	-9.18	0.0001	**OVCA2**	10	0.0001
RNU6-738P	-9.04	0.0001	**SPTSSA**	10	0.0001
DGCR5	-8.97	0.0001	**ADGRF1**	10	0.0001
RNU6-11P	-8.91	0.0001	**GLDN**	10	0.0001
RNU6-7	-8.74	0.0001	**CYTH2**	9.52	0.0001
OR10G8	-8.66	0.0001	**STX16**	8.62	0.0001
LINC00698	-8.66	0.0001	**ESRP1**	7.65	0.0001
DGKG	-8.52	0.0001	**AQP9**	7.58	0.01
RPL11	-8.26	0.0001	**RSC1A1**	7.41	0.0001
EMC10	-7.70	0.0001	**MCEMP1**	7.13	0.02
RNASE4	-7.57	0.01	**COL6A3**	7.10	0.0001
UBL4A	-7.52	0.0001	**LYZ**	6.98	0.0001
TUBGCP3	-7.37	0.01	**MSN**	6.41	0.0001
RNU6-25P	-7.28	0.0001	**RPL26P28**	6.27	0.03
LINC01354	-7.28	0.01	**TBC1D5**	6.19	0.01
ZMYND10	-7.20	0.0001	**KRT6A**	6.12	0.01
HSPA1L	-7.14	0.02	**NFKBIZ**	5.87	0.0001
LINC02318	-7.11	0.02	**CDC42SE2**	5.83	0.0001
AKR1B1	-6.93	0.01	**CCDC6**	5.80	0.0001
FGFR1OP	-6.92	0.02	**NR4A2**	5.58	0.03
EYA1	-6.89	0.0001	**PCMTD1**	5.56	0.02
NFATC1	-6.81	0.02	**KRT6B**	5.44	0.04

LINC01145	-6.71	0.02	**PTP4A1**	5.40	0.0001
SIRPB1	-6.65	0.03	**APOBEC3F**	5.37	0.01
DUSP18	-6.59	0.01	**ZNFX1**	5.33	0.0001
C1orf194	-6.56	0.01	**CXCL9**	5.31	0.03
PLXNB1	-6.56	0.02	**UTP20**	5.30	0.03
ITGB1BP1	-6.56	0.02	**TMED10**	5.27	0.01
CYP2C18	-6.46	0.03	**POLR2B**	5.25	0.02
LRRN2	-6.46	0.03	**NFE2L2**	5.22	0.02
RNF25	-6.40	0.03	**IL6ST**	5.21	0.01
UBE3D	-6.40	0.03	**CHD1**	5.14	0.01
PSMD3	-6.36	0.02	**PARP6**	5.13	0.01
PI4KA	-6.34	0.01	**TMEM131**	5.13	0.02
RPUSD2	-6.34	0.03	**LCP2**	5.05	0.03
L3MBTL3	-6.34	0.02	**CBLB**	5.03	0.05
MOSPD3	-6.32	0.01	**CD44**	5.00	0.01
DNAAF1	-6.31	0.03	**ANXA1**	4.94	0.01
CD226	-6.28	0.03	**ELAVL1**	4.92	0.04
DHCR7	-6.28	0.04	**PHF3**	4.91	0.03
TCF3	-6.24	0.04	**SLC38A1**	4.91	0.03
RNU6-9	-6.22	0.04	**FAM168B**	4.83	0.03
PCAT1	-6.22	0.04	**MED13**	4.81	0.04
SCN11A	-6.21	0.04	**ARAP2**	4.74	0.01
TICAM1	-6.21	0.04	**PRPF8**	4.73	0.03
DEXI	-6.16	0.04	**NSD1**	4.71	0.05
MXD4	-6.15	0.00	**NCOA7**	4.69	0.02
ZNF80	-6.14	0.02	**SNX27**	4.62	0.05
RRP36	-6.14	0.04	**ZHX1**	4.62	0.04
LINC01271	-6.14	0.04	**TMBIM6**	4.62	0.00
IPO9	-6.14	0.01	**UBAP1**	4.59	0.03
SMIM13	-6.06	0.04	**CEP295**	4.59	0.04
CFAP74	-6.02	0.04	**UBE3B**	4.54	0.05
MAP3K21	-6.01	0.03	**PHIP**	4.51	0.04
ZNF622	-6.01	0.04	**SORL1**	4.47	0.01
CTSH	-5.96	0.05	**ZNF275**	4.45	0.04
ERICH5	-5.95	0.02	**MBNL1**	4.44	0.02
E2F2	-5.95	0.04	**DENND4C**	4.43	0.05
FN1	-5.93	0.04	**KCMF1**	4.43	0.04
RPUSD3	-5.86	0.05	**KLF4**	4.41	0.05
GOLGA2P5	-5.86	0.05	**KIAA0040**	4.39	0.04
MYH9	-5.86	0.00	**MAP1LC3B**	4.36	0.05
PRMT7	-5.83	0.03	**ACSL1**	4.35	0.03
UGGT1	-5.81	0.03	**TAB2**	4.33	0.02
CCDC180	-5.80	0.05	**CBX5**	4.31	0.03
TSR3	-5.80	0.05	**RPS6KA3**	4.28	0.05
DOP1A	-5.79	0.00	**NFIB**	4.26	0.03
PUF60	-5.76	0.05	**TRIM29**	4.23	0.03
PHLPP1	-5.68	0.01	**VAMP3**	4.21	0.05
DNAJB2	-5.67	0.03	**CPD**	4.14	0.03
RIOX2	-5.67	0.02	**CHP1**	4.10	0.04
RNU6-8	-5.65	0.04	**KMT2C**	4.06	0.02
NSD2	-5.60	0.03	**PJA2**	4.00	0.03
DNAH12	-5.59	0.04	**SMARCC1**	3.99	0.05

ZFAND3	-5.58	0.01	**WDR1**	3.98	0.04
SYTL2	-5.56	0.01	**ABLIM1**	3.97	0.01
LINC00484	-5.52	0.05	**MXD1**	3.96	0.02
MAPRE3	-5.51	0.01	**DDX17**	3.92	0.04
UBE2D2	-5.51	0.02	**CFLAR**	3.89	0.04
RABGAP1L	-5.50	0.00	**IL1RN**	3.75	0.04
CHM	-5.50	0.04	**CD55**	3.71	0.02
B9D1	-5.48	0.03	**GNE**	3.67	0.04
MAPKAPK2	-5.48	0.01	**MALAT1**	3.64	0.02
BSPRY	-5.48	0.04	**AKAP13**	3.43	0.04
LAIR1	-5.47	0.02	**PNRC1**	3.29	0.04
DDX39B	-5.43	0.02			
SAE1	-5.36	0.04			
PPP1R13B	-5.34	0.01			
ISYNA1	-5.30	0.05			
DAP3	-5.27	0.04			
PAK6	-5.21	0.05			
CLIC5	-5.16	0.02			
GCN1	-5.11	0.04			
CAMSAP1	-5.10	0.03			
ATP5MF	-5.05	0.02			
LAYN	-5.01	0.04			
RPL10A	-4.99	0.02			
RBM15B	-4.97	0.02			
RPL10P9	-4.95	0.03			
MSL1	-4.95	0.02			
LZTFL1	-4.88	0.04			
NEK11	-4.79	0.03			
EPB41L5	-4.77	0.03			
PAK1	-4.68	0.01			
HMGCS1	-4.67	0.02			
ARF6	-4.62	0.04			
SYAP1	-4.52	0.04			
MKRN1	-4.43	0.03			
RPS3A	-4.40	0.02			
UFC1	-4.38	0.04			
PSMD4	-4.37	0.04			
MKNK2	-4.36	0.04			
GSN	-4.35	0.02			
RPS3AP26	-4.29	0.02			
ALCAM	-4.17	0.03			
ALDOA	-4.11	0.03			
FBP1	-4.11	0.04			
DLG3	-4.05	0.04			
EYA2	-3.99	0.04			
B3GNT5	-3.97	0.04			

* 56 specimens were excluded from this analysis due to poor alignment to the hg38 reference genome and/or poor FASTQ QC check percentages.

Supplemental Table 2. EBV (AGSiZ) Cellular Genes Commonly Perturbed Due to Infection with All Variants of SARS-CoV-2. SARS-CoV-2-infected Vero E6 cells co-cultured with EBV (AGSiZ)- harboring cells. Virus supernatants from infected Vero E6 and EBV cells were subjected to RNA extraction and RNA sequencing differential gene expression analysis.

Name	Log fold change	*p-value*	Log fold change	*p-value*	Log fold change	*p-value*
LOC112268261	-1.69	0.03	-1.6	0.04	-2.46	0.0001
RNU1-4	1.11	0.04	1.09	0.05	3.34	0.0001
ZNF687-AS1	1.49	0.03	1.65	0.02	1.5	0.03
LOC729867	3.44	0.02	3.18	0.03	3.48	0.01
CR2	-1.78	0.03	-1.7	0.04	-2.79	0.01
MAP4K3-DT	1.3	0.0001	1.24	0.0001	1.34	0.0001
LOC100507006	-1.69	0.0001	-2.44	0.0001	-1.75	0.0001
LOC105374841	-1.22	0.05	-1.49	0.02	-1.63	0.01
RGPD5	2.13	0.0001	3.15	0.0001	2.68	0.0001
PHOSPHO2-KLHL23	9.83	0.02	9.23	0.03	10.33	0.02
OSGEPL1-AS1	-1.12	0.03	-1.03	0.04	-1.97	0.0001
HSPE1-MOB4	-2.09	0.0001	-8.23	0.05	-8.2	0.04
C3orf35	-1.65	0.0001	-1.71	0.0001	-1.86	0.0001
ATRIP-TREX1	-3.52	0.0001	-2.18	0.0001	-1.53	0.0001
LOC105377106	3.54	0.01	2.9	0.05	4.27	0.0001
MYH15	-2.85	0.0001	-3.69	0.0001	-3.81	0.0001
LOC105374071	1.49	0.03	1.9	0.0001	1.36	0.05
SLC9B1	-1.58	0.02	-1.49	0.02	-1.44	0.02
LINC02615	-1.47	0.02	-1.38	0.03	-1.71	0.0001
TPPP	-1.28	0.02	-1.45	0.0001	-1.75	0.0001
LOC107986411	1.24	0.0001	1.47	0.0001	1.57	0.0001
TICAM2	1.57	0.0001	1.62	0.0001	1.36	0.0001
LOC107986513	-2.11	0.0001	-1.66	0.0001	-2.38	0.0001
C4B	-1.41	0.0001	-1.14	0.01	-1.12	0.01
LINC01590	-2.08	0.0001	-1.27	0.03	-1.41	0.01
C7orf57	-3.06	0.0001	-2.02	0.01	-1.59	0.03

SPATA48	-1.97	0.0001	-2.2	0.0001	-1.36	0.03
FBXO32	1.16	0.03	1.3	0.02	1.45	0.0001
ANKRD20A4	3.54	0.01	3.05	0.04	4.48	0.0001
LOC102724057	-2.4	0.0001	-2.88	0.0001	-1.49	0.04
LOC105376412	-3.08	0.03	-3	0.04	-3.12	0.03
SKIDA1	1.22	0.0001	1.34	0.0001	1.06	0.01
LOC107984214	-1.53	0.03	-3.89	0.0001	-2.56	0.0001
LIPJ	-4.46	0.0001	-2.19	0.0001	-2.33	0.0001
MCAM	1.54	0.0001	1.25	0.0001	1.65	0.0001
USP2	-1.92	0.04	-2.4	0.03	-1.98	0.04
AKAP3	3.34	0.0001	3.64	0.0001	2.73	0.01
FAM66C	3.09	0.0001	2.26	0.04	2.65	0.01
SPX	2.7	0.01	3.31	0.0001	3.5	0.0001
RASSF8-AS1	-2.68	0.0001	-1.63	0.02	-2.02	0.0001
LOC102724050	1.8	0.0001	1.49	0.02	1.41	0.03
LINC02384	1.12	0.0001	1.19	0.0001	1.02	0.0001
TGM1	-1.29	0.03	-1.71	0.0001	-2.07	0.0001
LINC01269	-1.73	0.0001	-3.39	0.0001	-2.26	0.0001
LOC105371084	-2.26	0.0001	-1.19	0.02	-1.55	0.0001
LOC112268174	-3.65	0.01	-1.7	0.04	-2.24	0.02
PDZD9	-1.8	0.0001	-1.52	0.01	-2.07	0.0001
SLX1A	4.93	0.0001	4.52	0.0001	3.86	0.0001
CKLF-CMTM1	-2.64	0.0001	-1.56	0.0001	-8.58	0.03
KRT23	1.56	0.0001	1.65	0.0001	1.56	0.0001
LOC107985010	-1.12	0.03	-1.83	0.0001	-1.65	0.0001
HCN2	1.03	0.05	1.26	0.01	1.03	0.05
LOC107987266	-4.31	0.0001	-1.21	0.05	-3.45	0.0001
CSNK1G2-AS1	-1.9	0.02	-2.77	0.01	-1.64	0.03
TGFBR3L	1.04	0.02	1.13	0.0001	1.24	0.0001
ZNF625-ZNF20	8.91	0.04	9.31	0.03	9.96	0.02
LOC101930071	1.24	0.0001	1.09	0.03	1.07	0.03
PTOV1-AS1	-1.53	0.03	-3.89	0.0001	-1.37	0.04
CDC27P11	-1.07	10.0001	-1.93	0.0001	-3.14	0.0001
LOC388813	1.22	0.05	1.65	0.0001	1.61	0.0001
LINC01700	1.81	0.0001	1.58	0.02	1.43	0.04
SIK1	4.87	0.0001	2.9	0.05	3.48	0.01
HSF2BP	4.15	0.0001	2.9	0.05	3.28	0.02

RIMBP3B	2.94	0.0001	2.08	0.0001	2.93	0.0001
MID1	-3.15	0.0001	-2.51	0.0001	-1.46	0.03
LINC01560	2.08	0.0001	2.48	0.0001	1.47	0.0001
CT45A3	-8.13	0.04	-8.14	0.05	-8.11	0.04
CT45A7	4.71	0.0001	4.04	0.0001	4.7	0.0001

Supplemental Table 3. KSHV (BC-1) Identified Commonly Perturbed Cellular Genes Due to Infection with All Variants of SARS-CoV-2. SARS-CoV-2-infected Vero E6 cells co-cultured with KSHV (BC-1)-harboring cells. Virussupernatantst from infected Vero E6 KSHV cells were subjected to RNA extraction and RNAseq differential gene expression analysis.

Name	Log fold change	*p-value*	Log fold change	P-value	Log fold change	*p-value*
LOC100288175	1.9	0.0001	1.99	0.0001	2.04	0.0001
MASP2	2.07	0.0001	2.74	0.0001	2.82	0.0001
RNU1-1	-1.54	0.0001	-9.96	0.01	-1.68	0.0001
RNU1-3	-1.81	0.0001	-6.11	0.0001	-1.21	0.0001
MICOS10-NBL1	-1.46	0.0001	-1.26	0.0001	-2.73	0.0001
LDLRAD2	-2.85	0.0001	-2.98	0.0001	-1.03	0.04
SPOCD1	1.38	0.02	1.76	0.0001	1.65	0.0001
TFAP2E	-1.11	0.0001	-1.06	0.0001	-1.35	0.0001
EDN2	-1.58	0.0001	-2.13	0.0001	-2.05	0.0001
ZSWIM5	1.33	0.0001	1.47	0.0001	1.79	0.0001
LOC105378728	-1.55	0.0001	-1	0.02	-1.62	0.0001
LOC102724416	-2.52	0.02	-3.56	0.01	-3.4	0.02
AP4B1-AS1	-1.57	0.0001	-1.96	0.0001	-2.32	0.0001
RNVU1-18	-1.91	0.0001	-6.83	0.0001	-1.4	0.0001
HIST2H3PS2	-1.79	0.0001	-2.26	0.0001	-4.01	0.0001
LIX1L-AS1	-1.68	0.05	-2.77	0.01	-2.05	0.03
HIST2H2BF	-1.57	0.0001	-2.44	0.0001	-2.07	0.0001
HIST2H2AA3	-1.18	0.0001	-2.02	0.0001	-2.04	0.0001
HIST2H2AA4	-1.13	0.0001	-1.88	0.0001	-2	0.0001
HIST2H2BE	-1.11	0.0001	-1.46	0.0001	-1.74	0.0001
CELF3	-1.13	0.0001	-1.54	0.0001	-2.17	0.0001

LOC105371450	-1.11	0.0001	-1.49	0.0001	-1.87	0.0001
LOC100505728	-2.1	0.01	-1.92	0.01	-1.49	0.04
LOC105371729	3.05	0.04	3.17	0.03	3.22	0.03
PYHIN1	1.57	0.01	1.3	0.04	1.55	0.02
VSIG8	-1.39	0.04	-1.53	0.02	-1.83	0.01
CFAP45	-1.04	0.0001	-1.58	0.0001	-2.34	0.0001
LINC01363	-1.02	0.05	-1.5	0.0001	-2.75	0.0001
LOC105371622	1.44	0.01	1.25	0.03	1.37	0.02
LOC646976	1.07	0.03	1.15	0.02	1.52	0.0001
LOC105371637	-1.18	0.02	-1.55	0.0001	-1.51	0.0001
KIAA1614	1.12	0.05	1.79	0.0001	1.93	0.0001
LINC00260	-1.61	0.0001	-1.13	0.02	-1.86	0.0001
LOC103021295	-1.09	0.0001	-1.46	0.0001	-10.64	0.01
IL19	1.45	0.0001	2.1	0.0001	1.97	0.0001
LOC105373117	-3.44	0.02	-1.7	0.05	-2.48	0.02
TSNAX-DISC1	-1.15	0.0001	-1.06	0.0001	-9.29	0.03
DISC1	1.58	0.0001	1.63	0.0001	2.29	0.0001
MAP10	1.31	0.0001	1.6	0.0001	1.55	0.0001
ATP6V1C2	-1.53	0.0001	-2.01	0.0001	-1.86	0.0001
FKBP1B	-1.48	0.03	-1.61	0.01	-1.66	0.02
LOC107985878	-2.2	0.02	-2.89	0.0001	-2.72	0.01
VAMP5	-1.53	0.0001	-1.17	0.01	-2.26	0.0001
LINC01955	-1.48	0.03	-2.4	0.0001	-4.1	0.0001
LOC100506076	-1.61	0.0001	-3.05	0.0001	-1.77	0.0001
DDX11L2	-1.05	0.02	-1.73	0.0001	-1.17	0.01
LOC107985780	-1.76	0.0001	-2.01	0.0001	-2.3	0.0001
PKP4-AS1	-1.25	0.0001	-1.21	0.0001	-1.9	0.0001
ZNF804A	1.2	0.0001	1.47	0.0001	1.63	0.0001
DNAH7	1.59	0.02	1.38	0.05	1.7	0.01
HSPE1-MOB4	1.83	0.0001	1.39	0.0001	2.59	0.0001
IKZF2	1.32	0.0001	1.26	0.0001	1.21	0.0001
LINC01963	1.04	0.0001	1.5	0.0001	1.86	0.0001
LOC101928156	-1.05	0.0001	-1.59	0.0001	-1.29	0.0001
COL6A3	2.65	0.0001	2.67	0.0001	2.15	0.0001
UBE2F-SCLY	-2.46	0.0001	-1.66	0.0001	-2.13	0.0001
C3orf20	1.65	0.05	2.28	0.0001	1.97	0.02
SH3BP5	2.11	0.0001	1.41	0.01	1.59	0.0001

LOC107986065	-2.49	0.0001	-1.33	0.02	-2.13	0.0001
LOC105376972	2.36	0.0001	2.02	0.01	2.34	0.0001
LOC105376981	-3.14	0.0001	-1.89	0.0001	-1.72	0.0001
LINC01981	-1.17	0.02	-2.61	0.0001	-1.57	0.0001
CCR5	1.13	0.0001	1.55	0.0001	1.94	0.0001
LOC105377103	-2.79	0.0001	-4.79	0.0001	-2.44	0.0001
ST3GAL6-AS1	2.16	0.0001	1.87	0.02	1.69	0.04
TIGIT	9.49	0.02	9.18	0.03	10.15	0.01
EFCAB12	-1.69	0.03	-1.35	0.05	-1.65	0.03
LOC107986023	3.42	0.02	3.28	0.02	3.46	0.02
LOC105374152	-1.02	0.02	-1.3	0.0001	-1.67	0.0001
SHOX2	1.49	0.0001	1.72	0.0001	1.99	0.0001
MECOM	1.01	0.0001	1.32	0.0001	1.52	0.0001
LOC107986153	-1.42	0.0001	-1.63	0.0001	-1.31	0.0001
ATP13A4	-1.47	0.0001	-1.16	0.02	-1.71	0.0001
UBXN7-AS1	-2.1	0.01	-1.92	0.01	-1.74	0.02
LOC100129931	-1.49	0.0001	-1.12	0.0001	-1.99	0.0001
LOC107986187	-3.3	0.02	-2.51	0.02	-2.34	0.03
TMPRSS11A	1.49	0.0001	1.35	0.01	1.48	0.0001
C4orf36	1.94	0.0001	1.41	0.01	1.31	0.03
SLC25A31	1.26	0.0001	1.01	0.03	1.3	0.0001
SLC7A11-AS1	-1.96	0.0001	-2.31	0.0001	-3.38	0.0001
LOC107986197	-1.15	0.0001	-1.51	0.0001	-1.24	0.0001
GASK1B	1.52	0.03	2.58	0.0001	2.11	0.0001
LOC101928383	-1.39	0.04	-1.75	0.01	-1.83	0.01
SLED1	-2.54	0.0001	-2.86	0.0001	-4.44	0.0001
CTNND2	-1.2	0.05	-1.69	0.0001	-1.32	0.04
OTULINL	1.93	0.02	1.79	0.03	2.46	0.0001
LOC646652	-3.47	0.0001	-1.36	0.0001	-2.71	0.0001
LOC101929704	2.42	0.0001	2.02	0.01	2.52	0.0001
CARD6	1.12	0.0001	1.14	0.0001	1.4	0.0001
LRRC70	-1.39	0.0001	-1.5	0.0001	-1.55	0.0001
LOC107986373	-2.82	0.0001	-8.2	0.04	-8.19	0.05
LOC112267942	1.38	0.02	1.85	0.0001	1.54	0.01

LOC107986352	-2.36	0.0001	-7.74	0.05	-1.66	0.0001
LOC105379016	-4.04	0.0001	-3.21	0.0001	-3.29	0.0001
MTRNR2L2	-1.18	0.03	-2.44	0.0001	-2.26	0.0001
ACOT12	-1.19	0.04	-1.33	0.02	-2.45	0.0001
PRDM6	-2.2	0.02	-2.33	0.01	-3.64	0.01
MIR3936HG	2.24	0.01	2.74	0.0001	2.45	0.0001
PCDHGB7	3.12	0.0001	2.98	0.0001	3.16	0.0001
PCDHGA11	-2.42	0.0001	-1.44	0.0001	-4.58	0.0001
PCDHGC3	-8.37	0.04	-3.83	0.0001	-3.66	0.0001
DOK3	1.26	0.0001	1.67	0.0001	1.6	0.0001
FLT4	-1.95	0.0001	-2.4	0.0001	-3.18	0.0001
LOC107986553	2.05	0.03	3.22	0.0001	3.48	0.0001
BLOC1S5	1.81	0.0001	1.12	0.0001	2.07	0.0001
LOC105374947	-1.04	0.03	-1.67	0.0001	-1.91	0.0001
HIST1H2BD	-1.4	0.0001	-1.58	0.0001	-1.7	0.0001
HIST1H3G	-1.16	0.01	-1.69	0.0001	-1.62	0.0001
LOC105374995	-1.97	0.0001	-2.93	0.0001	-2.52	0.0001
PPP1R10	1.06	0.0001	1.66	0.0001	1.96	0.0001
LOC105375018	-3.3	0.0001	-1.91	0.0001	-1.32	0.04
HSPA1A	-1.33	0.0001	-1.52	0.0001	-1.29	0.0001
HSPA1B	-1.02	0.0001	-1.11	0.0001	-1.14	0.0001
LOC107986588	-1.17	0.02	-2.61	0.0001	-2.22	0.0001
EGFL8	-1.68	0.0001	-1.21	0.0001	-1.57	0.0001
SPDEF	-2.02	0.0001	-1.7	0.0001	-2.39	0.0001
LOC401261	1.72	0.0001	1.34	0.0001	1.82	0.0001
RSPH9	1.66	0.02	1.83	0.0001	1.39	0.05
B3GAT2	-1.54	0.0001	-1.17	0.0001	-1.89	0.0001
FILIP1	2.8	0.0001	3.41	0.0001	3.79	0.0001
LOC101927365	1.85	0.0001	1.71	0.01	1.63	0.02
FRK	2.03	0.0001	2	0.0001	2.22	0.0001
SUMO4	-1.78	0.0001	-1.42	0.0001	-2.32	0.0001
CLDN20	-1.26	0.03	-1.54	0.0001	-1.37	0.02
PRR18	-2	0.01	-1.57	0.03	-3.84	0.0001
LOC107986673	-1.44	0.0001	-1.22	0.0001	-1.51	0.0001
LOC105375113	-2.24	0.0001	-1.54	0.0001	-1.72	0.0001
LOC107986755	-1.43	0.04	-1.35	0.05	-1.65	0.03
ETV1	1.16	0.02	1.19	0.02	1.01	0.05

C7orf31	1.17	0.0001	1.41	0.0001	1.4	0.0001
LOC105375213	1.69	0.0001	2.19	0.0001	2.33	0.0001
LOC105375215	-1.78	0.02	-1.66	0.02	-2.06	0.01
LOC102724723	-4.14	0.0001	-4.26	0.00013	-4.1	0.0001
LOC101928421	1.12	0.03	1.02	0.04	1.59	0.0001
YAE1	1.08	0.0001	1.42	0.0001	1.4	0.0001
COBL	1.77	0.0001	1.34	0.0001	1.15	0.01
LOC105375301	2.56	0.0001	2.68	0.0001	2.6	0.0001
TMBIM7P	3.18	0.03	3.28	0.02	3.22	0.03
STAG3	-2.26	0.0001	-1.51	0.0001	-2.69	0.0001
PVRIG2P	3.54	0.0001	2.67	0.0001	2.08	0.0001
LOC101927632	1.37	0.04	1.71	0.0001	1.48	0.02
ZASP	-10.47	0.01	-10.47	0.0001	-10.46	0.01
RELN	3.18	0.03	4.28	0.0001	3.22	0.03
IQUB	-1.17	0.02	-1.94	0.0001	-1.13	0.02
WEE2-AS1	1.49	0.0001	2.02	0.0001	2.14	0.0001
LOC112267988	-2.3	0.01	-2.99	0.0001	-3.74	0.0001
ZNF596	1.01	0.0001	1.47	0.0001	1.76	0.0001
LOC105377785	1.44	0.03	1.76	0.0001	1.48	0.02
ANGPT2	-1.51	0.0001	-2.64	0.0001	-1.67	0.0001
SCARA3	-2.22	0.0001	-3.88	0.0001	-2.75	0.0001
PKIA-AS1	-1.6	0.0001	-1.74	0.0001	-1.43	0.0001
RBM12B-AS1	-1.76	0.0001	-1.07	0.0001	-1.78	0.0001
LOC105375652	-1.87	0.0001	-3.12	0.0001	-1.96	0.0001
LOC107986959	1.23	0.0001	1.94	0.0001	2.33	0.0001
LOC105369147	1.1	0.0001	1.38	0.0001	1.31	0.0001
ZFPM2-AS1	1.44	0.01	1.72	0.0001	2.05	0.0001
MAPK15	-1.33	0.0001	-1.08	0.01	-2.11	0.0001
LOC107986985	1.06	0.0001	1.43	0.0001	1.47	0.0001
LOC107986989	2.89	0.0001	2.47	0.02	3.01	0.0001
LOC107987047	2.9	0.05	3.75	0.0001	4	0.0001
LOC107986998	-4.49	0.0001	-1.96	0.0001	-1.6	0.0001
ANKRD20A4-ANKRD20A20P	1.01	0.04	1.77	0.0001	1.55	0.0001

ZNF658	1.73	0.0001	1.65	0.0001	2.07	0.0001
LOC100507346	-1.29	0.0001	-1.53	0.0001	-1.3	0.0001
CCDC180	1.7	0.02	2.19	0.0001	2.15	0.0001
SUSD1	1.05	0.0001	1.13	0.0001	1.15	0.0001
HSPA5	-1.19	0.0001	-1.35	0.0001	-1.48	0.0001
LOC107987135	-1.19	0.0001	-3.86	0.0001	-13.54	0.0001
LOC100996574	-1.33	0.0001	-1.17	0.01	-2.65	0.0001
SLC2A6	-1.59	0.0001	-1.89	0.0001	-1.26	0.02
LOC105376311	-1.33	0.0001	-1.47	0.0001	-1.98	0.0001
RNF224	3.31	0.02	2.91	0.04	3.76	0.0001
LOC100499489	1.25	0.04	1.93	0.0001	1.89	0.0001
LINC00993	-1.09	0.0001	-1.14	0.0001	-1.09	0.0001
LINC00839	2.3	0.0001	2.4	0.0001	2.73	0.0001
SGMS1-AS1	3.36	0.0001	2.95	0.0001	3.23	0.0001
SPOCK2	3.23	0.0001	2.74	0.0001	2.73	0.0001
KLLN	1.33	0.0001	1.36	0.0001	1.49	0.0001
FAS-AS1	-1.54	0.0001	-2.5	0.0001	-2.51	0.0001
IFIT3	1.21	0.0001	1.3	0.0001	1.51	0.0001
IFIT5	1.34	0.0001	1.82	0.0001	1.82	0.0001
LOC102723665	1.4	0.01	1.68	0.0001	2.48	0.0001
LOC105378480	-1.19	0.0001	-1.4	0.0001	-1.54	0.0001
LOC692247	-5.14	0.0001	-2.83	0.0001	-2.24	0.0001
DRD4	-1.45	0.0001	-1.83	0.0001	-1.3	0.0001
LOC105376512	-1.08	0.02	-1.63	0.0001	-1.23	0.01
HOTS	-2.24	0.0001	-3.09	0.0001	-4.39	0.0001
CARS-AS1	-1.6	0.0001	-2.28	0.0001	-4.18	0.0001
OR51J1	-1.36	0.03	-2.16	0.0001	-1.51	0.02
LOC105376591	-1.07	0.03	-1.55	0.0001	-2.49	0.0001
RCN1	-1.06	0.05	-4.62	0.0001	-1.79	0.0001
LOC107984328	-1.24	0.02	-1.37	0.0001	-1.08	0.04
LOC107984329	-2.19	0.0001	-3.47	0.0001	-2.32	0.0001
LRRC55	1.06	0.0001	1.04	0.0001	1.33	0.0001
SLC43A3	-1.4	0.0001	-2.22	0.0001	-2.88	0.0001
LOC105369313	-1.76	0.0001	-1.2	0.02	-1.4	0.01
CNTF	2.26	0.04	2.36	0.03	2.3	0.04
BEST1	-1.42	0.0001	-1.25	0.0001	-1.19	0.0001
LBHD1	1.03	0.0001	1.16	0.0001	1.66	0.0001
LOC105369332	-1.46	0.0001	-3.02	0.0001	-2.08	0.0001

PPP1R14B-AS1	-1.24	0.02	-1.14	0.03	-1.08	0.04
RIN1	1.53	0.0001	1.46	0.0001	2.17	0.0001
ARAP1-AS2	-1.65	0.0001	-1.03	0.03	-3.31	0.0001
DYNC2H1	3.44	0.0001	2.98	0.0001	2.84	0.0001
ELMOD1	2.38	0.0001	2.03	0.0001	2.56	0.0001
TMPRSS5	-2.31	0.0001	-1.52	0.0001	-2.27	0.0001
LOC100652768	-1.38	0.0001	-1.2	0.0001	-1.16	0.0001
POU5F1P3	-1.68	0.0001	-1.73	0.0001	-1.64	0.0001
LINC02449	3.31	0.02	3.28	0.02	3.09	0.03
LOC105369669	1.24	0.03	1.4	0.01	1.66	0.0001
LOC105369690	-1.75	0.0001	-1.85	0.0001	-1.83	0.0001
FLJ13224	-2.18	0.0001	-3.27	0.0001	-1.57	0.02
KRT80	2.9	0.05	2.91	0.04	3.35	0.02
LOC112268097	-1.05	0.02	-1.62	0.0001	-1.17	0.01
PCBP2-OT1	-1.71	0.0001	-2.01	0.0001	-2.01	0.0001
GPR182	-1.49	0.0001	-1.41	0.0001	-1.86	0.0001
DDIT3	-1.58	0.0001	-2.25	0.0001	-2.85	0.0001
PPP1R12A-AS1	2.26	0.04	2.36	0.03	2.92	0.0001
NTS	-2.35	0.0001	-1.22	0.0001	-1.95	0.0001
LOC101929204	-2.11	0.0001	-2.25	0.0001	-1.08	0.02
RAD9B	-1.87	0.01	-1.53	0.02	-1.35	0.04
LOC105370274	1.66	0.02	1.38	0.05	1.89	0.0001
LOC105370365	-1.69	0.03	-3.09	0.0001	-1.96	0.02
POTEM	1.64	0.0001	1.53	0.0001	1.49	0.0001
LOC101929718	-1.3	0.0001	-1.76	0.0001	-2.44	0.0001
TMEM253	1.93	0.0001	1.1	0.05	1.58	0.0001
LINC00641	1	0.0001	1.38	0.0001	1.93	0.0001
LOC107984660	2.76	0.0001	2.35	0.0001	2.74	0.0001
BCL2L2-PABPN1	1.64	0.0001	2.01	0.0001	1.46	0.0001
LOC101927045	1.41	0.0001	1.36	0.0001	1.58	0.0001
HIF1A-AS2	-1.1	0.0001	-1.65	0.0001	-1.89	0.0001
FNTB	1.66	0.0001	1.09	0.0001	1.83	0.0001
LOC100289511	-1.13	0.0001	-1.81	0.0001	-1.4	0.0001
LOC101928462	1.25	0.01	1.15	0.02	1.11	0.03

LOC101929107	-1.02	0.02	-1.23	0.0001	-1.56	0.0001
DIO3	-1.38	0.0001	-1.24	0.0001	-1.78	0.0001
LOC107984763	-1.65	0.0001	-1.21	0.01	-1.13	0.02
STRC	-1.21	0.0001	-1.43	0.0001	-1.25	0.0001
LOC107984741	-1.31	0.0001	-1.24	0.0001	-2.01	0.0001
LOC112268150	-1.71	0.0001	-1.91	0.0001	-1.88	0.0001
ONECUT1	1.59	0.02	1.45	0.04	1.48	0.03
MNS1	2.92	0.0001	2.09	0.0001	1.69	0.04
LINC00926	-1.59	0.0001	-1.2	0.02	-1.26	0.02
LOC112268147	-1.5	0.0001	-2.61	0.0001	-4.63	0.0001
LOC105370877	-2.24	0.0001	-2.67	0.0001	-2.08	0.0001
SENP8	1.16	0.0001	1.07	0.0001	1.24	0.0001
LOC105370970	-2.24	0.0001	-3.04	0.0001	-2.87	0.0001
LOC105370982	-3.56	0.01	-2.22	0.02	-3.52	0.01
HSP90B2P	-2.86	0.0001	-2.44	0.0001	-2.82	0.0001
LOC101927751	1.06	0.03	1.82	0.0001	1.74	0.0001
LOC729652	1.22	0.0001	1.25	0.0001	1.74	0.0001
LOC112267907	-1.59	0.0001	-1.73	0.0001	-1.4	0.01
EIF3CL	-1.45	0.0001	-1.52	0.0001	-1.48	0.0001
LOC101928215	-2.26	0.0001	-3.35	0.0001	-4.1	0.0001
SLX1B	-1.69	0.0001	2	0.0001	1.96	0.0001
HERC2P4	-1.1	0.0001	-1.2	0.0001	-1.48	0.0001
RRAD	-1.43	0.04	-1.35	0.05	-3.84	0.0001
ARLNC1	-2.9	0.0001	-1.6	0.0001	-1.88	0.0001
LOC100129617	-1.68	0.0001	-2.95	0.0001	-2.98	0.0001
IRF8	1.86	0.0001	1.28	0.0001	1.72	0.0001
CENPBD1	1.11	0.0001	1.26	0.0001	1.45	0.0001
LOC100506388	-3.97	0.0001	-2.23	0.0001	-3.94	0.0001
DBIL5P	3.23	0.0001	3.31	0.0001	3.46	0.0001
LOC105371481	-1.91	0.0001	-1.19	0.0001	-2.51	0.0001
LOC105371592	2.7	0.0001	2.8	0.0001	1.87	0.05
SPNS2	1.25	0.04	1.67	0.0001	1.6	0.0001
MIR497HG	-1.38	0.0001	-1.23	0.0001	-1.12	0.0001
ATP1B2	-1.64	0.0001	-1.29	0.0001	-1.89	0.0001
GRAP	-1.8	0.03	-1.93	0.02	-2.72	0.01

LOC100287072	2.28	0.0001	1.97	0.0001	2.09	0.0001
TBC1D3	-1	0.0001	-1.3	0.0001	-1.03	0.0001
LINC02594	-4.06	0.0001	-1.75	0.01	-1.83	0.01
LOC105371814	1.92	0.0001	1.71	0.02	2.32	0.0001
FLJ45513	-3.44	0.02	-3.56	0.01	-1.92	0.04
LINC01977	-1.12	0.03	-1.95	0.0001	-1.96	0.0001
LOC100294362	-1.24	0.0001	-1.22	0.0001	-1.56	0.0001
LINC00482	-2.17	0.0001	-1.48	0.01	-4.33	0.0001
LOC105376866	-2.86	0.0001	-1.47	0.04	-2.27	0.01
LOC105372098	1.51	0.02	1.37	0.03	1.48	0.02
LOC107987258	-3.15	0.03	-3.27	0.02	-2.19	0.05
ZNF554	1.16	0.0001	1.57	0.0001	1.54	0.0001
CACTIN-AS1	-1.12	0.01	-1.88	0.0001	-2.57	0.0001
TINCR	-2.24	0.0001	-1.15	0.0001	-2.85	0.0001
NRTN	-1.27	0.03	-1.57	0.01	-2.38	0.0001
USHBP1	1.3	0.05	1.55	0.01	1.34	0.04
LOC105372337	-3.14	0.0001	-1.34	0.04	-1.83	0.01
LOC105372345	-1.57	0.0001	-1.44	0.0001	-3.18	0.0001
ZBTB32	1.85	0.0001	2.15	0.0001	1.99	0.0001
LOC100505585	-1.27	0.01	-1.05	0.02	-1.04	0.03
SRRM5	3.89	0.0001	3.58	0.01	3.46	0.02
KCNN4	-1.12	0.0001	-1.21	0.0001	-2.08	0.0001
ZC3H4	1.02	0.0001	1.47	0.0001	1.66	0.0001
DHDH	-4.36	0.0001	-1.33	0.02	-3.41	0.0001
LOC400710	-2.72	0.0001	-3.18	0.0001	-1.77	0.0001
KLK4	-1.18	0.0001	-1	0.0001	-1.73	0.0001
ZNF765-ZNF761	-3.3	0.0001	1.97	0.0001	2.5	0.0001
LAIR1	1.58	0.0001	1.16	0.0001	1.28	0.0001
LILRB1	2.71	0.01	3.12	0.0001	3.08	0.0001
MZF1-AS1	1.12	0.0001	1.43	0.0001	1.46	0.0001
LOC107985403	-1.41	0.0001	-1.49	0.0001	-2.09	0.0001
EFCAB8	-1.01	0.0001	-1.01	0.0001	-1.3	0.0001
DBNDD2	-2.91	0.0001	-8.28	0.03	-8.28	0.05
LOC105372647	2.26	0.04	2.36	0.03	2.17	0.05
MOCS3	1.14	0.0001	1.34	0.0001	1.62	0.0001
STX16-NPEPL1	-1.42	0.0001	-3.9	0.0001	-3.98	0.0001
LOC105372708	1.75	0.0001	1.76	0.0001	2.61	0.0001

RNA5-8SN2	-8.48	0.04	-8.48	0.03	-8.47	0.04
RNA5-8SN1	-5.14	0.0001	-8.32	0.03	-8.32	0.05
LOC105372733	2.23	0.0001	1.87	0.02	2.57	0.0001
LOC105372791	1.39	0.02	1.3	0.02	1.31	0.03
SMIM11A	-10.88	0.0001	-10.88	0.0001	-10.87	0.0001
LOC102725065	-2.38	0.03	-2.51	0.02	-3.26	0.02
LINC01547	1.09	0.0001	1.55	0.0001	1.41	0.0001
LOC107985497	-1.18	0.02	-1.69	0.0001	-1.51	0.0001
LOC112268297	-2.49	0.0001	-1.64	0.0001	-1.66	0.01
KIAA1671	1.04	0.0001	1.21	0.0001	1.33	0.0001
LOC105372976	1.66	0.02	2.03	0.0001	2.3	0.0001
LOC105373014	-3.05	0.0001	-2.63	0.0001	-3.94	0.0001
ELFN2	-1.2	0.0001	-1.6	0.0001	-1.33	0.0001
NFAM1	1.42	0.0001	1.08	0.0001	1.26	0.0001
LOC101927393	-1.61	0.02	-2.01	0.0001	-1.83	0.01
LOC105369161	-1.78	0.02	-1.92	0.01	-2.46	0.0001
LOC107985556	-1.79	0.0001	-1.22	0.0001	-1.75	0.0001
LOC105373098	-1.96	0.04	-2.09	0.03	-2.48	0.02
CD99_1	-2.3	0.01	-3.9	0.0001	-3.74	0.0001
CXorf21	1.78	0.0001	1.64	0.0001	1.68	0.0001
MID1IP1-AS1	2.76	0.0001	3.05	0.0001	3.03	0.0001
LOC107985678	-2.46	0.0001	-2.38	0.0001	-2.68	0.0001
ZNF41	1.12	0.0001	1.12	0.0001	1.24	0.0001
USP27X-AS1	1.83	0.05	2.1	0.02	2.19	0.02
IQSEC2	1.84	0.02	1.95	0.02	2.4	0.0001
PFKFB1	1.79	0.0001	1.26	0.02	1.4	0.0001
LOC105373311	-1.33	0.0001	-1.47	0.0001	-1.29	0.0001
LOC105373323	-1.7	0.0001	-3.21	0.0001	-1.49	0.01
LOC107987374	-1.49	0.0001	-1.5	0.0001	-2.51	0.0001
LOC105379272	-1.12	0.04	-1.26	0.02	-1.22	0.03
LOC105379554	-4.22	0.0001	-4.34	0.0001	-1.51	0.02

4.7.2 Data Availability Statement

Specific patient datasets generated and analyzed during the current study are available upon reasonable request and governed as protected health information (PHI) following federal Health Insurance Portability and Accountability Act (HIPAA) regulations.

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4.7.4 Conflicts of Interest

The authors declare no conflicts of interest.

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CHAPTER 5

5. EARLY INSIGHTS AND PRELIMINARY FINDINGS

INTO THE MOLECULAR NEUROPATHOGENESIS OF LONG-COVID

5.1 Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) remains a focal point of medical research due to its multifaceted impact on human health and evolving clinical manifestations. Long-COVID can affect a broad demographic and manifests in a variety of symptoms and complications. Current estimates suggest that 10-12 % of infected individuals and up to 70% of hospitalized cases may develop long-COVID, with a higher prevalence between the ages of 36 and 50. The condition impacts multiple organ systems and can cause new-onset diseases (*e.g.*, T2DM, ADEM, and CFS).

This study aimed to comprehensively analyze SARS-CoV-2 variants in Nevada (NV, USA) and investigate the determinants of long-COVID, defined as ongoing, relapsing symptoms present for four or more weeks after the acute phase of infection. Our primary research question focused on identifying variables that can illuminate the severity and pattern of neurological sequelae (*e.g.*, fatigue, brain fog, anxiety, depression, loss of taste and smell, *etc.*) in adults diagnosed with long-COVID. We analyzed nasopharyngeal (NP) swab samples from 114 patients, 57 of those with confirmed long-COVID, along with 57 age-, sex-, and comorbidity-matched controls collected at Renown Regional Medical Center (RRMC, Reno, NV, USA) between June 2020 and June 2022. NGS analyses were performed using RT-qPCR and RNA-Seq analysis.

Our results suggest a putative link between viral co-infections and prolonged illness; further, SARS-CoV-2 variants that exacerbate COVID-19 severity were identified. Variants causing severe COVID-19 may influence the prevalence and spectrum of those seen in long-COVID patients and their presenting clinical

symptomology. These findings contribute valuable insights into the complexities of SARS-CoV-2 infections and pave the way for targeted therapeutic interventions, guiding future research and therapeutic interventions. We reviewed the literature for current pathophysiology, risk factors, and demographic distribution of long-COVID and integrated these insights to frame our understanding and guide our future clinical study directions.

5.1.1 Keywords

long-COVID, fMRI, Serum Proteins, NP Swab, RNA Seq, Variants

5.2 Introduction[1]

Long-COVID (*i.e.*, Post-Acute Sequelae of SARS-CoV-2 [PASC]) can affect anyone who has had COVID-19 infection from SARS-CoV-2, regardless of age or health status. It is currently estimated that up to 25% of COVID-19 patients (about 230 million people worldwide) experience severe symptoms which affect their daily life.[1-4] Although most people who contract COVID-19 experience mild to moderate symptoms and recover without long-term effects, a significant proportion go on to develop long-COVID.[1-3] Long-COVID incidence is estimated to be prevalent among 50-70% in hospitalized cases, and 10-30% in non-hospitalized cases.[5-7] Long-COVID is prevalent in all age groups, with the highest percentage of diagnoses between 36 and 50 years.[8] While multiple factors (*e.g.*, acute phase disease severity) contribute to PASC, there are a few posited factors that may increase the risk of developing long-COVID (*e.g., severity of initial COVID-19 illness, pre-existing medical conditions, immune system response, and delayed or incomplete recovery*). Long-COVID can manifest as a complex set of symptoms involving cardiopulmonary, gastrointestinal, endocrine, psychiatric,

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[1]**Abbreviations**

AAV, Adeno-Associated Virus; ACE2-R, Angiotensin-Converting Enzyme 2 Receptor; ADA, Americans With Disabilities Act; ADEM, Acute Disseminated Encephalomyelitis; B, Beta; BBB, Blood-Brain Barrier; BEI, Biodefense And Emerging Infections; BSL, Biosafety Level; CDC, Centers For Disease Control And Prevention; COVID-19, Coronavirus Disease 2019; CNS, Central Nervous System; CFS, Chronic Fatigue Syndrome; CMM, Center for Molecular Medicine; CRC, Clinical Research Center; DMEM, Dulbecco's Modified Eagle Medium; DNA, Deoxyribonucleic Acid; dsDNA, Double-Stranded DNA; EMEM, Eagle's Minimum Essential Medium; ELISA, Enzyme-Linked Immunosorbent Assay; FBS, Fetal Bovine Serum; fMRI, Functional Magnetic Resonance Imaging; GDPR, General Data Protection Regulation; GABA, gamma-aminobutyric acid; HCoVs, Human Coronaviruses; HBMEC, Human Brain Microvascular Endothelial Cells; HR, Hazard Ratio; IL, Interleukin; IFA, Immunofluorescence Assay; IRB, Institutional Review Board; kyn, Kynurenine; MOI, Multiplicity of Infection; NGS, Next-Generation Sequencing; NIH, National Institutes of Health; NP, Nasopharyngeal; NSPHL, Nevada State Public Health Lab; NV, Nevada; PASC, Post-Acute Sequelae of SARS-CoV-2 Infection; PBMC, Peripheral Blood Mononuclear Cell; PHI, Patient Health Information; RNA, Ribonucleic Acid; RPMI, Roswell Park Memorial Institute; RRMC, Renown Regional Medical Center; SARS-Cov-2, Severe Acute Respiratory Syndrome Coronavirus 2; TNF-α, Tumor Necrosis Factor-Alpha; T2DM, Type 2 Diabetes Mellitus

and neurological systems (*e.g.*, fatigue, brain fog, anxiety, depression, loss of taste and smell, *etc.*) – symptoms often persisting three months beyond the initial infection.[9-12] Long-COVID can also cause new-onset conditions (*e.g.*, T2DM, ADEM, and CFS).[9,13] SARS-CoV-2 will likely persist, mutate, and cause more suffering to American lives and the economy for years; these adverse long-COVID outcomes are a clear example of this imminent long-term burden.

Our comprehensive literature review analyzed current findings on the pathophysiology, risk factors, and demographic distribution of long-COVID, integrating insights from an array of global studies to frame our understanding of this condition within the existing body of knowledge. This review highlights the urgent need for continued research into the varied presentations and mechanisms underlying PASC.

Building on this, our study sought to contribute to this gap in knowledge by examining long-COVID patients. Our primary research question focused on identifying variables that can illuminate the severity and pattern of neurological sequelae in adults diagnosed with long-COVID. To this end, we analyzed nasopharyngeal swab samples from 114 patients, including 57 confirmed long-COVID cases and 57 age-, sex-, and comorbidity-matched controls. The samples were collected at Renown Regional Medical Center (RRMC, Reno, NV, USA) from June 2020 to June 2022. NP Swab specimens were subjected to RT-qPCR and SARS-CoV-2 variant analysis. We will take these insights and investigate whether specific SARS-CoV-2 variants can provide physiological insights on a diagnostic and qualitative level, especially concerning disease severity and neurological sequelae in adults diagnosed with long-COVID.

5.3 Materials and Methods

5.3.1 Study Overview

We have collected NP swabs of COVID-19 patients (deidentified) at the outpatient and inpatient clinics of RRMC since March 2022. After confirming SARS-CoV-2 positivity through RT-PCR, demographic information (PHI) was retrieved and entered into our repository. Patients who have developed long-COVID, confirmed by the pulmonologist and diagnosed with the long-COVID ICD10 code, were entered into a separate database and assigned to the long-COVID cohort. At their initial exposure, the NP swabs of those long-COVID patients will be pulled from our repository and subjected to SARS-CoV-2 whole genome sequencing (WGS). Our laboratory has a dedicated room for extracting genomic RNA from SARS-CoV-2 positive NP swabs using the QIAamp Viral RNA Mini Kit (QIAGEN).[14] A fraction of this RNA was used for RT-PCR of SARS-COV-2 RNA using the TaqPath COVID-19 kit (FDA-approved).

5.3.2 Clinical Study

For our ongoing long-COVID trial, we will continue to collect demographic information on COVID-19 patients, including vaccination status and symptomatology data of patients developing long-COVID. Such data from a similar number of COVID-19 patients without long-COVID symptoms (controls), matched for age, gender, and ethnicity, along with the duration of sample collection (to normalize for the circulating variants) and symptomatology of the first exposure of SARS-CoV-2 will be obtained for

correlative studies. We expect to determine a few key findings, such as the frequencies of SARS-CoV-2 variants causing long-COVID, which will be evaluated (*i.e.*, whether any variants have caused increased levels of long-COVID by comparing with the prevalence in patients without long-COVID), the symptomatology of the first exposure to SARS-CoV-2 compared between long-COVID and without long-COVID patients, any specific symptoms or co-morbid factor(s) contributing to long-COVID disease states, whether vaccination reduces the symptoms of COVID-19, *etc.* Long-COVID diagnosis is confirmed by a doctor based on the reported symptoms and recorded into the patient chart with the ICD10 code, respectively.

5.3.3 Whole Genome Sequencing

SARS-CoV-2 WGS was done following treating the RNA with DNase I (QIAGEN) and concentrated using RNeasy Minelute spin columns (QIAGEN). Concentrated samples will be converted into Illumina-compatible sequencing libraries with a QIAseq FX Single Cell RNA Library kit (QIAGEN). RNA samples were annealed to QIAseq FastSelect-HMR probes (QIAGEN) to reduce the amplification of human ribosomal RNA. After removing traces of DNA, reverse transcription was used to generate cDNA using hexamer primers, followed by isothermal linear amplification. Amplified DNA was then enzymatically sheared to an average insert size of 300 bp and Illumina-compatible dual-indexed sequencing adapters. The adapter-ligated sample was amplified with six cycles of PCR, and the SARS-CoV-2 genome was enriched with the myBaits kit and coronavirus-specific biotinylated probes (Arbor Biosciences). Samples

were sequenced using an Illumina Next-seq mid-output (2 x 75). The generated FASTQ files from the sequencer will be analyzed for variants through our bioinformatics pipeline.[14-18]

5.3.4 Statistical Methods

Data analyses were conducted using Python (v3.12) using several well-documented and used Python libraries. The Pandas library (https://pandas.pydata.org/) was used for pre-processing and subsequent analysis, providing the necessary data structures and functions for structured data manipulation. Figures were generated using the comprehensive plotting library (Matplotlib, https://matplotlib.org/). Numerical computations were performed (NumPy, https://numpy.org/). Additional analyses were performed using Prism 10.0 software (GraphPad Inc., San Diego, CA, USA), and *p*-values were calculated using 2-way ANOVA. A *p - value* of $p < 0.05$ was considered significant (*), and a $p < 0.01$ was considered highly significant (**).

5.4 Preliminary Results

5.4.1 Patient Demographics and SARS-CoV-2 Variant Analyses

Our long-COVID cohort consisted of 114 patients; 57 confirmed long-COVID were evaluated against 57 age-, sex-, and comorbidity-matched non-long-COVID patients (controls) (**Table 1**). The unique prevalence of key SARS-CoV-2 variants in long-COVID subjects were identified (BA.5.26, BA.4.1, BQ.1, BQ.1.2, BQ.1.1, BA.1.25, BA.5.3.4, and BQ.1.1) associated in long-COVID patients (**Figure 1**). SARS-CoV-2 variants that were uniquely prevalent in non-long-COVID cohorts were also identified (BA.2.9, BF.15, BA.5.1.27, BA.2.12, and BA.2.3). There were variants (BA.2.12.1, BA.2, BF.7, BA.5.1, BA.5.5, BA.5.2, BA.2.13, and BA.5.2.1) that had mixed prevalence between the long-COVID and non-long-COVID cohort. These preliminary findings suggest a putative link between certain SARS-CoV-2 variants exacerbating COVID-19 severity and influencing the spectrum of long-COVID symptoms.

Table 1. Cohort Demographic Descriptive Statistics. Descriptive Statistics of Cohort. Severity classification is defined as: 0, Asymptomatic; 1, Mild; 2, Moderate; 3, Severe; and 4, Critical.

Long-COVID (n=57)		**Non-long-COVID (n=57)**	
Males	24 (42%)	**Males**	28 (44%)
Females	33 (58%)	**Females**	32 (56%)
Mean Severity (0-4)	2.98	**Mean Severity (0-4)**	2.11
Mean Ct Value	18.93	**Mean Ct Value**	20.3

Figure 1: Comparative Distribution of SARS-CoV-2 Variants Between Cohorts. Association of specific SARS-CoV-2 variant frequencies seen in each group, long-COVID Cohort, and Controls (non-long-COVID). SARS-CoV-2 variant frequencies in the long-COVID cohort (blue) and non-long Covid (pink). Unassigned variants were excluded from the plot. Common variants seen in both cohorts are depicted in hot pink in the center. Controls were determined by 1:1 matching against individual long-COVID patients by gender, age, and ethnicity.

5.4.2 long-COVID-19 Symptoms and Disease Severity

The most common symptoms of long-COVID in our cohort were lingering cough, shortness of breath, sore throat, fatigue, and some cases of brain fog (**Figure 2**). These symptoms are consistent with other reports studying long-COVID.[19] Some long-COVID

symptoms, primarily persistent cough, and shortness of breath, start to present immediately after clearance of acute infection; however, more severe symptoms (*e.g.*, brain fog, chronic fatigue, and headaches) present months after recovery from SARS-CoV-2. Notably, cough, encompassing acute and chronic presentations, emerged as the most frequently reported symptom, followed by shortness of breath. Nasal congestion, including general congestion, was the third most common symptom, followed by fatigue and weakness; this underscored the prevalent impact of respiratory ailments on systemic exertion levels. Similarly, sore throat and broad respiratory issues, including wheezing and pneumonia, and ear-related symptoms, including congestion, pain, and discharge, indicated significant upper and lower respiratory tract involvement. Headaches were also reported, with sinus-related complications, such as sinus infections and congestion. Diarrhea was reported on three separate occasions, suggesting gastrointestinal disturbances concurrent with respiratory conditions. Other symptoms such as fever, nausea, vomiting, dizziness, confusion, runny nose, malaise, and postnasal drip showed a lower frequency. These findings illustrate symptomatology that is predominantly respiratory, with frequent systemic manifestations that merit further clinical investigation to elucidate their etiologies and potential interconnections.

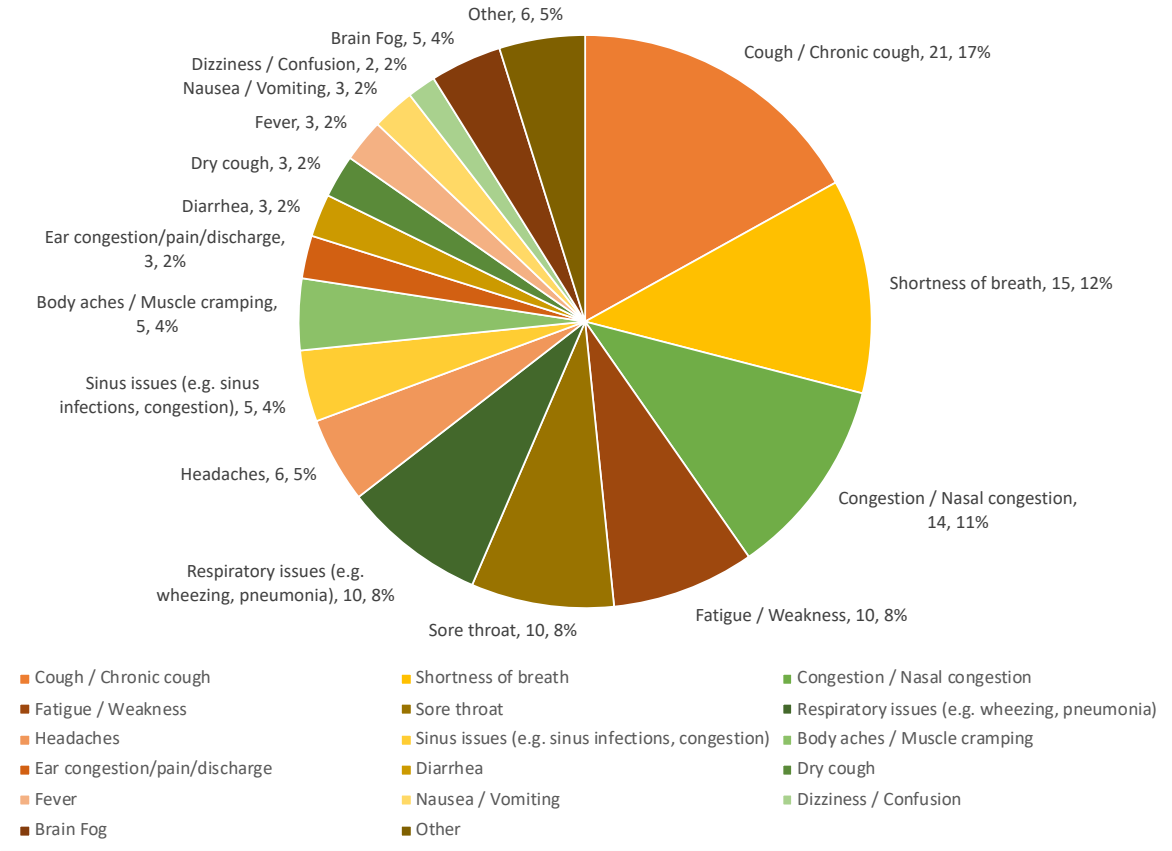

Figure 2. Distribution of Symptoms in long-COVID Cohort. A comprehensive symptom frequency analysis revealed the predominance of respiratory-related symptoms.

5.4.3 Symptom Duration Preceding long-COVID Diagnoses

The mean duration of the first onset of long-COVID symptoms following RT-PCR confirmation of SARS-CoV-2 infection ranged from weeks to months, with the mean duration in our cohort of ~49 days (**Figure 3**). We also determined the frequencies of SARS-CoV-2 variants associated in both long-COVID patients and compared with age-, sex-, ethnicity-matched SARS-CoV-2-infected individuals who did not develop long-COVID symptoms.

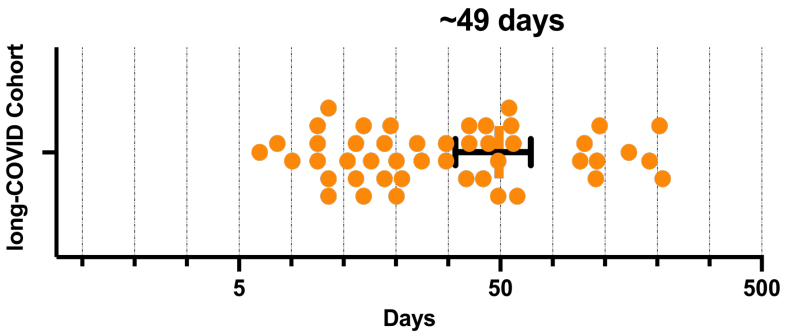

Figure 3: Mean Number of Days from Initial COVID-19 Infection to long-COVID Diagnosis RT-PCR-positive COVID-19 diagnosis was required to confirm SARS-CoV-2 infection—the distribution of days from COVID-19 infection to long-COVID diagnosis (6 to 360 days). The mean days for long-COVID diagnosis was -49 days (SD 60).

5.4.4 Disease Severity of Initial COVID-19 Infection

An important question in long-COVID is who will develop PASC and whether any specific genes, pathways, and markers get activated during initial SARS-CoV-2 exposure and persist in contributing to long-COVID. We know that SARS-CoV-2, like other coronaviruses, is a large positive-sense single-stranded RNA (ssRNA) virus, which is sensed by the host cell pattern recognition receptors (PRRs) and responds by activating several anti-viral pathways critical for early defense against viral invasion.[20,21] This yields a broad presentation of the host's immune response to handle infection and clear the virus. We analyzed the disease severity of initial COVID-19 infection and compared these severities between the long-COVID and non-long-COVID cohorts (**Figure 3**).

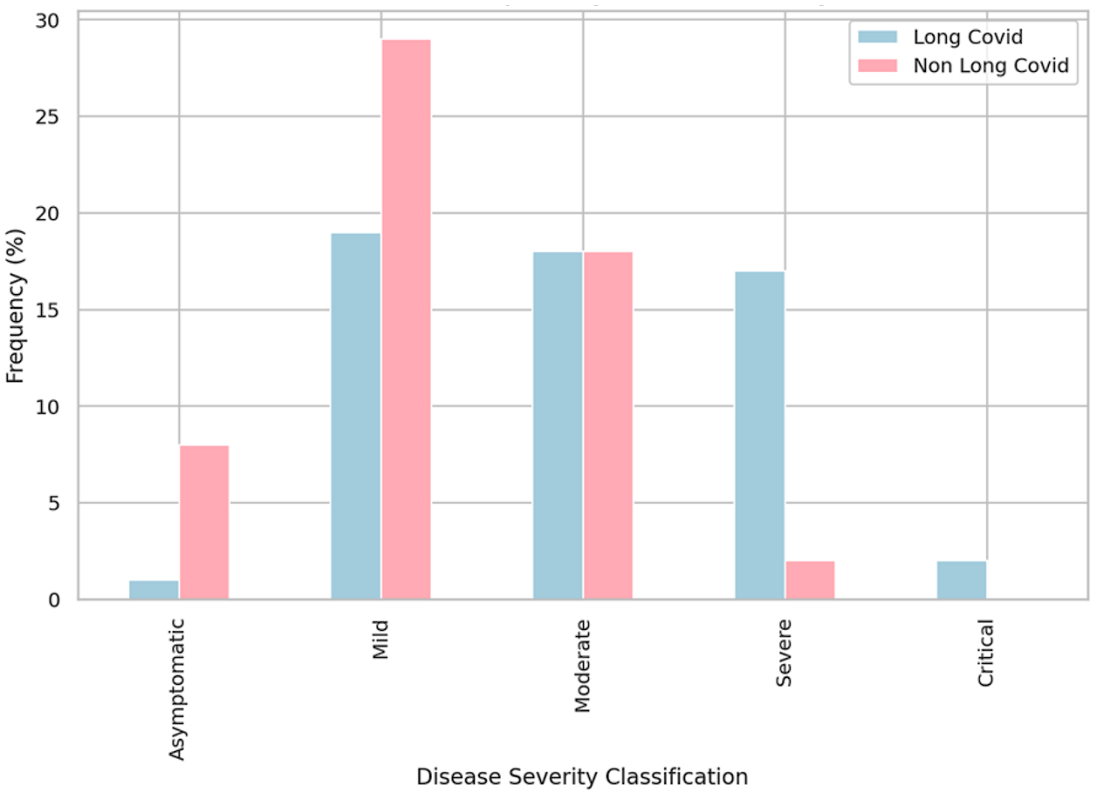

Figure 3. Distribution of Disease Severity. This plot visualizes the distribution of disease severity in long-COVID (blue) and non-long-COVID (pink) cohorts. Disease severity classification categories were used, including Asymptomatic, Mild, Moderate, Severe, and Critical. *Disease severity was obtained from primary COVID-19 infection.

5.5 Discussion

5.5.1 Significance of long-COVID

Our monitoring of SARS-CoV-2 in the community, done through sequencing, analyzed about ~ 3% of all specimens collected at the Washoe County Health District (WCHD) and RRMC. These specimens are analyzed to track the introduction of any variants of concerns (VOCs). Seen in this study were key SARS-CoV-2 variants specifically prevalent in long-COVID patients; thus, it presents the case for the need for continued surveillance and analyses leading to the production of novel findings that will lead to the development of evidence-based clinical guidelines for managing long-COVID symptoms and complications, which will significantly impact patient and public health outcomes. The emergence of long-COVID as a condition affecting individuals recovering from COVID-19 highlights the need for a comprehensive understanding of its etiopathogenesis. This lack of knowledge has led to a significant burden on patients and healthcare systems worldwide. The pathophysiology underlying long-COVID is complex and multifactorial.

5.5.2 Role of the Immune System

Several factors contribute to its development, including residual damage or viral proteins from acute infection, persistent immune system activation, and underlying comorbidities.[12,22-25] There are significant gaps in our understanding of the underlying mechanisms of long-COVID and the potential role of persistent circulating SARS-CoV-2

spike/S1 proteins in contributing to neurological symptomology, which is more prevalent in long-COVID patients and causes long-lasting impacts to patients.[22,26-29] Recent studies have identified levels of the polypeptide that makes up the SARS-CoV-2 spike protein (S1) and immune markers (*e.g.*, IL-6, IL-1β, and IL-18) to be of particular interest associated with long-COVID, which may lead to an identification of underlying mechanisms and novel treatment avenues for long-COVID.[25,30,31] Evaluation of these key pathways and inflammatory biomarkers is expected to identify cellular and molecular determinants altered during long-COVID, which may provide insight into the determinants of PASC. The pathway plays a significant role in cellular energy production, mediating immune responses and neuroinflammation through metabolites.[32]

Our group has demonstrated that severely and critically ill COVID-19 patients exhibit diarrhea due to an elevated level of serotonin in their plasma after SARS-CoV-2 infection.[33] This was done by collecting NP swabs and serum samples from COVID-19 patients with varying disease severity at our hospital, RRMC. SARS-CoV-2 positivity of those patients was determined by rt-PCR in the NP swabs. The levels of anti-SARS-CoV-2 antibodies and cytokines were quantified in the plasma, which showed elevated levels of IL-6 and serotonin among severely ill patients.[33]

Kyn pathway metabolites are correlated with the severity of COVID-19, suggesting that they could serve as useful biomarkers for acute, long- and post-COVID-19 diagnostics.[34] Similarly, the kyn pathway may contribute to CFS,[35,36] which has common abnormalities with long-COVID. These studies suggest that the kyn pathway may lead to CFS and long-COVID pathogenesis. High levels of inflammation,

coagulation, immune response impairment, *etc.*, in individuals with pre-existing comorbidities (*e.g.*, diabetes) could aggravate viral infection and symptoms.[37] For instance, since endogenous ACE-2 regulates glucose uptake and insulin sensitivity,[38] a virus-mediated dysregulation of insulin signaling could further aggravate long-COVID symptoms in individuals with pre-existing comorbidities (*e.g.*, diabetes, HTN, Metabolic Syndrome, *etc.*).

5.5.3 Implications of Circulating S1/S2 Spike Protein

SARS-CoV-2 spike protein consists of two separate polypeptides (S1 & S2), of which S1 binds to ACE2-R, producing cleavage (via host furin-like proteases) and subsequent circulation of S1 into the serum.[25,30,39] Recent data suggests that other receptors may also be able to bind to S1 in other parts of the body, further highlighting the importance of investigating the role of circulating S1 in the multi-organ etiology of long-COVID.[26] This highlights the potential significance of circulating spike protein in long-COVID symptomology.

It is understood that spike/S1 binding to ACE-2R is critical for SARS-CoV-2 pathogenicity and transmissibility.[40,41] Circulating S1 is produced following binding with ACE2-R, undergoing conformational change, and subsequently cleaved into circulation.[39] This process is facilitated by furin proteases (cleavage) and increases the amount of spike binding to ACE2-R.[42] Johnson et al. have shown that loss of the furin cleavage site on the SARS-CoV-2 virus attenuates viral pathogenesis *in vivo*.[42] Paiardi et al. also

demonstrated this attenuation effect using heparin to prevent furin-mediated spike cleavage.[39] Moreover, histamine was shown to potentiate spike entry into endothelial cells, though this mechanism is likely histamine and histamine receptor signaling-dependent.[43] These interactions highlight the tight regulation of the spike/S1/ACE-R receptor binding domain and their effects on overall viral pathogenesis. Furthermore, other potential receptors for spike protein entry (*e.g.*, TMPRSS2 & NPR1) have been suggested as alternative entry routes for S1.[44]

There have been recent studies involving *in vitro* experiments as well as post-mortem human and mouse tissue experiments.[22,28,45-47] Swank et al. analyzed plasma samples from a cohort of previously infected COVID-19 patients, including those diagnosed with post-acute sequelae of SARS-CoV-2 infection (PASC, or long-COVID), and found the concentration of SARS-CoV-2 antigens (S1-spike, full-length spike glycoprotein, and nucleocapsid) were detectable in approximately 65 % of the PASC patients several months after SARS-CoV-2 infection. However, S1 was quantified to a lesser degree than full-length spike and nucleocapsid with higher S1 and nucleocapsid levels in hospitalized and severe cases.[25] Additionally, Patterson et al. found that non-classical monocytes, a subset of monocytes involved in inflammation, were significantly elevated in patients with PASC up to 15 months after initial infection compared to healthy controls.[30] This study also detected the presence of the S1 protein in non-classical monocytes from severe COVID-19 patients and PASC patients, indicating that these cells may be a source (or potential indicator) of inflammation in long-COVID.[30]

5.5.4 long-COVID Symptoms

Long-COVID patients experience persistent symptoms that significantly impair their quality of life (*e.g.*, brain fog, fatigue, headaches, insomnia, memory issues, episodic disorders, cerebrovascular events, sensory and motor deficits, and mental health changes).[9,11,12,48-51] Furthermore, it is now recognized that long-COVID is an ADA-recognized disability, which highlights the urgent need to identify the underlying mechanisms driving this condition.[52]

As the COVID-19 pandemic continues to impact communities worldwide, it is essential to understand this disease's long-term consequences and develop effective interventions to manage and treat long-COVID patients. SH-SY5Y cells show the capability of SARS-CoV-2 infection through a noncanonical mechanism involving spike-neuropilin-1 (NRP-1) interaction.[53] Furthermore, SH-SY5Y cells were investigated for mRNA expression levels of NRP-1 and ACE2, as well as transmembrane serine protease 2 (TMPRSS2), a cell surface protein involved in spike protein priming following ACE2-R binding.[44] However, whether ACE2, TMPRSS2, or NRP-1 are involved in S1-induced long-COVID pathogenesis is unknown. Thus, by identifying the molecular markers involved in spike-and S1/S2-induced long-COVID symptoms, this research could pave the way for developing novel interventions and treatments to improve patient outcomes and QoL

5.5.5 CNS Involvement in long-COVID

Recent neuroimaging studies indicate that SARS-CoV-2 can attack the central nervous system, leading to structural and functional changes in the brain.[54-57] Structural and functional differences have also been observed in COVID survivors,[58-62] potentially contributing to sequela in long-COVID (*e.g.*, fatigue, lack of attention, sleep disturbance, delayed recovery of smell/taste, *etc.*). While most neuroimaging studies have focused on brain correlates of various symptoms, several studies explored the use of functional connectivity as a neurobiological indicator.[63,64] Functional connectivity has also been used to predict healthy individuals' vulnerability to stress, depression, and negative effects following the pandemic.[65-68]

Functional connectivity is typically measured with resting-state fMRI and analyzed in a way that estimates the temporal synchronization of activities across distinct brain regions. When brain networks are analyzed as a graph of connected elements, one can reveal the presence of distinct systems consisting of brain areas highly connected within the system but less so with areas outside the system. A fine balance of within- and between-system connectivity is essential for network operations that support cognitive functions.[69] Reduced network segregation (*i.e.*, decreased within-system connectivity and increased between-system connectivity) has been considered a neural dedifferentiation commonly associated with aging.[70] A potential link between reduced network segregation, sensorimotor performance, and changes in the brain's major inhibitory neurotransmitter, gamma-aminobutyric acid (GABA).[71] Reduced GABAergic inhibition in the primary motor cortex (M1) has indeed been indicated in patients who recovered

from COVID-19 with fatigue and dysexecutive syndrome.[72] Interestingly, a recent study reported that the system segregation of the frontoparietal control and default mode networks modulate the impact of perceived stress on COVID-19-related changes in mental health.[73]

5.6 Clinical Study Future Directions

Collectively, these studies suggest that there may be a relationship between the SARS-CoV-2 S1/spike protein and the development of long-COVID symptoms, highlighting the need for further research to understand the underlying mechanisms fully. The significance of circulating S1/S2 in the serum is notwithstanding precursory SARS-CoV-2 infection or the necessary co-factors associated with viral entry, replication, *etc.* In our study, we will build upon these preliminary findings to investigate the role of S1 in the etiology of long-COVID neurological symptoms and explore the identification of novel therapeutic targets and pathways for management and treatment. The findings of these studies provide important insights into the factors contributing to the severity of COVID-19 and long-COVID, which are valuable for developing effective treatments and prevention strategies. We hypothesize that SARS-CoV-2 infection leads to an accumulation and circulation of S1, which triggers various cellular and molecular processes contributing to the maintenance of neurologic symptomology seen in long-COVID**.** The findings will provide much-needed insight into the cellular and molecular effects of spike/S1 (specifically in neuronal and CNS microvascular cells).

We hypothesize that the innate immune response to SARS-CoV-2 is different in certain individuals, leading to some patients developing long-COVID and that there may be certain common pathways (*e.g.*, overactive immune responses producing pro-inflammatory cytokines) contributing to these differences. We expect to determine these *early* changes (markers) during the primary infection by analyzing the transcriptomic profile of SARS-CoV-2 infected nasal epithelial cells in the NP swabs collected from

long-COVID subjects at primary infection. Comparing the transcriptomic profiles of the SARS-CoV-2 infected cells in the NP swabs from initial exposure of non-long-COVID individuals may provide a differential pattern to identify the etiologic markers of long-COVID.

This project will focus solely on investigating long-COVID etiologies and biomarker(s). We will continue to collect the PHI of additional long-COVID patients with similarly matched controls to determine the effects of SARS-CoV-2 variants, symptoms, vaccination, and comorbidities during acute infection in developing PASC. If available, individuals identified with a long-COVID diagnosis from RRMC will also have their primary infection specimen analyzed. Matched controls (those who have not developed long-COVID) with similar disease severity or symptoms during acute infection, as well as age-, gender-, and ethnicity-matched subjects, will be used for comparison.

5.7 Appendix

5.7.1 Data Availability

Data used in this study are available from the corresponding author upon reasonable request. Protected health information (PHI) is governed under HIPAA regulations.

5.7.2 Funding

We would like to thank the NV INBRE for their support.

5.7.3 Conflicts of Interest

The authors declare no conflicts of interest.

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CHAPTER 6

6. INVESTIGATING THE QUALITY OF LIFE OF PATIENTS UNDERGOING CLINICAL TRIAL TREATMENT FOR GLIOMAS DURING THE COVID-19 PANDEMIC - A RETROSPECTIVE COHORT STUDY

6.1 Abstract

The pandemic has led to reductions in cancer diagnoses, patient enrollment, and antitumor treatment, particularly in the first three months, causing significant distress, anxiety, and depression among glioma patients. While changes in medical management strategies for glioma patients have helped to mitigate these challenges, little is known about the effect of the pandemic and these changes on the quality of life of these patients.

The study examines a cohort of clinical trial patients from the University of Utah Department of Neurosurgery who underwent surgery for glioma and completed QoL questionnaires pre-operatively and then at various intervals up to 12 months post-operatively. Patients were categorized into pre-COVID and COVID cohorts based on the timing of their surgery and completion of the QoL surveys. Our main objective of the study was to examine changes in patient QoL due to the COVID-19 pandemic and identify variables that may have negative effects on QoL and patient health outcomes.

Preliminary results indicate that there were no significant differences in QoL measures between the pre-COVID and COVID-19 cohorts, suggesting that the pandemic did not significantly impact the reported QoL outcomes in this cohort of glioma patients. We discuss the complexities of managing gliomas during the pandemic and the need for updated guidelines and adaptations to neurosurgical and neurooncological practices. Although we identified a few areas of improvement, we highlight the resilience of the patient care protocols and the healthcare system in maintaining the standard of care for glioma patients during the pandemic.

Future directions of this study are to assess the safety and feasibility parameters from pre- and intra-operative MR image-guided resection of gliomas, evaluate the effect of pre-and intra-operative MR image-guided extent of resection (EOR) maximization on overall and progression-free survival, and investigate the impact of novel MR imaging techniques, tumor biomarkers, and intra-operative MR data on patient QoL and outcomes.

6.2 Introduction[1]

The COVID-19 pandemic has significantly impacted the quality of life (QoL) and outcomes of glioma patients. There has been a reduction in new cancer diagnoses, patient enrollment, and antitumor treatment; this was most severe in the first three months of the pandemic.[1,2] Still, the effects of the pandemic have had a profound impact on the QoL and outcomes of glioma patients, with significant distress, anxiety, and depression reported among these patients.[3-5] The pandemic has also led to changes in the management of glioma patients, emphasizing individualized treatment plans to balance treatment benefits with infection risk.[6] Amid the pandemic, the benefit of urgent surgery

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[1]**Abbreviations**

AE, Adverse event; ALT, Alanine aminotransferase; ANCOVA, Analysis of covariance; ANOVA, Analysis of variance; APTT, Activated partial thromboplastin time, AST, Aspartate aminotransferase; AV, Atrioventricular; β-HCG, Beta-human chorionic gonadotropin; BID, Twice daily; BLQ, Below limit of quantification; BMI, Body mass index; BP, Blood pressure; BUN, Blood urea nitrogen; Ca++, Calcium; CBC, Complete blood count; CFR, Code of Federal Regulations; CHF, Congestive heart failure; CI, Confidence interval; Cl-, Chloride; CNS, Central nervous system; CR, Complete response; CT, Computed tomography; CTCAE, Common Toxicity Criteria for Adverse Events; CV, Coefficient of variation; CYP, Cytochrome P450; COVID-19, Coronavirus Disease 2019; D/C, Discontinue; ECOG, Eastern Cooperative Oncology Group; eCRF, Electronic case report form; DLT, Dose Limiting Toxicity; ECG, Electrocardiogram; FACS, Fluorescence Activated Cell Sorting; FDA, Food and Drug Administration; FDG-PET, Fluorodeoxyglucose (FDG)-positron emission tomography (PET); GCP, Good Clinical Practice; GFR, Glomerular filtration rate; GGT, Gamma glutamyl transferase; GLP, Good laboratory practice; HBsAg, Hepatitis B surface antigen; HBV, Hepatitis B virus; HCO3-, Bicarbonate; HCV, Hepatitis C virus; HIV, Human immunodeficiency virus; IC50, Half maximal inhibitory concentration; IEC, Independent ethics committee; IND, Investigational New Drug; INR, International normalized ratio; IRB, Institutional review board; IU, International unit; IV, Intravenous, intravenously; LDH, Lactate dehydrogenase; LLQ, Lower limit of quantitation; MedRA, Medical Dictionary for Drug Regulatory Activities; MRI, Magnetic resonance imaging; MRSD, Maximum recommended starting dose; MTD, Maximum tolerated dose; NOAEL, No-observed-adverse-effect level; NOEL, No-observed-effect-level; PD, Pharmacodynamic(s); PFS, Progression Free Survival; PK, Pharmacokinetic(s); PO, Per os (administered by mouth); PR, Partial response; PT, Prothrombin time; PTT, Partial thromboplastin time; QC, Quality control; RBC, Red blood cell; QD, Once daily; QTc, QT interval corrected; SAE, Serious Adverse Event; SD, Standard Deviation or Stable Disease; T1/2, Terminal Elimination Half-Life; T3, Triiodothyronine; T4, Thyroxine; Tmax, Time Oof Maximum Observed Concentration; TID, Three Times Daily; TSH, Thyroid-Stimulating Hormone; ULN, Upper Limit Of Normal; ULQ, Upper Limit of Quantitation; UV, Ultraviolet; WBC, White Blood Cell

to resect gliomas needed to be balanced with the risk of exposing patients and staff to COVID-19 infection.

Gliomas are primary brain tumors that encompass various subtypes, including low-grade astrocytoma and oligodendroglioma (LGG, WHO grade II), anaplastic astrocytoma and anaplastic oligodendroglioma (AA, AO, WHO grade III), and glioblastoma (GBM, WHO grade IV).[7] Among these, GBM is the most common malignant brain tumor, with a median survival of approximately 14 months.[7] Gliomas exhibit significant heterogeneity, involving various molecular pathways and tumor invasion mechanisms characterized by diverse genetic and epigenetic profiles (*e.g.,* EGFR, IDH1, 1p19q, MGMT, PTEN, MIB-1 proliferation index, ATRX, p53, BRAF, and IDH2 mutations).[7]

A considerable number of gliomas are located in or near eloquent areas, and although the removal of the tumor would be the ideal answer, aggressive resection has the potential to lead to significant and severe postoperative neurological deficits and consequences, which often impact patient QoL and functioning.[8] Thus, to preserve the patient's QoL while simultaneously protecting neurological function and maximizing the extent of resection, neurosurgeons have developed several techniques to help balance these risks and benefits of approaching glioma treatment. One of the most significant predictors of QoL in glioma patients is postoperative function (*i.e.*, Karnofsky Performance Status [KPS]), which can indicate significant impacts on survival outcomes in glioma patients.

We examined a small cohort of clinical trial patients from the University of Utah Department of Neurosurgery (Salt Lake City, Utah, USA). These patients underwent surgery for glioma and completed QoL questionnaires (Functional Assessment of Cancer Therapy [FACT] and MD Anderson Symptom Inventory [MDASI]) administered pre-operatively and at 1, 3, 6, and 12 months post-operatively. Karnofsky's Performance Status (KPS) score was also recorded by the physician, thus allowing for the evaluation of all three metrics (KPS, FACT, and MDASI) concerning the overall QoL evaluation for these patients. We categorized patients into those who had surgery and completed all of the QoL questionnaires before March 2022 (Pre-COVID cohort) and those who were operated on before COVID and completed the QoL surveys during COVID-19 (COVID Cohort).

6.2.1 Study Purpose and Objectives

The purpose of this retrospective, unblinded clinical trial was to assess safety and feasibility parameters from pre- and intra-operative MR image-guided resection of gliomas (DCE-MRI, DSC-MRI, 2-HG MR spectroscopy, and DWI/ADC histograms) that may guide EOR maximization of gliomas and lead to a minimization of patient complications.[9] The study aimed to assess the effect of preoperative and intraoperative MR image-guided EOR maximization of gliomas on overall and progression-free survival and QoL, as well as correlate various tumor and peritumoral areas assessed with MR imaging techniques with 2-HG expression and hypoxia biomarkers and RNA expression signatures from intraoperatively sampled tissue.[9] The impact of novel MR

imaging techniques, tumor biomarkers, and intraoperative MR imaging on patient QoL were also evaluated. These results provide new insights into the molecular and genomic variants of GBM, the impact of intra-tumor heterogeneity on tumor progression, and treatment response.[10,11] The ultimate goal was to evaluate patient QoL outcomes and the effect of other variables to understand the response to treatment better and investigate whether COVID-19 impacted QoL and patient outcomes.

6.3 Methods

6.3.1 Overview

This study utilized a retrospective clinical trial dataset from the Department of Neurosurgery at the University of Clinical Neurosciences Center Utah (Salt Lake City, UT, USA). A total of 51 participants were eligible for inclusion in the trial, consisting of 34 patients enrolled in the study and operated on before COVID and 17 who were enrolled and operated during COVID-18. Gliomas consisted of contrast-enhancing and non-contrast-enhancing lesions on imaging. Participants found to have non-gliomas were excluded. Patients were assessed for survival every 90 days (± 30 days) and until 12-months postoperatively. Survival status was determined via the patient's medical record. Survival follow-up visits were conducted remotely in some cases.

6.3.2 Study Selection

Patients were identified through a review of the University of Utah EPIC electronic medical record (EMR) for patients newly admitted to the neurosurgery service and through referrals to the Neuro-oncology clinic to evaluate a suspected glial lesion. All patients admitted to the University of Utah Health Sciences Center with suspected glioma were eligible for enrollment in the trial (**Table 1**). All patient data was stored in a HIPAA-compliant, password-encrypted database (OnCore and RedCap databases). Patients were distributed into suspected low- and high-grade glioma groups, where specific imaging modalities were subsequently acquired. Patients who did not agree to

enrollment in the trial continued to be treated with the standard of care and used as a comparison against recruited patients to ensure no biased selection. Interim data analysis and reporting and data safety monitoring were performed according to the policy of the University of Utah Institutional Review Board.

6.3.3 Inclusion and Exclusion Criteria

For inclusion, patients must have signed the informed consent form, be 18 years or older, and correctly follow study protocols and follow-up procedures. They have a suspected diagnosis of glioma (grades II, III, or IV). Preoperative MR perfusion was mandated for those with enhancing tumors, while preoperative MR 2-HG spectroscopy was required for individuals with non-enhancing tumors. Furthermore, eligible patients were indicated for standard-of-care surgical resection, radiation, and chemotherapy following the standard-of-care guidelines. For inclusion, Karnofsky performance status scores of at least 60 and a life expectancy of more than 12 weeks were required.

Patients were excluded if they did not have a prior diagnosis of intracranial glioma or had other malignancies requiring systemic therapy within the next three years. Those with any contraindications for receiving 6000 Gy of radiation to the brain were also excluded. Patients that had an immediate need for palliative interventions for primary disease (*e.g.*, impending herniation), as well as those with significant medical histories (*e.g.*, evidence of bleeding, diathesis, coagulopathy, a history of intracerebral abscess within six months before Surgery [Day 0], undergoing a major surgical

procedure, open biopsy, or significant traumatic injury within 28 days before Day 0), were also excluded. Pregnant females and those unable to undergo MRI imaging with contrast were removed from the study and replaced.

6.3.4 Description of Consent Processes

Patients were enrolled and identified through the EPIC electronic medical database or neurooncology clinic referral. Patients were categorized as inpatients or outpatients. After a review of the patient's medical record and imaging for the inclusion criteria, patients were contacted to consent to the trial. The time between identification and enrollment was around 1-2 days. All consenting was performed before preoperative imaging. During the study, patients who previously consented to LAR to regain their capacity to make informed consent were reconsented at that time for continuing in the trial; this reconsent occurred in the hospital post-treatment, during follow-up in the clinical setting, or remotely. The risks and benefits of continuing in the trial at the time of reconsenting were thoroughly discussed.

6.3.5 Study Outline

*One Day -14 to Surgery (Day 0), p*atients were screened, enrolled, and consented to the clinical trial through a list of directly admitted patients to the neurosurgery service or through evaluations through the neuro-oncology clinic. Female patients of

childbearing age (18-45 years) underwent a urine or serum pregnancy test to exclude pregnancy before enrollment. Female patients of childbearing potential were advised to use effective contraceptives (*e.g.*, abstinence, oral contraceptives, the contraceptive patch, the contraceptive ring, condoms, etc.) if sexually active and continuing with this trial. Patients were categorized into Group A (*contrast enhancing)* and Group B (*non-contrast enhancing*). They underwent standard-of-care preoperative MR imaging (*e.g.*, T1, T2, FLAIR, contrasted T1, DWI/ADC map, and stereotactic sequence). MR perfusion (Group A) and 2-HG spectroscopy with a minimum of 3 voxels placed within and around the suspected glioma and a contralateral control voxel (Group B) are non-standard-of-care techniques. Patients underwent neurological evaluation and administration of the QoL metrics (*e.g.*, MDASI, FACT). Standard surgical treatment was performed, and an intraoperative MRI was performed to evaluate for residual tumor. Post-MRI resection was performed on a case-by-case basis at the attending physician's discretion.

Biopsies for Group A patients included areas with 1) Abnormal preoperative T1 contrast enhancement (suspected tumor) and 2) Abnormal intraoperative T1 contrast enhancement, MR perfusion, and DWI-ADC MRI. Biopsies for Group B patients included areas with 1) Abnormal preoperative T2 and FLAIR signal abnormalities (suspected tumor), 2) Areas with preoperative 2-HG voxels from the central T2/FLAIR signal area, 3) Abnormal intraoperative T2, FLAIR and DWI-ADC signal abnormalities, 4) Areas with intraoperative 2-HG voxels, and 5) Areas with new within 1 cm of the resection bed defined as the end of the T2/FLAIR signal border.

On Days 1 to 7, patients underwent evaluation in the neurocritical ICU (NCCU) or on the floors as needed for postoperative recovery. At this time, the risk of adverse or serious events with this clinical trial was the same as with any other craniotomy for tumor resection. Standardized postoperative complications assessment was performed before discharge. Any unusual increase in adverse or serious events within the study compared to standard tumor resection led to a study halt until the cause was identified; moreover, the study was terminated when the increased rate of adverse events could not be remedied. Any halting or termination of the study was discussed with the IRB. Patients underwent a standard postoperative MRI to serve as a baseline for evaluating tumor recurrence at future follow-ups.

On Days 30-60 (±30 days), patients returned for follow-up and underwent neurological evaluation and QoL assessments (MDASI, FACT). For patients unable to return to the clinic (due to geographical or insurance issues or if the recommended standard of care follow-up schedule differed from the follow-up schedule dictated for this study), QoL assessments were mailed to the patient for completion, and a follow-up call was completed to assess for complications and survival status.

On Days 120, 180, and 365 (± 30 days), patients returned for follow-up and underwent neurological evaluation and QoL assessments (MDASI, FACT). A surveillance MRI was scheduled as standard for tumor follow-up, if applicable. MRI results and neurological exam notes were requested from the patient's treating physician if applicable. For patients unable to return to the clinic (due to geographical or insurance issues or if the recommended standard of care follow-up schedule differed from the

follow-up schedule dictated for this study), QoL assessments were mailed to the patient for completion, and a follow-up call was completed to assess for complications and survival status.

6.3.6 Statistical Methods, Data Analysis, and Interpretation

This feasibility study aimed to evaluate whether these advanced imaging modalities can be used intraoperatively. Our initial power calculation (equivalence two-sided T-test, power of 80 %, alpha = 0.05, equal variance in primary outcome measurement, and 10 % difference between groups) yielded a minimum requirement of 19 patients. Pilot data from retrospective clinical studies was obtained to perform a power analysis of postoperative complications resulting in 1-month complications. T-tests will be used to analyze parametric data with correction for multiple comparisons, while nonparametric data will be analyzed using the Chi-squared test.

Comparison of pre- and post-operative EOR from group A will involve comparison of contrast-enhancing regions of interest, while areas of T2/FLAIR signal will be compared for group B. Comparison of EOR and patient complications will be evaluated as a continuous variable by linear regression and as a discrete measure by logistic regression. Other variables will be evaluated by T-test and Chi-square test when appropriate. Univariate and multivariate analysis will identify factors that predict EOR, patient complications, and QoL. Patient complications will also be compared to QoL assessments. Survival analysis will be performed using Kaplan- Meier and Cox

regression analyses. Samples stained by immunohistochemistry and ELISA will be quantified and analyzed. Target proteins and genes will be evaluated by Western blot and RT-PCR.

The dataset was processed using Python (v3.12), with the pandas library (pandas v1.3, https://pandas.pydata.org/) employed for data manipulation and cleaning. Initial processing was conducted. Descriptive statistics were calculated to determine the mean and standard deviation of the QoL scores at each point in time. Individual patient trajectories were plotted to visualize the range of responses over time. Additionally, summary plots were generated (seaborn v1.11, https://seaborn.pydata.org/). To ensure the robustness of the longitudinal analysis, the Python library (statsmodels, v1.12, https://www.statsmodels.org/) was utilized to perform mixed-effects linear regression models, accounting for repeated measures within patients over time. This approach allowed for assessing changes in QoL metrics while controlling for the within-patient correlation of repeated observations.

6.3.7 *Ethical Considerations*

The Institutional Review Board at the University of Utah reviewed and approved the study protocol. All procedures performed were following the ethical standards of the institutional research committee. Informed consent was obtained from all individual participants included in the study. All data was deidentified before analysis.

6.4 Results

6.4.1 Overview

The study included 51 adults with suspected glioma (**Table 1**) at the Department of Neurosurgery at the University of Clinical Neurosciences Center Utah (Salt Lake City, UT, USA). Patients underwent standard surgical resection followed by radiation and chemotherapy as per the current standard of care. Complication rates (neurological deficit at one year) were 10–25 %, depending on EOR (*i.e.*, patients with lower EORs were expected to have a ~10 % complication rate while patients with greater EORs were expected to have a ~25 % complication rate).

Table 1. Descriptive Statistics of Study Population and Glioma Characteristics

n = 51	**Pre-COVID**	**During COVID**	**Total Count (%)**
Gender			
Male	23 (45)	5 (10)	28 (56)
Female	11 (22)	12 (24)	23 (45)
Diagnosis			
Glioblastoma, WHO Grade IV	5 (10)	13	18 (36)
Oligodendroglioma, WHO Grade II	(3)	7	10 (20)
Anaplastic Astrocytoma, WHO Grade III	2 (4)	4 (8)	6 (12)
Diffuse Astrocytoma, WHO Grade II	1 (2)	5 (10)	6 (12)
Other Diagnoses	1 (2)		
Astroblastoma		1 (2)	1 (2)
Glioneuronal Tumor		1 (2)	1 (2)
Metastatic Adenocarcinoma	1 (2)	1 (2)	2 (4)
Frontal cortical Dysplasia and Moderate Chronic Astrogliosis	-	1 (2)	1 (2)
Anaplastic Oligodendroglioma, WHO Grade III	-	1 (2)	1 (2)
Astrocytoma, WHO Grade II	1 (2)	-	1 (2)
Anaplastic Ependymoma, WHO Grade III	1 (2)	-	1 (2)
Anaplastic Astrocytoma, WHO Grade II	1 (2)	-	1 (2)
Anaplastic Astrocytoma, WHO Grade IV	1 (2)	-	1 (2)

Complications			
Weakness	4 (4)	-	4 (4)
DVT	3 (3)	-	3 (3)
Intraoperative Neurological Injury	2 (4)	-	2 (4)
Dysarthria	1 (2)	1 (2)	2 (4)
Cognitive disturbance	2 (4)	-	2 (4)
Cognitive Deficit	2 (4)	-	2 (4)
Aphasia	1 (2)	1 (2)	2 (4)
Dysesthesia	1 (2)	-	1 (2)
Paresthesia	1 (2)	-	1 (2)
Hemiparesis	1 (2)	-	1 (2)
Hypocalcemia	1 (2)	-	1 (2)
Hemorrhage	1 (2)	-	1 (2)
SMA syndrome	1 (2)	-	1 (2)
Atrial fibrillation	1 (2)	-	1 (2)
Dysphagia	1 (2)	-	-
Ataxia	-	1 (2)	1 (2)
Seizure	1 (2)	-	1 (2)

6.4.2 Glioma Pathologies in Cohort

The analysis of the pathology diagnoses within the dataset revealed a diverse range of glioma subtypes, with a notable concentration in certain categories (**Figure 1**). WHO Grade IV Glioblastoma was the most prevalent (24 %, n = 12). This was followed by WHO Grade II Diffuse Astrocytoma and WHO Grade II Oligodendroglioma (12 % and 10 %, n = 6 and 5, respectively). WHO Grade III Anaplastic Astrocytoma (8 %, n = 4) and oligodendroglioma were less frequent, each contributing to smaller percentages of the total. The data visualization, rendered in shades of purple, provided a clear and distinct representation of the distribution of diagnoses, adhering to the preferred PRISM palette's neutrals. The standardization process ensured that all data were accurately categorized, facilitating a comprehensive analysis of the pathology spectrum present in the dataset.

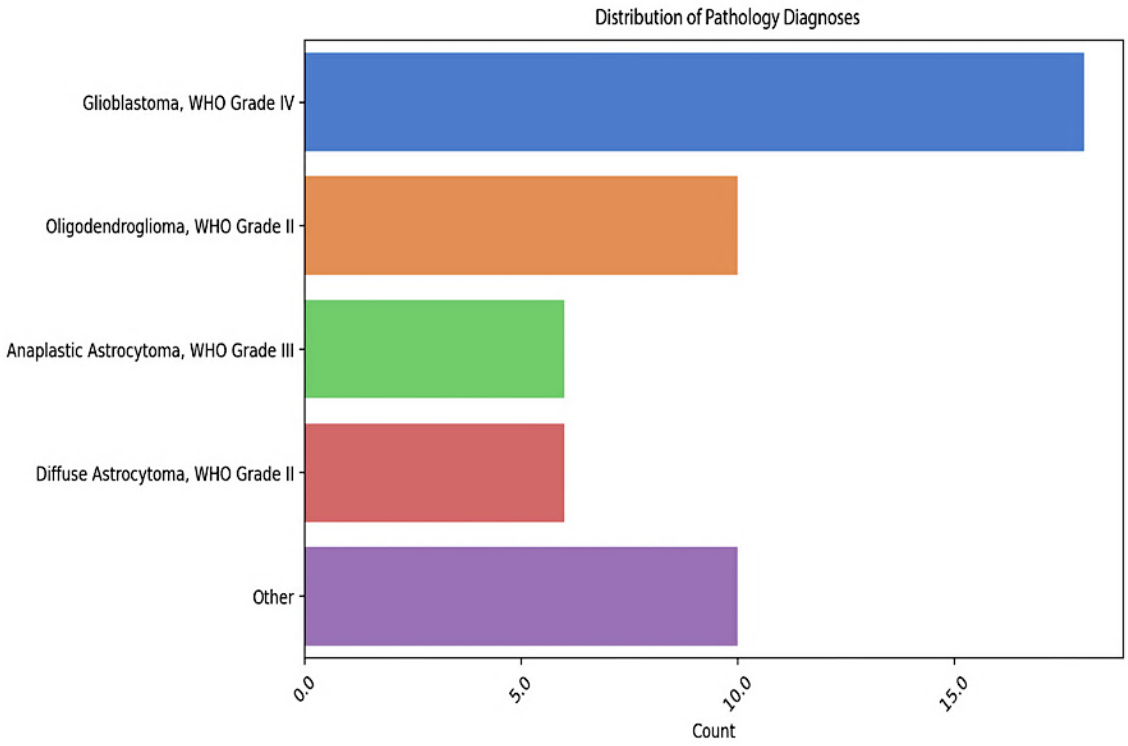


Figure 1. Distribution of Glioma Pathology Diagnoses. The bar chart represents the frequency of each glioma subtype within the dataset. The "Other" category includes pathologies with a single occurrence (Astroblastoma, Glioneuronal Tumor, Anaplastic Astrocytoma [WHO Grade IV], Metastatic Adenocarcinoma, Front Cortical Dysplasia with Moderate Chronic Astrogliosis, Anaplastic Oligodendroglioma [WHO Grade III], Astrocytoma [WHO Grade II], Anaplastic Ependymoma [WHO Grade III], and Anaplastic Astrocytoma [WHO Grades II and IV].

In our retrospective analysis, we stratified glioma patients into two cohorts based on the timing of their operations relative to the COVID-19 pandemic—those who underwent surgery before the emergence of the pandemic (pre-COVID) and those who were operated on during the pandemic (COVID-19). Mean KPS, FACT, and MDASI scores were computed from preoperative to 12-month postoperative intervals for each

cohort (**Figure 2**). The analysis was stratified into two cohorts: Pre-COVID and during COVID-19. The mean MDASI scores (symptom severity and interference**)** indicate that the QoL followed a similar trajectory for both cohorts (**Figure 2A**). There were observable differences in the scores at specific time points, suggesting potential impacts of the pandemic on patient-reported outcomes. The FACT scores (cancer-related QoL) showed a distinct pattern for each cohort (**Figure 2B**). The pre-COVID cohort's scores appeared to improve more consistently over time compared to the COVID-19 cohort, which may reflect the additional challenges faced during the pandemic. The KPS scores (functional status) also demonstrated a divergence between the two cohorts where the COVID-19 cohort exhibited a more variable recovery trajectory (**Figure 2C**).

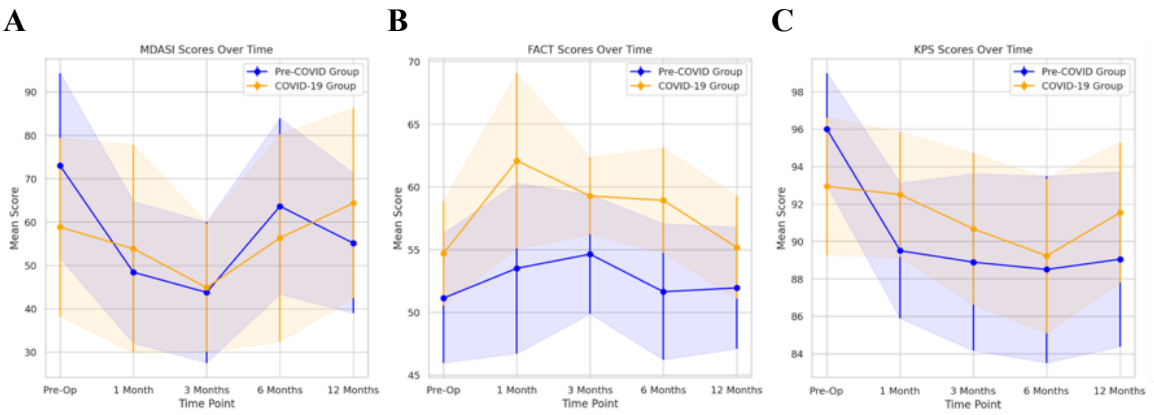


Figure 2 Mean QoL Questionnaire (MDASI, FACT, and KPS) Scores Over Time by Cohort. This figure illustrates the trend of mean MDASI (**A**), FACT (**B**), and KPS (**C**) scores from pre-operation to 12 months post-operation. The scores are separated by cohorts, with the pre-COVID cohort (blue) and the COVID-19 cohort (orange). 95% confidence bands are shaded per group. Individual and mean KPS, FACT, and MDASI scores are displayed in **Supplemental Figure 1**

Kaplan-Meier estimates were generated to assess overall and progression-free survival in the two cohorts (**Figure 3**). The median overall survival for the cohort was 21 months (682 days in the pre-COVID cohort and 345 days in COVID-19 cohort). 1-year survival probability were about the same in both cohorts (75 vs. 74 %). These results suggest that there were no significant differences in overall survival probabilities between patients treated pre-COVID and during COVID, supporting the conclusion that the timing of treatment relative to the pandemic did not adversely affect overall survival .

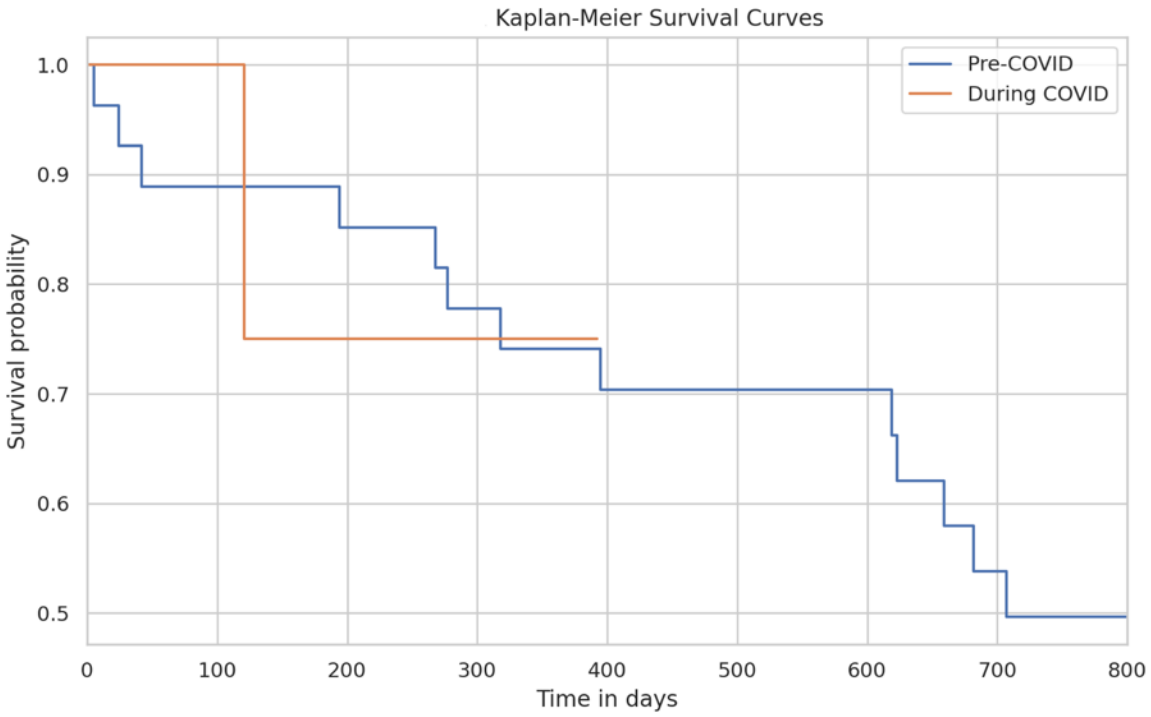


Figure 3. Kaplan-Meier Survival Curve: Estimated Survival Probabilities Over Time. This curve depicts the estimated survival probabilities for glioma patients over time, measured in days. Each downward step in the curve corresponds to an event, which in this context is a death. The plot provides a visual summary of the survival experiences within the cohorts (pre-COVID, blue; during COVID, orange).

We assessed the QoL variables (KPS, FACT, and MDASI scores), collected pre-operatively and at 1, 3, 6, and 12 months post-operatively (**Table 2**). The analysis revealed no statistically significant differences in QoL measures between the two cohorts across all time points. For KPS scores, preoperative measures were similar between the pre-COVID and COVID-19 groups (Mean = 94.1, SD = 8.57 vs. 92.9, 7.72; $p = 0.624$). This pattern persisted at 1-month (87.9, 9.78 vs. 92.5, 6.83; $p = 0.074$), 3-months (87.7, 10.7 vs. 90.7, 8.0; $p = 0.319$), 6-months (88.6, 13.0 vs. 89.2, 7.6; $p = 0.839$), and 12-months (88.9, 11.5 vs. 91.5, 6.9; $p = 0.372$) postoperative time points. FACT and MDASI scores followed similar non-significant trends at all time points. These findings suggest that the constraints of the pandemic did not significantly or notably negatively impact the reported QoL outcomes in this cohort of glioma patients.

Table 2. **Comparative Analysis of QoL Variables at Visits.** Mean scores between pre-COVID and During COVID-19 were computed and compared using t-tests.

Time Point	Measure	Pre-COVID Mean	Pre-COVID Std Dev	COVID-19 Mean	COVID-19 Std Dev	*p-value*
Preop	**KPS**	94.1	8.57	92.9	7.72	0.624
	FACT	52	11.57	54.7	8.47	0.358
	MDASI	77.8	51.31	58.9	41.99	0.189
1 month	**KPS**	87.9	9.78	92.5	6.83	0.0742
	FACT	57.4	13.04	62	12.85	0.355
	MDASI	63.1	39.79	53	44.03	0.524
3 months	**KPS**	87.7	10.7	90.7	7.99	0.319
	FACT	55	8.61	60	6.06	0.109
	MDASI	55.42	34.48	45	29.28	0.470
6 months	**KPS**	88.6	13.0	89.2	7.6	0.839
	FACT	66	11.33	58	7.38	0.102
	MDASI	89	41.67	56.3	42.28	0.505
12 months	**KPS**	88.9	11.5	91.5	6.89	0.372
	FACT	53	10.35	55.17	7.23	0.662
	MDASI	55	32.97	64	38.64	0.508

6.5 Discussion

6.5.1 Overview of QoL Assessments During the COVID-19 Pandemic

Gliomas are a heterogeneous group of primary brain tumors with significant molecular heterogeneity and complex genetic profiles.[5] Patients with gliomas are particularly vulnerable due to their relative immunocompromised status from previous radiation and chemotherapy treatments; moreover, the postoperative mortality rate is higher in patients with cancer than those with benign diseases.[12] Amid the pandemic, the benefit of urgent surgery to resect GBM needed to be balanced with the risk of exposing patients and staff to COVID-19 infection.[12] This balance created a challenging issue for healthcare providers and patients worldwide. Other management strategies for gliomas during the pandemic included proposed algorithms that individually suggest tailoring surgery, radiotherapy (RT), and chemotherapy to increase survival rates and QoL while reducing the risk of COVID-19 exposure.[13] There is strong evidence to support the importance of minimizing hospital contact for glioma patients during a pandemic; however, this risk mitigation must come without delay to the diagnosis and treatments[13]

The KPS is a standardized tool that evaluates a patient's capacity to perform everyday tasks and activities.[14] It scores patients on a scale of 0 to 100, with 100 representing perfect health and 0 indicating death. It is a comprehensive measure of a patient's overall health status, considering physical and psychological well-being. It has been extensively validated in different patient populations and is widely used in clinical trials and observational studies. Strengths of the KPS include its simplicity and broad applicability to various diseases and health conditions; however, it is a subjective

measure and can vary depending on who makes the assessment (*e.g.*, a physician, a nurse, or the patient). It also lacks sensitivity to slight changes in a patient's condition. It may not accurately reflect the patient's QoL, focusing more on physical functioning and less on psychological or social well-being. In our study, the pre-COVID cohort's KPS scores appeared to improve more consistently over time compared to the COVID-19 cohort, which may reflect the additional challenges faced during the pandemic.

The individual patient trajectories depicted considerable variability, with some patients maintaining stable QoL scores over time while others experienced significant changes. This could be attributed to the disruptions in healthcare services and support systems during the pandemic. Overall, the results suggest that the COVID-19 pandemic has had a measurable effect on the quality of life and functional recovery of glioma patients. Further research is warranted to explore the underlying factors contributing to these differences and to develop strategies to mitigate the impact of such global health crises on patient outcomes.

The MDASI assessment is a multi-symptom patient-reported outcome measure that assesses the severity of multiple symptoms common to cancer patients and the interference of these symptoms with daily functioning.[3,15,16] In our study, MDASI scores decreased from preoperative levels, implying a reduction in symptom burden. The MDASI includes thirteen core items measuring symptom severity and six measuring symptom interference with daily activities. Strengths of the MDASI include its focus on symptoms relevant to cancer patients (*e.g.*, the inclusion of symptom severity, interference items, and its good psychometric properties). Limitations of the MDASI

include its reliance on patient self-report, which can be influenced by factors such as mood, attentiveness, and recall biases. They may also not capture all symptoms relevant to a specific patient or tumor type.

The FACT assessment is a comprehensive set of quality-of-life questionnaires specifically designed for use with cancer patients.[17] In our study, the FACT scores showed a more pronounced decrease post-operatively, suggesting a potential decline in QoL following treatment. FACT measures multidimensional aspects of QoL, including physical, social/family, emotional, and functional well-being. Strengths of the FACT include its multidimensional approach to quality-of-life assessment, ease of use, and extensive validation in various cancer populations. It also includes cancer-specific modules relevant to specific types of cancer or treatments *(e.g.,* GBM). The limitations of the FACT include its length, which may be burdensome for some patients, and its reliance on patient self-reporting. Given the strengths and weaknesses of each QoL measure, using them in combination can provide a comprehensive and robust assessment of patient QoL. The KPS provides an overall measure of patient functioning, the MDASI focuses on symptom severity and interference, and the FACT comprehensively assesses various aspects of QoL.

Studies have also highlighted the need for guidelines and adaptations in neurosurgical practice during the pandemic.[5] The insights gained from this health crisis are valuable for healthcare workers worldwide, especially neurosurgeons and neuro-oncologists. Offering uninterrupted oncological surgical service during difficult times such as the pandemic requires prompt and strict regulations that can serve patients

without compromise. Thus, analyzing the effect of the pandemic on their QoL is essential for making regulatory and clinical changes aimed at improving overall QoL, ensuring that certain key factors contributing to worsening QoL are not missed. We hope to contribute to these efforts by providing a comprehensive analysis of the impact of COVID-19 on the QoL of glioma patients and offering insights into the management of Neurosurgery and other healthcare services during critical and hectic times such as the pandemic, which we hope will extend into the future.

6.5.2 Clinical Trial Future Directions

Clinical trials, in general, have also been affected by the pandemic.[2] Neuroendovascular clinical trials reported that enrollment was suspended at 78 % of sites due to COVID-19.[2] Thus, several strategies have been employed to maintain recruitment in glioma clinical trials during the pandemic. Remote electronic consent (e-Consent) has been implemented to allow patients to provide informed consent remotely, minimizing face-to-face interactions that increase the risk of COVID-19 transmission.[18] Remote prescreening and screening activities can be conducted through phone or email, allowing research teams to identify potential participants and prepare for trial restarts once quarantine measures are lifted.[19] Telehealth and virtual consultations have increased during the pandemic, allowing for remote patient monitoring and follow-up visits.[20] Flexible institutional review board policies are crucial to support clinical trials during the pandemic, as they allow for rapid amendments to protocols and new strategies to maintain patient safety and data integrity.[20] Remote monitoring offers an alternative to

traditional on-site monitoring practices, allowing research sites to share information with monitors through various methods.[20]

The management and treatment of gliomas require an increased extent of resection, newer MR perfusion techniques, and the identification of biomarkers.[5] Newer MR perfusion techniques, such as dynamic contrast-enhanced (DCE-MRI) and dynamic susceptibility contrast magnetic resonance imaging (DSC-MRI), have been developed to assess cerebral blood flow, vascularity, and tumor permeability.[21] Studies using DCE-MRI have shown that cerebral blood volume and flow correlate with poor outcomes, responsiveness to radiation, and tumor expression of vascular endothelial growth factor (VEGF).[21] Perfusion MRI parameters, such as cerebral blood volume and relative apparent diffusion coefficients, have been used to predict tumor aggressiveness and glioma recurrence.[21] These dynamic MRI techniques can potentially guide surgical resection and improve the understanding of tumor behavior.[21]

MR perfusion can improve the discrimination of tumor-containing regions from the normal brain.[9] Mutation of the Krebs cycle enzyme isocitrate dehydrogenase 1/2 (IDH1/2) results in the generation of the oncometabolite D-2-hydroxyglutarate (2-HG), which is found in a high percent of LGG and is associated with improved survival of patients with this mutation.[9] 2-HG can be detected by MR spectroscopy, opening the prospect of predicting patient outcomes in a noninvasive manner and using this biomarker to guide resection, treatment efficacy, and patient prognosis.[9] Recent studies have shown that IDH mutations are early events in the development of astrocytoma and oligodendroglioma.[9] Additionally, IDH mutations have been associated with a distinct

hypoxia/angiogenesis transcriptome signature that can be imaged with perfusion MRI in human glioma.[9] This suggests that MR perfusion imaging can provide valuable information about the tumor microenvironment and guide clinical treatment decisions.[9]

Intraoperative MRI (iMRI) has been used as an adjuvant in improving overall tumor resection while reducing patient deficit.[22] iMRI allows for evaluating tumor areas, avoiding critical structures, and assessing possible surgery complications during surgical resection.[22] The overall improvement of patient treatment with iMRI has not been uniform; thus, there is a need for more uniform radiological methods for assessing pre- and post-surgical tumor volumes.[22] Dynamic MR techniques and iMRI technology are groundbreaking tools in the direct surgical treatment of gliomas.[22] These dynamic MRI techniques can guide surgical resection and improve the understanding of tumor behavior.[21] MR perfusion can improve the discrimination of tumor-containing regions from the normal brain.[9]

The extent of resection EOR has been shown to improve patient prognosis in glioma treatment.[23,24] However, there is a lack of consensus regarding the safety of extensive EOR, prospective studies on EOR, and novel MR-based methods for guiding glioma resection.[7] Both low-grade and high-grade gliomas benefit from increased EOR, with studies demonstrating a significant advantage associated with > 98 % resection of tumors.[24] However, the impact of EOR on patient outcomes has been evaluated in retrospective and heterogeneous studies. Increased EOR has been shown to correlate with improved survival from low- and high-grade gliomas; however, there remains a lack of consensus regarding the safety of extensive EOR, prospective studies evaluating EOR,

and novel MR-based methods for guiding glioma resection. We plan to evaluate pre- and intra-operative MR perfusion, MR spectroscopy, and DWI/ADC MRI modalities during standard surgical resection of gliomas. Intra-operative tumor sampling was performed using iMRI-guided frameless stereotactic localization. Detailed outcomes on the role of preoperative and intraoperative MR imaging in guiding EOR to balance maximal tumor removal against the risk of postoperative deficits, focusing on minimizing complications and optimizing patient outcomes and overall survival, as well as the correlation between MR imaging techniques and molecular biomarkers, including 2-HG expression, hypoxia biomarkers, and RNA expression patterns are pending.

Genomic studies have identified distinct subtypes of primary GBM, including proneural, mesenchymal, classical, and neural subtypes, each characterized by specific alterations in genes and molecular pathways; however, the heterogeneity of glioblastoma contributes to the difficulty in treating the disease and understanding its behavior.[25] There is a need for further investigation, particularly in the context of newer dynamic MRI studies and understanding of glioma biomarkers and transcriptome signatures.[24] Sampled tissue was assessed for glioma biomarkers and RNA expression patterns (in a smaller subset of patients) and correlated to pre- and intra-operative MR features. The impact of novel MR imaging techniques on the correlation of tumor biomarkers and patient outcomes, as well as the impact of COVID-19 on QoL metrics, will be investigated.

Assessing biomarkers and RNA expression patterns in tumor samples provides valuable insights into glioma biology.[5] Specialized MRI techniques and intraoperative MR imaging can improve glioma treatment and tumor behavior.[5] These biomarkers can

predict patient prognosis and improve treatment stratification in gliomas.[26] Commonly mutated genes in glioblastoma include EGFR, PDGFR, p53, PTEN, MGMT, and IDH1/2.[26] MGMT methylation and IDH1/2 mutation have demonstrated independent improvement in prognosis and enhanced therapeutic efficacy with chemoradiotherapy and surgical resection.[26] IDH1/2 mutation, in particular, has been associated with improved overall patient prognosis and enhanced surgical resection outcomes.[26] Preoperative evaluation of 2-HG production by MR spectroscopy has been suggested to delineate low- and high-grade gliomas and improve tumor volume resection.[26]

6.6 Conclusion

Our retrospective analysis revealed a nuanced landscape of glioma diagnoses and their implications on patient outcomes during the COVID-19 pandemic. Despite the challenges imposed by the pandemic, our findings suggest that the QoL metrics, including KPS, FACT, and MDAIS scores, did not significantly differ between the pre-COVID and COVID-19 patient cohorts. This underscores the resilience of the patient care protocols and the adaptability of the healthcare system in maintaining the standard of care for glioma patients. Moreover, our investigation into the correlation between tumor characteristics, surgical interventions, and patient outcomes provided valuable insights into the complex interplay between tumor biology and treatment strategies. Notably, our cohort's distribution of glioma subtypes highlighted the prevalence of high-grade glioblastoma, which informed the subsequent analysis of survival outcomes.

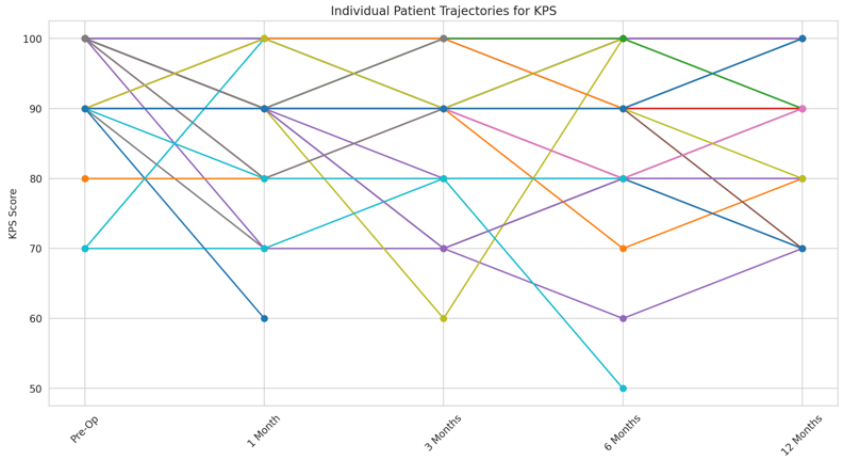
The utilization of advanced pre- and intra-operative MR image-guided techniques showcased a commitment to optimizing the EOR while minimizing patient complications. Meanwhile, the analysis of survival data through Kaplan-Meier curves indicated the overall survival did not significantly deviate from existing benchmarks in glioma treatment. The descriptive statistics of complications further corroborated the safety of the surgical procedures, with low rates of neurological and systemic complications observed. The COVID-19 pandemic has significantly impacted clinical trials for glioma treatment, including recruitment.[1] Various strategies have been employed to maintain recruitment in glioma clinical trials during the pandemic; however, the long-term impact of the pandemic on glioma clinical trials and recruitment remains to

be seen. This study contributes to the ongoing discourse on managing glioma amidst a global health crisis, offering evidence of sustained clinical efficacy and patient-centered care. Future research should expand upon these findings with larger cohorts and more granular data, further refining the understanding of glioma management in unprecedented circumstances.

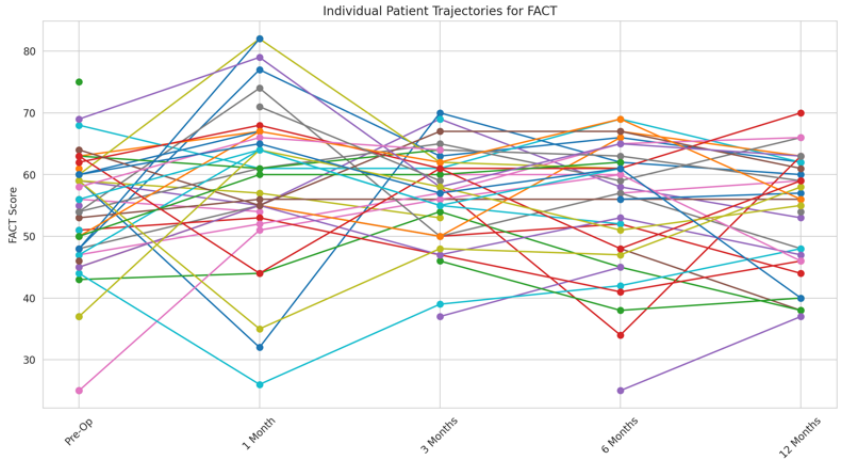
6.7 Appendix

6.7.1 Supplemental Data

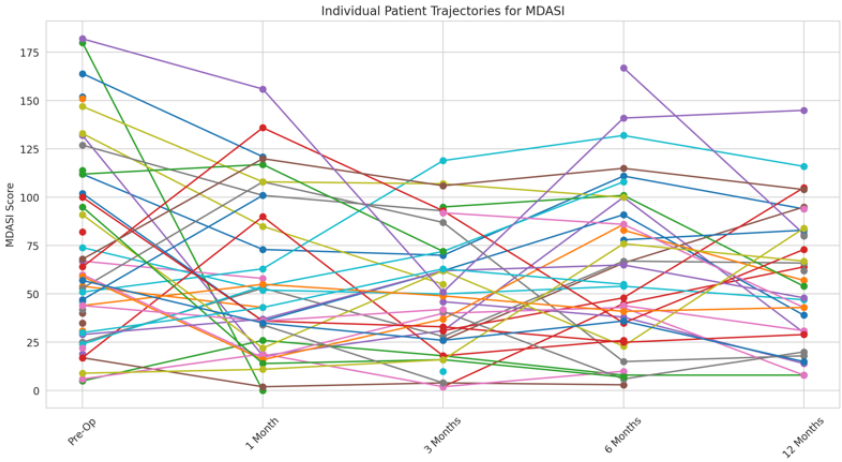
A



B



C



Supplemental Figure 1. Individual Patient QoL Metrics Analysis. The plots show individual patient scores for KPS (**A**), FACT (**B**), and MDASI (**C**) over time, with each patient's trajectory represented by a line.

6.7.2 Acknowledgments

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6.7.3 Data Availability

Specific patient datasets generated and analyzed during the current study are available upon reasonable request and governed as protected health information (PHI) following federal Health Insurance Portability and Accountability Act (HIPAA) regulations.

6.7.4 Conflicts of Interest

The authors declare no conflicts of interest.

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CHAPTER 7

7. CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Main Conclusions*

Our comprehensive analysis has revealed the dynamic prevalence of SARS-CoV-2 variants in the region over a two-year period, underscoring the virus's evolving nature. We have identified the relative prevalence of local circulating SARS-CoV-2 variants over 24 months. Of those highly prevalent variants in our region (Top 5) and the most prevalent in our clinical cohort (Top 5), we investigated the relationships between these variants and their relationship with disease severity and other factors involved in long-COVID. The study yielded critical insights into age-related susceptibility, the exacerbating role of comorbidities, and the identification of five key SARS-CoV-2 variants that significantly contribute to disease progression and severity. Additionally, our research has illuminated the notable presence of HHVs in COVID-19 patients, prompting a pioneering investigation into how SARS-CoV-2 may trigger the reactivation of these latent viruses. We found that SARS-CoV-2 can reactivate HHVs from their latent (dormant) state to their lytic (active, replicating) state, which may have a compounding effect on disease severity. We identified key cellular and molecular genes and pathways involved in these processes. Furthermore, we elucidated the role of the COVID-19

***Abbreviations**

AAV, Adeno-Associated Virus; ACE2-R, Angiotensin-Converting Enzyme 2 Receptor; ADA, Americans With Disabilities Act; B, Beta; BBB, Blood-Brain Barrier; BEI, Biodefense And Emerging Infections; BSL, Biosafety Level; CDC, Centers For Disease Control And Prevention; CNS, Central Nervous System; DMEM, Dulbecco's Modified Eagle Medium; DNA, Deoxyribonucleic Acid; dsDNA, Double-Stranded DNA; EMEM, Eagle's Minimum Essential Medium; FBS, Fetal Bovine Serum; HCoVs, Human Coronaviruses; HBMEC, Human Brain Microvascular Endothelial Cells; HR, Hazard Ratio; IL, Interleukin; IFA, Immunofluorescence Assay; MOI, *Multiplicity Of Infection;* NGS, Next-Generation Sequencing; NIH, National Institutes Of Health; NP, Nasopharyngeal; PASC, Post-Acute Sequelae of SARS-CoV-2 Infection; RNA, Ribonucleic Acid; RPMI, Roswell Park Memorial Institute; SARS-Cov-2, Severe Acute Respiratory Syndrome Coronavirus 2; TNF-α, Tumor Necrosis Factor- α

pandemic on the quality of life of patients undergoing treatment for gliomas. We found that the pandemic had minimal effect on the outcomes of these patients, though certain key variables should be investigated to improve patient outcomes in the post-COVID era.

These insights provided us with insights to investigate these SARS-CoV-2 variants, underlying comorbidities, disease severity, underlying HHVs, and neuropathology involved in long-COVID outcomes. Our study has unveiled key preliminary insights, revealing a marked increase in the prevalence of certain SARS-CoV-2 variants, notably in patients with comorbidities such as diabetes, hypertension, and metabolic syndrome, which correlate with more severe primary COVID-19 infections and a mean delay of 49 days from symptom onset to diagnosis. These are broken down below by chapter topic.

7.1.1 Wastewater-Based Epidemiology for SARS-CoV-2 Variant Analysis

Detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral genome in wastewater has proven useful for tracking virus prevalence trends within the community. Wastewater-based epidemiology (WBE), or surveillance, emerges as a precise tool for the early detection of emergent and circulating variants, offering a proactive advantage in outbreak response and management.[1-13] Site-specific monitoring of SARS-CoV-2 variants provides valuable information on the community's prevalence of new or emerging variants. Our multisite sampling strategy enabled us to pinpoint regional disparities in viral prevalence and to identify emergent variants ahead of the

upsurges associated with two dominant Omicron waves and showed that near-proximity regional differences in virus prevalence exist (*i.e.*, different variants are more prevalent in certain areas).

We sequenced the genomic RNA of viruses present in the wastewater samples. We analyzed the prevalence of SARS-CoV-2 variants and other respiratory viruses for one year to account for seasonal variations. The samples were collected weekly from the Reno-Sparks metropolitan area between November 2021 and November 2022. This study confirmed that wastewater monitoring of SARS-CoV-2 variants can be used for community surveillance and early detection of circulating variants and supports WBE as a complement to clinical respiratory virus testing as a healthcare response effort. Further supporting the effectiveness of WBE in detecting and tracking the spread of SARS-CoV-2 and its variants was the absence of the requirement for persons to self-report or depend on testing infrastructure, as our samples are community-pooled wastewater, which provides better insight into community virus prevalence and supports WBE as a sufficient and acceptable alternative to clinical-based testing surveillance. Contrasting other respiratory viruses' seasonal patterns, our findings demonstrate SARS-CoV-2's year-round persistence, highlighting its unique and resilient genetic diversity. Through secondary analysis, we further identified antimicrobial resistance (AMR) genes in the same wastewater samples and found WBE to be a feasible tool for community AMR detection and monitoring.

Overall, our findings highlight the values of WBE for monitoring the presence and dynamics of SARS-CoV-2 in an urban setting, employing multiple fields and

expertise in the process (*e.g.*, virology, bacteriology, epidemiology, public health research, and surveillance). We emphasize that WBE can be an essential tool for monitoring the transmission of pathogens between humans, animals, and the environment, providing valuable insights into the community prevalence of SARS-CoV-2 variants and other respiratory viruses and AMRs. Building on these findings, future research could expand the use of WBE for early detection of other viral diseases or environmental contaminants. Additionally, expanding the geographical scope of WBE could provide a broader understanding of SARS-CoV-2 prevalence and variant distribution.

7.1.2 RNA Sequencing of COVID-19 Patient NP Swabs

Our retrospective cohort study probed whether SARS-CoV-2 infection instigates the reactivation of HHVs, potentially exacerbating COVID-19 symptomatology. We sought to evaluate the influence of specific SARS-CoV-2 variants on COVID-19 severity and their association with patient characteristics and environmental wastewater samples in Northern Nevada. A retrospective analysis was performed on 1,800 nasopharyngeal swabs and 1,400 biorepository samples. Sequencing data was analyzed alongside patient health variables, and wastewater samples were sequenced from the Truckee Meadows Water Reclamation Facility (TMWRF) for the study period from November 2021 to March 2023. The study revealed variant-specific effects on COVID-19 severity, age, and comorbidity-related implications. Significant correlations and trends were seen between WBE and clinical data, notably the lag time from WW identification to clinical case

diagnoses, implicating WBE for early pathogen detection in community surveillance efforts. Our findings underscore the role of SARS-CoV-2 variants in disease severity and offer valuable perspectives for targeted public health interventions. Future studies could delve deeper into these correlations by conducting longitudinal studies on patients with specific SARS-CoV-2 variants to monitor changes in disease severity over time.

7.1.3 Underlying Herpesviruses Augmenting COVID-19 Symptoms

We investigated the potential role of SARS-CoV-2 in triggering the reactivation of HHVs in patients with COVID-19 and the potential effects of this reactivation on the severity of COVID-19 symptoms. Additionally, we used an *in vitro* cell culture model to study the mechanisms behind this potential relationship at a molecular level. We noted a relationship between SARS-CoV-2 and HHVs and the potential sequela of reactivation during COVID-19. Reactivation of HHVs during COVID-19 infection may contribute to increased severity of symptoms. The broad spectrum of clinical symptoms observed in SARS-CoV-2 infected individuals ranges from no symptoms to critical illness and death. The influence of latent viruses on the severity of COVID-19 remains an enigmatic aspect of COVID-19 pathology, warranting further exploration. We sought to determine whether SARS-CoV-2-infection triggered the reactivation of HHVs, which may contribute to the increased COVID-19 symptom severity through a retrospective cohort study.

The predominant SARS-CoV-2 clade was used to classify the COVID-19 NP Swab specimen cohorts into Wuhan (Wuhan-Hu-1), Delta (B.1.617.2), or Omicron

(BA.1). RNA-Seq metagenomics analysis of these specimens showed high NT rPM for SARS-CoV-2 signatures, confirming positive COVID-19 status and SARS-CoV-2 infection. Some specimens also showed significant co-infection with other respiratory pathogens (*e.g.*, Influenza A, Klebsiella pneumoniae, *etc.*). Analysis of key HHV genes via transcriptome mapping revealed elevated levels of BZLF1 lytic genes in COVID-19 patients with severe and critical symptomology; furthermore, analysis of the cellular gene through RNA-Seq identified 234 differentially expressed genes in HHV-positive COVID-19 patients. These genes were found to be associated with a variety of biological processes and molecular functions, including regulation of immune response, cell adhesion, response to hypoxia, RNA metabolism and processing, RNA splicing and translation, DNA repair, and replication. Furthermore, *in vitro* reactivation of key immediate-early lytic genes using EBV- and KSHV-harboring cell lines following SARS-CoV-2 infection validated our hypothesis on the possible link between HHV reactivation during SARS-CoV-2 infection.

Collectively, these results help to establish that the reactivation of HHVs due to SARS-CoV-2 may contribute to worsened COVID-19 severity. The next step could be investigating the mechanisms behind this reactivation and whether similar interactions exist with other latent viruses. This could lead to developing therapeutic interventions that target these mechanisms. Identifying key immediate-early lytic genes in severe COVID-19 patients and the increased prevalence of HCMV and HSV viruses in these patients suggests that HHVs play a role in COVID-19 disease progression. Research elucidating the mechanisms of interaction between the SARS-CoV-2 and HHVs may

contribute to findings of novel targets and pathways that may contribute to potential therapeutic opportunities that may arise from targeting them. By better understanding the role of HHVs in COVID-19 pathogenesis, we can develop more effective treatment strategies for patients with pre-existing HHV infections.

7.1.4 long-COVID Neuropathogenesis and Molecular Markers

When investigating the pathogenesis of long-COVID, a condition characterized by persistent neurological symptoms among a proportion of SARS-CoV-2 infected individuals, two underlying mechanisms are highlighted that may be at play: host immune system dysregulation and resultant neuroinflammation leading to direct invasion of the central nervous system (CNS) by the SARS-CoV-2 virus or its components (*e.g.*, S1 spike protein). We utilized next-generation RNA sequencing of COVID-19 patient specimens to understand these mechanisms better. Our cohort of patients with a confirmed diagnosis of long-COVID provided a rich dataset, including specific variant identification and comprehensive patient health variables, to explore the persistent effects of the virus. The associated spike glycoprotein was deduced based on the specific variant identified.[14]

The overarching goal of the study was to elucidate insights into the prevalence of specific SARS-CoV-2 variants in long-COVID patients, investigate the literature to understand and summarize the current literature on these variants' spike protein characteristics and provide insights into potential targets and pathways that may be used

in the diagnosis and intervention of long-COVID. Our research endeavors to enhance the QoL for those afflicted with long-COVID by pinpointing therapeutic targets and elucidating the pathways that could lead to effective interventions. To build on this research, other studies should investigate the long-term health implications of specific variants seen in long-COVID and explore potential treatment options for patients with long-COVID.

7.1.5 COVID-19 Effect on Outcomes of Glioma Clinical Patients

The COVID-19 pandemic has significantly impacted clinical trials for glioma treatment, including recruitment.[15] Various strategies have been employed to maintain recruitment in glioma clinical trials during the pandemic; however, the long-term impact of the pandemic on glioma clinical trials and recruitment remains to be seen. This study contributes to the ongoing discourse on managing gliomas amidst a global health crisis through evaluating impacts of COVID-19 on the QoL and outcomes of glioma patients. offering evidence of sustained clinical efficacy and patient-centered care, while simultaneously investigating areas for improvement of glioma treatment and tumor behavior via analyses of MRI data (*e.g.,* intraoperative MR imaging) and glioma tumor biomarkers and genetic properties.

Our retrospective analysis revealed a nuanced landscape of glioma diagnoses and their implications on patient outcomes during the COVID-19 pandemic. Our preliminary findings suggest that the QoL metrics, including KPS, FACT, and MDAIS scores, did not

significantly differ between the pre-COVID and COVID-19 patient cohorts. This underscores the resilience of the patient care protocols and the adaptability of the healthcare system in maintaining the standard of care for glioma patients. Moreover, our investigation into the correlation between tumor characteristics, surgical interventions, and patient outcomes provided valuable insights into the complex interplay between tumor biology and treatment strategies. Notably, our cohort's distribution of glioma subtypes highlighted the prevalence of high-grade glioblastoma (GBM), which informed the subsequent analysis of survival outcomes. Moreover, the analysis of survival data through Kaplan-Meier curves indicated the overall survival in pre- and post-COVID-19 cohorts did not significantly deviate from existing benchmarks in glioma treatment. The descriptive statistics of complications further corroborated the safety of the surgical procedures, with low rates of neurological and systemic complications observed.

Future research will expand upon these findings and incorporate more granular data, further refining the understanding of glioma management in unprecedented circumstances. Preoperative MR perfusion and 2-HG spectroscopy were obtained as patients underwent standard surgical resection, standard radiation, and chemotherapy. Thus, using these advanced pre- and intra-operative MR image-guided data to further improve EOR quantification will aid in minimizing patient complications. Assessing biomarkers and RNA expression patterns in tumor samples will provides valuable insights into glioma biology. The findings of this study can help guide clinical treatment, design future therapies, and improve understanding of the tumor microenvironment. Analyses of these data points are to be determined as the trial is still ongoing.

7.2 Understanding the Neuropathogenesis of long-COVID: Study

Long-COVID has been shown to affect anyone who has had COVID-19, regardless of age or health status; a significant proportion of COVID-19 patients have developed long-COVID or had symptoms of COVID persist 1 to 3 weeks beyond initial infection (250 million people worldwide, about one-third of whom have had COVID-19).[16-18] Among those with lingering symptoms, hospitalized patients have the highest incidences (50–70 %) as compared to non-hospitalized cases (10–30 %) and vaccinated cases (10–12 %).[19-21] Long-COVID is prevalent in all age groups, with the highest percentage of diagnoses between the ages of 36 and 50 years.[22] While multiple factors (*e.g.*, acute phase disease severity) contribute to PASC, there are a few posited factors that may increase the risk of developing long-COVID (*e.g., severity of initial COVID-19 illness, pre-existing medical conditions, immune system response, and delayed or incomplete recovery*). Long-COVID can manifest as a complex set of symptoms (*e.g.*, fatigue, brain fog, anxiety, depression, loss of taste and smell, *etc.*) involving cardiopulmonary, gastrointestinal, endocrine, psychiatric, and neurological systems.[23-26] Long-COVID also causes new-onset conditions (*e.g.*, type 2 diabetes [T2DM], acute disseminated encephalomyelitis [ADEM], and chronic fatigue syndrome [CFS]).[23,27] SARS-CoV-2 will likely persist, mutate, and cause more suffering to American lives and the economy for years; these adverse long-COVID symptoms are a clear example of this imminent long-term burden.

While multiple factors (*e.g.*, acute phase disease severities) contribute to PASC, a few posited factors may increase the risk of developing long-COVID – the severity of

initial COVID-19 illness (*i.e.*, more severe acute COVID-19 disease) and those with pre-existing medical conditions (*e.g.*, diabetes, obesity, and cardiovascular disease). While these factors may increase the risk of developing long-COVID, it is important to note that there are no established predictors of long-COVID and even healthy individuals without severe COVID-19 illness or pre-existing medical conditions can develop long-COVID.[28] This makes long-COVID an enigmatic disease condition without a clear etiology, which poses challenges for therapeutic interventions. The Immune system's response to the SARS-CoV-2 virus may also play a role in developing long-COVID, although more research is needed to understand this fully. Studies should focus on investigating the effects of SARS-CoV-2 on the immune system and how this contributes to the development of long-COVID. More research is also needed to identify potential biomarkers and genetic factors that may predispose individuals to long-COVID, which could help identify and manage individuals who are at higher risk for developing long-COVID.

There is a need for in-depth investigation on the impact of specific SARS-CoV-2 variants on long-COVID. Studies should investigate the relationship between specific variants of the virus and the clinical outcomes of COVID-19 patients. This could provide valuable insights into the virulence and transmissibility of different variants and inform public health measures. Furthermore, there is a need to explore the potential role of underlying latent viral diseases, such as herpesvirus, KSHV, and EBV, in augmenting COVID-19 symptoms. Understanding the interactions between these latent viral infections and SARS-CoV-2 could help explain the variability in COVID-19 symptoms

and inform treatment strategies. Another area of future research is the study of SARS-CoV-2 infection in neonatal infants and its potential impact on neurological development. Research should focus on understanding how SARS-CoV-2 affects the neonatal nervous system and the long-term consequences of infection. This could help develop interventions to mitigate the neurological effects of SARS-CoV-2 in neonates. Overall, future research should aim to improve our understanding of long-COVID, the long-term impact of certain SARS-CoV-2 variants, the role of latent viral infections, and the effects of SARS-CoV-2 on neonatal neurological development. This knowledge will be crucial in developing effective strategies for preventing, diagnosing, and treating long-COVID.

An important question surrounding long-COVID is predicting who will develop long-COVID/PASC and whether any specific genes/pathways/markers get activated during initial SARS-CoV-2 exposure and persist in causing/contributing to long-COVID. We know that SARS-CoV-2, like other coronaviruses, is a large positive-sense single-stranded RNA (ssRNA) virus, which is sensed by the host cell pattern recognition receptors (PRRs) and responds by activating several anti-viral pathways critical for early defense against viral invasion.[29,30] We hypothesize that the innate immune response to SARS-CoV-2 differs in certain individuals, leading to some patients developing long-COVID and that certain common pathways (*e.g.*, overactive immune responses producing pro-inflammatory cytokines) may contribute to these differences. We expect to determine these early changes (markers) during the primary infection by analyzing the transcriptomic profile of SARS-CoV-2 infected nasal epithelial cells in the NP swabs collected from long-COVID subjects at primary infection. Comparing the transcriptomic

profiles of the SARS-CoV-2 infected cells in the NP swabs from initial exposure of non-long-COVID individuals may provide a differential pattern to identify the etiologic markers of long-COVID.

7.2.1 Future Directions long-COVID Study

Moving forward, we aim to collect blood samples from long-COVID patients' and their age-, gender-, ethnicity-, and comorbidity-matched controls and to measure the levels of SARS-CoV-2 spike glycoprotein and other immunological markers, enriching our understanding of the condition. Our planned longitudinal study will delve into the analysis of inflammatory biomarkers (*e.g.*, SARS-CoV-2 S1/S2-specific immunoglobulins (IgA, IgG, and IgM antibodies), type-I/II interferons, monocytes (CD14, CD16), and interferon-stimulated genes) to decode their correlation with the neurological manifestations in long-COVID patients. Recent studies have shown a prolonged circulation of S1-spike and nucleocapsid antigens in the plasma of PASC/long-COVID patients, which may induce neuroinflammatory pathways leading to neurocognitive symptoms.[31,32] Thus, we will also determine the levels of SARS-CoV-2 spike glycoprotein in the serum and correlate these levels with dates of COVID-19 infection and last vaccination or booster. We aim to longitudinally collect peripheral blood mononuclear cell (PBMC) and serum samples from these patients during their long-COVID diagnosis and perform proteomics analysis to investigate protein markers in the blood to differentiate what is significantly different in long-COVID versus non-long-COVID. Our goal is to draw meaningful correlations between serum proteomic profiles

and neurological symptoms. We will also factor patients' diabetic status and serum protein biomarkers into our analyses and explore the impact of diabetic and inflammatory mechanisms on long-COVID-associated changes in the brain. By delineating the differences in serum proteins among individuals with long-COVID compared against matched non-long-COVID controls (age-, sex-, and ethnicity-matched), we may identify early and specific determinants of long-COVID, which will provide insight into the treatment of PASC conditions.

We will perform a longitudinal analysis of specific inflammatory biomarkers (*e.g.*, cytokines, immunoglobulins, IL-6, TNF-α, kynurenine, *etc.*) and correlate their presence with the neurological complications experienced by long-COVID patients. This may help to identify potential therapeutic targets to alleviate these complications. We will employ various innovative techniques, including proteomics, enzyme-linked immunosorbent assay (ELISA), and multiplex cytokine analysis, to measure and analyze these biomarkers in our subjects. PASC patients in our cohort diagnosed based on their symptoms with the ICD-10 code will be recruited for this study. These patients will come to our clinical research laboratory at the School of Medicine for a blood draw under our approved protocol (IRB). Collected blood will be processed to isolate serum and PBMCs in the Verma laboratory for the proteomic analysis and evaluation for molecular diagnostic targets in long-COVID. PBMCs will be subjected to transcriptomic analysis to identify differentially expressed genes as biomarkers of PASC.

Our collaborator (Dr. Fang Jiang, Institute of Neuroscience, UNR) has plans to recruit PASC patients for brain-fMRI imaging studies and cognitive evaluation tests to

understand the neurological states among long-COVID patients. fMRI data will quantify the functional connectivity between various pairs of brain regions, revealing altered (*i.e.*, pathological) functional connectivity insights.[33] This collaborative team is poised to investigate the potential neurophysiological mechanisms underlying these observed changes, aiming to integrate neuroimaging data with neurochemical insights for a comprehensive analysis.

Our goals are to delineate the differences in altered serum proteins among individuals with PASC, which may lead to identifying the determinants (*i.e.*, underlying molecular diagnostic targets/markers) of long-COVID; moreover, by associating the neurological complications/sequelae that occur, will provide novel insights into the neuropathology of long-COVID and pave the way for novel molecular diagnostics and targeted therapies. The levels of serum proteins and cellular genes in PBMCs, which can be predictors of long-COVID, are useful clinically and can be implemented with standard procedures alongside rt-qPCR testing. The assessment and correlation of disease severity and neurological complications with inflammatory biomarkers may allow for the generation of prediction models for neurological complications in long-COVID patients. We will also further gain an understanding of underlying neurological activity changes in different brain regions during long-COVID progression.

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CHAPTER 8

8. PERSONAL PERSPECTIVES

8.1 About Me

My long-term goal has been to become an academic clinician-scientist-surgeon trained in neurosurgery. I have been fortunate to come to this conclusion early on in my education due to the support and guidance of my many mentors. As a first-generation college and medical student, most of my learning has come through advice from my mentors and overcoming adversity. I was fortunate to enter the UNR Med B.S., M.D. Program with about three years of college credits, enabling me to devote extra time to extracurricular activities, including clinical and basic science research. Through these opportunities (clinical, research, and extra-curricular), I have accumulated a wide variety of experiences and trainings that have prepared me to pursue my goals.

My first research experiences began early in my undergraduate career, months apart from each other, with Dr. Subhash Verma and Dr. Randy Jensen. I had a formative experience in Dr. Jensen's laboratory in the PathMaker program at the Huntsman Cancer Institute. These experiences have been critical towards my goal of becoming a successful independent investigator. These works resulted in presentations at local and national conferences. At UNR, I developed expertise in innovative microbiology techniques, which allowed me to become a productive and valuable team member in the laboratory. As my project developed with Dr. Verma, we applied for and attained pilot grant funding from the NV INBRE (NIH P20 GM103440). This work resulted in the genomic sequencing of a novel Zika virus (ZIKV) strain, and the successful isolation of neuroglial cancer stem cells for further experiments. I presented our results at local and national conferences and even won runner-up for best research at our medical school's annual

research day in 2019. This project further resulted in published work by the lab regarding ZIKV's role in cellular pathway dysregulation.

When observing Dr. Jensen in Utah, I saw a new angle of academia that incorporated healthcare. I would see Dr. Jensen perform surgery and participate in research activities, thus seeing first-hand bench-to-bedside clinical translational research. At the University of Utah and Huntsman Cancer Institute, clinical sciences take place in an energetic and collaborative atmosphere, which piqued and solidified my interest in pursuing a career in neurosurgery and academia. Under Dr. Jensen's guidance, I have established a neurosurgery chapter at UNR, associated with the American Association of Neurological Surgeons (AANS), to foster new clinical research opportunities and inspire students in neuroscience (Reno has no neurosurgery residency program). Following graduation from this M.D., Ph.D. program, I will apply to an academic training program and begin my training in neurosurgery.

Between receiving my bachelor's degree from UNR and matriculating into medical school, I had six months that I used to return to the University of Utah for clinical research experience. I also spent time at the University of Utah Hospital Department of Neurosurgery, exploring the various subspecialties within neurosurgery. Through this experience, I established lasting relationships with the residents and attending doctors. Since beginning medical school, these relationships have fostered an outlet for me to keep my hand in neurosurgical clinical research experiences (*e.g.,* large database analyses, case reports, case series, clinical trial reporting, and systematic literature reviews), which have resulted in numerous peer-reviewed publications.

8.2 Why the University of Nevada, Reno School of Medicine

I committed to UNR Med the second I was admitted to the UNR Med BS/M.D. program during my senior year of high school. I was introduced to Dr. Verma early in my undergraduate career. He was well-known on campus as a productive, independent investigator who operates one of the most productive research labs at UNR Med. Dr. Verma's track record has successful students, an established research environment, and unmatched mentorship. I chose the Department of Microbiology and Immunology (and further the Cellular and Molecular Biology graduate track) due to the constant interaction between the scientists and clinicians, opportunities for exchanging ideas and techniques, meetings, journal club, invited speaker seminar series, and honestly, the people here. I believe this diversity has provided me with an unparalleled opportunity for success.

As I sought out research experience during my undergraduate career in 2017, Dr. Verma was recommended to me by both Dr. Kenyon and my B.S., M.D. program director as a successful and motivated investigator at UNR Med. It was clear early on that Dr. Verma's investment and dedication to training, mentoring, and challenging his students to excel was unmatched. He carefully adapts his mentoring style to each student based on their learning style and needs. Additionally, Dr. Verma's work on infectious diseases and viruses, established collaborations with many successful investigators, and a productive laboratory with all necessary equipment, reagents, and guidance solidified the success of my projects and career development. Further, Dr. Verma was eager to collaborate with other investigators like Drs. Karsy and Jensen, though outside of his direct field, highlighting his commitment to my goals and aspirations to become a neurosurgeon

physician-scientist. After finalizing my plans to pursue my M.D., Ph.D., it was a clear and easy choice to continue working with Dr. Verma.

As a first-generation college and medical student, I was often unsure about the next steps. I often relied on individuals like Dr. James Kenyon, who have surpassed every level of mentorship I thought was possible. Throughout my undergraduate career, meetings with Dr. Kenyon would always result in me leaving his office excited to pursue a new opportunity he had either planned for me or told me about–this has not changed even as a medical student. His immense passion for mentoring and guiding students to the next level has resulted in my success today; he has been genuinely invested in my academic success, for which I am forever in debt. Additionally, the guidance and advice throughout this submission of my F30 and his vision for the UNR Med M.D., Ph.D. program are a sentiment to Dr. Kenyon's commitment to UNR as a whole – I was the first F30 applicant from UNR Med, which we hope will pave the way for increased applicants in the years to come as I implement Dr. Kenyon's vision.

Acknowledging that movement from institutions is normal and is often encouraged to provide a broad insight into teachings, ideas, and opportunities, in pursuit of my M.D., Ph.D. began, my mentors and I explored the possibility of a temporary leave-of-absence in pursuit of my Ph.D., particularly at the University of Utah, as UNR Med is currently not NIH-MSTP funded. This idea, however, resulted in issues concerning the practical matter of this pursuit, such as which credits transfer per the transfer agreement *(i.e.,* the University of Utah would not accept some UNR credits, and *vice versa*; though possible, this arrangement would result in extended training time and

would require me to take more credits to fulfill the requirements. Thus, I stayed at UNR Med and pursued applying for my own NIH-F30 Fellowship award. Though this application received a “not discussed” result, it was an accomplishment to submit one of the most challenging funding applications for students pursuing an M.D., Ph.D.

I am especially appreciative of both Drs. Karsy and Jensen as they have understood and encouraged the need for me to integrate clinical experiences into my graduate and medical training years as I pursue this career in academic neurosurgery. They reflect my ambitions, and I look forward to their guidance throughout my M.D., Ph.D. training and beyond. I aspire to become a mentor like Drs. Jensen and Karsy, as their devotion to their patients, trainees, and colleagues is second to none. I have had the honor to be able to interview and examine patients with them, interpret scans, and participate in surgery and critical care of patients with advanced neurological pathologies and trauma. There is no neurosurgery program at UNR Med; however, due to the immense support from the University of Utah and their entire Department of Neurosurgery, this pursuit of a multi-institutional M.D., Ph.D. training plan has been a reality – We have garnered a newfound interest in Neurosurgery and the neurosciences here in Reno. Both institutions and their respective programs are committed to the education and development of their students and the next generation of leaders in science and medicine. I have experienced multiple occasions of productive discussion, which have led to much personal and academic success and growth. As a student with the privilege to be a student scholar at both institutions, I am a part of a large family of motivated and scientific colleagues.

8.3 Early Academic Experiences in Research: Guiding My Future

Being a first-generation student presented a subset of challenges and some advantages – this would yield a constant state of excitement, curiosity, fear, and determination. The novelty did not deter me; it fueled my quest to explore, improve, and seek the next level. For me, the absence of the "traditional" roadmap or familial guidance in the academic field was both a challenge and an opportunity. I had to chart my course and learn from each step and stumble. I had to find mentors, build my support system, and create my success story. Still, because the world is defined by complex anatomy and intricate connections, my pursuit of this career in neurosurgery was no small feat – more impossible. Somehow, I have made it this far on this journey, which has been less a tale of academic achievement but rather a journey shaped by those who have devoted time and energy to me. Only then does my determination, learning, and pursuit of this career lead to anything. Meeting Dr. Verma is one example of a step in this journey. I have had a solid foundation that I have carried throughout my education since joining the Verma Lab. The opportunities to engage in innovative research, the wisdom shared during countless hours of guidance, and seeing the passion and drive that Dr. Verma embodied daily became the cornerstones of my academic growth. It was with Dr. Verma that I conducted and completed my first-ever research poster. To this day, the research I conducted back then continues to follow me.

As a first-year undergraduate interested in research, I met Dr. Verma while exploring biomedical research. His excitement about his research endeavors and willingness to take me under his wing inspired me to pursue research. While working

under Dr. Verma, I had primary responsibility and completed a main project on Zika Virus (KIKV) and took part in the completion of Kaposi's Sarcoma Herpesvirus (KSHV) projects. Although the first few months were spent training and learning new protocols through shadowing, I was introduced to essential standard molecular biology techniques, including DNA isolation, PCR, DNA transformation, and Western blots. Alongside collaboration with a team of Graduate Students and Postdoctoral Fellows, I participated in departmental activities, including Journal Club and poster symposiums where I felt like a scientist through my interactions (*i.e.,* representing our lab's progress and networking) which reinforced my desire to one day have a project of my own to present.

Shortly after beginning work in Dr. Verma's lab in the Fall of 2016, receiving training, and getting my feet wet with various laboratory procedures, experiments, and equipment, my mentor, Dr. Kenyon, presented me with an opportunity for cancer research in the upcoming summer of 2017. I was accepted and transferred to Utah to participate in the PathMaker Summer Cancer Research Program. It was here I met Dr. Randy L. Jensen. I completed this research under the supervision of Drs. Randy L. Jensen and David L. Gillespie. My primary research project followed the future directions outlined in Dr. Michael Karsy's Master's Thesis; little did I know Dr. Karsy would end up on my dissertation committee six years later.

The PathMaker program included a two-week intensive research training course introducing students to cancer research. This was a program that would change the trajectory of my life. Upon completion of training, I requested a match with Dr. Randy L. Jensen to work with an M.D., Ph.D. clinician-scientist. I learned that Dr. Jensen performs

brain surgery and research, where his lab's research focuses on understanding the mechanisms of angiogenesis of both benign and malignant brain tumors with an emphasis on innovation in novel therapeutics and imaging techniques. The overarching goal of my summer project was to compare the efficacy of gene silencing by a lentiviral-based CRISPR-Cas9 construct designed to knockout HIF-1α against an adenovirus-mediated Hif-1α targeting shRNA construct. We used cell culture, fluorescence imaging, DNA extraction and purification, and PCR techniques. I also worked with Dr. David L. Gillespie in Dr. Jensen's laboratory; together, we performed microsurgery on rats, which involved stereotactic tumor injection and subsequent radiotherapy.

This animal surgery was my first experience with translational science. It was thrilling handling the rats and performing my first surgery, where I dissected the brain to harvest tissue samples. In addition to my research, I attended rotations with Dr. Jensen in the clinic and operating room. I saw first-hand how Dr. Jensen's basic science and animal model research applied to human therapies to alleviate patients' suffering amidst clinical trial treatments. My research during the PathMaker program concluded in multiple local and national poster and oral presentations and further presented opportunities for me to pursue clinical research with the department as a first-year medical student. Collectively, this day in the life of a neurosurgery physician-scientist experience was a profound experience seeing how an M.D., Ph.D. operates daily. My collaboration with Dr. Jensen and the Department of Neurosurgery, time spent researching and shadowing at the hospital, and witnessing momentous advancements at HCI have influenced my career pursuit of becoming a physician-scientist.

Dr. Karsy guided me through my PathMaker summer project – coincidentally, it turns out that I was continuing the future directions outlined in his master's thesis. While working with Dr. Karsy, I completed many projects studying various subspecialties within neurosurgery. We have several ongoing projects, most combining database analyses of various procedures, patient risk factors, and complications within different demographics and subspecialties of neurosurgery. Other research by Dr. Karsy generates models that use algorithms and machine learning to identify and predict procedural outcomes using patient demographics and known risk factors. I plan to participate in developing more publications with Dr. Karsy. Dr. Karsy has been a monumental role model for me over the past seven years and one of the primary residents I shadowed during my visits to Utah. I have been fortunate to gain valuable insight into the lifestyle and sacrifice required of a physician-scientist through my relationship with Dr. Karsy.

These experiences with Dr. Karsy and Dr. Jensen have allowed me to immerse myself in the relevant literature and clinical nuances completely. I've learned to better manage my time with heavy workloads, school requirements, extracurriculars, and research deadlines. In addition, I have cultivated the ability to work diligently with meticulous detail as I have been able to experience the research process from start to finish. I appreciate the work involved in the scientific process, from bench to bedside. They prepared me for the commitment and performance needed in the M.D., Ph.D. program, which will continue to develop as I progress in my career.

Utah became more than a place; it was a chapter where I met most of my mentors, including many well-known and phenomenally successful neurosurgeons. I also met

many scientists who showed me how much this basic science research can impact patients. In Utah, I embraced the novelty of new possibilities and challenges – where I explored, improved, and sought the next level. I would constantly find myself attending morning rounds at 5:30 am, spending all day shadowing, watching surgeries, tending to patients in the Neuro Critical Care Unit (NCCU) until 7 pm, only to stay up watching more videos, or working on research, and sometimes even spending the night in the NCCU conference room working on homework – only to find myself asleep on my laptop on the table, with a blanket put on over me by a generous soul.

The experiences with everyone at the Utah Department of Neurosurgery developed for me a crucible that would not only motivate me on my hardest days but also sharpen my academic path and build my character. I connected with some of the most talented minds in the field, engaged in groundbreaking research, and explored the vistas of neurosurgery. I learned to navigate the steep learning curves, much like the mountainous terrains of Utah, facing failures, learning from them, and moving forward. I forged collaborations with surgeons and scientists across the U.S., enriching my perspective and honing my skills. I engaged in conferences, seminars, and workshops, contributing to papers and projects that added value to the field. These experiences expanded my horizons and taught me to approach problems from various angles, allowing me to create a network of professionals who continue to influence my career today. Each research project, each clinical case, and each interaction with a mentor was a step towards reaching the peak of my potential – one that I will never reach; simply aspiring to pursue this *peak* is all one needs to keep going.

Upon my return to Dr. Verma's laboratory, I continued to shadow and gain experience with new techniques such as fluoresce in-situ hybridization (FISH) and fluorescence and electron microscopy. In December 2017, I developed an independent project combining my neurosurgery and infectious disease research interests. It was funded by a Pilot Grant award from the NV INBRE (NIH P20 GM103440). During this project, we sought to validate our cell line, understand the genetic makeup of a novel strain of Zika Virus, and understand pathways in differentiated *vs.* stem cells to elucidate therapeutic targets. We also conducted experiments to study the cytopathic effect of this new strain against glioblastoma stem cells *in vitro*. We found that the novel strain of Zika Virus had twice the virulence of the originally isolated strain and that it encoded a smaller-sized viral envelope protein from the originally isolated Zika Virus. During this time, I gained skills in performing biosafety level (BSL) 2+ cell culture techniques, lab notebook etiquette, designing analytical figures, and scientific writing. Working year-round in Dr. Verma's laboratory enabled me to apply what I was learning in my classes to my work on my projects in real time.

I went on to write my undergraduate BS/M.D. thesis based on my project with Dr. Verma, which resulted in various local and national posters and oral presentations. I experienced a collaborative and exciting research environment and felt motivated to contribute to science. I was recently awarded the second-best overall research poster for this ZIKV work at the annual UNR Med Research Day symposium. I have aided other students in completing their Ph.D. dissertations and undergraduates with their graduation thesis.

8.4 A Guiding Force: Mamba Mentality

My passion for neurosurgery found its roots in an unlikely hero – Kobe Bryant. Kobe's unwavering dedication, focus, and unyielding aspiration to be the best in his field resonated with my ambitions after being introduced to the field. The Mamba Mentality (described in *Mamba Mentality How I Play,* by Andrew D. Bernstien) was more than just a mantra; it evolved into a way of life. Kobe's relentless practice, his determination never to settle for mediocrity, and his constant striving for perfection in his craft are all reflected in my approach to my life. I suddenly found myself - a first-generation Pakistani-American– in a position to apply for and pursue an M.D., Ph.D. degree. The Mamba Mentality is not confined to the basketball court; it is about being the best version of oneself – constant growth, learning, and pushing past the boundaries. It has been instrumental in shaping my interests, from rock climbing and mountain biking to taking up new hobbies like snowboarding and even extending into my work as I now delved into integrating artificial intelligence and machine learning algorithms within neurosurgery. My mentors as leaders have guided me in these pursuits.

Kobe's approach to his craft taught me that the road to excellence is never easy but worth traveling. It requires sacrifice, hard work, and an unbreakable will to keep going despite countless failures. His Mamba Mentality taught me that failure is not a stopping point but a stepping stone toward success. Embodying this mentality has driven me through countless sleepless nights, endless research, and clinical challenges – all in pursuing my dreams and aspirations, pushing my boundaries, finding my potential, and becoming the best version of myself.

8.5 Conclusion: A Tapestry of Faith, Fate, and Excellence

My journey is a complex tapestry woven from the threads of relentless dedication, an unbreakable pursuit of excellence, faith in my abilities, and an intricate dance with destiny – One in which I always feel like I owe everyone, those that I have met already and those that I have yet to meet (*i.e.,* my future patients), to give it my all. One of the reasons I am still pursuing this career after so many years is because after presenting my research with Drs. Jensen and Verma, I had received a note - I still do not know who this note came from (**Figure 3**). The values I cherish are a combination of those of my mentors, those who have shaped me, and the principles that have driven me from within, which, when combined, form the fabric of a path that is uniquely my own.

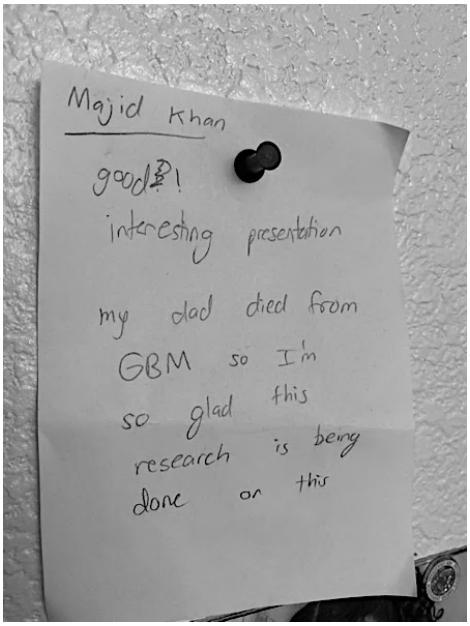


Figure 3. Reason. I received a note from an unknown individual following a presentation for my undergraduate thesis, which compiled the research I had completed with Drs. Verma and Jensen. I discussed the basic science advances and future treatment options for glioma patients, such as those diagnosed with Glioblastoma (GBM).

I hope my story resonates with others, especially the next generation (our future), embodying a symphony of determination, lifelong learning, and an unwavering commitment to innovation and improving human lives. I am excited as I look into the future; the road ahead is filled with exciting challenges, incredible opportunities, and infinite potential. Kobe Bryant's Mamba Mentality continues to be a beacon guiding me toward achieving greatness in my personal life and professional career. Reminded that this journey has no summit because the quest for excellence is endless – a continuous climb where every peak conquered reveals a new horizon to explore.

I am reminded of the little things - for instance, in my sophomore year of high school, we were tasked to draw our favorite part of the body (**Figure 4**). Even a decade ago, it seemed like I must have known what I wanted to do for the rest of my life.

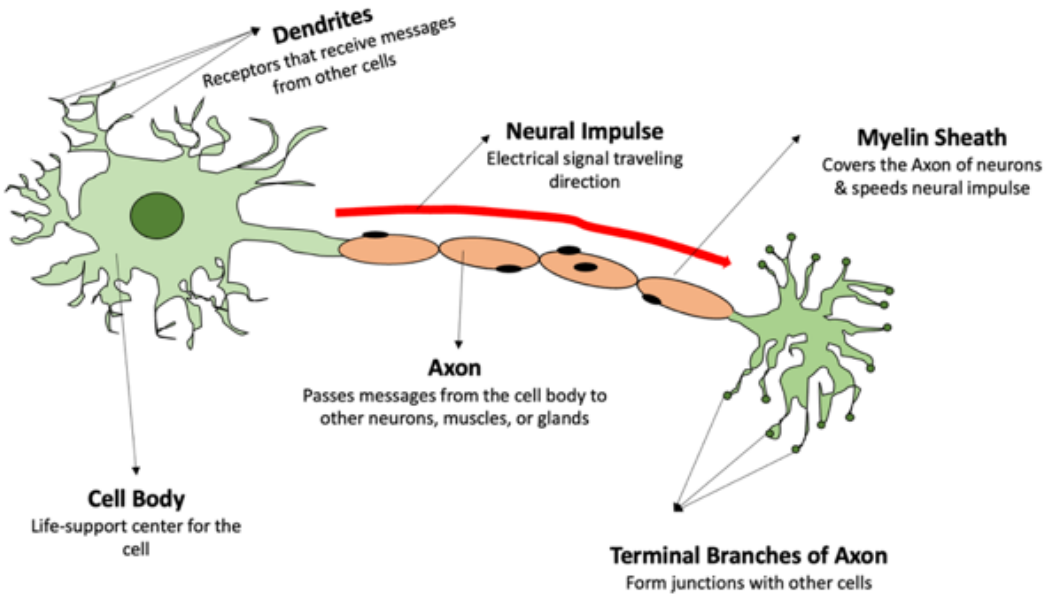


Figure 4. Illustration of a Neuron Completed in High School. This composition was made in Microsoft PowerPoint for my Biology class. The last saved date is November 3, 2013, titled "Khan Majid - Neuron PowerPoint Drawing Attempt.pptx."

The complex interplay of my aspirations, the intricate connections I have come across and formed along the way, and my belief in pushing beyond conventional boundaries has carved a story that transcends academic achievement. – The story is that numerical achievements or scores do not define your potential. Rather, your faith, hard work, ability to get back up repeatedly, a spirit filled with gratitude, and the support of mentors, loved ones, and a community of inspiring minds.

With the Mamba Mentality coursing through my veins, I embrace what lies ahead, confident that pursuing excellence is a path worth traveling, no matter the terrain. “The most important thing is to try and inspire people so they can be great at whatever they want to do,” said Kobe Bryant. I hope my story will inspire others to embark on their journeys towards excellence, knowing that failures are not setbacks but stepping stones, lessons learned, and challenges to overcome. I now step forward into my journey’s next step – which has only just begun.

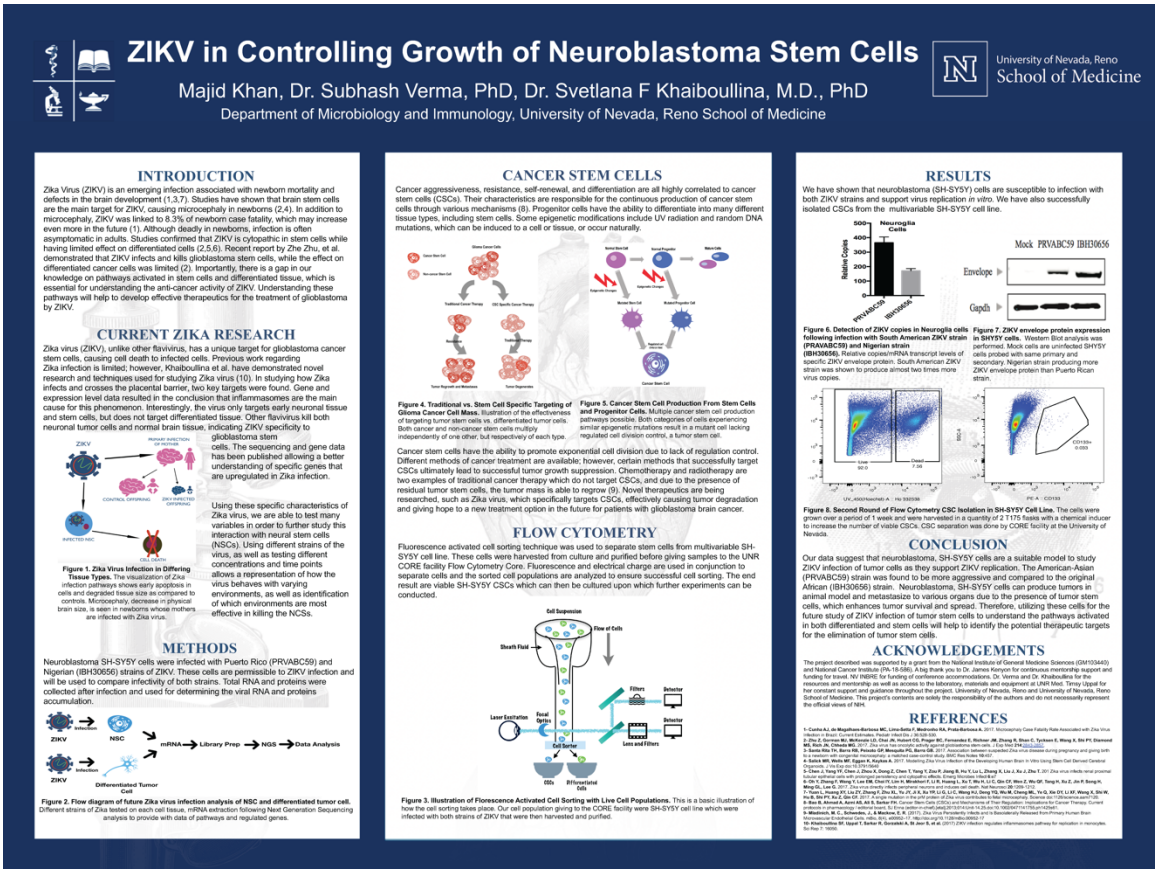
8.6 Appendix

8.6.1 Poster: Undergraduate Research Project in Verma Lab

ZIKV IN CONTROLLING GROWTH OF NEUROBLASTOMA STEM CELLS

Authors

M. Khan; S. Verma PhD; S. F. Khaiboullina, MD., Ph.D.



Acknowledgments: The project described was supported by a grant from the National Institute of General Medicine Sciences (GM103440) and the National Cancer Institute (PA-18-586). Thank you to Dr. James Kenyon for continuous mentorship support and funding for travel. Dr. Verma and Dr. Khaiboullina for the resources, mentorship, and access to the laboratory, materials, and equipment at UNR Med. Dr. Timsy Uppal for her constant support and guidance throughout the project. University of Nevada, Reno, and University of Nevada, Reno School of Medicine.

ABSTRACT

INTRODUCTION: Zika Virus (ZIKV) is an emerging infection associated with newborn mortality and defects in brain development (1,3,10). Studies have shown that brain stem cells are the main target for ZIKV, causing microcephaly in newborns (2,4,6). In addition to microcephaly, ZIKV was linked to 8.3% of newborn case fatality, which may increase even more in the future (1). Although deadly in newborns, infection is often asymptomatic in adults (5). Studies confirmed that ZIKV is cytopathic in stem cells, with limited effect on differentiated cells (2,7,8). A recent report by Zhu et al. showed that ZIKV infects and kills glioblastoma stem cells, while the effect on differentiated cancer cells was limited (2). Importantly, there is a gap in our knowledge of pathways activated in stem cells and differentiated tissue, which is essential for understanding the anti-cancer activity of ZIKV. Understanding these pathways will help develop effective therapeutics for treating glioblastoma by ZIKV.

MATERIAL AND METHODS: Neuroblastoma SH-SY5Y cells were infected with Puerto Rico (PRVABC59) and Nigerian (IBH30656) strains of ZIKV. Total RNA and proteins were collected at selected time points after infection and used to determine the viral RNA and protein accumulation. Additionally, the presence of viral proteins was shown using an immunofluorescence assay (IFA), and the production of infectious virions was determined by a plaque-forming assay.

RESULTS: We have shown that neuroblastoma and SH-SY5Y cells are susceptible to infection with ZIKV strains and support virus replication.

CONCLUSION: Our data suggest that neuroblastoma and SH-SY5Y cells are suitable models for studying ZIKV infection of tumor cells as they support ZIKV replication. Neuroblastoma, SH-SY5Y cells can produce tumors in animal models and metastasize to various organs due to the presence of tumor stem cells, which enhances tumor survival and spread. Therefore, using these cells for the future study of ZIKV infection of tumor stem cells will help identify the potential therapeutic targets for eliminating tumor stem cells.

8.6.2 Poster: PathMaker Scholars Research in Jensen Lab

KNOCKOUT OF HYPOXIA-INDUCED FACTOR 1A IN MENINGIOMA AND GLIOMA CELL LINES VIA SHORT HAIRPIN RNA INTERFERENCE AND THE CRISPR-CAS9 SYSTEM

Authors

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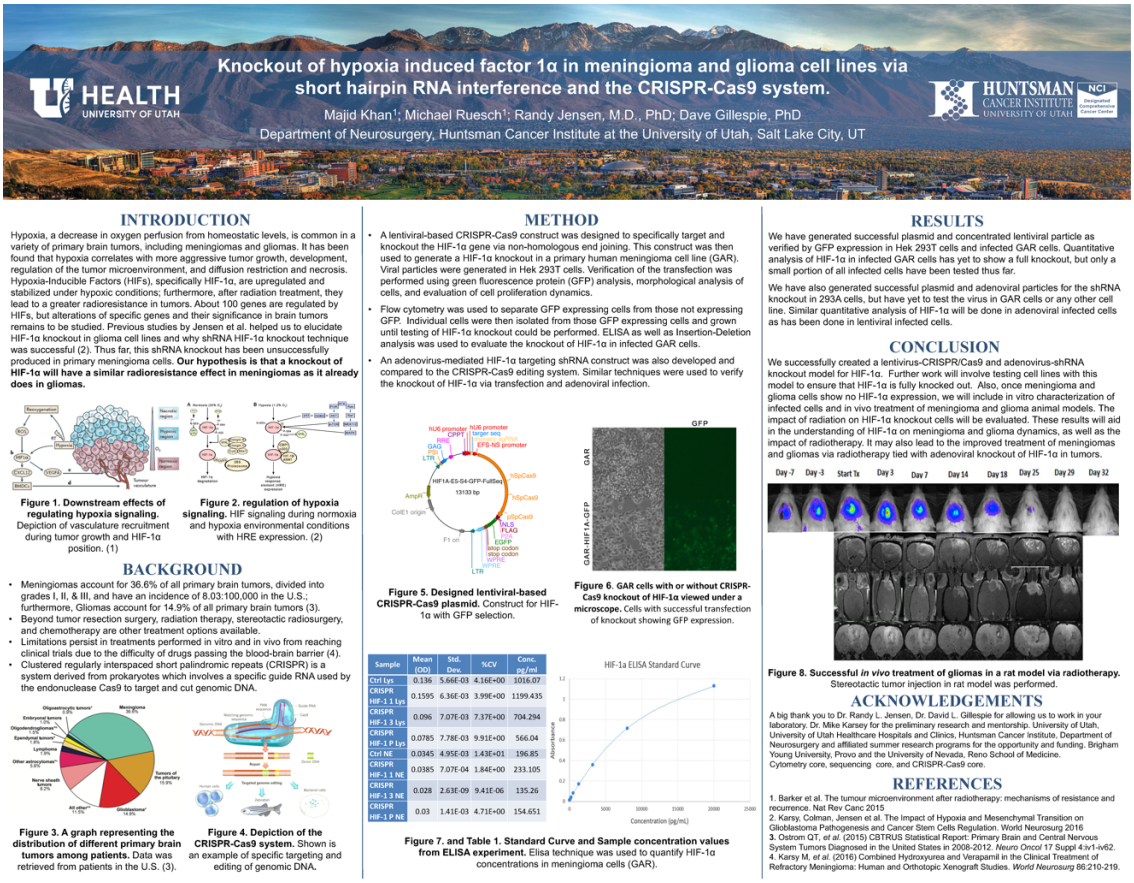

Acknowledgments: Thank you to Dr. Randy L. Jensen and Dr. David L. Gillespie for allowing us to work in your laboratory. Dr. Karsy for the preliminary research and mentorship. University of Utah, University of Utah Healthcare Hospital, Huntsman Cancer Institute, Department of Neurosurgery, and PathMaker research program for the opportunity and funding. Thank you to Brigham Young University, Pro Cytometry, Sequencing, and CRISPR-Cas9 Cores.

ABSTRACT

INTRODUCTION: Hypoxia, a decrease in oxygen perfusion from homeostatic levels, is common in a variety of primary brain tumors, including meningiomas and gliomas. It has been found to correlate with more aggressive tumor growth, development of both meningiomas and gliomas, regulation of tumor microenvironment, diffusion restriction, and necrosis. Hypoxia-inducible factors (HIFs), specifically HIF-1α, are upregulated under hypoxic conditions and after radiation treatment, leading to greater radioresistance in tumors. Earlier work in our lab used a HIF-1α & shRNA knockdown in a glioma primary cell model, which has been unsuccessfully produced in primary meningioma cells.

METHODS: A lentiviral-based clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 construct was used to generate a HIF-1α & knockout in a primary human meningioma cell line (GAR). Viral particles were generated in HEK-293T cells. The transfection was verified using green fluorescence protein (GFP) analysis, morphological analysis of cells, and evaluation of cell proliferation dynamics. An adenovirus-mediated HIF-1α targeting shRNA was also developed and compared.

RESULTS: We have generated a successful plasmid and concentrated viral particle as verified by GFP expression in HEK-293T and infected GAR cells. Generation of a HIF-1α targeting shRNA adenovirus was confirmed by immunofluorescence analysis of virus-producing HEK-293T cells and PCR analysis of generated plasmids. Quantitative analysis of HIF-1α levels was determined via a HIF-1α-based ELISA to determine knockout efficiency.

DISCUSSION: We successfully created a lentivirus-CRISPR/Cas9 and adenovirus-shRNA knockout model for HIF-1α. Further work will include in vitro characterization of infected cells and in vivo treatment of meningioma and glioma animal models. The impact of radiation on HIF-1α knockout cells will be evaluated. These results aid our understanding of HIF-1α and radiotherapy effects on meningioma and glioma dynamics.

Khan et al. *ResearchGate* (2017).

http://dx.doi.org/10.13140/RG.2.2.29654.63047

CHAPTER 9

9. APPENDIX – OTHER PUBLISHED WORKS

9.1 Personal Remarks

This dissertation embodies my commitment to innovative methodologies to advance human health across medical conditions and research domains. As I find myself at the threshold of an academic career filled with countless questions, I remain exhilarated by the boundless opportunities that lie ahead; a never-ending pursuit of knowledge. My ambition is not merely to be a spectator but an active contributor to the breakthroughs that will shape our understanding and treatment of disease in my anticipated roles as a physician, scientist, and surgeon.

The mentorship of Dr. Michael Karsy has profoundly influenced my medical school trajectory. Under his guidance, I have had many opportunities to engage in extensive research endeavors encompassing diverse neurosurgical topics using various research methodologies. These efforts have yielded fruitful outcomes, with several projects garnering acceptance for publication in peer-reviewed journals.

While rooted in neurosurgery, these experiences have endowed me with an expansive set of skills and perspectives. This diverse skill set has been instrumental in exploring my dissertation – from the epidemiological progression of SARS-CoV-2 to the intricate molecular mechanisms contributing to disease severity and the consequent clinical ramifications of neurological sequelae in long-COVID pathogenesis. It is this confluence of research and clinical insight that I aim to harness to drive advancements in medical practice, patient care, and improved health outcomes.

The convergence of my graduate studies with the Neurosciences and the field of Neurosurgery has illuminated the vast potential and infinite opportunities to address disease through multifaceted and novel approaches. This interconnection is a vivid illustration of the broader unity between our environment and the nervous system, revealing how a pathogen like SARS-CoV-2 might unexpectedly pave the way for innovative brain cancer therapies.

As such, this dissertation is not only a record of past achievements but also a record of the promise that I am making to humanity through my future career. It stands as a bridge between the solid foundations of current medical knowledge and the exciting, yet-to-be-discovered territories that await our exploration and contribution.

9.2 Published Manuscripts

SYSTEMATIC REVIEW OF RESEARCH, MENTORSHIP, AND CAREER RESOURCES FOR MEDICAL STUDENTS PURSUING NEUROSURGICAL TRAINING

Authors

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Carter *et al. World Neurosurgery* **179**, 28-36 (2023).

https://doi.org/10.1016/j.wneu.2023.07.133

ABSTRACT[1]

BACKGROUND: Medical students are important team members and will be potential healthcare providers in neurosurgery or other medical fields. We performed a systematic review evaluating studies assessing medical student guidance, mentorship, and career development. The study aimed to identify best practices and acknowledge gaps for improvement.

METHODS: A systematic review of 586 research studies evaluating important aspects of medical student career development in neurosurgery was performed. Studies were analyzed for evidence supporting specific strategies to foster career development.

RESULTS: 45 identified studies were categorized into eight categories, including: 1) medical student interest groups, 2) student fellowships and institutional programs, 3) research and observership funding, 4) medical student research and scholarship, 5) student-led interest groups, 6) student mentorship, 7) educational resources, as well as 8) diversity, equity, and inclusion for medical students. Studies supported the significant positive impact of career resources for medical students, often resulting in higher publication quantities, increased interest in the field, and greater ease of matriculation into neurosurgical residency. One central gap included limited formal opportunities at many institutions, including medical schools, without neurosurgery programs. Another gap included an absent structure to many forms of mentorship and delayed engagement of medical students in neurosurgical training, which significantly impacts career interests. Current resources for these aspects of career development are listed.

DISCUSSION: These studies highlight the current endeavors to encourage medical student careers; however, ample gaps and missed opportunities were also identified. Further work at both institutional and national levels is needed to improve the current environment. Tremendous contribution by students, residents, and faculty in neurosurgery towards the development of medical student careers in neurosurgery. Nonetheless, there is a need for further work on this issue.

CONNECTOMIC NETWORKS AND THEIR IMPACT ON CLINICAL OUTCOMES IN GLIOMA TREATMENT: A REVIEW

Authors

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Rawanduzy *et al. Indian Journal of Neurosurgery* **12**, 116-131 (2023).

https://doi.org/10.1055/s-0043-1771214

ABSTRACT[2]

The emerging field of connectomics has provided an improved understanding of the structural and functional organization of the human brain into large-scale brain networks. Recent studies have helped define the canonical neurological networks and outline how considering their presence may aid in surgical decision-making in brain tumor patients. Gliomas represent one of the most common types of brain tumor and often involve displacement and/or infiltration of neurological pathways, suggesting an opportunity to use connectomics maps to improve patient morbidity and mortality based on oncofunctional goals.

This review aims to provide a working knowledge of important neurological networks, examine the use of networks in surgical planning, and describe the current literature discussing the impact of these networks on clinical outcomes in glioma resection.

ANTERIOR SKULL BASE OUTCOMES AND COMPLICATIONS: A PROPENSITY SCORE–MATCHED EVALUATION OF AGE AND FRAILTY AS MEASURED BY MFI-5 FROM THE ACS-NSQIP DATABASE

Authors

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Bauer *et al. Indian Journal of Neurosurgery* (2023).

https://doi.org/10.1055/s-0043-1770908

ABSTRACT[3]

BACKGROUND: Frailty is increasingly recognized as a predictor of surgical outcomes; however, its utility in anterior cranial fossa (ACF) surgery remains unclear. We analyzed whether age and frailty independently predict outcomes after ACF surgery using a retrospective cohort study.

METHODS: The American College of Surgeons National Surgical Quality Improvement Program database was queried by Current Procedural Terminology codes for ACF procedures from 2005 to 2020. Cases included open approaches, endoscopic approaches, and all tumor types except for pituitary adenoma. A propensity score–matched data set was analyzed via multiple logistic regression.

RESULTS: Unmatched multivariate analysis of ACF cases demonstrated that severe frailty (modified 5-item frailty index [mFI-5] 3) was independently associated with having any (odds ratio [OR] 1⁄4 3.67) and minor (OR 1⁄4 5.00) complications (both $p < 0.001$). Analysis of individual mFI-5 components demonstrated poor functional status was significantly associated with any (OR 1⁄4 3.39), major (OR 1⁄4 3.59), and minor (OR 1⁄4 3.14) complications (all $p < 0.001$). After propensity score matching, only age was modestly impactful on minor complications (OR 1⁄4 1.02) and extended length of stay (eLOS) (OR 1⁄4 1.02) ($p < 0.001$). Frailty did not maintain its predictive ability after matching. Non-independent functional status, as a subcomponent of mFI, maintained significant predictive ability for any (OR1⁄44.94), major (OR1⁄44.68), and minor (OR 1⁄4 4.80) complications and eLOS (OR 1⁄4 2.92) (all $p < 0.001$).

CONCLUSION: After propensity score matching, age demonstrated a greater ability to predict postoperative complications in ACF surgery than frailty. Rather than age or frailty, functional status served as a better outcome predictor and potential guide for patient counseling. Further validation of these findings in multicenter or disease-specific studies is warranted, as well as aims to improve functional status in ACF surgery preoperatively.

MODELING MENINGIOMAS: OPTIMIZING TREATMENT APPROACH

(TEXTBOOK CHAPTER)

Authors

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Jensen *et al. Neurosurgery Clinics of North America* **34** (2023).
https://doi.org/10.1016/j.nec.2023.04.001

Khan *et al. Neurosurgery Clinics of North America* **34**, 479-492 (2023).
https://doi.org/10.1016/j.nec.2023.02.014

ABSTRACT[4,5]

PREFACE: Meningiomas are among the most common intracranial tumors. Despite their relatively benign nature, multimodal treatment is often required to treat them because of their invasive nature, varying location, and lack of medical or chemotherapy availability. Over the past decade, advances in imaging, diagnostics, and therapeutics have improved the contemporary care of patients with meningiomas. In this issue, we examine the contemporary updates in genomics, epigenomics, pathologic diagnosis, imaging, medical management, and surgical approaches used to treat meningiomas. Special considerations are made for meningiomas in the spine and skull base, with articles describing the unique features of managing these tumors. Preoperative embolization of meningioma is an ongoing debate discussed in this issue.

Decision-making regarding incidentally found meningiomas is also explored. In addition to standard therapies, such as surgery, radiosurgery, and radiotherapy, we also discuss novel treatment options, including immunotherapy and targeted therapy. Management of atypical, malignant, and treatment-refractory meningiomas is explored, and controversies are outlined. The role of radiotherapy in higher-grade meningiomas is a commonplace question for practicing neurosurgeons and is discussed in detail. Emerging advanced neuroimaging techniques, including pre-operative characteristics that may predict the consistency of meningiomas, are also reviewed. This collection is rounded out with a review of meningioma models and an in-depth examination of measures of meningioma patient outcome and value-based care. Taken as a whole, these articles provide cutting-edge information to improve the care of patients with meningiomas.

CHAPTER: Preclinical meningioma models offer a setting to test molecular mechanisms of tumor development and targeted treatment options but historically have been challenging to generate. Few spontaneous tumor models in rodents have been established. Still, cell culture and *in vivo* rodent models have emerged along with artificial intelligence, radiomics, and neural networks to differentiate the clinical heterogeneity of meningiomas. We reviewed 127 studies using the PRISMA guideline methodology (Preferred Reporting Items for Systematic Reviews and Meta-Analyses), including laboratory and animal studies, which addressed preclinical modeling. Our evaluation identified that meningioma preclinical models provide valuable molecular insight into disease progression and effective chemotherapeutic and radiation approaches for specific tumor types.

IMMUNOHISTOCHEMICAL PROFILING AND STAGING IN ESTHESIONEUROBLASTOMA: A SINGLE-CENTER COHORT STUDY AND SYSTEMATIC REVIEW

Authors

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Henson *et al. World Neurosurgery* **170**, e652-e665 (2023).

https://doi.org/10.1016/j.wneu.2022.11.094

ABSTRACT[6]

OBJECTIVE: Esthesioneuroblastoma (ENB) is a rare sinonasal malignant neoplasm with 40 % 5-year survival. Because of the rarity of the tumor, the optimal treatment and subsequent prediction of prognosis are unclear. We studied a modern series of patients with ENB to evaluate the association of immunohistochemical (IHC) markers and clinical stages/grades with outcomes.

METHODS: A single-center retrospective review of patients with ENB treated during a 25-year period was performed. A systematic literature review evaluating the prognostic benefits of current staging systems in evaluating survival outcomes in ENB was undertaken.

RESULTS: Among 29 included patients, 25 (85 %) were treated surgically at our institution, with 76 % of those endoscopically resected; 7 (24.1 %) received chemotherapy, and 18 (62.1 %) received radiation therapy. The 5-year overall survival (OS) was 91.3 %, and the 10-year OS was 78.3 %. Progression-free survival at 5 and 10 years was 85.6 % and 68.2 %, respectively. 36 distinct IHC markers were used to diagnose ENB but were inconsistent in predicting survival. A systematic literature review revealed predictive accuracy for OS using the Kadish, TNM, and Hyams staging/grading systems was 68 %, 42 %, and 50 %, respectively.

CONCLUSIONS: This study reports the 5- and 10-year OS and progression-free survival in a modern series of patients with ENB. No traditional IHC marker consistently predicted outcomes. Some novel-reviewed markers show promise but have yet to enter clinical mainstream use. Our systematic review of accepted staging/grading systems also demonstrated a need for further investigation due to limited prognostic accuracy.

PROPENSITY SCORE-MATCHED ASSESSMENT OF ENDOSCOPIC VERSUS MICROSCOPIC APPROACHES IN THE MANAGEMENT OF PITUITARY ADENOMAS

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Findlay *et al. Neurosurgery* **93**, 794-801 (2023).

https://doi.org/10.1227/neu.0000000000002497

ABSTRACT[7]

BACKGROUND: There is considerable controversy about which of the two operating modalities (microsurgical or endoscopic transnasal surgery) currently used to resect pituitary adenomas (PAs) is the safest and most effective intervention.

OBJECTIVE: To compare rates of clinical outcomes of patients with PAs who underwent resection by either microsurgical or endoscopic transnasal surgery.

METHODS: To independently assess the outcomes of each modality type, we sought to isolate endoscopic and microscopic PA surgeries with a 1:1 tight-caliper (0.01) propensity score-matched analysis using a multicenter, neurosurgery-specific database. Surgeries were performed between 2017 and 2020, with data collected retrospectively from 12 international institutions on four continents. Matching was based on age, previous neurological deficit, American Society of Anesthesiologists (ASA) score, tumor functionality, tumor size, and Knosp score. Univariate and multivariate analyses were performed.

RESULTS: Among a pool of 2,826 patients, propensity score matching resulted in 600 patients from 9 surgery centers being analyzed. Multivariate analysis showed that microscopic surgery had a 1.91 odds ratio (OR) ($p = 0.03$) of gross total resection (GTR) and shorter operative duration ($p < 0.01$). However, microscopic surgery also had a 7.82 OR ($p < 0.01$) for intensive care unit stay, 2.08 OR ($p < 0.01$) for intraoperative cerebrospinal fluid (CSF) leak, 2.47 OR ($p = 0.02$) for postoperative syndrome of inappropriate antidiuretic hormone secretion (SIADH), and was an independent predictor for longer postoperative stay ($\beta = 2.01$, $p < 0.01$). Overall, no differences in postoperative complications or 3- to 6-month outcomes were seen by surgical approach.

CONCLUSION: Our international, multicenter matched analysis suggests microscopic approaches for pituitary tumor resection may offer better GTR rates, albeit with increased intensive care unit stay, CSF leak, SIADH, and hospital utilization. Better prospective studies can further validate these findings, as matching patients for outcome analysis remains challenging. These results may provide insight into surgical benchmarks at different centers, offer room for further registry studies, and identify best practices.

ARE THERE RACIAL AND ETHNIC HEALTH DISPARITIES AMONG OUTCOMES AFTER ANTERIOR CRANIAL FOSSA SURGERY? A PROPENSITY SCORE-MATCHED AMERICAN COLLEGE OF SURGEONS NATIONAL SURGICAL QUALITY IMPROVEMENT PROGRAM STUDY

Authors

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Findlay *et al. Neurosurgery* **93**, 176-185 (2023).

https://doi.org/10.1227/neu.0000000000002397

ABSTRACT[8]

BACKGROUND: Race-based health care outcomes remain to be described in anterior cranial fossa (ACF) surgery.

OBJECTIVE: To determine whether race predicts worse outcomes after ACF surgery.

METHODS: A retrospective cohort study was performed using the American College of Surgeons National Surgical Quality Improvement Program data for 2005 to 2020. Current Procedural Terminology and International Classification of Diseases-9 codes were used to identify ACF tumor cases. Propensity score matching was performed to compare White and minority patients to assess the robustness of unmatched findings. A sub-analysis of pituitary adenoma (PA) resections was also performed.

RESULTS: In an unmatched analysis of 1370 patients who underwent ACF surgery (67.9 % White, 17.4 % Black, 6.6 % Asian/Pacific Islander, and 6.3 % Hispanic), minority groups had higher rates of comorbidities. Unmatched multivariate analysis found Hispanic patients bore a 1.86 odds ratio (OR) of minor complications, Black and Asian and Pacific Islander patients bore 1.49 and 1.71 ORs, respectively, for extended length of stay, and Black patients bore a 3.78 OR for urinary tract infection (UTI). The matched analysis found that minority patients had higher UTI rates ($p = 0.02$) and a 4.11 OR of UTI. In PA cases specifically, minority groups had higher comorbidities and length of stay in addition to their odds of extended length of stay (1.84 OR).

CONCLUSION: Although most ACF surgery outcomes were unaffected by race, minority groups had more minor postoperative complications than White patients, particularly UTI. Similar disparities were observed among PA cases. Higher rates of comorbidities may also have led to longer hospital stays. Further studies are needed to understand what actions might be necessary to address any race-associated health disparities in ACF surgery.

SYSTEMATIC REVIEW OF TREATMENT OPTIONS AND THERAPEUTIC RESPONSES FOR LESIONS OF THE SELLA AND ORBIT: EVIDENCE-BASED RECOMMENDATIONS

Authors

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Cutler *et al. World Neurosurgery* **173**, 136-145 e130 (2023).

https://doi.org/10.1016/j.wneu.2022.12.108

ABSTRACT[9]

OBJECTIVE: Inflammatory pathologies of the sella and orbit are rare but require prompt diagnosis to initiate effective treatment. Because uniform recommendations for treatment are currently lacking, we performed an evidence-based review to identify recommendations.

METHODS: We performed a literature search of the PubMed, Embase, and Web of Science databases to identify papers evaluating the treatment of inflammatory pathologies of the sella and orbit. We used PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines to define recommendations, specifically examining aggregated sample sizes, disease-specific patient follow-up, and clinical trials focused on inflammatory diseases of the sella and orbit.

RESULTS: A total of 169 studies were included and organized by disease pathology. Treatments for various pathologies were recorded. Treatment options included surgery, radiation, steroids, targeted treatments, immunomodulators, intravenous immune globulin, and plasmapheresis. Steroids were the most often employed treatment. Second-line management options and timing varied. Pathological diagnosis was highly associated with the treatment used. Most evidence was level 3 without available control groups, except for 13 trials in neuromyelitis optica with level 1 or 2 evidence.

CONCLUSION: This is the first evidence-based review to recommend specific treatments for pathologies of the orbit and sella. The reported data can help guide randomized clinical trials and provide resources for clinical management decisions based on the available evidence.

EVALUATING PITUITARY ADENOMAS USING NATIONAL RESEARCH DATABASES: SYSTEMATIC REVIEW OF THE QUALITY OF REPORTING BASED ON THE STROBE SCALE

Authors

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Khan *et al. Neurosurgical Review* **45**, 3801-3815 (2022).

https://doi.org/10.1007/s10143-022-01888-z

ABSTRACT[10]

BACKGROUND: National research databases have become more prevalent for studying various neurosurgical diseases. Despite the advantages of using large databases to glean clinical insight, variation remains in the methodology and reporting among studies.

METHODS: Using STROBE and RECORD guidelines, we evaluated the quality of reporting of the database literature investigating surgical management of benign pituitary adenomas. In this systematic review of the PubMed/MEDLINE database, we identified studies employing large national research databases of patients who underwent surgery for benign pituitary adenoma. We evaluated each of these studies using the STROBE-RECORD reporting guideline criteria to assess their quality.

RESULTS: A total of 42 studies from 2003 to 2020 were identified for inclusion. The two raters demonstrated a kappa = 0.228 with 84 % overall agreement. Commonly underreported criteria included bias (discussed in 56 % of studies), main result reporting (70 %), subgroup analysis (69 %), generalizability (68 %), and funding (57 %). In addition to the data sources/measurement criteria, these factors also had the largest discrepancies between reviewers. About 20 % of administrative database reviews did not accurately address bias or control for confounding variables. We found frequent underreporting of crucial information and criteria that can be challenging to identify and may limit large database studies of pituitary adenomas.

CONCLUSION: Improved reporting of certain criteria is critical to optimize reader understanding of large database studies. This would allow better dissemination and implementation of study findings, especially as the use of these research tools increases.

USE OF A SURGICAL STEPDOWN PROTOCOL FOR COST REDUCTION AFTER TRANSSPHENOIDAL PITUITARY ADENOMA RESECTION: A CASE SERIES

Authors

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Hunsaker *et al. World Neurosurgery* **152**, e476-e483 (2021).

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ABSTRACT[11]

OBJECTIVE: No established standard of care currently exists for the postoperative management of patients with surgically resected pituitary adenomas. Our objective was to quantify the efficacy of a postoperative stepdown unit protocol for reducing patient costs.

METHODS: In 2018-2020, consecutive patients undergoing transsphenoidal microsurgical resection of sellar lesions were managed postoperatively in the full intensive care unit (ICU) or an ICU-based surgical stepdown unit based on preset criteria. Demographic variables, surgical outcomes, and patient costs were evaluated.

RESULTS: Fifty-four patients (27 stepdown, 27 full ICU; no difference in age or sex) were identified. Stepdown patients were also compared with 634 historical control patients. The total hospital length of stay was no different among stepdown, ICU, and historical patients (4.8 ± 1.0 vs. 5.9 ± 2.8 vs. 4.4 ± 4.3 days, respectively, $p = 0.1$). Overall costs were 12.5 % less for stepdown patients ($p = 0.01$), a difference mainly driven by reduced facility utilization costs of -8.9 % ($p = 0.02$). The morbidity and complication rates were similar in the stepdown and full ICU groups. Extrapolation of findings to historical patients suggested that approximately $225,000 could have been saved from 2011 to 2016.

CONCLUSION: These results suggest that the use of a postoperative stepdown unit could result in a 12.5 % savings for eligible patients undergoing treatment of pituitary tumors by shifting patients to a less acute unit without worsened surgical outcomes. Historical controls indicate that over half of all pituitary patients would be eligible. Further refinement of patient selection for less costly perioperative management may reduce the cost burden for the healthcare system and patients.

PREDICTION OF READMISSION AND COMPLICATION IN PITUITARY ADENOMA RESECTION VIA THE NATIONAL SURGICAL QUALITY IMPROVEMENT PROGRAM (NSQIP) DATABASE

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Hunsaker *et al. Cureus* **13**, e14809 (2021).

https://doi.org/10.7759/cureus.14809

ABSTRACT[12]

INTRODUCTION: Pituitary adenomas are common intracranial tumors (incidence 4:100,000 people) with good surgical outcomes; however, a subset of patients shows higher rates of perioperative morbidity. Our goal was to identify risk factors for postoperative complications or readmission after pituitary adenoma resection.

METHODS: Using the National Surgical Quality Improvement Program database, we undertook a retrospective cohort study of patients who underwent surgery for pituitary adenoma in 2006-2018. The main outcome measures were patient complications and the 30-day readmission rate.

RESULTS: Among the 2,292 patients (mean age 53.3 ± 15.9 years), there were 491 complications in 188 patients (8.2 %). Complications and 30-day readmission have remained stable over time rather than declined. Unplanned readmission was seen in 141 patients (6.2 %). Multivariable analysis demonstrated that hypertension (OR = 1.6; 95 % CI = 1.1, 2.1; $p = 0.005$) and high white blood cell count (OR = 1.08; 95 % CI = 1.03, 1.1; $p = 0.0001$) were independent predictors of complications. Return to the operating room (OR = 5.9, 95 % CI = 1.7, 20.2, $p = 0.0005$); complications (OR = 4.1, 95 % CI = 1.6, 10.6, $p = 0.004$); and blood urea nitrogen (OR = 1.08, 95 % CI = 1.02, 1.2, $p = 0.02$) were independent predictors of 30-day readmission.

CONCLUSION: Using one of the largest datasets of pituitary adenoma patients, we identified the most critical perioperative factors for patient outcomes. One strength of this study is adjusting for cofactors that predict outcomes, which has not been done previously. Several patient biomarkers, namely white blood cell count and blood urea nitrogen, may serve as preoperative markers that might identify patients at higher risk. Control of blood pressure and renal disease may be perioperative management strategies that can impact the outcome.

IMPACT OF HOSPITAL VOLUME ON OUTCOME AFTER SURGICAL TREATMENT FOR HYDROCEPHALUS: A U.S. POPULATION STUDY FROM THE NATIONAL INPATIENT SAMPLE

Authors

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Khan *et al. Cureus* **13**, e13617 (2021).

https://doi.org/10.7759/cureus.13617

ABSTRACT[13]

INTRODUCTION: Hydrocephalus remains a common condition with significant patient morbidity; however, accurate accounting of the incidence of this disease, as well as of the impact of hospital volume on outcome, remains limited.

METHODS: The National Inpatient Sample was used to evaluate patients who underwent surgical treatment for hydrocephalus from 2009-2013. Patient demographics (*e.g.*, length of stay, disposition, charges) and the impact of hospital volume on outcomes were evaluated.

RESULTS: A total of 156,205 patients were identified. Ventriculoperitoneal (VP) shunting is the most common type of device (35.8 %), followed by shunt replacement (23.9 %). Hydrocephalus treatment charges were $332 million in 2009 and $418 million in 2013 nationally. High-volume hospitals had more routine discharges than lower-volume hospitals (65.7 % vs. 50.9 %, $p < 0.0001$), a trend that improved over time. Multivariate analysis confirmed that hospital volume was independently associated with routine disposition after adjusting for other factors such as patient age, length of stay, and shunt type. However, hospital volume showed a small association with length of stay ($\beta = -0.05$, $p = 0.0001$) and did not impact hospital charges on multivariate analysis.

CONCLUSION: This analysis provides a recent update on hydrocephalus epidemiology, trends, and outcomes nationally. Estimates from this study suggest that hydrocephalus is a common and costly problem. Hospital volume was, for the first time, shown to be associated with significant differences in patient outcomes.

LETTER: EVALUATING THE ROLE OF ADVANCED PRACTICE PROVIDERS IN NEUROSURGERY

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Khan *et al. Neurosurgery* **88**, E285-E287 (2021).

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ABSTRACT[14]

BACKGROUND: Recent estimates of the demand for neurosurgical services suggest there are staffing shortages, especially in rural communities. Despite the possibility of advanced practice providers (APPs) filling in these and other gaps, little is known about the specific roles of neurosurgical APPs.

OBJECTIVE(S): We conducted an anonymous survey to understand the current day-to-day roles of APPs in neurosurgery in terms of procedures, levels of autonomy, and overall job satisfaction.

METHODS: An anonymous electronic survey was distributed to neurosurgical APPs.

RESULTS: A total of 75 APPs (27 nurse practitioners and 48 physician assistants) responded from 28 states. APPs had an average work experience of 6.7 ± 5.2 years (median five years). Approximately 87 % of APPs responded from urban regions and 54 % from academic centers. The majority worked in inpatient hospitals (75 %) or outpatient clinics (73 %). APPs commonly participated in bedside (75 %) and OR (55 %) procedures. The vast majority felt valued (range 63 – 96 %) by their coworkers (*i.e.*, residents, fellows, attending surgeons, and other APPs). Overall, adequacy of training was a major barrier, with only 43 % reporting receiving some formal neurosurgery training (*e.g.*, Bootcamp, clinical rotation, *etc.*) and 63 % stating there were adequate resources for continued learning.

CONCLUSION: The involvement of neurosurgical APPs in procedures and patient care is substantial but has yet to be fully evaluated. Training, autonomy, scope of practice, and emerging roles for APPs are all critical issues as these clinicians are called to take on greater roles as healthcare demand for neurosurgical services rises. Future studies should more comprehensively address perceived value, roles for improvement, and additional demographics for neurosurgical APPs.

9.3 Published Abstracts

IMMUNOHISTOCHEMICAL PROFILING AND STAGING IN ESTHESIONEUROBLASTOMA: A SINGLE-CENTER COHORT STUDY AND SYSTEMATIC REVIEW

Authors

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Henson *et al. World Neurosurgery* **170**, e652-e665 (2023).

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ABSTRACT[15]

OBJECTIVE: Esthesioneuroblastoma (ENB) is a rare sinonasal malignant neoplasm with 40 % 5-year survival. Because of the rarity of the tumor, the optimal treatment and subsequent prediction of prognosis are unclear. We studied a modern series of patients with ENB to evaluate the association of immunohistochemical (IHC) markers and clinical stages/grades with outcomes.

METHODS: A single-center retrospective review of patients with ENB treated during a 25-year period was performed. A systematic literature review evaluating the prognostic benefits of current staging systems in evaluating survival outcomes in ENB was undertaken.

RESULTS: Among 29 included patients, 25 (85 %) were treated surgically at our institution, with 76 % of those endoscopically resected; 7 (24.1 %) received chemotherapy, and 18 (62.1 %) received radiation therapy. The 5-year overall survival (OS) was 91.3 %, and the 10-year OS was 78.3 %. Progression-free survival (PFS) rates at 5 and 10 years were 85.6 and 68.2 %, respectively. A total of 36 distinct IHC markers were used to diagnose ENB but were inconsistent in predicting survival. A systematic literature review revealed predictive accuracy for OS rates using the Kadish, TNM, and Hyam's staging/grading systems were 68, 42, and 50 %, respectively.

CONCLUSION: This study reports the 5- and 10-year OS and PFS in a modern series of patients with ENB. No traditional IHC marker consistently predicted outcomes. Some novel-reviewed markers show promise but have yet to enter clinical mainstream use. Our systematic review of accepted staging/grading systems also demonstrated a need for further investigation due to limited prognostic accuracy.

RACIAL AND ETHNIC HEALTH DISPARITIES AMONG TUMOR CASES OF THE ANTERIOR CRANIAL FOSSA: A PROPENSITY SCORE–MATCHED ACS-NSQIP STUDY

Authors

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Findlay *et al. Journal of Neurological Surgery Part B Skull Base* **84**, P048 (2023).

https://doi.org/10.1055/s-0043-1762269

ABSTRACT[16]

BACKGROUND: Race-based healthcare outcomes remain to be described in anterior cranial fossa (ACF) surgery. Objective: To determine whether race predicts worse outcomes after ACF surgery.

METHODS: A retrospective cohort study was performed using the American College of Surgeons National Surgical Quality Improvement Program data for 2005–2020. Current Procedural Terminology and International Classification of Diseases-9 codes were used to identify ACF tumor cases. Propensity-score matching was performed to compare white and minority patients to assess the robustness of unmatched findings. A subanalysis of pituitary adenoma (PA) resections was also done.

RESULTS: In an unmatched analysis of 1,370 ACF patients (67.9 % White, 17.4 % Black, 6.6 % Asian/Pacific Islander, 6.3 % Hispanic), minority groups had elevated comorbidity and urinary tract infection (UTI; $p = 0.02$) rates (Tables 1, 2). Unmatched multivariate analysis found Hispanic patients bore a 1.86 odds ratio (OR) of minor complications, Black and API patients bore 1.49 and 1.71 ORs, respectively, for an extended length of stay (eLOS), and Black patients bore a 3.78 OR for UTI (Table 3). The matched analysis found minority patients had higher comorbidities and UTI rates ($p = 0.02$) and a 4.11 OR of UTI (Tables 1–3). In matched PA cases specifically, minority groups had higher comorbidities and LOS in addition to eLOS odds (OR: 1.84).

CONCLUSIONS: Race-based health disparities impact ACF outcomes: minority groups had significantly more comorbidities and postoperative complications than white patients. Similar disparities were observed among PA cases. Further study is needed to understand why these imbalanced outcomes are seen and what actions may reduce race-associated health disparities in ACF surgery.

HSR21-052: PREDICTION OF READMISSION AND COMPLICATIONS AFTER PITUITARY ADENOMA RESECTION VIA THE NATIONAL SURGICAL QUALITY IMPROVEMENT PROGRAM (NSQIP) DATABASE

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Hunsaker *et al. Journal of the National Comprehensive Cancer Network* **19** (2021).

https://doi.org/10.6004/jnccn.2020.7731

ABSTRACT[17]

OBJECTIVES: Pituitary adenomas are common intracranial tumors (incidence 4:100,000 people) with good surgical outcomes; however, a subset of patients show higher rates of perioperative morbidity. Our goal was to identify relevant risk factors of postoperative complications or 30-day readmission following pituitary adenoma resection.

DESIGN: Retrospective database cohort study. Setting: National Surgical Quality Improvement Program (NSQIP) database

PARTICIPANTS: Patients who underwent surgery for pituitary adenoma in 2006-2018.

MAIN OUTCOME MEASURES: Patient complications and 30-day readmission rate

RESULTS: Among the 2,292 patients identified (mean age 53.3 ± 15.9 years), there were 491 complications in 188 patients (8.2 %). Complications and 30-day readmission have plateaued over time, even as the number of cases has substantially increased. Unplanned readmission was seen in 141 patients (6.2 %). Multivariable analysis demonstrated that hypertension (odds ratio [OR] = 1.6, 95 % confidence interval [CI] = 1.1, 2.1, $p = 0.005$) and high white blood cell count (OR = 1.08, 95 % CI = 1.03, 1.1, $p = 0.0001$) were independent predictors of complications. Return to the operating room (OR = 5.9, 95 % CI = 1.7, 20.2, $p = 0.0005$), complications (OR = 4.1, 95 % CI = 1.6, 10.6, $p = 0.004$), and blood urea nitrogen (OR = 1.08, 95 % CI = 1.02, 1.2, $p = 0.02$) were independent predictors of 30-day readmission.

CONCLUSION: We evaluated one of the largest pituitary adenoma datasets to identify the most critical perioperative factors for dictating patient outcomes. Several patient biomarkers, namely white blood cell count and blood urea nitrogen, may serve as preoperative markers to identify patients at higher risk. Control of blood pressure and renal disease may be perioperative management strategies that can impact outcomes.

QIM21-085: USE OF A SURGICAL STEPDOWN PROTOCOL FOR COST REDUCTION AFTER TRANSSPHENOIDAL PITUITARY ADENOMA RESECTION

Authors

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Hunsaker *et al. Journal of the National Comprehensive Cancer Network* **19** (2021).

https://doi.org/10.6004/jnccn.2020.7711

ABSTRACT[18]

OBJECTIVE: Pituitary tumors represent a range of diseases treated by neurosurgeons, with the most common being pituitary adenomas. Significant variation remains in these patients' treatment and postoperative care. We sought to quantify the efficacy of a postoperative stepdown unit protocol for reducing patient costs.

METHODS: Between 2018 and 2020, consecutive patients undergoing transsphenoidal microsurgical resection of sellar lesions were managed postoperatively with either full intensive care unit (ICU) care or surgical stepdown care in the ICU. Stepdown patients were assigned based on strict preset criteria: tumor < 2 cm, no cavernous sinus invasion, no use of a lumbar drain, and no prior surgery. Demographic variables and surgical outcomes were evaluated. Patient costs were derived from the Value Driven Outcome (VDO) database.

RESULTS: A total of 54 patients (27 stepdown vs. 27 full ICU) were identified with no difference in age or sex. The average length of stay in the stepdown unit was 2.7 ± 1.0 days vs. 4.1 ± 2.0 days in the ICU ($p = 0.004$). The total hospital length of stay was no different between stepdown and ICU patients (4.8 ± 1.0 vs. 5.9 ± 2.8 days, $p = 0.08$). No difference in surgical or perioperative complications was seen between groups. Overall costs were 12.5 % less for stepdown patients ($p = 0.01$), a difference mainly driven by reduced facility utilization costs of –8.9 % ($p = 0.02$) compared with other categories, including imaging, supplies/implants, pharmacy, labs, and other four services. Extrapolation of findings to a historical control of 326 patients suggested that approximately $225,000 could have been saved from 2011 to 2016.

CONCLUSIONS: These results suggest that the use of a postoperative stepdown unit could result in a 12.5 % savings for eligible patients undergoing treatment of pituitary tumors without worsened surgical outcomes. Over half of all pituitary patients treated would be eligible for this protocol. Further refinement of patient selection for less costly perioperative management may reduce the cost burden for the healthcare system and patients.

FAR LATERAL APPROACH TO THE VENTROLATERAL SKULL BASE: CASE SERIES

Authors

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Dioso *et al. Journal of Neurological Surgery Part B Skull Base* **84**, P118 (2023).

https://doi.org/10.1055/s-0043-1762339

ABSTRACT[19]

OBJECTIVE: Pituitary tumors represent a range of diseases treated by neurosurgeons, with the most common being pituitary adenomas. Significant variation remains in these patients' treatment and postoperative care. We sought to quantify the efficacy of a postoperative stepdown unit protocol for reducing patient costs.

METHODS: Between 2018 and 2020, consecutive patients undergoing transsphenoidal microsurgical resection of sellar lesions were managed postoperatively with either full intensive care unit (ICU) care or surgical stepdown care in the ICU. Stepdown patients were assigned based on strict preset criteria: tumor < 2 cm, no cavernous sinus invasion, no use of a lumbar drain, and no prior surgery. Demographic variables and surgical outcomes were evaluated. Patient costs were derived from the Value Driven Outcome (VDO) database.

RESULTS: A total of 54 patients (27 stepdown vs. 27 full ICU) were identified with no difference in age or sex. The average length of stay in the stepdown unit was 2.7 ± 1.0 days vs. 4.1 ± 2.0 days in the ICU ($p = 0.004$). The total hospital length of stay was no different between stepdown and ICU patients (4.8 ± 1.0 vs. 5.9 ± 2.8 days, $p = 0.08$). No difference in surgical or perioperative complications was seen between groups. Overall costs were 12.5 % less for stepdown patients ($p = 0.01$), a difference mainly driven by reduced facility utilization costs of –8.9 % ($p = 0.02$) compared with other categories, including imaging, supplies/implants, pharmacy, labs, and other four services. Historical extrapolation to 326 patients suggested ~$225,000 could have been saved from 2011 to 2016.

CONCLUSIONS: These results suggest that the use of a postoperative stepdown unit could result in a 12.5 % savings for eligible patients undergoing treatment of pituitary tumors without worsened surgical outcomes. Over half of all pituitary patients treated would be eligible for this protocol. Further refinement of patient selection for less costly perioperative management may reduce the cost burden for the healthcare system and patients.

THE IMPACT OF RURALITY ON TREATMENT AND OUTCOMES AMONG ADULT PATIENTS WITH SINONASAL MALIGNANCIES IN UTAH

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ABSTRACT[20]

INTRODUCTION: Occupational exposures (farming, construction, mining, metalwork), uninsured status, low-income status, and race and ethnicity have been identified as significant risk factors in sinonasal malignancies. Other social determinants of health (SDOH) – specifically related to rurality and access to health care – may play an unidentified role in the prognosis of sinonasal malignancies.

OBJECTIVE: To understand the role of rurality as a prognostic factor in the treatment course and outcomes of sinonasal malignancies.

METHODS: This is a retrospective cohort study using the Utah Cancer Registry (UCR) database of 229 patients diagnosed with sinonasal malignancies from 1973 to 2019. Patient demographics and socioeconomic statuses, tumor characteristics at diagnosis, treatment, and survival data were analyzed using multivariable models across Utah's 29 counties. County population density classifications include Frontier (< 6 people/sq mi), Rural (6–99 people/sq mi), and Urban (> 100 people/sq mi).

RESULTS: Of the 229 patients (61.1 % male, 94.8 % white, 95.2 % non-Hispanic), 20 (8.7 %) were from Frontier counties, 41 (17.9 %) from Rural counties, and 168 (73.4 %) from Urban counties. One hundred fifty-six tumors were squamous cell carcinomas (12 [60.0 %] Frontier, 27 [65.9 %] Rural, 96 [57.1 %] Urban), and 43 tumors were olfactory neuroblastomas (1 [5.0 %] Frontier, 6 [14.6 %] Rural, 36 [21.4 %] Urban). Patients from Frontier counties traveled an average of 98.4 miles (SD 60.0), Rural patients traveled an average of 44.4 miles (SD 52.1), and Urban patients traveled an average of 10.4 miles (SD 11.0) to receive definitive treatment. On average, Frontier patients had a faster treatment course, in months, when compared with Urban patients from diagnosis to surgery (0.4 [SD 0.7] vs. 0.8 [SD 1.6]), radiation (1.3 [SD 0.4] vs. 2.0 [SD 1.5]), and chemotherapy (1.5 [SD 0.5] vs. 1.77 [SD 1.2]) across squamous cell carcinomas and from diagnosis to surgery (0.3 [0.5] vs. 0.6 [1.0]) in neuroendocrine sinonasal tumors. Frontier patients showed longer survival in months when compared with Urban patients with squamous cell carcinomas (100.6 [86.9] vs. 72.5 [93.3]) and neuroendocrine tumors (110.0 [91.0] vs. 73.7 [96.3]).

CONCLUSION: These data suggest that while patients from Frontier counties are traveling farther to receive their care, statistically maintaining lower socioeconomic statuses, and experiencing increased SDOH barriers, this patient population is not receiving a lower standard of care. The treatment course of Frontier patients is seemingly expedited when compared with that of Urban patients. Frontier patients with sinonasal malignancies survive longer on average despite the SDOH barriers. These results can support providers and treatment teams in tailoring care for patients with specific SDOHs to achieve timely treatment and improved outcomes.

EVALUATING THE ROLE OF ADVANCED PRACTICE PROVIDERS IN NEUROSURGERY

Authors

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ABSTRACT[14]

BACKGROUND: Recent estimates of the demand for neurosurgical services suggest there are staffing shortages, especially in rural communities. Despite the possibility of advanced practice providers (APPs) filling in these and other gaps, little is known about the specific roles of neurosurgical APPs.

OBJECTIVE(S): We conducted an anonymous survey to understand the current day-to-day roles of APPs in neurosurgery in terms of procedures, levels of autonomy, and overall job satisfaction.

METHODS: An anonymous electronic survey was distributed to neurosurgical APPs.

RESULTS: A total of 75 APPs (27 nurse practitioners and 48 physician assistants) responded from 28 states. APPs had an average work experience of 6.7 ± 5.2 years (median five years). Approximately 87 % of APPs responded from urban regions and 54 % from academic centers. The majority worked in inpatient hospitals (75 %) or outpatient clinics (73 %). APPs commonly participated in bedside (75 %) and OR (55 %) procedures. The vast majority felt valued (range 63 – 96 %) by their coworkers (*i.e.*, residents, fellows, attending surgeons, and other APPs). Overall, adequacy of training was a major barrier, with only 43 % reporting receiving some formal neurosurgery training (*e.g.*, Bootcamp, clinical rotation, *etc.*) and 63 % stating there were adequate resources for continued learning.

CONCLUSION: The involvement of neurosurgical APPs in procedures and patient care is substantial but has yet to be fully evaluated. Training, autonomy, scope of practice, and emerging roles for APPs are all critical issues as these clinicians are called to take on greater roles as healthcare demand for neurosurgical services rises. Future studies should more comprehensively address perceived value, roles for improvement, and additional demographics for neurosurgical APPs.

EVIDENCE-BASED RECOMMENDATIONS ON THE WORKUP AND TREATMENT OPTIONS FOR INFLAMMATORY PATHOLOGIES OF THE SELLA AND ORBIT: A SYSTEMATIC REVIEW

Authors

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Cutler *et al. Journal of Neurological Surgery Part B Skull Base* **84**, S157 (2023).

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ABSTRACT[21]

BACKGROUND: Inflammatory pathologies of the sella and orbit are rare but require prompt diagnosis to initiate effective treatment and avoid long-term complications. Diagnosis and treatment of these pathologies have largely been guided by a clinician's personal experience. Uniform guidelines have yet to be established.

OBJECTIVE: Identify evidence-based recommendations for the treatment of inflammatory pathologies of the sell and orbit

METHODS: (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) PRISMA guidelines were utilized to screen inflammatory pathologies of the orbit and sella. Clinical trials, large case series, or retrospective/prospective cohort studies that involved clinical treatment and associated follow-up were included. Narrative reviews, case reports, preclinical studies, as well as studies focusing on trauma, infection, or inappropriate pathology were excluded.

RESULTS: A total of 169 studies were included and organized by disease pathology. Treatments for specific pathologies, such as surgery, radiation, steroids, targeted treatments, immunomodulators, intravenous immune globulin [JA1], and plasmapheresis, were noted and included. Steroids were the most often employed treatment; studies varied on 2nd line management options and timing. Outcomes measured by the studies included neurological, endocrinological, radiological, visual, and functional improvement and relapse rates. Pathological diagnosis highly impacted treatment selection, generating a treatment guideline. Level 3 evidence was present for most studies except for 13 trials in neuromyelitis optica with level 1 or 2 evidence.

CONCLUSION: This is the first evidence-based review of treatment recommendations for inflammatory pathologies of the orbit and sella. The presentation and workup of many of these pathologies are often homogeneous, adding to the diagnostic complexity of these cases. We present one of the first reviews to consolidate novel and effective treatments for these rare pathologies and a general workup. Trial endpoints, treatment length, dose, and patient selection varied widely across the studies in this review, making generalization of the findings difficult. Since several pathologies in this review stem from extracranial primary disease, future guidelines should also include appropriate screening and prevention in those with a primary inflammatory pathology outside of the skull base. Future guidelines will aim to improve our findings to guide treatment across the multidisciplinary team involved in these challenging cases.

ANTERIOR SKULL BASE OUTCOMES AND COMPLICATIONS: A PROPENSITY SCORE-MATCHED EVALUATION OF AGE AND FRAILTY AS MEASURED BY MFI-5 FROM THE ACS-NSQIP DATABASE

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ABSTRACT[22]

BACKGROUND: Frailty is increasingly recognized as a predictive factor of surgical outcomes—however, its utility in anterior cranial fossa (ACF) surgery has not been sufficiently examined. Objective: Analyze the independent predictive ability of age and frailty in postoperative complications following ACF surgery.

METHODS: The American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP) database was queried using current procedural terminology (CPT) codes for ACF procedures between 2005 and 2020. A propensity score-matched dataset was built and analyzed via multiple linear regression. To achieve more comparable datasets, a nearest-neighbor matching algorithm was performed via the MatchIt package in R. This matching was done with a 1:1 ratio, matching with equal ratios of CPT codes, and by the length of operation. Length of operation was chosen as a proxy for the complexity of a case, which could not be accounted for adequately with other variables included by the NSQIP dataset. Pituitary adenoma (PA) cases were similarly identified and analyzed for internal comparison.

RESULTS: A total of 1,651 ACF cases and 2,246 PA cases were identified from the NSQIP database (Tables 1 and 2). Following matching, the datasets contained 506 ACF cases and 706 PA cases. Unmatched multivariate analysis of ACF cases demonstrated severe frailty (mFI-5 3 and 4) was independently associated with having any complications (OR = 3.67) and minor complications (OR = 5.00; $p < 0.001$; Table 3). Analysis of individual mFI-5 components demonstrated poor functional status significantly associated with any complications (OR = 3.39), major complications (OR = 3.59), and minor complications (OR = 3.14; $p < 0.001$). Increasing age was not predictive of complications in ACF or PA cases. However, post-matched multivariate analysis demonstrated age was predictive of minor complications, with an OR of 1.02 per year increase in age and elongated length of stay (eLOS) (OR = 1.02; $p < 0.001$; Table 4). Frailty did not maintain its predictive ability post-matching. Non-independent functional status in ACF cases maintained significant predictive ability for any complications (OR = 4.94), major complications (OR = 4.68), minor complications (OR = 4.80), and eLOS (OR = 2.92; $p < 0.001$).

CONCLUSIONS: Following propensity score matching, age demonstrated an increased ability to predict postoperative complications in ACF surgery compared with mFI-5. Individual mFI-5 components, such as functional status, indicate potential risk factors that can be optimized before surgery for better outcomes.

SKULL BASE COMPLICATIONS AND 30-DAY READMISSION: AN EVALUATION OF THE ELDERLY FROM THE NATIONAL SURGICAL QUALITY IMPROVEMENT PROGRAM (NSQIP) DATABASE

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ABSTRACT[23]

OBJECTIVE: Older patient age is associated with worse outcomes and higher complication rates in a variety of neurosurgical diseases. Whether age has a similar impact on patient outcomes after skull base procedures has not been demonstrated.

METHODS: The American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP) database was used to evaluate patients who underwent anterior, lateral, and posterior fossa skull base procedures between 2005 and 2018.

RESULTS: A total of 17,980 patients from 2005 to 2018 were categorized by age into the following groups: < 65 (n = 13,132) or ≥ 65 years (n = 4,848). Complications occurred at a significantly higher rate in older patients (14 vs. 21 %, $p = 0.0001$), especially among anterior skull base, cerebellopontine angle, and craniopharyngioma cases. The 30-day unplanned readmission rate was also higher for older patients (9 vs. 11 %, $p = 0.0001$), along with a higher likelihood of disposition to skilled nursing facilities (2.4 vs. 8.4 %) or rehabilitation (7 vs. 12.6 %). Trends over time showed improvement in outcomes after skull base procedures but to a lesser extent in the elderly. Multivariate analysis revealed that age was independently associated with a higher risk of complications and 30-day readmission.

CONCLUSION: Our results indicate that elderly patients undergoing skull base surgical procedures have worse outcomes and a higher likelihood of readmission, in spite of adjusting for various medical comorbidities and frailty. The impact of age on outcome after skull base surgery also varies by surgical site and type. Greater attention to older patients and closer perioperative management may be helpful for improving outcomes.

PREDICTION OF READMISSION AND COMPLICATIONS IN PITUITARY ADENOMA RESECTION VIA THE NATIONAL SURGICAL QUALITY IMPROVEMENT PROGRAM (NSQIP) DATABASE

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ABSTRACT[24]

PURPOSE: Pituitary adenomas are common intracranial tumors (incidence 4:100,000 people) with good surgical outcomes; however, a subset of patients show higher rates of worsened perioperative morbidity. Understanding the relevant risk factors and whether outcomes have improved over time would improve understanding of this disease.

METHODS: We evaluated patients who underwent surgery for pituitary adenoma by using the National Surgical Quality Improvement Program (NSQIP) database. Patients treated in 2006–2018 were included. Patient complications and 30-day readmission were the primary outcomes.

RESULTS: Among the 2,292 patients identified (mean age: 53.3 ± 15.9 years), there were 491 complications in 188 patients (8.2 %). Complications and 30-day readmission have plateaued over time, even as the number of cases has substantially increased. Unplanned readmission was seen in 141 patients (6.2 %). Multivariable analysis demonstrated that hypertension (odds ratio [OR] = 2.9, 95 % confidence interval [CI] = 1.3–6.7, $p = 0.01$), return to the operating room (OR = 3.5, 95 % CI = 1.1–11.7, $p = 0.04$), length of stay (OR = 1.1, 95 % CI = 1.02–1.1, $p = 0.008$), and white blood cell count (OR = 1.13, 95 % CI = 1.05–1.2, $p = 0.003$) were independent predictors of complications. Return to the operating room (OR = 5.9, 95 % CI = 1.7–20.2, $p = 0.0005$), complications (OR = 4.1, 95 % CI = 1.6–10.6, $p = 0.004$), and blood urea nitrogen (OR = 1.08, 95 % CI = 1.02–1.2, $p = 0.02$) were independent predictors of 30-day readmission.

CONCLUSION: We evaluated one of the largest pituitary adenoma datasets to identify the most critical perioperative factors for dictating patient outcomes and show improvement in complication rates over time. Several patient biomarkers, namely white blood cell count and blood urea nitrogen, may serve as preoperative markers that could potentially identify higher-risk patients. Control of blood pressure may be a perioperative management strategy that can improve outcomes. Lastly, the impact of the surgeon on outcomes in this patient group is reemphasized.

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