Variable expression of Human Cytomegalovirus (HCMV) tegument genes among immunocompromised individuals and their response to different antiviral agents

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Submitted

by

Debsopan Roy

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2024

CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **Variable expression of Human Cytomegalovirus (HCMV) tegument genes among immunocompromised individuals and their response to different antiviral agents"** Submitted by Sri **Debsopan Roy**. who got his registered on **Department of Science and Biotechnology** for the award of **Ph. D. (Science)** Degree of Jadavpur University, is absolutely based upon his own work under the supervision of Dr. Nilanjan Chakraborty, Scientist-F, ICMR-NICED. and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

Nilanjan Chakraborty 3/10/2024

(Signature of the Supervisor(s) date with official seal)

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Declaration

I, Debsopan Roy confirm that the research work presented in this thesis is my own. Any information that has been derived from other sources has been indicated and referenced in the thesis.

Dedication

I dedicate this work to all the members of my family, my PhD supervisor and my lab mates without whose constant support and encouragement this task would have remained impossible.

“There is no greater gift you can give or receive than to honor your calling. It's why you were born and how you become most truly alive.”

Oprah Winfrey

Thesis Title: Variable expression of Human Cytomegalovirus (HCMV) tegument genes among immunocompromised individuals and their response to different antiviral agents

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Abstract of the thesis:

Human cytomegalovirus (HCMV) is a widespread virus that often goes unnoticed in healthy adults but can cause serious illness in those with weakened immune systems. This broad investigation was conducted in metropolitan hospitals near Kolkata and found that 51.57% of hospitalized neonates and 49.20% of immunosuppressed renal transplant recipients tested positive for HCMV. Many patients showed persistent HCMV infection after antiviral therapy. The study also investigated specific clinical manifestation Choroidal neovascularization (CNV) can lead to partial or complete blindness associated with HCMV-infection to understand the immunological alteration.

It was found that HCMV-induced CNV progression was prominent in the presence of AT2R-dependent angiogenesis, while in the absence of HCMV, AT1R-dependent CCL-5-mediated angiogenesis was documented. Significant increases in CCL-19, CCL-21 chemokine responses, and CCR-7 chemokine receptor activation were observed in HCMV-induced CNV patients compared to HCMV non-induced CNV groups. These findings suggest that ocular HCMV latency poses a significant risk factor for the progression of retinal neovascularisation through a distinct NFkB non-canonical immunological signalling pathway.

In a study of renal transplant patients, a significant number of persistent infectivity of HCMV was observed after the antiviral therapy. The research aimed to understand the impact of latent HCMV infection on renal rejection in an Eastern Indian cohort. Among HCMV-positive transplant patients, 16.45% experienced early rejection, while 27.84% experienced late rejection due to persistent HCMV infection. Clinical parameters revealed that rejection due to latent HCMV cases were associated with serious health complications.

The current synthetic medications have limitations such as toxicity, processing challenges within the body, and susceptibility to viral resistance. In this study, bioactive extracellular enzyme laccase isolated from mushrooms was tested with ganciclovir, a common antiviral drug used against HCMV. The study found that laccase has promising antiviral effects and can effectively inhibit HCMV replication by targeting novel inhibitory sites on the UL54 protein. Laccase has also been observed to act synergistically with ganciclovir to inhibit HCMV replication. The in silico analysis has also indicated that the laccase enzyme's novel binding site on HCMV DNA polymerase can effectively obstruct a crucial structural component of the UL54, preventing another significant accessory replication regulatory protein, UL44, from binding efficiently. This discovery could lead to innovative antiviral approaches targeting *Herpesviridae* and offer insights into regulating HCMV replication through host pathways manipulation.

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Acknowledgement

"Hope" is the thing with feathers -
That perches in the soul -
And sings the tune without the words -
And never stops - at all - *-Emily Dickinson*

Throughout my academic journey, I encountered significant obstacles that challenged my resolve. At times, I found myself grappling with the temptation to relinquish hope and abandon this path, In my subconscious, there has always been a profound longing to delve into uncharted territories of scientific exploration.My introduction to science occurred early in my life, but it took considerable time for my passion to fully develop. This ardor likely originated during my childhood, largely due to the guidance of exceptional educators such as Sri Rathindranath Sengupta, Sri Amitava Giri, and, most notably, my "Acharya-guru" Sri Swapan Kumar Mukhopadhyay. The latter consistently emphasized the importance of questioning rather than blindly accepting information without understanding the underlying facts. Upon making the decision to explore my passion, I embarked on the journey of experimental research with the goal of attaining a PhD. It is widely acknowledged that pursuing a PhD is a challenging endeavor, yet the support of those around you can make the process not only bearable but also achievable, and at times, even enjoyable. The support of one's supervisor is crucial in this process, and I am grateful for the guidance provided by my PhD supervisor, Dr. Nilanjan Chakraborty. His mentorship and dedication to scientific inquiry have been invaluable to me. Dr. Chakraborty fostered an environment that encouraged exploration of scientific inquiries and provided both support and constructive feedback, which greatly contributed to my growth as a researcher. His unwavering support and motivation, especially in times of setback, have been instrumental in my academic and professional development. I firmly believe that next to parents, teachers hold a position of great respect. Throughout my doctoral studies, Dr. Nilanjan Chakraborty has transcended the traditional role of a teacher. His unwavering support during life's challenges has been akin to that of a fatherly figure, standing by my side through every obstacle.

I extend my gratitude to the members of my laboratory for fostering a conducive work environment. I would like to give special recognition to Dr. Aroni Chatterjee, my Senior, who has been unwavering in his support since the inception of my journey. His guidance and unwavering support have been akin to that of a true mentor, providing me with the encouragement and knowledge necessary to venture into uncharted scientific realms. Both individuals have played instrumental roles in broadening my perspective and understanding the intricacies of life and academia, offering invaluable insights akin to a bird's eye view.

I would like to express my gratitude for the assistance and support provided by the Director of our institute, who facilitated the provision of necessary instruments and funds essential for the

completion of my research work. I am also thankful to the scientists of ICMR-Virus Unit and ICMR-NICED for their invaluable support during crucial times. Additionally, I extend my appreciation to numerous others, including the personnel in the accounts department and the security guards, whose contributions have greatly facilitated my work.

I express my deep gratitude to my family for their unwavering support throughout my personal and professional pursuits. Their unconditional love and profound understanding have been invaluable to me. Their presence during significant milestones has been instrumental in my achievements. I am thankful for their guidance in shaping me into a resilient, determined, and industrious individual. My profound appreciation extends to my parents and my spouse-suchismita, who have gracefully weathered my challenges, providing unwavering support through every twist and turn. Their encouragement, inspiration, and resilience have been pivotal in shaping my journey. They have been a constant source of strength, love, and joy throughout my doctoral pursuit. My father serves as the primary source of inspiration in my life. His unwavering encouragement propelled me onto this journey, and to this day, he steadfastly supports me in all endeavours. He is the kind of individual who remains inconspicuous yet diligently oversees the minutiae of the performance. When necessary, he steps forward to fortify my moral fortitude, akin to a sturdy pillar.

My research collaborators also deserve a special mention, especially, Dr. Lopamudra Mishra and Dr. Atanu Pal. They have helped me throughout my PhD work with all kinds of support, from patient selection, data collection, data analysis and so much more. I cannot explain my gratitude towards them in simple words.

I would like to express my sincere gratitude to the individuals in my life who continuously inspire me to strive for self-improvement and to contribute meaningfully to the advancement of society. Your support and encouragement have been instrumental in shaping my journey, and for that, I am profoundly thankful.

"I am a great believer in luck, and I find the harder I work the more I have of it"

-Thomas Jefferson, American Founding Father

List of Abbreviation:

HCMV- Human Cytomegalovirus

CNV- Choroidal Neo-Vascularization

AMD-Age related macular degeneration

VEGF- Vascular endothelial growth factor

IE-1- Immediately Early Gene-1

Ang-I/II- Angiotensin I/II

AT1R- Angiotensin II- Type-I

AT2R- Angiotensin II- Type-II

SGOT- Serum glutamic oxaloacetic transaminase

SGPT- Serum glutamic pyruvic transaminase

ALP- Alkaline phosphatase

GGT- Gamma glutamyltransferase

IFNγ- Interferon gamma

IL6 Interlukin 6

IL10- Interlukin 10

IL1β- Interlukin 1 beta

TGFβ- Transforming growth factor beta

CRP- C-Reactive protein

IL2- Interlukin 2

CCL 5- C-C motif chemokine ligand 5

CCL 19- C-C motif chemokine ligand 19

CCL 21- C-C motif chemokine ligand 21

CCR 5- C-C chemokine receptor type 5

CCR 7- C-C chemokine receptor type 5

IFI16- Interferon gamma inducible protein 16

ROC- Receiver-operating characteristic

AUC- Area under the curve

ANOVA- Analysis of variance

ELISA- Enzyme-linked immunosorbent assay

NCBI- National Centre for Biotechnological Information

ATCC- American Type Culture Collection

CC_{50}- cytotoxic concentration 50%

EC_{50}- Effective concentration 50%

PDB- Protein Data Bank

RMSD- Root Mean Square Deviation

RMSF- Root Mean Square Fluctuation

SPSS- Statistical Product and Service Solutions

Index

INTRODUCTION

History and Origin of Human cytomegalovirus

Human cytomegalovirus (HCMV) is a well-studied member of the Betaherpesvirinae virus sub-family. The initial discovery of a virus resembling HCMV occurred in 1881 when Ribbert observed unusually large cells in the kidneys and parotid gland of a stillborn child. At that time, the specific identity of HCMV was still undisclosed. Similar pathological analyses were shared Two renowned scientists Jesionek and Keiolemenoglou, some critical pathological observation of an 8-month-old child with serious complications in the lungs, kidney, and liver. Initially, there was a hypothesis that these cells could be indicative of a protozoan infection, leading to the presence of significant intranuclear inclusions [1]. Subsequent research identified the atypical and characteristic histological attribute as exhibiting an "Owl's eye" appearance, a cytological designation subsequently employed to describe the specific characteristics of HCMV infection. Lowenstein, a colleague of Ribbert in the laboratory, observed that in infants, numerous bulging cells in the parathyroid glands, after examination, were characterized with strange and unusual cell pathology [2]. The initial theory proposed that the agent responsible for driving this cellular deformity developed due to protozoan infection. Subsequently, Scientist Lipschuetz successfully identified the cells with intercellular deformities incorporated within the patient showing bubble shaped appearance. He critically observed these bodies varicella-zoster infected and herpes simplex infection.[3]. Hence, it was assumed that the hypertrophied cellular bodies can initiate viral infection, potentially related to other members of herpesvirideae. Although the nature of the new microorganism remained unknown, it was evident that the disease was prevalent in the population, particularly among children. A research study revealed that 26 out of 183 deceased infants, who had succumbed to various causes, exhibited similar types of inclusion bodies in their salivary glands [4]. In 1932, several rare and fatal congenital infections were observed, all characterized by calcification in the brain, small red or purple spots on the skin, and enlargement of the liver and spleen. These infections also involved cells with abnormal structures within the nucleus. It was during this time that the term "Cytomegalovirus" originated, derived from cytomegalic inclusion disease (CID) [5].The term "cytomegaly" originates from the Ancient Greek word "cyto-" meaning "cell" and the suffix "-megaly" from the word "megálos" meaning significant enlargement. This combination aptly describes the significant enlargement effect of cytomegaly on the cell. In the further course of scientific investigation one of the gorund breaking experiment was conducted by Fetterman , 1952 [6] where he made a cytological

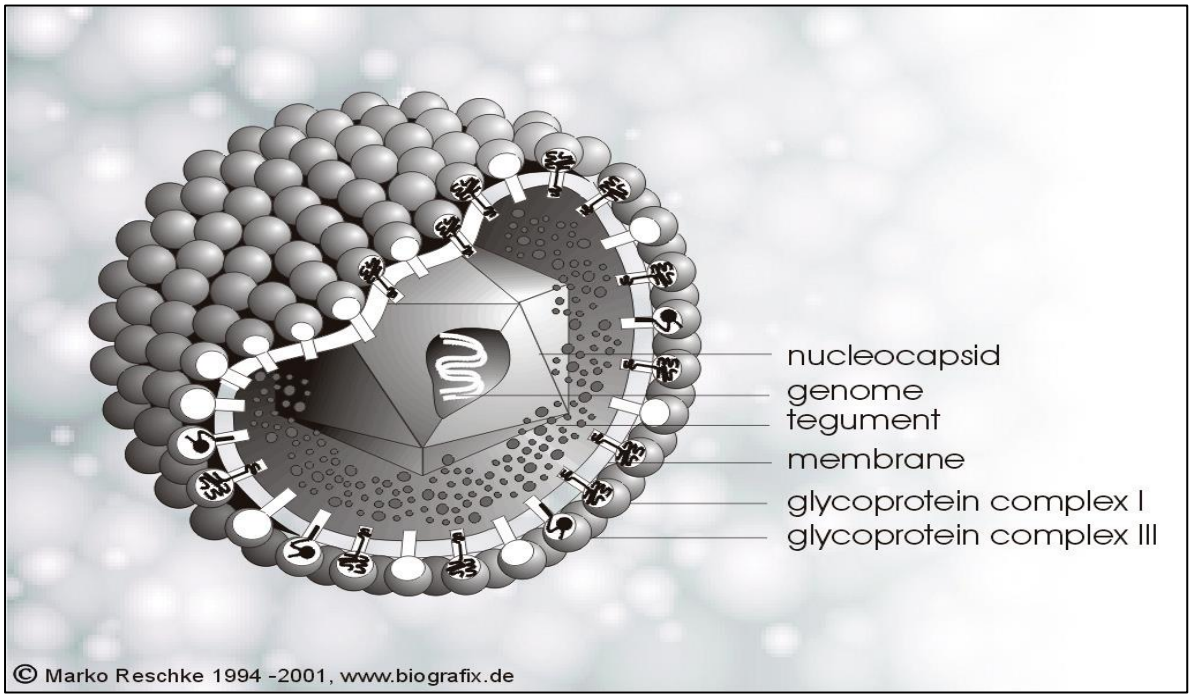


Figure 1: ***A schematic picture of the CMV structure. (Reproduced from the work of Dr. Marko Reschke, http://www.biografix.de/).***

preparation from the urine sediment of a suspected case, and made the first intravital diagnosis of CID. The subsequent findings suggested a possible link between CID and viral infection, i.e the discovery of cellular inclusion boddies approximately 199 nm , which mostly covered the intercellular orientation of CID- patient. Isolating and culturing HCMV would necessitate the initiation of human cell line maintenance strategies and techniques that enable the culturing of viruses in a controlled environment. Margaret Smith's groundbreaking work in 1955 involved the isolation of HCMV, a viral agent found in the human salivary gland that was able to grow in human cell cultures but not in mouse cell cultures. Initially, her findings were met with scepticism, as it was believed that the agent, she isolated might have been a contaminant from her concurrent work with mouse salivary glands. However, in retrospect, we now understand that this criticism was unfounded, as cytomegaloviruses are highly specific to particular species. Therefore, the possibility of contamination from murine cell culture is extremely unlikely [7]. The issue of cross-contamination remains a significant concern, as demonstrated by a study on the xenotropic murine leukemia virus-related virus (XMRV). Researchers isolated the virus from the kidney of a patient with combined immunodeficiency (CID). Furthermore, renowned biologists Thomas Weller and Wallace Rowe independently isolated the same virus, which was later identified as HCMV, from patients exhibiting similar symptoms around the same time. Weller proposed the name 'Cytomegalovirus' for the virus [8].

Systematic position Human cytomegalovirus

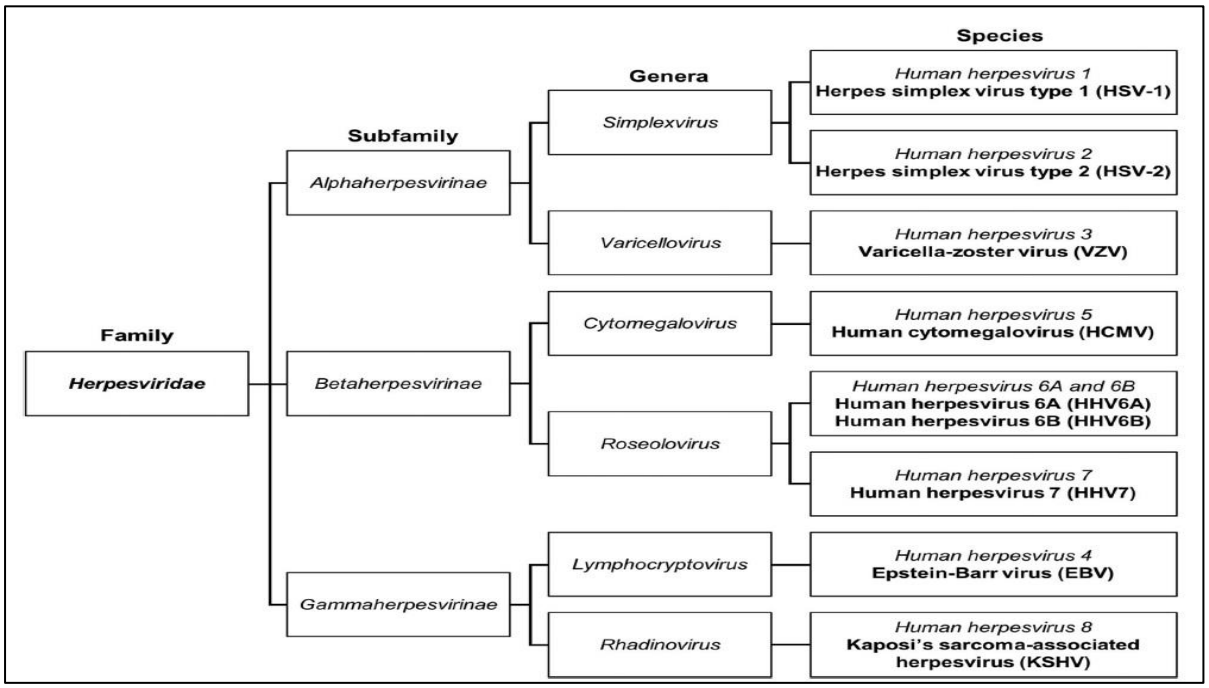


Figure 2: ***A flow-chart of Herpesviridae classification. (Reproduced from DOI: 10.1097/ICO.0000000000002425).***

HCMV, consists of nine viruses that can infect humans. These viruses are ancient and have evolved to be highly specific to their human hosts and their surroundings, indicating a long history of parallel evolution. These large DNA viral groups are separated into three subcategories: α-herpesvirideae (alpha), β- herpesvirideae(beta), herpesvirideae-γ. The differences in their phylogenetic structure likely began to emerge a few million years ago, and these three groups split from their nearest common ancestor about 200 million years ago. It's not just humans who can be infected by these viruses; species-specific herpesviruses have been found in birds, reptiles, fish, and many other vertebrates. Although serious morbidity is very uncommon, the ability to cause severe pathogenicity in specific circumstances and their species-specific nature make these viruses a dominant feature of the viral world. Herpesviruses are highly prevalent infections, with around 90% of individuals in higher-income countries testing positive for both varicella-zoster virus (VZV) and Epstein-Barr (EPV) infection by the age of 18. The elevated seroprevalence can be anticipated to some extent as a result of their adaptability to cause serious infection persistently, creating a substantial opportunity for different host transmission. Herpesviruses have been connected to long-term latent infection established by these viruses, which may be responsible for pathological processes related with ageing, including the beginning of Alzheimer's disease and immunosenescence, the progressive deterioration of the immune system.In a very small percentage of the population (0.2-1%), a 'persistent' latent infection can be taken to an extreme, as HHV6A and HHV6B (which integrate into telomeres) can infect germ cells. When these cells divide, half of the resulting gametes

will carry a copy of the viral genome. As a result, any offspring produced from these gametes will have a duplicate of the viral genome in every nucleated cell of their body.

Epidemiology of Human cytomegalovirus:

Human cytomegalovirus infections have been recognized in every human population that has been studied. HCMV infection is endemic without seasonal variation. HCMV is acquired early in life in most populations with the exception of people in economically well developed countries of northern Europe and North America. The acquisition patterns of human cytomegalovirus (HCMV) exhibit substantial variability contingent upon the geographic and socioeconomic milieu of the population, with seroprevalence typically escalating commensurate with age. In regions categorized as developing, HCMV acquisition occurs nearly ubiquitously during early childhood. Empirical investigations have revealed that a majority exceeding 90% of preschool children across South America, Sub-Saharan Africa, East Asia, and India exhibit seropositivity for HCMV antibodies. Conversely, seroepidemiological inquiries conducted within Great Britain and specific cohorts in the United States have discerned that less than 20% of children of comparable age evince seropositivity [9]. In a population survey conducted in Chengdu, China, it was observed that 60% of children aged 4 to 7 years were HCMV seropositive. Similarly, in Taipei, Taiwan, 58% of children aged 4 to 12 years, 61% of hospitalized paediatric patients from a low-income population in Rio de Janeiro, Brazil, and 56% of children aged 1 to 4 years in Jamaica were found to be HCMV antibody positive. In Finland, the HCMV seroprevalence rate increased from 27% in children 7 months of age to 41% in children 8 years of age in a cohort of children followed for 8 years. In a population survey in Parma, Italy, the age-specific HCMV seroprevalence increased from 28% in two-year-olds to 96% in 45–54-year-old residents. Similarly, in Spain, the CMV seroprevalence rate in children aged 2 to 5 years was 42%, increasing to 79% in adults aged 31 to 40 years.

In congenital HCMV infection (cCMV), it was initially believed that the severity and frequency of cCMV disease were greater when the mother experienced a primary infection which might be possible during the early stages of pregnancy.[10]. This assumption was partly influenced by the high prevalence of CMV antibodies in women of childbearing age in Sub-Saharan Africa, leading to the expectation that the impact of cCMV would be relatively low in this population. Recent large-scale studies conducted in Brazil have revealed that maternal immunity to cytomegalovirus (CMV) prior to conception does not offer protection against congenital CMV (cCMV) transmission to the foetus. It has been shown that non-primary infection—the reactivation of a latent CMV infection or reinfection with fresh strains of CMV during pregnancy—can result in deadly infection. Furthermore, it has been shown that when maternal CMV seroprevalence rises, so does the prevalence of congenital CMV infection.

[11]. Studies reveal that non-primary infection accounts for three-quarters of congenital cytomegalovirus (cCMV) infections, whereas primary infection is responsible for the remaining 25% of cases. According to data from a recent meta-analysis, high-income countries (HICs) with low CMV seroprevalence are thought to have lower rates of cCMV than low- and middle-income countries (LMICs), which have rates of the disease almost three times higher. By the time they are 5 or 6 years old, 85–95% of children in low- and middle-income countries (LMICs) are projected to be seropositive for CMV, and nearly all women of childbearing age test positive for the virus. [12,13]. Congenital cytomegalovirus (cCMV) infection is predicted to affect 0.67 percent of people worldwide. The prevalence rates of cCMV in Sub-Saharan Africa range greatly, from 1.4% in the Ivory Coast to 6% in Burkina Faso. Prevalence rates can differ significantly between specific nations; for example, 5.4% in The Gambia, 3.8% in Nigeria, 3% in Uganda, 2.9–3.6% in Kenya, and 2.5% in South Africa. It is noteworthy that all of these rates—which vary from 0.97% to 2.08%—beyond the average for low- and middle-income countries (LMICs), which is 1.42%, and even the worldwide prevalence rate of cCMV, which is 0.67%. [14,15,16].Congenital cytomegalovirus (cCMV) is more common in preterm and extremely low birthweight newborns than in term infants; rates in high-income countries (HICs) range from 1.5% to 4.8%. Notably, Sub-Saharan Africa has a dearth of published data on cCMV in preterm deliveries. The incidence of cCMV among infants exposed to HIV in utero is around 5%, but it varies between 6.5% and 11% in newborns with HIV acquired during postnatal care. [17,18]. It is pertinent to recognize that CMV-HIV co-infection has been linked to expedited infant HIV disease progression. Some of the identified risk factors for congenital cytomegalovirus (cCMV) transmission include lower socio-economic status, an earlier onset of sexual activity, and maternal age below 25 years. Additionally, factors such as caring for preschool children in the year before delivery, experiencing preterm labour, HIV co-infection, and other sexually transmitted infections during pregnancy are also associated with an increased risk of cCMV transmission.

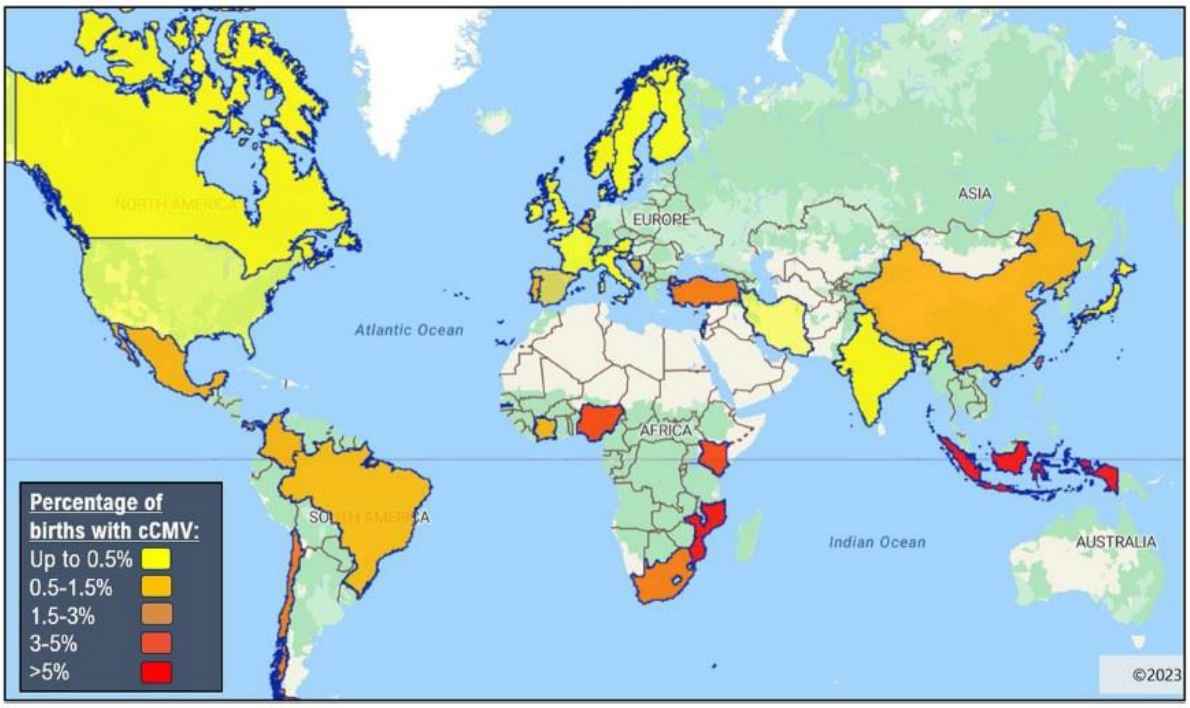


Figure 3: Global prevalence of congenital HCMV infection (*Reproduced from: Payne H and Barnabas S (2024) Congenital cytomegalovirus in Sub-Saharan Africa—a narrative review with practice recommendations. Front. Public Health 12:1359663. doi: 10.3389/fpubh.2024.1359663)*

Recent research on blood donors has revealed that the prevalence of CMV (Cytomegalovirus) antibodies remains very high in populations across Asia and Africa, with seropositivity rates ranging from 95% to 100%. In contrast, in Germany, the seropositivity rates for HCMV (Human Cytomegalovirus) among blood donors are notably lower, starting at 30% in young adults aged 18 to 20 years and increasing to over 70% in individuals over the age of 65. These findings shed light on the differing epidemiological patterns of CMV infection within different geographic regions. The precise mechanism of Human Cytomegalovirus (HCMV) transmission remains unclear, although it is believed to primarily involve direct contact with the bodily fluids of an infected individual. Age disparities may impact child behaviour, sexual activity, and lifestyle choices. Breastfeeding, group dynamics, unhealthy habits, and gender are all associated with increased susceptibility to HCMV. Modes of transmission encompass oropharyngeal secretions, urine, cervix and vagina secretions, semen, breast milk, blood products, and organ transplants. Notably, saliva has been identified as a carrier of the virus, posing a transmission risk. HCMV infection is especially notable as infected infants can continue to shed high levels of the virus for months to years' post-infection [19].

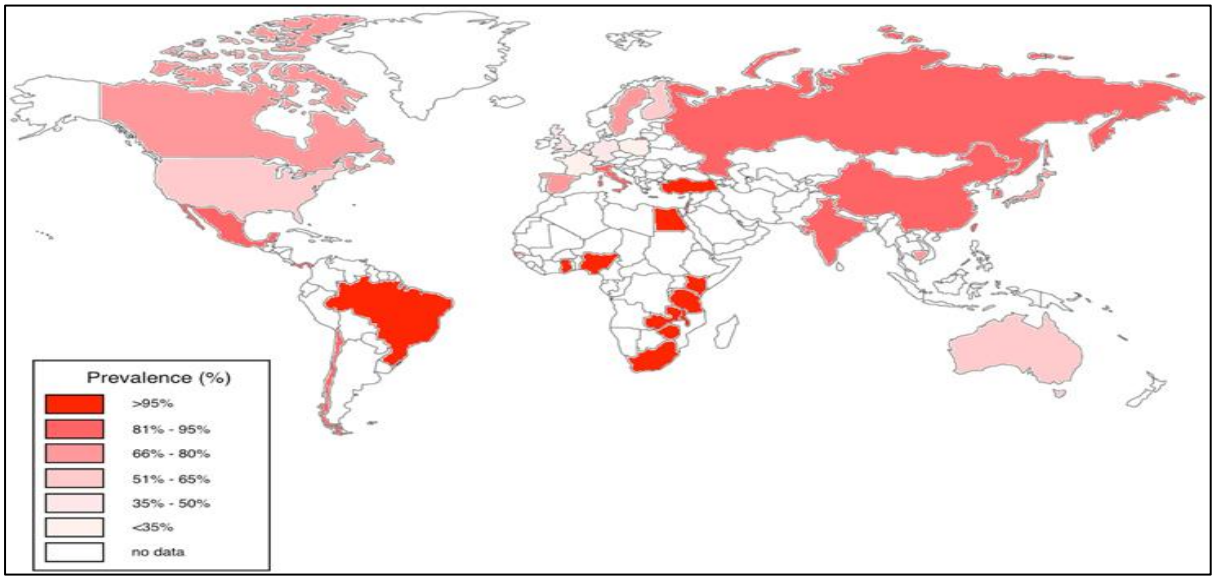


Figure 4: Global seroprevalence rate of HCMV infection among adults. HCMV is found in all geographic locations and all socioeconomic groups, and infects between 30% and 70% of adults in developed countries and 90-95% in developing countries. ***(Reproduced from: Adland E, Klenerman P, Goulder P, Matthews PC. Ongoing burden of disease and mortality from HIV/CMV coinfection in Africa in the antiretroviral therapy era. Front Microbiol. 2015 Sep 24;6:1016. doi: 10.3389/fmicb.2015.01016. PMID: 26441939; PMCID: PMC4585099).***

Long-term HCMV infection, lasting over six months after the initial exposure, can occur in both children and adults. Additionally, a significant proportion of individuals who have tested positive for HIV continue to experience regular diagnoses of the disease. An essential determinant of the frequency of congenital and perinatal HCMV infections is the seroprevalence among adult women of childbearing age. The occurrence of congenital HCMV is directly impacted by the prevalence of antibodies in the population. Research conducted in Europe and the United States has revealed that seropositivity rates among women aged 18-25 years range from 50% to 85%. In contrast, most women of reproductive age in lower-income countries have tested positive for HCMV antibodies. This disparity in seropositivity rates may contribute to variations in the incidence of congenital HCMV infections across different regions [20,21]. A research study conducted in the United States focused on pregnant women and discovered that the incidence of HCMV (Human Cytomegalovirus) was approximately 6% per year among young women from low-income families, while it was 2% among women from middle to high-income households [22].

Perinatal exposure to human cytomegalovirus (HCMV), including congenitally acquired infection, is a major contributor to the increased transmission of HCMV in the population. This is due to the fact that infected infants excrete a substantial amount of the virus for an extended period of time. One lesser-known mode of transmission of the virus is through breast milk. Studies have estimated that more than 80% of infants who are breastfed by infected mothers

become infected with HCMV as a result of breastfeeding. Additionally, similar to systemic infections, infants can carry the virus for an extended period through breastfeeding, thus playing a significant role in the transmission of the infection [23,24]. The human cytomegalovirus (HCMV) is prevalent in adolescents and adults, particularly at the onset of sexual activity. HCMV is found in high levels in semen and the cervix, indicating potential transmission through sexual contact. Infection can lead to mortality, with rates comparable to those of sexually transmitted diseases [25]. Studies have shown that individuals attending sex clinics, particularly homosexual men and women, are at higher risk of HCMV infection. Therefore, HCMV should be recognized as a sexually transmitted disease in adults, capable of being transmitted through sexual contact [24].

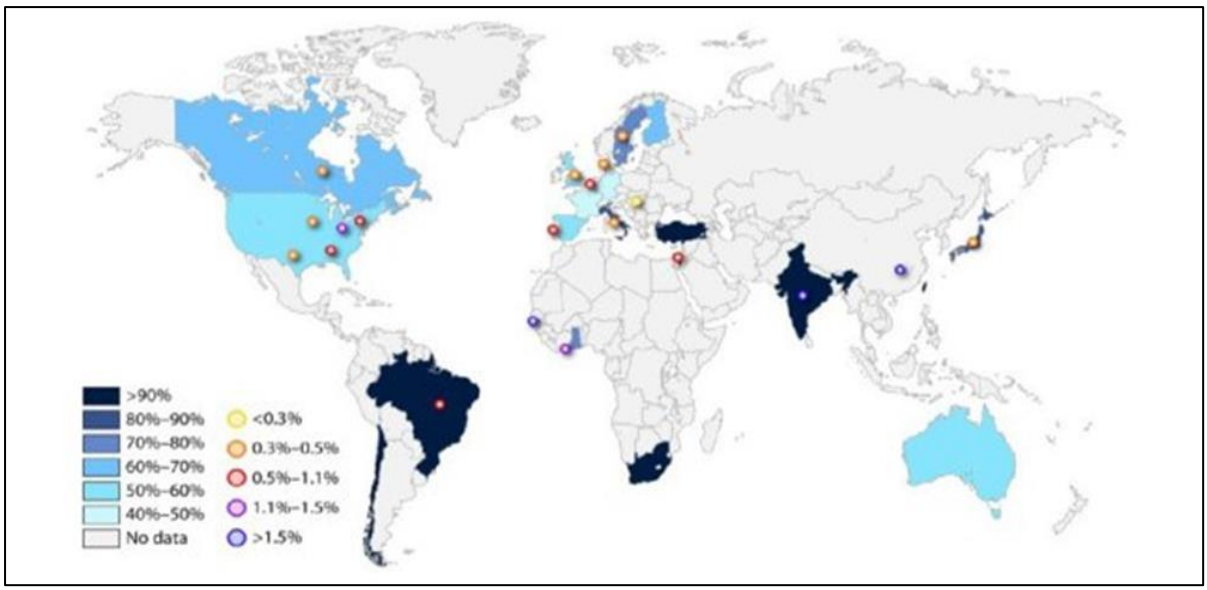


Figure 5: ***Global seroprevalence rates of HCMV among pregnant women and birth prevalence of congenital HCMV infection. HCMV is the most common congenital infection worldwide with estimated incidence of 0.2%–2.2% newborns. HCMV seroprevalence among pregnant women ranges 40% to >95% worldwide. (Reproduced from: Manicklal S, Emery VC, Lazzarotto T, Boppana SB, Gupta RK. The "silent" global burden of congenital cytomegalovirus. Clin Microbiol Rev. 2013 Jan;26(1):86-102. doi: 10.1128/CMR.00062-12. PMID: 23297260; PMCID: PMC3553672***

Opportunistic infections (OIs) are a significant concern for individuals living with human immunodeficiency virus (HIV). OIs in this population can be attributed to a wide range of pathogens, including bacteria, viruses, fungi, and protozoa. Among the human herpesviruses, human cytomegalovirus (HCMV) stands out as one of the most prevalent causative agents of OIs in people living with HIV. While HCMV infections are global in scope, they are more commonly observed in developed countries compared to developing nations [26.27,28].

In immunocompromised individuals, primary Human Cytomegalovirus (HCMV) infections generally do not show any symptoms and the virus often remains inactive during severe illness.

However, in people living with HIV (PLHIV), this inactive virus can become active again, leading to autoimmune diseases and causing significant morbidity and mortality [29,30]. In PLHIV, HCMV can cause serious and life-threatening infections such as retinitis, pneumonia, encephalitis, and enteritis. These infections can persist indefinitely and pose challenges to the immune system. Ongoing transmission of HCMV underscores the need for a targeted vaccine against the virus. The development of HCMV-specific IgM vaccines precedes the IgG vaccine. Even after the resolution of the initial infection, the production of CMV IgM can still be detected for an extended period. In addition to primary infections, IgM/IgG antibodies can also be detected during secondary infections, indicating a relapse or recurrence [31].

If Human Cytomegalovirus (HCMV) viremia is not identified early and treated properly, it can lead to a weakening of the immune system in people living with HIV (PLHIV). This weakened immune system can further increase susceptibility to HIV and ultimately result in the death of the individual [32,33,34]. There are two primary methods used to screen for HCMV in PLHIV: polymerase chain reaction (PCR) to detect the virus and enzyme-linked immunosorbent assay (ELISA) to test for immunity [35]. However, due to the delayed immune response to CMV in PLHIV, which can yield false negative results, PCR is the preferred diagnostic method for HCMV testing. Late diagnosis of CMV has been reported as an adverse event in PLHIV, emphasizing the importance of early disease detection for proper management and follow-up.

HCMV seroprevalence in India

A study from Pune has shown that seroprevalence of HCMV IgM among immunocompromised adult patients ranges between 15.3%. The IgG seroprevalence in Delhi as described by another study was 85.2%, whereas IgM seroprevalence was 13.4% in immunocompromised adult patients. A study from Maharashtra showed a seroprevalence of 83.24% for HCMV IgG and 9.46% for HCMV IgM among pregnant women. Another study from Kerala has showed an HCMV IgM seroprevalence of 22.03%, while another from Kashmir has shown the IgM prevalence to be 15.98%. A study from Punjab has reported 1.9% incidence of HCMV infection among new-borns whereas another study from Karnataka reported 2.6% incidence. In Eastern India and especially in West Bengal data regarding HCMV prevalence among immunocompromised patients is very much limited and no studies exist describing the prevalence and incidence estimation of HCMV among pregnant women or new-borns.

Structure of the cytomegalovirus virion

- **Genome structure**

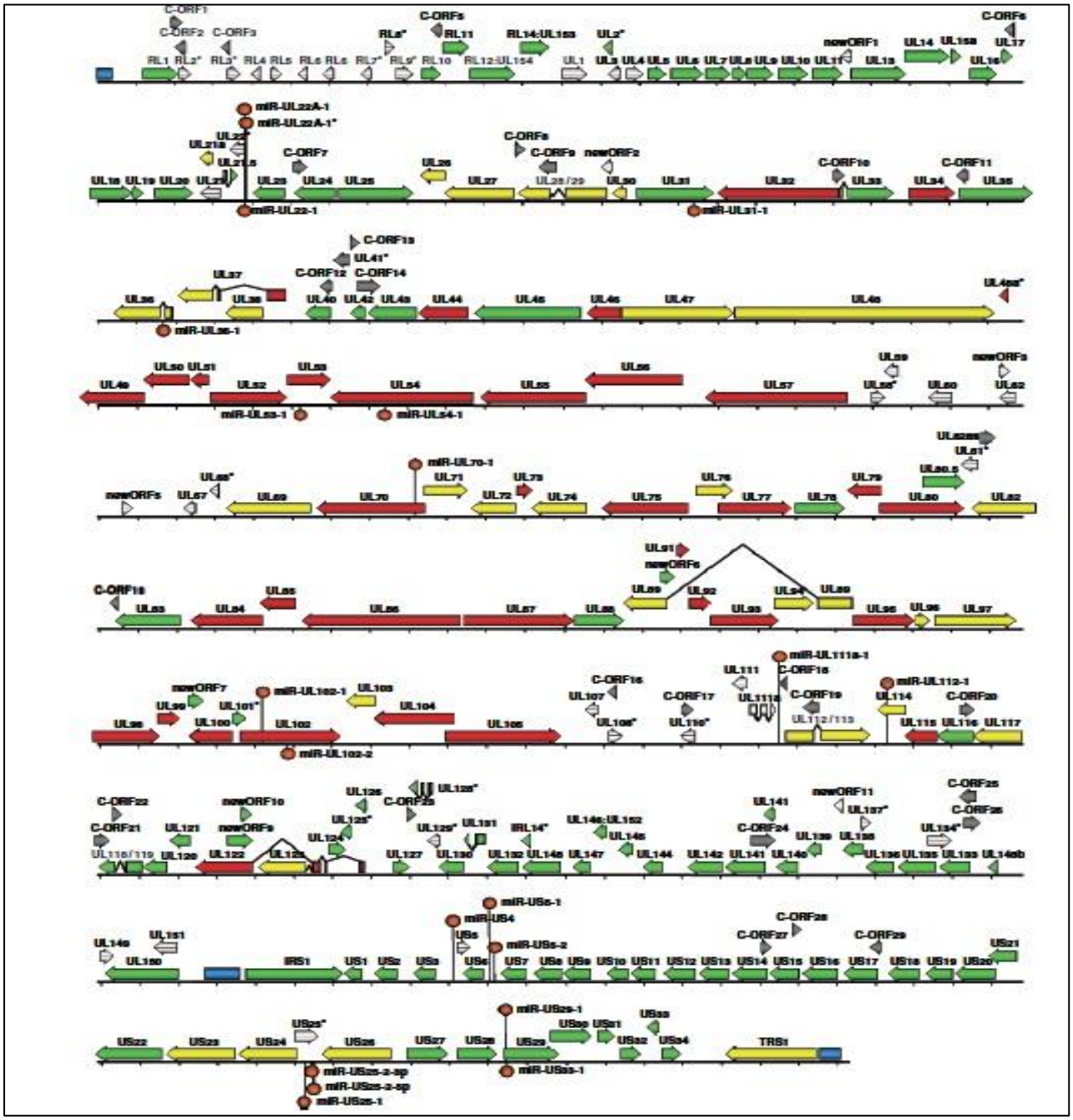


Figure 6: The color codes designate ORFs that are essential (*red arrows*), augmenting (*yellow arrows*) or nonessential (*green arrows*) for replication within cultured fibroblasts. *Gray arrows* represent ORFs that are have not yet been tested for function. The three *blue boxes* represent the repeat sequences found at the ends of the unique long and unique short regions. The *orange pins* designate the location of virus-coded miRNAs. Their placement above or below the sequence line designates the strand on which they are encoded. Each *tick mark* on the black sequence line represents 1 kb of DNA (Reproduced from *the work of Murphy et al. PNAS December 9, 2003 100 (25) 14976-14981).*

The centre of the HCMV virion consists of a double-stranded DNA that shows slight differences among various strains of the virus, typically around 230-235 kilobase pairs, making it the most sizable known human pathogen. The genetic material consists of two separate parts,

UL and US, which stand for "Unique Long" and "Unique Short" respectively. These sections are bordered by direct repeats (TRL and IRL bordering the UL region, TRS and IRS bordering the US region). The end and reproducible regions can duplicate all four forms of the HCMV genetic material. It is currently undetermined whether these isomers possess distinctive properties. HSV-1 mutants that still preserve the capacity to assume specific configurations exhibit no detrimental impact on their proliferation. The aforementioned specificity is only relevant to select cellular instances or in vivo scenarios, as evidenced by isolation tests excluding in vitro environments. Regrettably, the murine cytomegalovirus, extensively utilized as a model for investigating viral and pathogenic immune response in rats, is incapable of infecting internal cellular structures. The sequence repeats itself within the genome, making it unable to isomerize. The HCMV genome contains multiple proteins, with approximately 167 to over 200 open-source reads. The naming of the genes is contingent on the association of ORFs in the HCMV genome. Genes encoded in the US region are named accordingly, while those encoded in the UL region are denoted as UL1, UL2, and so on. For instance, genes in the US region are named US1, US2, and so forth. HCMV encompasses several families, including the US6 family (which features US6-11), indicating similar standards and related functions. The enhanced evolutionary strength of a virus can be attributed to the onset of regeneration and the selection of beneficial mutations within its genes. This process may lead to the loss of duplicate genes due to a scarcity of genetic material, ultimately resulting in a reduction in body size due to the large size of its genome. This phenomenon, known as "gene accordion," underscores the virus's ability to amplify its evolutionary robustness by regulating mutations while preserving adaptive substitutions. Initially observed in poxvirus, a large genome-sized dsDNA virus, this mechanism has also been identified in HCMV, highlighting its significance across different viral types.

- **Virion structure**

The icosahedral capsid of Human Cytomegalovirus (HCMV) is a highly intricate and complex structure, requiring a larger amount of genomic material compared to other herpes virus capsids. Despite this increased complexity, the mathematical symmetry of the HCMV capsid is remarkably similar to that of the Herpes Simplex Virus 1 (HSV-1) capsid. Recent strides in cryo-electron microscopy techniques have provided the means to develop more comprehensive and detailed models of HCMV capsids, offering valuable insights into their architecture and function. The capsid is a protein shell that contains four different proteins: the major capsid protein (MCP), the smaller capsid protein (SCP) [36,37], Tri1, and Tri2. It can be further divided into two types: Tri2A and Tri2B. The capsid is assembled from an icosahedral structure composed of 320 triplets, 12 pentons, and 150 hexagons, resulting in 16 triangulations. Additionally, the capsid-associated integument protein, pp150, binds to SCP, providing further support and stability to the capsid lining[38].

The viral envelope is surrounded by numerous viral envelope proteins, with up to 35 different proteins detected at various levels. These proteins interact with cellular proteins, bacteria, and cellular RNA molecules. When these viral envelope proteins come into contact with target cells, they carry out a diverse range of functions, such as inducing apoptosis and evading the host's immune system. These proteins are embedded in a bi-lipid membrane that contains various glycoproteins, including gB,gM,gN,gH,gL, and gO, oriented within UL128-131 ORF.[39]

Entry of HCMV into different cells

HCMV can infect a wide variety of cell types, including but not limited to epithelial, endothelial, and smooth muscle cells, as well as leukocytes. This broad cellular tropism is significant because it contributes to the diverse clinical manifestations and long-term consequences associated with HCMV infections. Additionally, the ability of HCMV to infect various cell types has important implications for its ability to evade the immune system and establish persistent infections. The tropism of HCMV for specific tissues is supported by empirical evidence from multiple investigations, which suggests that the expression and relative abundance of glycoproteins within the virion envelope are linked to this phenomenon. Our understanding of how Human Cytomegalovirus (HCMV) enters cells is primarily based on studies focused on its entry into fibroblast cells. In addition, our knowledge of HCMV entry is also informed by our understanding of how Herpes Simplex Virus (HSV) enters cells.

- **Virus binding to the surface of host cells**

During the process of fibroblast infection, the human cytomegalovirus (HCMV) virion initially attaches to the cell surface through the interaction of the glycoprotein M (gM) / glycoprotein N (gN) complex with glycoprotein B (gB). This gB protein binds to heparin sulphate proteoglycans (HSPG), which are host glycoproteins characterized by covalently attached heparin sulphate chains. There are 17 known HSPGs in humans, which can be categorized into three groups: membrane-bound HSPGs (including syndecan 1-4, glypican 1-6, CD44, betaglycan, and neuropilin-1), secreted extracellular matrix HSPGs (such as Perlecan, Agrin, and Collagen XVIII), and secretory vesicle HSPG serglycine. HSPGs regulate cellular processes, act as co-receptors for tyrosine kinases, and protect chemokines, cytokines, and growth factors from proteolysis. Mutations in these proteins lead to diseases, including Simpson-Golabi-Behmel disease, characterized by abnormal physical development and excessive growth and weight gain due to disruption of the hedgehog developmental pathway by the glypican-3 mutation Knobloch type I syndrome is linked to mutations in collagen XVIII and results in severe vision impairments. Studies in mice have revealed that null alleles of enzymes involved in heparan sulphate chain synthesis and addition, such as Glcat1 or Ext1/2, are lethal during embryonic or perinatal stages, highlighting their critical role. These enzymes

not only act as receptors for HCMV but also serve as receptors for other viruses, including and human T-cell leukemia virus type 1, hepatitis C virus, HSV-1 & 2, [40]

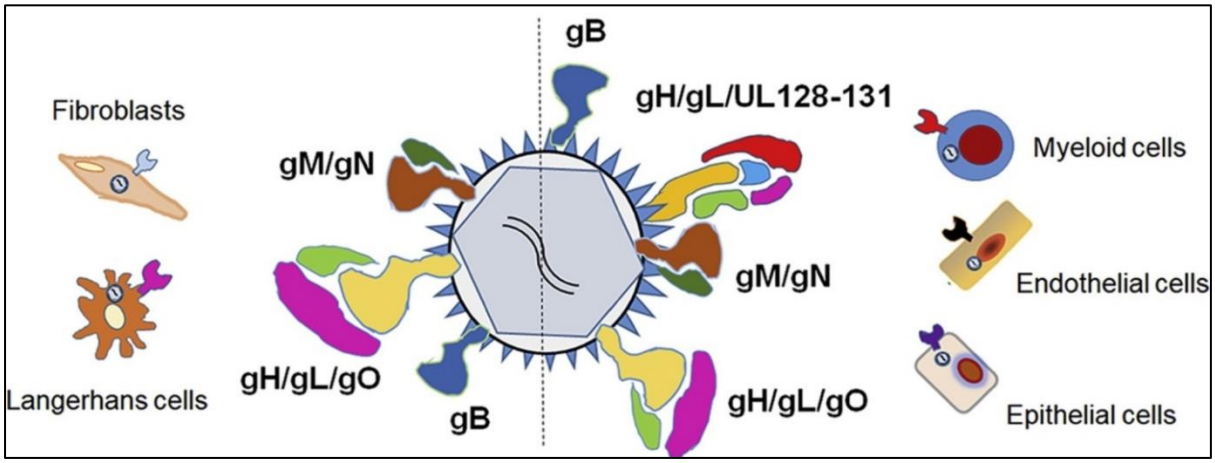


Figure 7- ***Glycoprotein complexes of HCMV: Reproduced from: Sandonís V, García-Ríos E, McConnell MJ, Pérez-Romero P. Role of Neutralizing Antibodies in CMV Infection: Implications for New Therapeutic Approaches. Trends Microbiol. 2020 Nov;28(11):900-912. doi: 10.1016/j.tim.2020.04.003. Epub 2020 May 21. PMID: 32448762***

- **Interactions with additional cell surface receptors**

After the initial adsorption of heparin sulphate proteoglycans (HSPG), human cytomegalovirus (HCMV) relies on specific interactions between virally encoded glycoproteins and cellular receptors for successful entry into host cells. These interactions involve the HCMV trimer complex consisting of glycoproteins gH, gL, and gO, and the cellular receptor platelet-derived growth factor receptor alpha (PDGFRα).[41] Additionally, interactions occur between integrins αvβ3 and glycoprotein B (gB), and potentially between the epidermal growth factor receptor (EGFR) and gB, although this latter interaction is subject to debate. This sequential binding of receptors culminates in a pH-independent fusion of viral and cellular membranes, a process regulated by the gB fusion protein, which facilitates the delivery of viral capsid and envelope proteins into the host cell [42, 43, 44]. The process of membrane mixing relies on proteins functioning as enzymatic catalysts to overcome the "hydration force," which is the primary kinetic barrier when the distance between membranes decreases below 20 Å. Lipid bilayer fusion occurs in a stepwise manner, starting with the outer layers of each bilayer joining together to form a hemi fusion intermediate, followed by the fusion of the distal layers to establish a fusion pore for the transfer of membrane contents.

The fusion activity of the gB protein is dependent on the presence of the gH/gL complex. While gB trimers are essential for the entry of the virus into cells and for cell proliferation, they are not necessary for attachment, assembly, or release of the virion. Interestingly, it appears that

the receptor binding activity of gB may not be required, as gB-null viruses can still enter cells expressing gB. Furthermore, tetraspanin CD151 has been found to play a role in mediating this entry step, with other members of the tetraspanin family being involved in the entry of other viral infections (for example, CD81 is crucial for the entry of hepatitis C virus into liver cells)[45]. As our understanding of human cytomegalovirus (HCMV) infiltration into fibroblasts continues to evolve, it has become clear that Thy-1, a member of the immunoglobulin superfamily, also impacts the early stages of HCMV entry through a mechanism that is not yet fully understood. However, knocking down Thy-1 using siRNA or introducing soluble Thy-1 inhibits viral entry. This molecule is known to interact with integrin αvβ3 and is also expressed in progenitor cells in various HCMV-targeted cells such as fibroblasts, endothelial cells, and CD34+ cells. Although pH-independent fusion is the most extensively studied model for fibroblast infection, macro-pinocytosis infiltration has also been observed. This suggests that HCMV may utilize different mechanisms to enter the same types of cells, or there may be strain-specific variations that impact the virus's entry into the cell [46] .

- **Entry into other cell types**

The infection of different cell types happens through various methods and relies on the existence of another glycoprotein complex known as the pentameric complex gH / gL / UL128-131. This complex alters fibroblasts over extended periods, causing the virus to be unable to effectively infect endothelial, epithelial, and dendritic cells, such as polymorphonuclear leukocytes. Furthermore, it hinders the generation of cell-free virions, instead managing cell proliferation effectively. The investigation of HCMV entry at latency sites is commonly replicated using CD14+ monocytes in vitro. It has been demonstrated that Paxilin-dependent macropinocytosis can impede the infection of these cells and is closely linked to EGFR signaling.[47] When EGFR function is inhibited through pharmacological approaches, like using receptor blocking antibodies, latent infection in these cells becomes less efficient. The capsid necessitates increased nuclear transport into CD14+ cells, which traverse the trans-Golgi network and recycle endosomes approximately 3 days post-infection[48]. This differs from the process in epithelial cells and fibroblasts, where it takes 30 minutes. In monocytes, the significance of these latency findings is considered to be largely unrelated, as the same process persists for 4-8 hours in CD34+ cells, which are the site of long-term HCMV latency in vivo.

- **Events following cell penetration**

Upon entry into the cytoplasm, the virus disassembles, allowing the capsid and tegument proteins to travel separately into the nucleus. Tegument proteins are crucial for promoting lytic infections by hindering cellular antiviral responses and stimulating viral gene expression. Among the tegument proteins, pp65 (UL83) and pp71 (UL82) are extensively studied.

Specifically, the pp65 protein disrupts IFI16 viral DNA surveillance by binding to the pyrin domain of IFI16, thereby impeding oligomerization and subsequent signaling activities [49]. The pp71 protein plays a role in evading the cell's antiviral defence mechanism by targeting and breaking down hDaxx, a key component of PML bodies that can inhibit viral gene transcription[50]. Functionally, pp71 is similar to VP16 in this aspect. Additionally, the discovery of the HCMV homolog of the ubiquitin-specific cysteine protease (USP) in HSV-1, known as pUL48, revealed its association with newly formed virions, indicating its role in infecting cells. Research on the effects of virion-induced pUL48 has been scarce, but it has been observed that viruses lacking the UL48 gene are less efficient in transporting their genetic material to the cell nucleus. Additionally, pUL48 may impede NF-κB signalling during the later stages of infection by breaking down polyubiquitin chains associated with K48 and K63 in RIP1 [51,52,53]. This function allows pUL48 to regulate inflammatory signaling in response to infection, similar to the role played by similar proteins in other herpes viruses. Successful delivery of the capsid to the nucleus is dependent on an intact microtubule network, as demonstrated by the inhibition of HCMV in the presence of the microtubule-depolymerizing agent nocodazole.

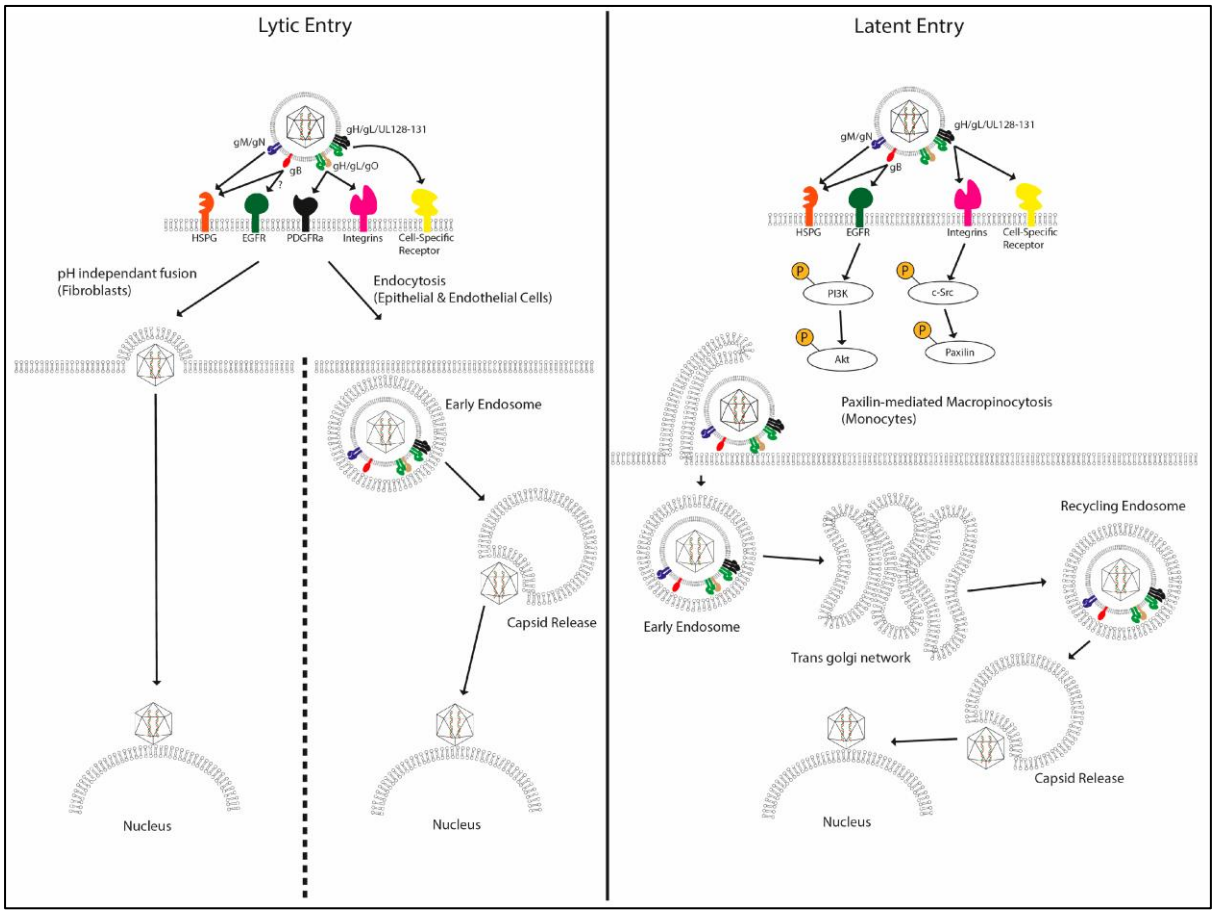


Figure 8: Post effect of HCMV entry into different cellular systrem: In fibroblast cells process of lytic infection, viral glycoproteins gM/gN and gB bind to heparan sulfate proteoglycans (HSPGs) to initiate a series of cell-specific interactions with cell surface receptors. This interaction triggers

specific signaling pathways that initiate virus entry. Following this, the viral membrane fuses with the cellular membrane, allowing the direct release of viral contents into the cytoplasm...The endocytic pathway in epithelial cells is utilized for viral entry into the cell, whereas paxillin-mediated macropinocytosis is used when infecting CD14+ monocytes. After that, there is a prolonged trafficking process involving the trans Golgi network and recycling endosomes before the genetic material is transported to the nucleus. ***(Reproduced from Murray, Matthew J., Nicholas E. Peters, and Matthew B. Reeves. 2018. "Navigating the Host Cell Response during Entry into Sites of Latent Cytomegalovirus Infection" Pathogens 7, no. 1: 30. https://doi.org/10.3390/pathogens7010030)***

- **Tissue compartmentalization of HCMV in human hosts**

An unmistakable feature of HCMV infections is the widespread dissemination across diverse host tissue compartments and cell types. In response to the considerable variability associated with HCMV infections, researchers have actively pursued investigations into potential differences in variability among viral samples from various compartments, both in terms of overall variability and specific genetic or genomic sequences. Rigorous quantitative assessments of variability have unequivocally revealed substantial variances in variability levels between compartments, with clear distinctions observed between blood populations and those found in urine or intraocular regions. The differences in drug resistance genotypes observed in some patients, such as with ganciclovir (GCV) resistance, are not evenly distributed in patient compartments during treatment with drug-sensitive strains and blood drug resistance present in the cerebrospinal fluid. The mechanisms for this compartmentalization are not yet clear, but there are at least two proposed hypotheses. First, it may result from the distribution of stochastic processes. For instance, when viral populations experience bottlenecks during transmission, this can lead to an increased proportion of population mutations driven by random frequency fluctuations, known as genetic drift. These stochastic changes have the potential to significantly alter the composition of populations compared to the original source. Second, compartmentalization can be attributed to natural selection within the distribution. Mutants that are better suited to the specific environment, such as those with enhanced cellular tropism, may come to dominate the population within that particular tissue. While some studies have investigated these models, there is emerging evidence suggesting that both bottlenecks and selection could account for observed compartmentalization. However, further research in larger patient populations is necessary to fully understand these mechanisms.

Events of HCMV DNA replications:

The process of lytic replication, which involves the initiation of DNA replication at a specific section of the genome known as oriLyt, originates within the UL region of the genome. Through the analysis of transient co-transfection replication, it has been determined that a total of 11 viral genes are essential for ensuring efficient DNA replication [54,55]. Notably, six of these genes (UL44, UL54, UL57, UL70, and two additional genes) are common to all herpesviruses and are commonly referred to as the 'core replication proteins'. It is worth noting that these core proteins from other herpesviruses have the remarkable capacity to facilitate efficient DNA replication from a different herpesvirus oriLyt, provided that the initiator factor for that specific oriLyt (UL84 in the case of HCMV) is present [56]. The IE2 protein has a significant impact on driving DNA replication, as it contains numerous binding sites within the HCMV oriLyt. The replication process of the HCMV genome becomes robust approximately 24 hours after the infection, and it is believed to progress through circularization and concatemer formation. The DNA synthesis is primarily facilitated by the viral polymerase UL54, which interacts with the polymerase accessory factor UL44.The helicase-primase complex, which includes the proteins UL105 and UL70, works together to perform essential functions in the replication of viral DNA. This complex is responsible for unwinding the double-stranded viral DNA and creating a primer for the initiation of DNA replication by the UL54 protein [57]. After the concatemers, or linked copies of the viral genome, are generated, they can be cleaved to produce individual genomes. These individual genomes are then ready to be packaged into capsids, which are protein containers that protect the viral genetic material.

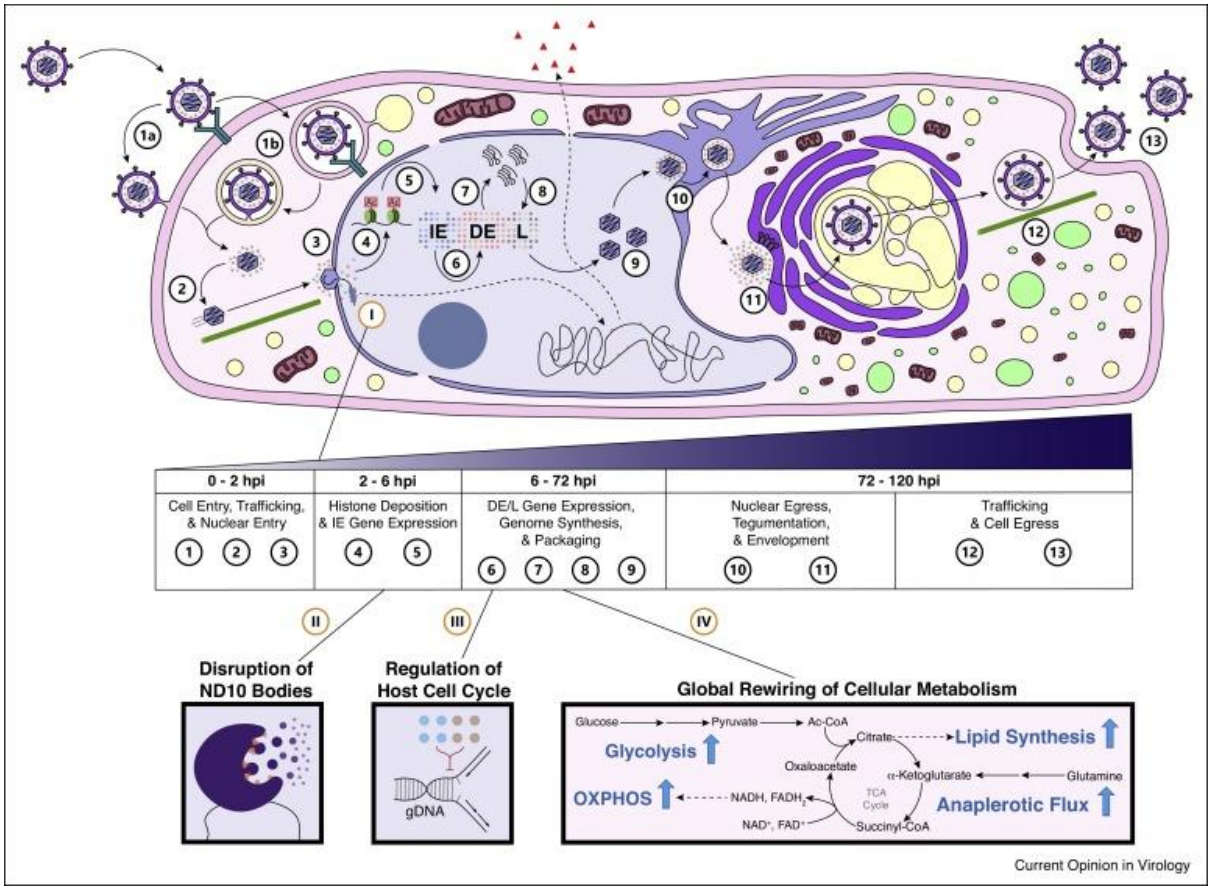


Figure 1. HCMV replication cycle associated with cellular changes: (I) DNA-binding proteins (Dark blue) shift from the nucleolus to the nuclear periphery, where they bind the incoming viral genome. This induces cytokine expression (Red triangle) from the host genome and represses viral gene expression, while viral proteins inhibit these DNA sensors. (II) The genetic material of HCMV interacts with nuclear domain 10 (ND10) bodies, which naturally inhibit the expression of viral genetic material. Proteins produced by HCMV break down ND10 structures in the initial phases of infection to alleviate this inhibition. (III) The cell cycle is delayed at the G1/S boundary by viral proteins in order to inhibit competition for nucleotides.(IV) HCMV causes an overall increase in cellular metabolic pathways to sustain various stages of its replication process, including oxidative phosphorylation (OXPHOS). (*Reproduced from Matthew D Tyl, Cora N Betsinger, Ileana M Cristea,2022,Virus–host protein interactions as footprints of human cytomegalovirus replication,Current Opinion in Virology,Volume 52,135-147,doi-10.1016/j.coviro.2021.11.016).*

Upon entering the host cell, the virion interacts with specific transmembrane proteins to facilitate its internalization [58]. The virion then releases its capsid and accompanying tegument proteins into the cytoplasm using one of two methods: direct fusion at the cell's plasma membrane or receptor-mediated endocytosis. Subsequently, the capsid travels along microtubules toward the cell's nucleus. Once at the nucleus, the capsid attaches to the nuclear pore and introduces its stored genome into the host cell. The viral genome becomes associated with histones in a replication-independent manner. Post-translational modifications of histones, such as lysine acetylation (Ac), play a crucial role in remodelling viral nucleosomes and facilitating the expression of immediate-early genes, which represent the first set of genes expressed in the characteristic temporal cascade of gene expression associated with herpesvirus infections. During the initial stages of human cytomegalovirus (HCMV) replication, immediate-early (IE) proteins are responsible for activating the expression of delayed-early (DE) genes, which play a crucial role in genome replication. These DE proteins are essential for the replication of the HCMV genome. As the replication process progresses, late genes become highly expressed, leading to the production of viral capsid proteins and glycoproteins. Subsequently, the newly synthesized genomes are packaged into viral capsids. These capsids then acquire their first layer of tegument proteins and exit the nucleus by budding through both nuclear membranes. Following this, the capsid obtains its second layer of tegument proteins and becomes enveloped at a newly formed proviral organelle, known as the virion assembly complex. This organelle is composed of components from the secretory system. Once the virions are formed, they are transported to the plasma membrane. Upon reaching the plasma membrane, the virions egress from the cytoplasm and spread to other cells, continuing the cycle of infection [59].

Diversity of Human Cytomegalovirus in Human Hosts

Research over 30 years ago showed significant genetic diversity in HCMV among individuals using RFLP, indicating variability between hosts.[60]. The accuracy of these findings was subsequently verified through focused resequencing of genetic sites, with a particular focus on glycoproteins such as gB, gN and gO, and also by analysing complete genome data from low passage isolates and clinical samples [61]. Recent studies have revealed significant genetic diversity of human cytomegalovirus (HCMV) within individual hosts. Intra-host diversity studies have primarily focused on HCMV glycoproteins, utilizing techniques such as PCR-RFLP, DNA sequencing, single-stranded conformational polymorphism, and heteroduplex mobility analysis to identify variability [62]. However, these studies face limitations due to technological constraints, as well as challenges related to low sampling depth and limited genome coverage. Nevertheless, consistent findings indicate that mixed infections contribute to a substantial portion of HCMV cases in the general human population, including congenital infections in infants, individuals with HIV/AIDS, transplant recipients, and immunocompetent children and adults [63,64]. A key study in 2011 utilized ultra-deep pyrosequencing to investigate the gO, gN, and gH loci of HCMV-infected transplant recipients, revealing mixed infections in all patients, with up to 6 genotypes observed in a single patient [65]. Deep sequence of HCMV genome obtained from a glioblastoma multiform (GBM) tumour showed a high level of genetic diversity, including hypervariable loci with all four nucleotides observed at various positions in the viral genome. Genomic analysis combined with high-throughput sequencing has revealed that HCMV diversity is not restricted to specific loci but is distributed across the entire genome, with nearly every open reading frame (ORF) showing noticeable intrahost genetic diversity [66].

Role of chemokines during HCMV infection

Chemokines and their receptors play a key role in the development of various vascular diseases. They regulate cellular migration and activation by binding to G-protein-coupled receptors [67]. Chemokines are categorized into four families based on conserved Cys motif, including the C, CXC, and CX3C chemokines. They are produced at specific inflammation sites and attract monocytes., Chemokines play a crucial role in attracting T cells and B cells to specific sites, promoting the production of various cytokines and growth factors, and enhancing cell adhesion to the vascular endothelium. They are secreted during all stages post transplantation, including injury, tissue rejection, and healing processes. The absence of chemokines has been linked to long-term tissue graft acceptance, while their presence contributes to allogeneic graft rejection.

CMV infection alters various host cellular machinery associated with the advancement of TVS. It increases the adhesive properties of endothelial cells and upregulates the expression of RANTES in smooth muscle cells and fibroblasts, promoting cellular infiltration sites [68]. HCMV infection of human smooth muscle cells induces migration dependent upon the binding

of the virus-encoded chemokine receptor US28 with the CC chemokines RANTES or MCP-1. HCMV upregulates host cytokine and chemokine responses and encodes viral IL-10 and other CC chemokines to regulate cellular immunomodulatory activity [69]. The effects of viral chemokines on the expression of different disease manifestations are still unclear.

Role of cytokines in case of HCMV induced diseases

In most organ transplantations, the time of viral exposure can be calculated, so cytokine responses due to HCMV viremia are studied. After replication begins, type-I cytokine expression is observed, leading to the upregulation of IFNγ, IL18, IL-6, and chemokine IP-10 secretion. Among these, IL-6 shows the most significant response to acute HCMV infection. Cytokine production by HCMV infection is initiated by simultaneous triggering of Pattern Recognition Receptors (PRRs), Toll-like receptor 2, cytoplasmic DNA sensor STING, and DNA binding protein [70]. In vivo models have shown that mice with defective PRRs have reduced cytokine expressions. Both MCMV and HCMV can be recognized by PRRs, contributing to significant roles in HCMV-induced cytokine expression. Evidence suggests that HCMV replication induces the expression of pro-inflammatory cytokines by directly interacting with host cellular responsive elements and activating host immune responses[71,72]. Additionally, secretion of type I cytokines plays protective roles during acute HCMV infection. However, unregulated T-cell responses induced by type I cytokines during MCMV infection can cause tissue damage and lead to MCMV hepatitis and other CMV-related diseases. The presence of MCMV has been linked to organ rejection in cardiac transplant patients. Reactivations of MCMV suggest that viral infection could trigger cytokine responses, activating the host's immunity and leading to graft rejection [73]. Further studies have shown that HCMV induces the production of IP-10 during infection, serving as a marker for allograft rejection. HCMV can infect multiple tissue types simultaneously, with specific HCMV regulatory genes being activated based on the tissue type. This immunological information suggests that continuous reactivation events may occur, re-stimulating the host's immune system. HCMV contributes to inflammation in vulnerable patients through continuous gene transcription and reactivation, leading to immune recognition and cytokine development. It is involved in cardiac issues like atherosclerosis, inducing the production of virus-specific cytotoxic CD4+ T-cells. HCMV US-28, a homolog of a chemokine receptor, also contributes to inflammation. HCMV can cause damage to endothelial cells, leading to vascular diseases and tissue damage [74]. Cytokines such as IL-6, TNFα, and IL-1β play a role in HCMV reactivation and gene activation, contributing to the progression of the disease. Recent data suggests that innate antiviral immunity may be imping by over production of pro-inflammatory cytokines. Not only can that inadequate accumulation of IL-6 also lead to induced NK cell death., thus, direct and indirect association of inflammatory cytokines promote gradual progression of viral replication, causing damage to peripheral tissue organization[75,76].

Aim and Objectives of the study

The major reason for choosing this study was the inadequacy of sensitive data sets on *Human Cytomegalovirus* (HCMV) prevalence and infectivity in India. Congenital HCMV infection is one of the leading causes of mortality and birth defects in new-borns as well as the leading cause of opportunistic **omit** infections in immunosuppressed renal transplant patients. Worldwide estimates have pointed out towards a very high prevalence rate of HCMV among the neonatal and immunocompromized population in developing countries but still there is no established guidelines in our country's health framework to regularize the screening of this virus. This has led to a continued lack of awareness of HCMV among health care workers and the public. It is expected that our work will be able to highlight the prevalence pattern and clinical characteristics of HCMV infection in the eastern region of the country making the medical practitioners aware of what to expect. Furthermore, as HCMV is known to exhibit tissue tropism and intra/inter-host genetic variations among its clinical strains, leading to continuous challenges on existing antiviral therapy due to huge variations in structural mutation in HCMV major replication regulatory protein. So I intended to find out possible antiviral meditational approaches by using a major ethnobotanical source mushrooms and deciphering what exact biomolecules responsible for effectively regulating the viral replication as well as understand how these regulatory pattern through *in vitro* and *in silico* approaches.

1. Different clinical manifestations associated with HCMV infection among immunocompromised groups. (New-born children and Renal Transplant patients)
2. Investigate a new therapeutic approaches by using different medicinal edible mushrooms and decipher the mode of action of the suitable bioactive compounds against HCMV infection *in vitro* conditions
3. To decipher the probable mode action of the selective bioactive compounds against clinical HCMV isolates by *in silico* method.

Chapter-1

Current prevalence of Human cytomegalovirus infections within different patient cohorts in West Bengal - Pilot Investigation

1.1 Background: Human cytomegalovirus (HCMV) is a widespread and prevalent infection that affects a vast majority of individuals at some point in their lives. As a member of the herpes virus family, HCMV has the ability to establish a latent infection within the human body and can reactivate periodically throughout the individual's lifetime. The global prevalence of HCMV varies considerably. In developed countries, studies have shown that HCMV affects between 30% and 80% of the adult population. On the other hand, in less developed countries, the prevalence of HCMV is even higher, with rates exceeding 90% among the adult population.[1]

Primary Human Cytomegalovirus (HCMV) infection in individuals with a healthy immune system typically does not result in severe illness. However, it may lead to a mononucleosis-like syndrome characterized by symptoms such as tiredness, lymphadenopathy (enlarged lymph nodes), and fever, similar to those experienced during primary Epstein–Barr virus (EBV) infection [2]. This syndrome may include other symptoms such as sore throat, muscle aches, and loss of appetite. The impact of human cytomegalovirus (HCMV) infection during pregnancy is well-documented, and it can have serious consequences for the developing foetus. Both primary and recurrent HCMV infections during pregnancy have been linked to significant congenital complications, including sensory or neurological impairment. Maternal HCMV seropositivity, indicating previous exposure to the virus, is a major risk factor for congenital HCMV infection in the offspring [3].

Furthermore, population-based prediction modelling studies that consider the likelihood of a primary infection in seronegative mothers have suggested that reinfection or reactivation in seropositive mothers accounts for the majority of congenital HCMV cases. This means that even mothers who have previously been exposed to HCMV are at risk of transmitting the virus to their unborn child through reactivation or reinfection. Additionally, these studies indicate that the majority of observed HCMV-related hearing loss in infants is attributed to reinfection or reactivation in seropositive mothers [4].

The direct effects of a Human Cytomegalovirus (HCMV) infection within transplant patients can lead to either acute or chronic infectious disease syndromes. These may present as symptoms such as persistent fever, decreased white blood cell count (leukopenia), and inflammation of the liver (hepatitis), among others. The outcome of these direct effects depends on the past exposure and experience of both the transplant donor and the recipient with the virus, as well as the specific immunosuppressive treatment regimen used [5]. HCMV infection

after transplantation can cause damage to the vasculature, leading to a range of complications. These complications may include rejection of a renal allograft, atherosclerosis (the build-up of plaque in the arteries), and thrombotic microangiopathy (the formation of blood clots in small blood vessels). It's important to monitor transplant patients closely for signs of HCMV infection and its potential complications in order to provide timely and appropriate medical intervention [6,7].

However, there was a significant lack of follow-up investigations of HCMV infections before and after the antiviral therapy in all of the previously mentioned population groups in the Indian scenario, so the primary objective of this study was to find out the exact prevalence pattern of HCMV infectivity and the possibility of HCMV disease recurrence among these patient groups in metropolitan areas of Kolkata.

1.2 Material and Method:

Sample collection

Ethylenediaminetetraacetic acid (EDTA) anticoagulated peripheral blood samples (1-3 mL) were collected from patients during both the initial observation and follow-up studies. The blood was collected in vacutainer tubes and processed immediately. The serum was separated from the whole blood through centrifugation at 1000x g for 10 minutes. Following centrifugation, the serum was rapidly frozen at −80°C and stored until further processing.

DNA isolation from serum

DNA was extracted from the blood serum using the QIamp DNA Mini Kit (Qiagen Inc., Hilden, Germany; 51106) following the manufacturer's protocol. After extraction, the remaining serum was carefully stored at -80°C for future use. The concentration of the extracted DNA was determined by measuring the optical density (OD) values using a spectrophotometer.

HCMV Qualitative PCR for virus detection

Primers were designed on the conserve sequence of primers in the UL 83 and gB regions of the HCMV genome using the Primer3 online software and referred to the HCMV AD169 strain genome as a reference. The primers were sourced from Eurofins Genomics India Pvt. Ltd. The specific forward and reverse primers for UL 83 were 5'-GGG ACACAA CAC CGT AAA GC-3' and 5'-GTC AGC GTT CGT GTT TCCCA-3', respectively. For the UL 55 (gB) region, the forward and reverse primers were 5'-GGTCTTCAAGGAACTCAGCAAGA-3' and 5'-CGGCAATCGGTTTGTTGTAAA-3', respectively.

For each 25 µL PCR reaction mixture, I used 12.5 µL of 2X master mix (Emerald Amp GT PCR Master Mix, TAKARA), 1.5 µL of each forward and reverse primer (10 µM Conc.), 6.6 µL of sterile water, and 2 µL of DNA samples. The thermal cycling process began with a

denaturation step at 95°C for 5 mins, followed by 35 cycles of 95°C for 45 seconds, 57.5°C for 30 seconds, and 72°C for 45 seconds. Finally, there was a final extension at 72°C for 5 mins.

Real-time PCR quantification of HCMV viral load

A quantitation standard curve was achieved by using six 10-fold serial dilutions of a standard HCMV DNA with known viral load (copies/ml) purchased from ATCC. A conserved partial region of the HCMV UL 75 (gH) gene was amplified in each case and Ct value was measured in a real time PCR instrument (ABI 7500- Applied Biosystems). The forward and reverse primers were as follows: 5'-CGTGGAAGATGACCGAAGAT-3' and 5'-ATCGGCCACACTTTAACCAG-3' respectively. The standard DNA concentration was also calibrated by spectrophotometry at 260 nm. A 25ul of total volume of reaction mixture was made with 5ul of DNA, 12.5ul of 2x master mix containing SYBR green (TB green premix Ex Taq, TAKARA), gH forward/reverse primers and water. The cycling conditions were as follows, denaturation at 94ºC for 5 mins, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and final extension at 72°C for 7 mins.

Statistical analysis

Results were expressed as mean ± standard deviation, unless otherwise indicated. Differences between groups (Male and Female) were compared by two-way analysis of variance (ANOVA) when distributions were normal. Bonferroni method was used with ANOVA for comparing between individual groups. The level of significance (P value) was set at 5%. All P values were two tailed. All statistical analyses were carried out with SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). All graphs were prepared using graph pad prism software

1.3 Result:

Table 1.1- prevalence and clinical characteristics of HCMV infected adult and children population.

		CMV Status	
	Total sample	**Positive (%)**	**Negative (%)**
Study population			
Children			
Male	278	121(43.52)	97(34.89)
Female	102	63(61.76)	39(38.23)
Adults			
Male	171	97(56.72)	74(43.27)
Female	79	26(32.91)	53(67.08)
Clinical Parameters			
Children			
Hepatic Cholestasis	49	12(24.48)	37(75.51)
Congenital nephrotic syndrome	**41**	**23(56.09)**	**18(43.90)**
Microcephaly	29	14(48.27)	15(51.72)
Ventriculomegaly	**33**	**17(51.51)**	**16(48.48)**
Chorioretinitis	**56**	**37(66.07)**	**19(33.92)**
Pneumonitis	**78**	**56(71.79)**	**22(28.20)**
Cerebral palsy	29	7(24.13)	22(75.86)
Hydro-nephrosis	21	6(28.57)	15(71.42)
Lympho adenopathy	18	8(44.44)	11(61.11)
Hydrocephaly	**19**	**10(52.63)**	**9(47.36)**
Cerebral atrophy	26	7(26.92)	19(73.07)
Congenital cataracts	37	16(43.24)	21(56.75)
Pulmonary hypertension	42	19(45.23)	23(54.76)
Hepatosplenomegaly	**68**	**49(72.05)**	**19(27.94)**
Thrombocytopenia	**73**	**42(57.53)**	**31(42.46)**
Adults:			
Retinitis			
Diarrhea	**81**	**65(80.24)**	**16(19.75)**
Urinary tract infection	**76**	**59(77.63)**	**17(22.36)**
Fever	**83**	**69(83.13)**	**14(16.86)**
Diabetes	89	46(51.68)	43(48.31)
Pneumonia	51	30(58.82)	21(41.17)
Hematuria	**79**	**64(81.01)**	**15(18.98)**

Chronic kidney injury	**91**	**73(80.21)**	**18(19.78)**
Skin rashes	**43**	**28(65.11)**	**15(34.88)**

During the initial investigation, hospital admitted 380 children and 250 adult HCMV suspected patients (mostly selected from immunosuppressive patients undergone to renal transplantation patients) were selected for understanding the epidemiological and demographic infectivity of the virus in the local metropolitan area of Kolkata. It has been observed that among the children patients 196 (51.57%) were found to be HCMV positive. In case of adult population, it was observed that the HCMV positivity rate is slightly lower where 123 patients out of 250 (49.20%) were found to be true HCMV positive by qualitative estimation. Clinical parameters were distinctively assessed for HCMV positive and negative groups.

In the case of understanding clinical manifestations in HCMV infected children. Hepatosplenomegaly (72.05%), Pneumonitis (71.79%), and Chorioretinitis (66.07%) were the major clinical manifestations observed in HCMV-infected groups. Whereas Thrombocytopenia (57.53%), Congenital nephrotic syndrome (56.09%), Ventriculomegaly (51.51%), Hydro-cephaly (52.63%) were found to be moderately associated with the HCMV infection. In case of adult HCMV-infected patients as most of the samples collected from renal transplanted patients so a very specific renal associated symptoms were visible in these groups. Chronic renal injury (80.21%), Hematuria (81.01%) and Urinary tract infection (77.63%) were found to be directly associated with acute HCMV infection in renal transplanted patients but fever (83.13%), Diarrhea (80.24%) and skin rashes (65.11%) were also found to be significantly prominent in HCMV infected populations.

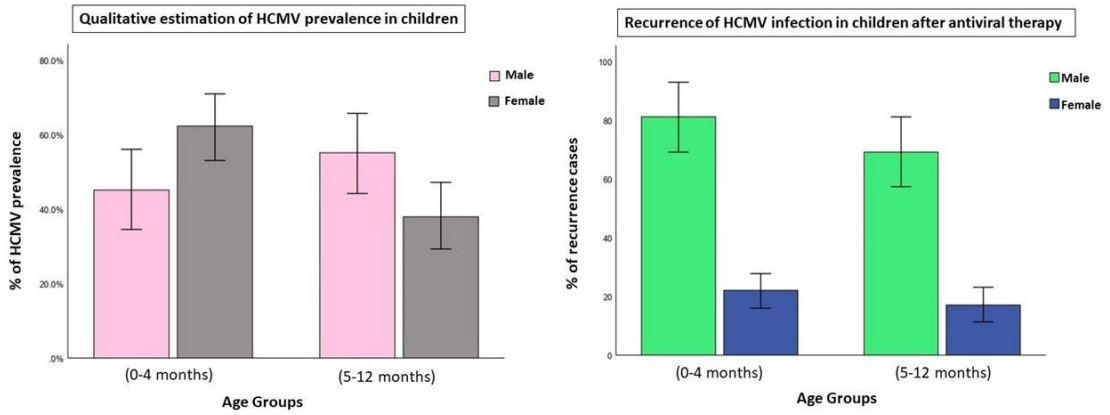


Figure 1.1- Qualitative estimation of HCMV infection among children in initial observation (left) and recurrence of infection after receiving antiviral therapy on the initially detected HCMV-infected patients (right). The patient groups were divided into two age categories. The % of infectivity cases was determined with $\pm$ SD.

The subjected patient groups were tested initially at the time of hospitalisation as well as the patients were followed up there months after the initial observation. In this research during the follow-up analysis we have observed that in both children and adult HCMV infected patient groups after the successive antiviral therapy the complete cure of viral infection were not visible in a significant number of cases. So we tried to assess the demographic pattern of HCMV recurrence after antiviral therapy in different patient groups.

Initially, it was observed that in the infected children population, male and female patients were 121(43.52%) and 63 (61.76%), respectively. Meanwhile, in the adult population, 97 (56.72%) were male and 26 (32.91%) were females infected with distinctive HCMV infection. In the initial observation of children, it was observed that 43% male and 61% female were infected with HCMV with less than 4 months older. Whereas 58% male and 39% female were infected and aged between 4 to 12 months. During the follow up study it was observed that a significant persistence of HCMV infection observed. It was found that 80 % and 20% of previously determined male and female patients within 0-4 months of age persisted with chronic HCMV infection as well as 68% and 18% of male and female patients, respectively, were shown the same results aged older than four months. **(Figure 1.1)**

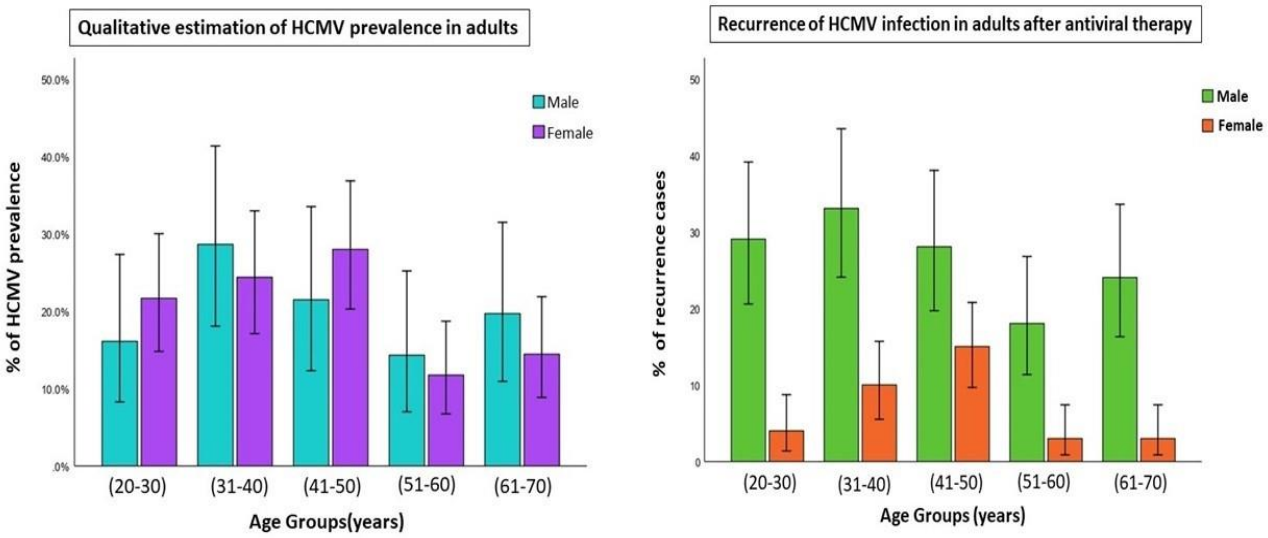


Figure 1.2 Qualitative estimation of HCMV infection among adult patients in initial observation (left) and recurrence of infection after receiving antiviral therapy on the initially detected HCMV-infected patients (right). The patient groups were divided into five age categories. The % of infectivity cases was determined with $\pm$ SD

As the adult population was selected from renal transplanted patient groups so after the initial days of renal transplantation no significant HCMV positivity variations were observed among male and female groups but it was observed that men within the age of 31-40 years and female within 41-50 years were found to be mostly HCMV positive after the initial days of transplantation. Understanding the recurrence pattern of HCMV infectivity, we have found a

very significant result where male transplanted patients with previous HCMV infection carried forward through HCMV antiviral therapy were prone to be much more persistent HCMV infection compared to their female counterparts. In the wide age range groups from 20-70 years, male patients showed a maximum number of HCMV positivity in the antiviral-treated patients.**(Figure 1.2)**

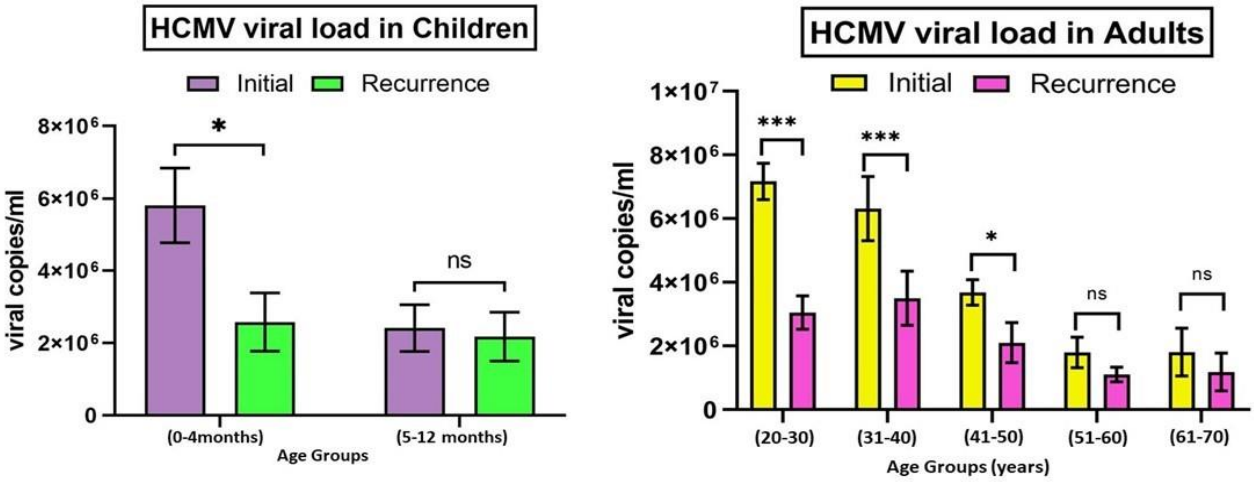


Figure 1.3- Comparative analysis of HCMV viral load among initially tested and recurred antiviral treated children (left) and adult (right) patients in different age groups. The comparison was carried out with a standard viral curve prepared from AD169 lab strain DNA molecule. The comparison of each group were done by two-way ANOVA followed by Bonferroni analysis. [The significant groups were determined as p <0.001=, <0.01 =**, <0.1= *, and not-significant= ns]***

Comparison of HCMV viral copies of the same previously detected and recurred HCMV-infected patients showed a very distinctive result. In children between 0 to 4 months of age initial HCMV viral load were found to be significantly high (5.75 X 10^6 copies/ml) in respect to recurred cases (3.29 X 10^6 copies/ml), whereas in 5-12 age groups HCMV viral load was found to be above 2 X 10^6 copies/ml in both initial and recurred cases with no significant variations. In case of adult population during the initial screening stage 20-30 age group (6.96 X 10^6 copies/ml), 31-40 age groups (2.9 X10^6 copies/ml) and 41-50 age groups (3.88 X10^6 copies/ml) significant elevation of HCMV viral loads in comparison to recurred cases on the respective groups. In the 51-70 age group of patients no significant variations were observed.

1.4 Discussion:

In this pilot investigation, a very unique observation was found in both children and adult group of patients that is other than the major clinical parameters fever and pneumonia were very much associated with acute HCMV infection which are not distinctively a symptomatic character of this disease. Not only that a major observation was that after the successive therapeutic strategies majority of the patients in the study cohort still persisted with chronic HCMV

infection detected during the three months follow up analysis. These observation leads to assume us that there might be a huge possibility drug resistance of the circulating strains. HCMV has been known to exhibit a tendency in lytic to latency switch so our observation can also elicit that there might be a possibility of conversion of lytic replication cycle of HCMV to lysogenic phase to bypass the adverse effect of antiviral drugs on HCMV DNA polymerase.

HCMV can symptomatically or asymptomatically infect different hosts and undergoes differential changes in clinical symptoms depending on the internal environment of the host as well as simultaneously adapt to its surroundings so we wanted to find out if there is any molecular immunological changes happening within the patients who persisted with HCMV infection.

As the life cycle switching and replication steps are very much driven by signalling transduction of different HCMV tegument protein so we focused our study to find out the role of tegument protein HCMV DNA polymerase

Chapter 2

The advancement of latent HCMV induced retinal choroidal neovascularization (CNV) and immunological signaling in neonatal patients

2.1: Background:

Retinal choroidal neovascularization (CNV) is characterized by the growth of abnormal blood vessels in the retina, leading to fluid leakage and vision impairments. It is associated with various serious ocular complications.[1]. It was found that significant CNV progression can impair the visualization and overall ocular health of newborns, necessitating prompt medical intervention. [2]. Age-related macular degeneration (AMD) responsible for primary of blindness and enduring ocular impairment in populations with aged individuals.[3, 4]. The precise mechanisms underlying adverse retinal conditions are currently not fully understood. Research involving *in-vivo* system indicates that regulatory pathways related to immune system may lead to changes in cellular morphology within the retinal environment.[5]. The mouse model of laser therapy induced choroidal neovascularization (CNV) indicates that the growth of new blood vessels, triggered by different angiogenic factors in the endothelial cells, ultimately resulted in retinitis development. [6]. Lymphatic tissue growth is initiated by alterations in various pro- and anti-angiogenic growth factors. Among these factors is VEGF, which is a pro-angiogenic factor secreted by pigment cells in retinal epithelial cells.[7]. Immune signalling related to VEGF can result in development of numerous blood vessels with uncontrolled growth extending into the retina from the choroid which ultimately form CNV or choroidal neovascularization.[8]. Children can experience choroidal neovascularization (CNV) due to several factors of congenital infections as well as several other parameters involving inflammatory responses and retinal myopia.[9].

HCMV is a ds-DNA containing virus is associated with significant health complications in various demographic groups and has been the subject of extensive scientific inquiry. [10]. The progression of HCMV disease depends on the activities of IE-1, glycoproteins, and several late response US genes. Reactivation of latent HCMV can lead to retinal complications in neonates. [11].

The process of angiogenesis involves intricate cellular mechanisms driven by migration and proliferation followed by the morphogenesis of endothelial tissues. In the context of HCMV infection, it is well-established that there is an augmentation of motility followed by proliferation of endothelial tissues which leads to the formation of angiogenic channels.[12,13]. Human cytomegalovirus infectivity elicits a cellular response by engaging angiogenesis-dependent receptors and modulating immune signalling protein (cytokines) activity. Vascular endothelial growth factor (VEGF) stimulates neovascularization by activating Ang II (angiotensin II)-

mediated signal transduction pathways, thereby promoting angiogenesis.[14]. Factors related to angiogenesis have the potential to reformation of new retinal blood vessel, which can trigger inflammatory responses, differentiation of endothelial cells, and the attraction of macrophages and responsive T-cells to the affected area. [15,16]. Ang II triggers reactions through G protein-coupled receptors called AGTR2 (Angiotensin II receptor type-II), comprising AT1R and AT2R. Studies indicate that angiogenesis mediated by AT1R may lead to a notable rise in cytokines which are mostly pro-inflammatory in nature. The AT2R receptor was discovered to exhibit antagonistic effects, resulting in reduced angiogenesis.[18]. According to a recent investigation, it was found that the AT1R receptor activation facilitates the positive regulation of the NFκB signalling pathway, resulting in the overexpression of various cytokines. [19]. The NFκB protein serves as a transcriptional regulator, which generally resides in cytoplasm as an inactive state bounded by inhibitor proteins such as IκBα or IκBβ. It has been well reported that the transcriptional regulation of NFκB initiated when these inhibitors[20].

HCMV possesses numerous genes relating to immune evasion which induces immunological imbalance and simultaneously, facilitating the establishment of viral latency within the host.[21]. The current understanding of inflammatory connection in clinical perspective in respect to retinal neovascularization contributed by HCMV latency is still lacking. Similarly, the comprehension of angiogenesis by Ang type II regulating the molecular dynamics of NFκB signalling pathways. This investigation seeks to contrast the alterations of immunological responses between retinal choroidal neovascularization and HCMV-infections in order to enhance our knowledge of disease pathology.

2.2. Methodology

Selection of Patients:

Infants aged between 28-42 were admitted to the hospital with suspected Human Cytomegalovirus (HCMV) infection, and Congenital Nasal Valve (CNV) were included in the research. The sample collection process received approval from the Institutional Ethics Committee in compliance with the 1964 Helsinki Declaration. The study comprised four groups: (R+/H+)- Group-I consisted of infants with both HCMV infection and CNV, (R-/H+) Group-II included HCMV infected infants without CNV, (R+/H-) Group-III comprised CNV infants with without HCMV infection and (R-,H-) Group-IV consisted of healthy individuals. Samples were obtained from the patients during the hospital admission, and After a period of two months, additional follow-up assessments were carried out.

Patient criteria for the study:

The study patients selected who were tested negative to other specific symptomatic chronic illnesses and any infections caused by bacterial or viral pathogens, with the exception of HCMV

(human cytomegalovirus). The research focused on neonatal patients who were actively infected with HCMV, and they received treatment with ganciclovir at a dosage of 6mg per kilogram at 12-hour intervals for a period of up to 4 weeks. It's important to note that patients who were in a moribund state were discarded from this study.

Collection of samples:

The PBMC (peripheral blood mononuclear cells) were isolated from initial and follow-up patients' blood in 5-10 mL EDTA collection tubes. The blood samples were processed through centrifugation at 1000g (10 minutes) for PBMC separation from the rest of the blood components. The PBMC obtained after centrifugation was carefully collected and then stored at -80°C for further utilization in downstream experiments.

Cytokine ELISA:

Serum Transforming growth factor beta (TGF-β), Interlukin 10 (IL10), Interlukin 6 (IL-6), Vascular cell adhesion molecule 1 (VCAM-1), Interlukin 8 (IL8) were assessed using ELISA method kits made by G-Biosciences, Abcam Biotech, both of Cambridge, UK, and Geno Technology Inc., of the United States. human cytokines, and only at extremely low blood quantities, were identified by these tests asper instructed by the manufacturer.

Preliminary detection of HCMV infection using the pp65 antigenemia assay:

The infection status of HCMV, determined by qualitative assessment of a matrix protein pp65 in leukocytes. This measurement was performed using the Millipore HCMV pp65 Antigenemia-kit with freshly collected sample. The initial sample was collected at the onset of the study, and follow-up analysis were done after the administration of antiviral drugs.

Quantitative assessment of viral quantity using major tegument genes.:

The QIamp DNA Blood Mini Kit (from Qiagen Inc. in Hilden, Germany reference number 51106) was utilized to extract complete DNA from isolated PBMC. PCR Primers for qualitative and quantitative analysis were specifically designed based on the UL 83 gene to confirm early HCMV infectivity [22] and viral attachment HCMV major surface glycoprotein (B) [23] by using the primer 3 online tools[24]. Details of Primer UL83, UL55 (gB), and latency related gene US28 as well as PCR protocols are mentioned in *Chapter 1*.

Expression of mRNA transcript from of PBMC by quantitative PCR:

Histopaque lymphocyte separation buffer, specifically Sigma-Aldrich-HISTOPAQUE-1077, was utilized to separate lymphocytes from the patient's blood. The isolated cells then underwent RNA isolation using TRIzol reagent from Invitrogen, a division of Thermo Fisher Scientific, Inc. The m-RNA extraction was further processed by converting it into complementary DNA (cDNA) using TAKARA Prime script cDNA extraction kit. The resulting complementary DNA were subsequently quantified in SYBR green dye-based real-

time PCR protocol. To ensure accuracy, a cellular internal housekeeping gene, GAPDH, was employed as the baseline transcript. Quantitative estimation of messenger RNAs were meticulously determined using the livak $2^{-\Delta\Delta Ct}$ method by analyzing the expression ratio of target gens with the internal control among different experimental study group.

Statistical analysis:

The study results were documented in the format of mean ± SD. In cases where results distributions exhibited normality, we utilized unpaired t-tests and one-way analysis of variance (ANOVA) with Bonferroni correction to assess differences among the different group. Significance level (P value) was set at 5%, with all P values being two-tailed. Every statistical experiments were performed using the SPSS (version 25.0). Additionally, graphical representations of the statistical analyses were created using GraphPad Prism (Ver 8).

2.3 Results

Investigation of neonatal patients exhibiting choroidal neovascularization (CNV) in the presence of acute human cytomegalovirus (HCMV) infection.:

The symptomatic apperance of CNV in R+/H- and R+/H+ patients included unilateral eyesight and Inflammatory retinochoroidopathy. Meanwhile, in R-/H+ patients, more commonly observed clinical symptom was Hepatosplenomegaly associated with HCMV. Both HCMV pp65 assay (antigenemia) and DNA copies were calculated at the early and post medicational therapy. It was found in the antigenemia assay that the R+/H+ group consisted of significantly higher (103$\pm$8 cells) of HCMV infected cells compared to R-/H+ (68$\pm$7 cells) ($p<0.001$). Additionally, log viral DNA copies were comparatively high in R+/H+ (6.779$\pm$0.36) in comparison to R-/H+ (5.589$\pm$0.55) ($p<0.001$). After a two-month follow-up, persisted HCMV-infested cells were measured in R+/H+ (27+4 cells) and R-/H+ (6+2 cells) along with the quantification of viral load of late response US-28 genes of 4.121$\pm$0.31 and 3.202$\pm$0.32 log copies/ml respectively, following post anti-HCMV treatment**. (Table 2.1)**

Table 2.1: Demographic distributions of different patients are arranged according to respective groups. Group-I with active Human Cytomegalovirus (HCMV) infection and retinal Choroidal Neovascularization (n=20); Group-II with HCMV infection without any retinal infection but acute HCMV infection (n=15); Group-III with neonates with retinal choroidal neovascularization with no HCMV infection. Group-IV(n=15) patients indicated as HCMV negative control with no retinal choroidal neovascularization (Healthy Control). HCMV viral load and antigenemia assay have been calculated by analysing Mean+ SD followed by student t-test between R+/H+ and R-/H+ during the initial as well as follow-up period.

		Gender		Eye laterality	
Patient Demographic features	**Age in days (Mean range)**	**Male (%)**	**Female (%)**	**Unilateral**	**Bilateral**
Group-I (n=20)	**17-26**	9 (45 %)	11 (55%)	13 (65%)	7 (35%)
Group II (n=15)	**20-24**	8 (53%)	7(46%)		
Group-III (n=15)	**23-29**	7 (46%)	8 (53%)	9 (60%)	6 (40%)
Group IV (n=15)	**22-38**	9 (60%)	6(40%)		

Clinical characterization

Features associated to CNV			**Features associated to HCMV infection**		
	R+/H- (n=15) (%)	**R+/H+ (n=20) (%)**		**R-/H+ (n=15) (%)**	**R+/H+ (n=20) (%)**
Optic disc hamartoma	1 (6%)	1(5)	**Hepatosplenomegaly**	9(60)	8 (40%)
Inflammatory retinochoroidopathy	7(46%)	8(40)	**Cholestasis**	4(26)	6 (30%)
chorioretinal scar	3 (20%)	6(30)	**Microcephaly**	3(20)	4 (20%)
multifocal choroiditis	4 (26%)	7(35)	**Fever**	10(66)	9(45 %)
Idiopathic choroidal neovascularization	4 (26%)	5(25)	**Thrombocytopenia**	7(46)	11 (55%)
High myopia	5 (33%)	7(35)	**hyperbilirubinemia**	4(26)	8 (40%)
			Biliary atresia	2(13)	5 (25%)

***HCMV viral load (*Log copy number/ml)**

		Mean±SD	**95% confidence interval**		**P value**
			Lower	**Upper**	
Initial study	***R+/H+***	6.779±0.36	6.576	6.981	<0.001
	R-/H+	5.589±0.55	5.284	5.894	
Follow up study after drug intervention	***R+/H+***	4.121±0.31	3.945	4.298	<0.001
	R-/H+	3.202±0.32	3.123	3.481	

Antigenemia Assay

No. of infected cells in 2X10^5 Leukocytes	**(Mean± SD)**		**95% confidence interval**		**P value**
			Upper	**Lower**	
Initial study	***R+/H+***	103±8	108	99	<0.001

	R-/H+	68±7	72	63	
Follow up study after drug intervention	***R+/H+***	27±4	29	25	<0.001
	R-/H+	6±2	7	5	

Biochemical profiling of serum constituents:

The analysis of biochemical constituents such as albumin, SGPT, bilirubin and SGOT revealed notable variations between the R+/H+ compare to other study population. Specifically, SGPT concentrations were significantly higher in the R+/H+ in respect to the R-/H+ patients, indicating a possible positive correlation between SGPT and CNV status associated with HCMV infection**.(Table 2.2)**

Table-2.2: Group-wise comparison of the biochemical constituents of the selected neonates.Data represented as Mean±SD values and were calculated on R+/H+ (N=20) samples from R-/H+, R+/H- and R-/H- (with each group n=15). Confidence level were calculated through one way ANOVA followed by post-hoc analysis by Bonferroni corelation coefficient for understanding the significant variation with respect to R+/H+

Parameter	**Study groups**	**Mean value ± standard deviation**	**Confidence (95%)**		**Significance**
			Upper	**Lower**	
Bilirubin (mg/dl)	**R+/H+**	5.94 ± 0.32			Constant
	R-/H+	5.42 ± 0.28	0.784	0.263	<0.001
	R+/H-	3.01 ± 0.25	3.173	2.672	<0.001
	R-/H-	3.06 ± 0.22	3.119	2.618	<0.001
Albumin (gm/dl)	**R+/H+**	4.94± 0.32			**Constant**
	R-/H+	3.78 ±0.22	1.293	0.923	<0.001
	R+/H-	1.74 ±0.26	3.439	2.970	<0.001
	R-/H-	2.10 ±0.17	3.075	2.605	<0.001
SGOT (IU/L)	**R+/H+**	275.69 ± 13.76			Constant
	R-/H+	217.75 ± 22.99	71.801	44.081	<0.001
	R+/H-	54.57 ± 12.46	234.981	207.261	<0.001
	R-/H-	35.10 ± 4.79	254.454	226.735	<0.001
SGPT (IU/L)	**R+/H+**	209.71 ± 24.47			Constant

	R-/H+	150.52 ± 19.31	75.157	43.209	<0.001
	R+/H-	39.62 ± 6.50	186.063	154.116	<0.001
	R-/H-	41.44 ± 7.25	184.243	152.296	<0.001
CRP (mg/L)	**R+/H+**	1.29 ± 0.32			Constant
	R-/H+	0.57 ± 0.17	0.922	0.510	<0.001
	R+/H-	1.75 ± 0.16	-0.257	-0.669	<0.001
	R-/H-	0.99 ± 0.12	0.506	0.094	<0.001
Creatinine (mg/dl)	**R+/H+**	2.06 ± 0.23			Constant
	R-/H+	1.82 ± 0.47	0.635	-0.153	0.598
	R+/H-	1.46 ± 0.43	0.992	0.204	0.001
	R-/H-	1.51 ± 0.55	0.945	0.157	0.002
GGT (IU/L)	**R+/H+**	35.20 ± 3.51			Constant
	R-/H+	36.62 ± 2.74	2.269	-5.108	1.000
	R+/H-	35.66 ± 4.61	3.222	-4.155	1.000
	R-/H-	34.26 ± 4.76	4.628	-2.748	1.000
Glucose (nmol/L)	**R+/H+**	4.74 ± 0.34			Constant
	R-/H+	4.40 + 0.63	0.872	-0.199	0.551
	R+/H-	4.72 ± 0.62	0.551	-0.520	1.000
	R-/H-	4.11 ± 0.70	1.154	0.825	0.015
Urea (nmol/L)	**R+/H+**	5.99 ± 0.18			Constant
	R-/H+	5.93 ± 0.24	0.302	-0.190	1.000
	R+/H-	5.76 ± 0.32	0.472	-0.020	0.090
	R-/H-	5.94 ± 0.27	0.288	-0.201	1.000
Sodium (mEq/L)	**R+/H+**	139.86 ± 7.22			Constant
	R-/H+	137.23 ± 5.38	7.891	-2.638	1.000
	R+/H-	140.80 ± 2.41	4.324	-6.204	1.000
	R-/H-	136.36 ± 5.79	8.764	-1.764	0.449
	R+/H+	149.61 ± 6.02			Constant

Cholesterol (mg/dl)	**R-/H+**	123.87 ± 2.53	29.660	21.820	<0.001
	R+/H-	131.64 ± 4.25	21.893	14.052	<0.001
	R-/H-	123.75 ± 1.85	29.776	21.936	<0.001
Triglyceride (mg/dl)	**R+/H+**	157.02 ±8.73			Constant
	R-/H+	159.60 ± 10.83	7.204	-12.354	1.000
	R+/H-	162.12 ± 12.60	4.684	-14.874	0.963
	R-/H-	162.83 ± 10.02	3.971	-15.588	0.663
HDL (mg/dl)	**R+/H+**	53.93 ± 3.45			Constant
	R-/H+	39.14 ± 4.15	18.712	10.854	<0.001
	R+/H-	44.42 ± 4.69	13.438	5.581	<0.001
	R-/H-	40.84 ± 4.69	17.012	9.154	<0.001
LDL (mg/dl)	**R+/H+**	64.85 ± 2.29			Constant
	R-/H+	65.55 ± 1.89	3.542	-5.479	1.000
	R+/H-	62.91 ± 9.28	6.182	-2.839	1.000
	R-/H-	65.53 ± 2.27	3.562	-5.459	1.000

Profiling of immunological constituents of different patient groups:

In the early observation during initial screening, VCAM-1 and VEGF cell proliferative cytokines along with IL-6 and IL-8 were highly expressed in the R+/ H- patients ($p<0.001$) compared to others. In the R+/H+ patients there was a notable increase in TGF-β concentration, indicating a correlation which positively favouring CNV progression associated with HCMV infectivity. This profiling of immunoregulators expressed a decrease in cytokine expression during acute HCMV infection. **(Table-2.3)**

Table 2.3: Initial group-wise comparison of the immunological active constituents of the selected neonates. Quantitative ELISA Data represented as Mean±SD values and were calculated on R+/H+ (n=20) samples from R-/H+, R+/H- and R-/H- (in each group n=15). Confidence level were calculated through one way ANOVA followed by post-hoc analysis through Bonferroni corelation coefficient for understanding significant variation with respect to R+/H+.

	Parameter	**Mean+SD**	**95% confidence**		**Significance**
			Upper	**Lower**	

VCAM-1 (pg/ml)	**R+/ H+**	795.75±69.50			**Constant**
	R+/ H-	1073.70±44.87	-234.32	-321.64	<0.001
	R-/H+	801.72±21.65	37.66	-49.87	1.000
	R-/H-	809.30±23.12	30.08	-57.25	1.000
IL-8 (pg/m)	**R+/ H+**	26.87 ± 2.94			**Constant**
	R+/ H-	33.40 ± 1.88	-4.063	-8.980	<0.001
	R-/H+	19.84 ±3.21	9.489	4.573	<0.001
	R-/H-	14.93 ± 2.16	14.405	9.489	<0.001
IL-6 (pg/ml)	**R+/ H+**	52.75 ± 7.16			**Constant**
	R+/ H-	82.27 ± 5.55	-24.543	-34.492	<0.001
	R-/H+	31.06 ± 3.44	26.674	16.723	<0.001
	R-/H-	33.26 ± 3.44	24.463	14.514	<0.001
VEGF (pg/ml)	**R+/ H+**	48.94±4.67			**Constant**
	R+/ H-	83.49±7.52	-29.579	-39.524	<0.001
	R-/H+	26.15±3.76	27.762	17.817	<0.001
	R-/H-	28.31±4.70	25.593	15.648	<0.001
TGF-β (pg/ml)	**R+/ H+**	1163.05±91.67			**Constant**
	R+/ H-	869.26±28.06	347.75	239.82	<0.001
	R-/H+	912.93±45.23	304.08	196.15	<0.001
	R-/H-	864.94±19.61	352.07	244.15	<0.001

In a follow-up investigation conducted at 2-month intervals, the expression of VEGF slightly increased in the R+/H+ patients. However, the concentrations of Transforming Growth Factor Beta (TGF-β) (p=1.000), IL-6 (p=1.000), IL-8 (p=0.738), and VCAM-1 (p=0.239) were statistically insignificant when comparing the R+/H- and R+/H- population. **(Table-2.4)**

Table 2.4*: Follow-up group-wise comparison of the immunological active constituents of the selected neonates. Quantitative ELISA Data represented as Mean±SD values and were calculated on R+/H+ (n=20) samples from R-/H+, R+/H- and R-/H- (in each group n=15). Confidence level were calculated through one way ANOVA followed by post-hoc analysis through Bonferroni corelation coefficient for understanding significant variation with respect to R+/H+*

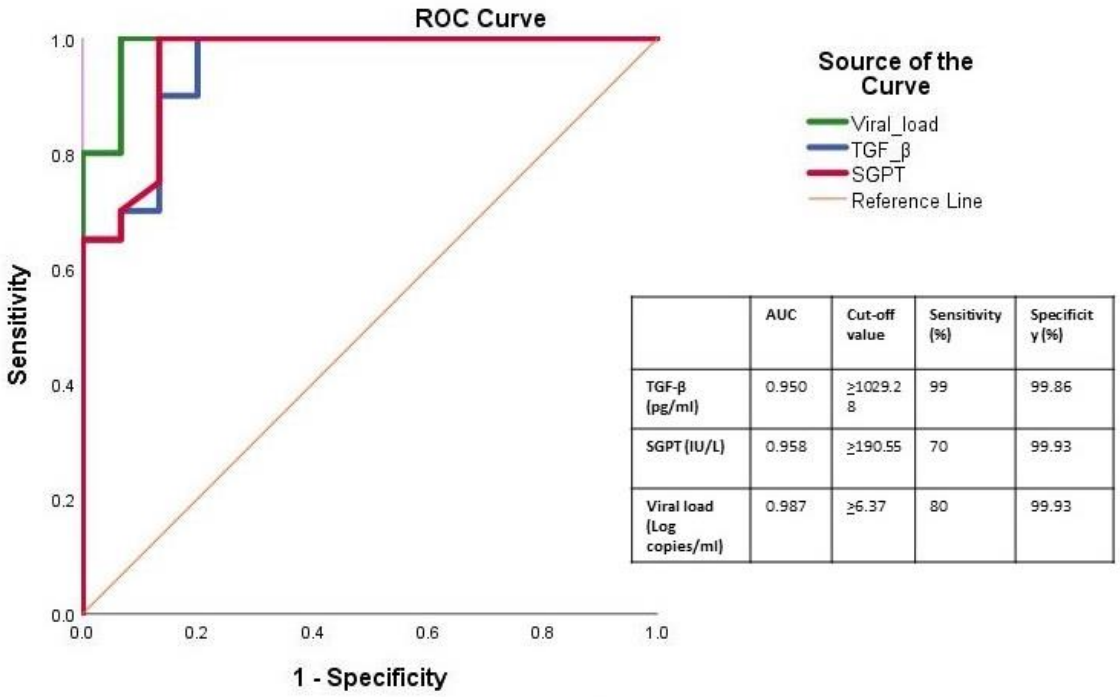
Parameter	Study groups	Mean value ± standard deviation	95% confidence		Significance
			Upper	Lower	
TGF-β (pg/ml)	**R+/H+**	839.20+29.14			**Constant**
	R+/H-	838.68+35.05	30.079	-29.022	1.000
	R-/H-	856.59+27.99	12.155	-46.946	0.682
	R-/H+	804.16+34.92	64.585	5.483	0.012
VCAM-1 (pg/ml)	**R+/H+**	1096.20+57.96			**Constant**
	R+/H-	1127.49+55.36	9.33	-71.92	0.239
	R-/H-	810.23+17.78	325.59	245.34	<0.001
	R-/H+	805.84+18.68	330.99	249.73	<0.001
IL-6 (pg/ml)	**R+/H+**	84.11+7.57			**Constant**
	R+/H-	87.25+7.70	3.083	-9.355	1.000
	R-/H-	43.27+4.89	47.062	34.623	<0.001
	R-/H+	56.17+5.74	34.156	21.717	<0.001
IL-8 (pg/m)	**R+/H+**	31.22+2.26			**Constant**
	R+/H-	32.67+2.71	1.081	-3.988	0.738
	R-/H-	15.58+2.14	18.178	13.107	<0.001
	R-/H+	19.79+3.64	13.966	8.895	<0.001
VEGF (pg/ml)	**R+/H+**	92.85±5.15			**Constant**
	R+/H-	111.74±15.16	-11.241	-26.548	<0.001
	R-/H-	25.90±3.73	74.606	59.298	<0.001
	R-/H+	24.92±3.78	78.589	60.272	<0.001

Determination of Cut-off value of choroidal neovascularisation with co-relation to HCMV infectivity:

Receiver Operating Characteristic curve of critical serum biochemical and immunological parameters were constructed between two groups: R-/H+ and R+/H+, to determine the threshold

value of the predictive selected biomarker that provides the greatest specificity and sensitivity. Among the assessed markers, Serum Glutamic Pyruvic Transaminase (SGPT) and Transforming Growth Factor Beta (TGF-β) demonstrated notably high predictive capabilities, as evidenced by their respective Area Under the Curve (AUC) values of 0.958 (95% CI 0.896-1.000) and 0.950 (95% CI 0.883-1.000). Furthermore, the analysis of HCMV viral levels in the R+/H+ and R-/H+ groups showed strong predictive ability, with an AUC of 0.987 [95% CI 0.957-1.000], taking into account the noteworthy fluctuations. These findings highlight the potential of biochemical parameter on SGPT and immunological parameter TGF-β, along with HCMV viral copies as promising distinctive biomarkers for the evaluated conditions. (**Figure-1)**

Figure-1: ROC curve of biochemical and immunological biomarkers for distinctive co-relation of CNV progression associated with HCMV infectivity.



	AUC	Cut-off value	Sensitivity (%)	Specificity (%)
TGF-β (pg/ml)	0.950	≥1029.28	99	99.86
SGPT (IU/L)	0.958	≥190.55	70	99.93
Viral load (Log copies/ml)	0.987	≥6.37	80	99.93

<u>Expression of Ang-II receptor in HCMV-associated CNV progression</u>:

In evaluating each group's mRNA expression pattern changes of both AT1R and AT2R (Ang-II receptors) against the negative control R-/H- as the baseline, a noteworthy elevation ($p<0.006$) in AT1R expression was exclusively identified in the R+/H- group. However, no substantial changes in AT2R expression fold were observed in either population based on initial early screening.(**Table 2.5**).

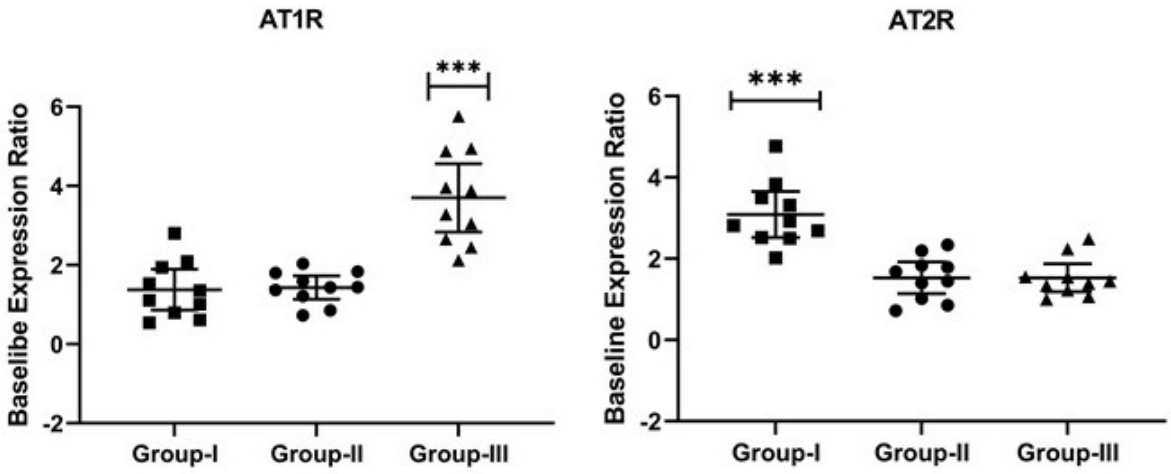
Table 2.5: Initial group-wise comparison of m-RNA transcript of AT1R and AT2R receptor in R+/H+, R-/H+,R+/H- with respect to control group R-/H-. The expression were represented by the change of expression fold ($2^{-\Delta\Delta Ct}$) in respect to internal cellular control GAPDH. Confidence levels were calculated through ANOVA followed by post hoc analysis using the

Bonferroni correlation coefficient for understanding the significant variation with respect to R-/H-.

Initial study					
Marker	**Mean ± SD**		**95% confidence interval**		**Significance (P value)**
			Upper	Lower	
AT1R	R+/H+	0.861±0.13	-0.286	0.764	0.564
	R-/H+	0.866±0.20	-0.291	0.781	0.559
	R+/H-	1.565±0.56	-0.990	0.006	-0.139
AT2R	R+/H+	0.848±0.16	-0.243	0.671	0.545
	R-/H+	0.974±0.21	-0.369	0.997	0.420
	R+/H-	0.893±0.23	-0.288	0.847	0.500
Follow up study					
Marker	**Mean ± SD**		**95% confidence interval**		**Significance (P value)**
			Upper	Lower	
AT1R	R+/H+	1.380±0.56	-1.184	0.521	0.424
	R-/H+	1.432±0.43	-1.236	0.420	0.371
	R+/H-	3.701±0.95	-3.505	<0.001	-1.897
AT2R	R+/H+	3.091±0.47	-2.694	<0.001	-1.488
	R-/H+	1.54±0.53	-1.135	0.100	0.071
	R+/H-	1.60±0.68	-1.136	1.000	0.070

After meticulous follow-up observation, it is evident that patients in group III (R+/H-) exhibit a comparatively higher AT1R gene expression in R+/H+ and R-/H+ ($p<0.001$). This clearly demonstrates the association between HCMV infection and increased AT1R gene expression. Furthermore, we have compelling evidence that in R+/H+ patients, AT2R expression was significantly overexpressed in comparison to both R+/H- and R-/H+ patients ($p<0.001$). In the context of Human Cytomegalovirus (HCMV) infection, it is evident that angiogenesis is primarily stimulated by the Angiotensin II type 1 receptor (AT1R) pathway in the absence of infection. However, in instances of persistent latent HCMV infection, there is a notable activation of the Angiotensin II type 2 receptor (AT2R) pathway, indicating a shift in the regulatory mechanisms of angiogenesis, indicating distinct mechanisms of angiogenesis regulation. **(Figure-2).**

Figure 2: Follow-up observation with a group-wise comparison of R+/H+(I), R-/H+ (II) and R+/H- (III). The expression was represented by the change of expression fold ($2^{-\Delta\Delta Ct}$) in respect to internal cellular control GAPDH. Relative expression was represented in respect to the baseline value of R-/H- (IV) patients. Significant groups were represented with p<0.001 (***)



<u>Comparison of different major pro-inflammatory and anti-inflammatory cytokines of initially collected samples:</u>

The expression of mRNA transcript was done from initially collected samples which are mainly responsible for inducing the activity of inflammatory cytokines TNF-α, IL-2, IL-6, IFN-γ, IL-1β, IL-8 and as well as IL-10 and IL-4 responsible for reducing inflammation among three study groups with baseline comparison to R-/H-. In this study, it was found that in patients with both retinopathy (R+/H-), a significant elevated expression of the major pro-inflammatory cytokines except IL-2 ($p<0.001$). This result strongly suggests that pro-inflammatory cytokines are directly associated with choroidal neovascularization (CNV) and not with human cytomegalovirus (HCMV) infection. Furthermore, we noted that only one pro-inflammatory cytokine IL-2 expression were significantly high in R-/H+ ($p<0.001$). On the other hand, analysis of anti-inflammatory cytokines was significantly high in patients with HCMV-associated retinopathy (R+/H+) (group-I), as well as in patients with only viral infection (R-/H+), when compared to patients with retinopathy (R+/H-). During acute human cytomegalovirus (HCMV) infection, there is an observed increase in the expression of anti-inflammatory cytokines. This elevation in anti-inflammatory cytokines suggests the initiation of a distinct immune regulation during HCMV-induced neovascularization. (**Figure-3; Table-2.6).**

Figure 3: initial observation of cytokine expressions through m-RNA transcript with a group-wise comparison of R+/H+ (I), R-/H+ (II) and R+/H- (III). The expression was represented by the change of expression fold ($2^{-\Delta\Delta Ct}$) in respect to internal cellular control GAPDH.

Relative expression was represented in respect to the baseline value of R-/H- (IV) patients. Significant groups were represented with p<0.001 (***)

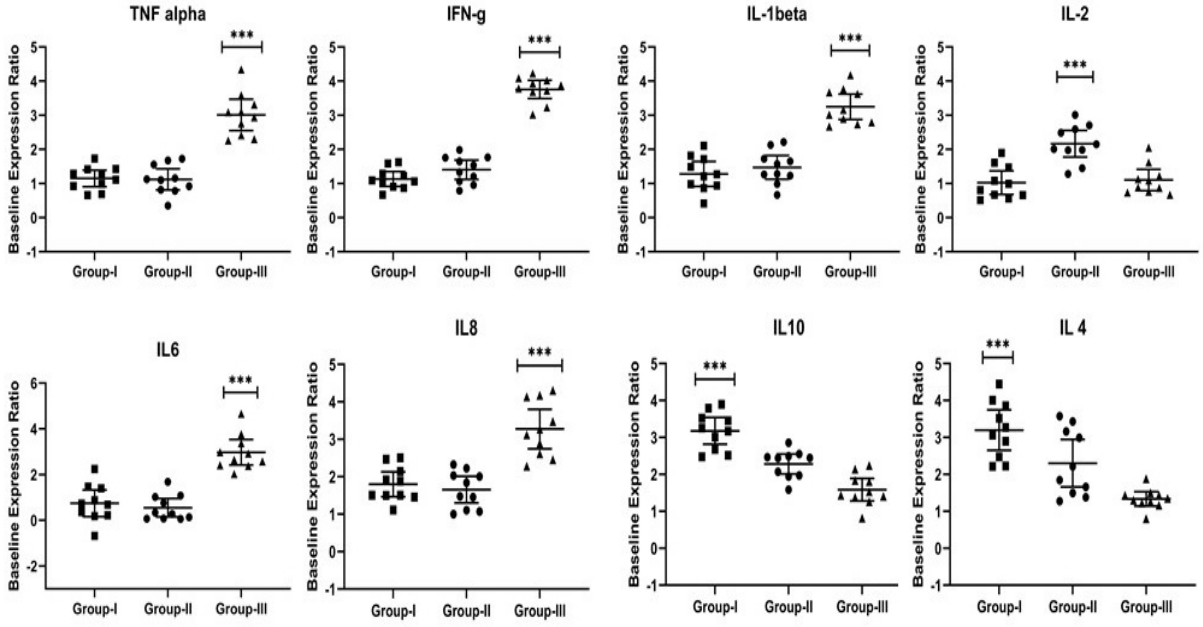


Table 2.6: Initial group-wise comparison of m-RNA transcript of different cytokines in R+/H+, R-/H+, R+/H- with respect to control group R-/H-. The expressions were represented by the change of expression fold ($2^{-\Delta\Delta Ct}$) in respect to internal cellular control GAPDH. Confidence levels were calculated through ANOVA followed by post hoc analysis using the Bonferroni correlation coefficient for understanding the significant variation with respect to R-/H-.

Immunological Marker	Mean ± SD		Significance (P-Value)	95% confidence	
				Lower	Upper
TNF-α	Group-I (R+/H+)	1.15±0.34	0.764	-0.615	0.312
	Group-II (R-/H+)	1.12±0.43	0.855	-0.586	0.341
	Group-III (R+/H-)	3.01±0.64	<0.001	-2.477	-1.549
IFN-γ	Group-I (R+/H+)	1.13±0.30	0.657	-0.476	0.208
	Group-II (R-/H+)	1.40±0.39	0.167	-0.747	-0.062
	Group-III (R+/H-)	3.76±0.37	<0.001	-3.102	-2.417
IL-1β	Group-I (R+/H+)	1.28±0.50	0.472	-0.773	0.208
	Group-II (R-/H+)	1.47±0.48	0.062	-0.965	0.017
	Group-III (R+/H-)	3.25±0.52	<0.001	-2.743	-1.761
IL-2	Group-I (R+/H+)	1.02±0.48	1.000	-0.504	0.448
	Group-II (R-/H+)	2.16±0.54	<0.001	-1.643	-0.689

	Group-III (R+/H-)	1.10±0.43	1.000	-0.581	0.371
IL-6	Group-I (R+/H+)	0.74±0.81	1.000	-0.455	0.958
	Group-II (R-/H+)	0.54±0.5	0.363	-0.259	1.154
	Group-III (R+/H-)	2.97±0.77	<0.001	-2.683	-1.269
IL-8	Group-I (R+/H+)	1.80±0.46	0.095	-1.365	-0.236
	Group-II (R-/H+)	1.65±0.49	0.067	-1.220	-0.091
	Group-III (R+/H-)	3.27±0.74	<0.001	-2.839	-1.711
IL-10	Group-I (R+/H+)	3.17±0.50	<0.001	-2.726	-1.519
	Group-II (R-/H+)	2.28±0.37	0.036	-0.809	0.028
	Group-III (R+/H-)	1.58±0.42	0.869	-0.958	1.125
IL-4	Group-I (R+/H+)	3.19±0.76	<0.001	-2.367	-1.476
	Group-II (R-/H+)	2.30±0.90	0.023	-0.519	0.017
	Group-III (R+/H-)	1.33±0.27	0.625	-0.428	1.185

Expression of different chemokines and the respective chemokine binding receptors to understand the crosstalk between HCMV dependent retinal CNV :

Expression of selective chemokines were measured from initially collected patient samples. It was observed that expression of three chemokines CCL-21, CCL-19, and CXCL-12 were insignificant in R+/H+, R-/H+ and R+/H- compared to R-/H- patient populations. However, in R+/H- patient showed significant elevation of CCL-5 m-RNA transcript (group-III) ($p<0.001$) compare to all other study groups. In the follow-up analysis of the same chemokines, it was observed that CCL-21 and CCL-19 expressed significantly higher in R+/H+ groups in comparison to other groups. Whereas significant elevation of CXCL12 were also found in R+/H+ along with R-/H+ patient groups. Similar to the initial observation, R+/H- also showed CCL-5 elevation in the follow-up study. The result indicates that CCL-5 distinctively have a positive regulatory role in choroidal neovascularization (CNV), while the elevation of other chemokines indicates a potential positive correlation between persistent HCMV associated CNV progression. (**Figure 4; Table-2.7**)

Figure-4: initial and follow-up observation of selective chemokines through m-RNA transcript with a group-wise comparison of R+/H+ (I), R-/H+ (II) and R+/H- (III). The expression was represented by the change of expression fold ($2^{-\Delta\Delta Ct}$) in respect to internal cellular control GAPDH. Relative expression was represented in respect to the baseline value of R-/H- (IV) patients. Significant groups were represented with p<0.001 (***) and P<0.01(**)

respectively.

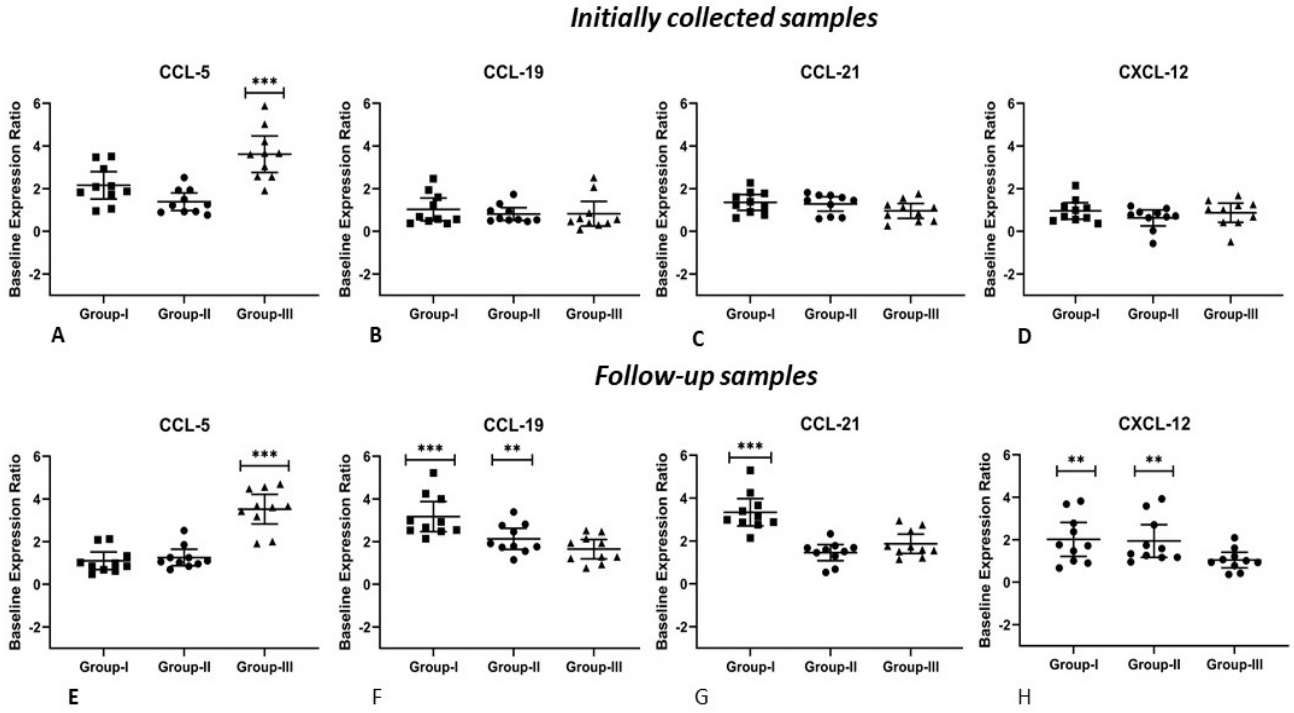


Table 2.7: Group-wise comparison of m-RNA transcript of different chemokines in R+/H+, R-/H+, R+/H- with respect to control group R-/H-. The expressions were represented by the change of expression fold ($2^{-\Delta\Delta Ct}$) in respect to internal cellular control GAPDH. Confidence levels were calculated through ANOVA followed by post hoc analysis using the Bonferroni correlation coefficient for understanding the significant variation with respect to R-/H-.

Initial admitted samples					
Immunological Marker	**Mean ± SD**		**Significance (P-Value)**	**95% confidence**	
				Lower	**Upper**
CCL-5	Group-I	2.06±0.89	0.436	-1.098	0.328
	Group-II	1.99±0.57	0.851	-1.289	0.509
	Group-III	3.61±1.19	<0.001	-3.517	-1.719
CCL-21	Group-I	1.35±0.52	0.212	-0.839	0.124
	Group-II	1.28±0.47	0.433	-0.767	0.195
	Group-III	0.96±0.48	1.000	-0.444	0.519
CCL-19	Group-I	1.03±0.74	1.000	-0.691	0.626
	Group-II	0.81±0.41	1.000	-0.473	0.844
	Group-III	0.82±0.80	1.000	-0.487	0.830

CXCL-12	Group-I	0.962±0.53	1.000	-0.513	0.588
	Group-II	0.63±0.52	0.377	-0.183	0.918
	Group-III	0.87±0.63	1.000	-0.422	0.679
Follow Up study					
CCL-5	Group-I	1.11±0.57	1.000	-1.014	0.802
	Group-II	1.26±0.54	1.000	-1.167	0.649
	Group-III	3.56±1.01	<0.001	-3.665	-1.849
CCL-21	Group-I	3.34±0.89	<0.001	-3.021	-1.659
	Group-II	1.45±0.52	0.303	-1.137	0.224
	Group-III	1.87±0.63	0.098	-1.533	-0.191
CCL-19	Group-I	3.17±0.98	<0.001	-2.939	-1.412
	Group-II	2.13±0.69	0.002	-1.897	-0.369
	Group-III	1.65±0.63	0.118	-1.414	0.113
CXCL-12	Group-I	2.019±1.11	0.025	-1.936	-0.101
	Group-II	1.944±1.07	0.042	-1.862	-0.026
	Group-III	1.04±0.51	1.000	-0.963	0.871

It is widely recognized that CCR-5 receptors can effectively allow different chemokines to bind with the cleft but specifically allow CCL-5 chemokines to initiate downstream signal transduction for cell proliferation.[26] whereas it was distinctively found that CCR-7 only allow CCL-19 as well as CCL-21 to effectively attach and initiate further signalling cascade [27]. We aimed to investigate the interaction of CCR-5 and CCR-7 with their corresponding chemokines in contrast to HCMV associated and non- associated neovascularization. In the initially collected samples, no significant elevation of expressivity on both these receptors was observed in any study groups, but in the follow-up investigation, it was observed that the CCR-5 receptor was significantly overexpressed within R+/H- groups as well as R+/H+ patient populations. But in case of CCR-7 the significant elevation was observed only in R+/H+ population. This significant elevation of CCR-7 directly positively correlated with the expression of respective chemokines which strongly suggests that HCMV associated CNV progression might be intrigued in R+/H+ population. (**Figure 5**; **Table-2.8**).

Figure-5: Follow-up observation of selective chemokine receptors through m-RNA transcript with a group-wise comparison of R+/H+ (I), R-/H+ (II) and R+/H- (III). The expression was represented by the change of expression fold ($2^{-\Delta\Delta Ct}$) in respect to internal cellular control

GAPDH. Relative expression was represented in respect to the baseline value of R-/H- (IV) patients. Significant groups were represented with p<0.001 (***)

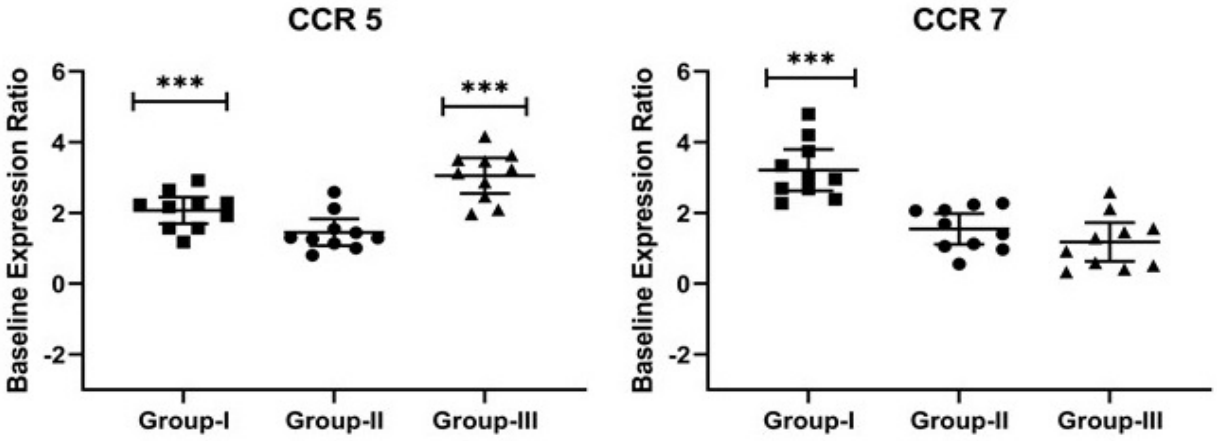


Table 2.8: A comparative follow-up analysis of relative m-RNA expression ratio of different Chemokine receptors analysed by Real time PCR. The Mean+/-SD has been calculated and One way ANOVA was performed to estimate the significance among the R+/H+ (group-I), R-/H+ (group-II) and R+,H- (group-III)).

Follow up study					
Chemokine Receptor	**Mean ± SD**		**Significance (P-Value)**	**95% confidence interval**	
				Lower	**Upper**
CCR-5	Group-I	2.07±0.52	<0.001	-0.511	-1.637
	Group-II	1.45±0.63	0.137	-0.109	-1.101
	Group-III	3.06±0.70	<0.001	-1.495	-2.621
CCR-7	Group-I	3.21±0.81	<0.001	-1.500	-2.929
	Group-II	1.54±0.60	0.187	0.167	-1.262
	Group-III	1.17±0.76	1.000	0.535	-0.893

Alteration of NF-kB canonical and non-canonical pathways in HCMV associated CNV progression:

Previous study reported that NF-kB signalling pathway always regulated by CCL-5 overexpression (28). So we focused on comprehending the signalling switching of canonical and non-canonical NF-kB activation pathways to get a distinctive view on immunological cascade. To get a proper class switching of the signalling pathway we conducted our focus on NFκB activator and inhibitory proteins. For canonical NFkB activation a ratio of two activator proteins IKKα (CHUK) and IKKβ (IKBKB) with two separate inhibitors of IKBA and IKBB gene transcripts were measured [(CHUK+IKBKB) / IKBA or IKBB]. Whereas for non-canonical NFkB activation a combinational effect of MP3K14 gene transcripts with CHUK activator was measured [CHUK+MP3K14]. In the initially collected samples from R+/H-

patients an elevated expression of NFkB canonical pathways [(CHUK+IKBKB) / IKBA and (CHUK+IKBKB) / IKBB] were observed in comparison to HCMV associated CNV progressive group R+/H+.In the initial study, insignificant elevation of NFkB non-canonical gene transcripts were observed when compared with housekeeping gene. However, during follow-up investigation conducted after the antiviral therapy, it was observed that the HCMV associated CNV group (R+/H+) showed a notably elevated expression ($p<0.001$) of the non-canonical signalling compared to only CNV patients devoid of HCMV infection. This finding provides valuable insight into the potential impact of combined exposure to R+/H+ on the expression of these genes and suggests a potential association with the NFκB non-canonical pathway. (**Figure 6; Table 2.9)**.

Figure 6: Comparing the alteration of NFkB canonical and noncanonical pathways activation in Initial and follow-up patient samples among R+/H+ (group-I) and R+/H- (group-III). The expression was represented by the change of expression fold ($2^{-\Delta\Delta Ct}$) in respect to internal cellular control GAPDH. Relative expression was represented in respect to the baseline value of R-/H- (IV) patients. Significant groups were represented with p<0.001 (***)

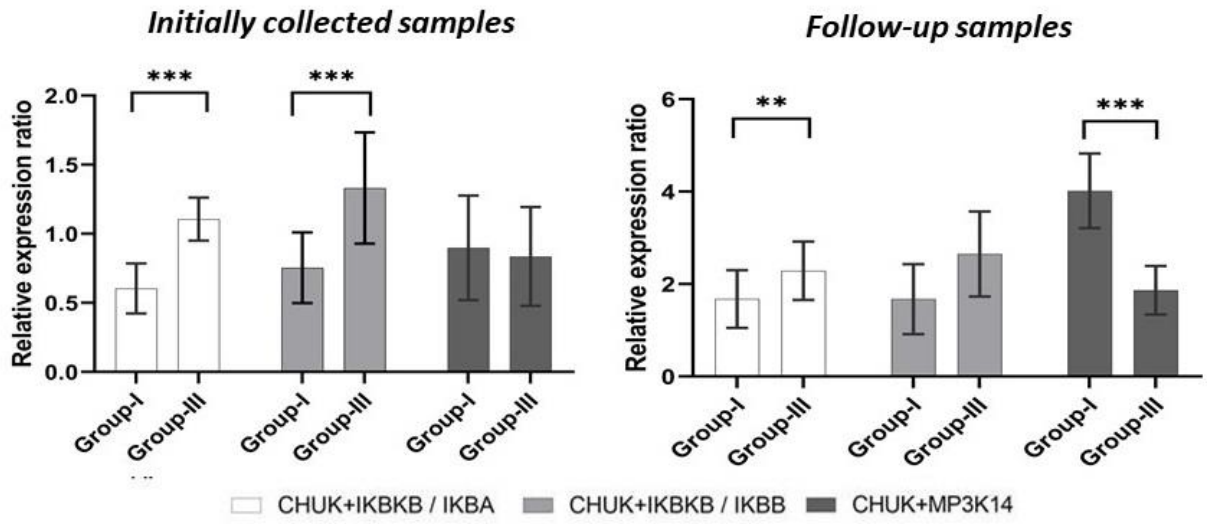


Table 2.9: A comparative analysis of relative m-RNA expression ratio of different NFκB signalling pathway markers of both initial and follow up samples analysed by Real time PCR. Mean ± SD values were calculated and independent sample-t test were performed between R+/H+ and R+/H- groups to estimate the significance of the signalling pathways.

Initial study					
Relative m-RNA expression	**Group**	**Mean ± SD**	**Significance (P value)**	**95% confidence interval**	
				Upper	**Lower**
(CHUK+IKBKB)/IKBA	Group-I	0.604±0.18	<0.001	0.075	-0.660
	Group-III	1.106±0.15			
(CHUK+IKBKB)/IKBB	Group-I	0.754±0.25	0.001	-0.893	-0.259
	Group-III	1.331±0.40			
CHUK+MP3K14	Group-I	0.898±0.37	0.708	-0.283	0.408
	Group-III	0.836±0.35			
Follow Up study					
Relative m-RNA expression	**Group**	**Mean ± SD**	**Significance (P value)**	**95% confidence interval**	
				Upper	**Lower**
(CHUK+IKBKB)/IKBA	Group-I	1.679±0.62	0.04	-1.201	-0.018
	Group-III	2.28±0.63			
CHUK+IKBKB)/IKBB	Group-I	1.675±0.75	0.18	-1.767	-0.187
	Group-III	2.652±0.97			
CHUK+MP3K14	Group-I	4.019±0.81	<0.001	1.512	2.793
	Group-III	1.867±0.53			

2.4 Discussion

The main objective of this study was to understand the immunological changes of persistent HCMV-associated CNV progression in neonates to decipher the critical cellular biomarkers related to pathogenically induced retinal angiogenesis. In previous studies it was reported that in case of non-pathological choroidal retinitis vascular endothelial growth factor (VEGF) generally overexpressed and initiates CXCL-12 and CCL-5 mediated inflammation which induces retinal angiogenesis.[29-31]. Based on our observations, we have found that the hypothesis holds true in the R+/H- (group-III) patients. In HCMV-associated CNV patients

(R+/H+), we initially observed no significant inflammation. However, we found that high levels of cytokine TGF-β and increased viral DNA copies might serve as predictive biomarkers in these patients. Notably, in the R+/H+ patient groups the expression of chemokine CCL-5 was not found insignificant. This suggests that an alternative pathway may have contributed to the delayed progression of CNV despite persistent HCMV infection in these patients.

Previous studies also reported that angiogenesis-II (Ang-II) receptor mediated CCL-5 chemokine interaction generally favoured the angiogenesis in endothelial system. This finding provides critical viewpoint to understand the CNV progression in patients with HCMV-infection. [32,33]. Based on our comprehensive investigation, we have gathered compelling evidence indicating that the R+,H- group exhibits heightened angiogenesis through AT1R-mediated CCL-5 activation followed by CCR-5 overexpression. On the other hand, the R+,H+ group showcases an overexpression of AT2R, which leads to distinct variations in angiotensin signalling and contributes to elevation of completely different chemokines like CCL-21 and CCl-19 which were only expressed significantly only in the persistent HCMV infectivity cases. Notably, our findings also revealed that due to the successive elevation of completely different chemokines, a distinctively separate chemokine receptor CCR-7 was expressed eventually.,

In previous studies it was reported that NFkB canonical pathway actively regulated by CCL-5 dependent signalling cascade, while most of the cases CCL-21 and some minor cases CCL-19 are involved in modulating the NFκB non-canonical pathway. These intricate molecular mechanisms shed light on the diverse effects of angiotensin signalling and chemokine expression in different patient groups. [34-37]. So, to analyse the crosstalk of the NFkB signalling system in response to the different immuno-regulatory proteins, we checked the m-RNA transcripts of chemokines and the respective receptors and found that upregulation of receptor CCR-7 positively corelated with upregulation of CCL-21 and CCL-19, leading to the initiation of the NFkB non-canonical pathway. This cascade of molecular signal transduction events was previously reported in an investigation, highlighting the complex interplay between cytokine expression and NF-kB signalling, shedding light on potential regulatory mechanisms within this pathway. [38]. Within the canonical NFκB signalling pathway, the equilibrium of positive regulator gene expression involving CHUK and IKBKB and negative regulator gene expression involving IKBA and IKBB transcripts is of paramount importance for the initiation of NFκB activation. Similarly, in the non-canonical NFκB pathway, the relative expression levels of distinctive gene transcript MAP3K14 are solely required with CHUK genes which play a significant role in modulating the pathway activity. This balance of gene expression ultimately determines the activation or inhibition of the NFκB signalling pathway, which in turn regulates various cellular processes. In patients with R+/H- status, the progression of CNV leads to activation of the canonical NFkB pathway, while in R+/H+ patients, there is significant activity in the non-canonical NFκB signalling system. This investigation also reported that R+/H+

patients might experience CNV progression slightly delayed because the early stages of HCMV infection are prone to downregulating the secretion of pro-inflammatory cytokines and simultaneously upregulating anti-inflammatory responses. It is possible to theorize that detection of early health conditions can be achieved through the analysis of different biochemical and immunological markers and the regulatory constituents of NFkB canonical or non-canonical signalling cascade. This comprehensive research also revealed that retinal neovascularization associated with persistent Human Cytomegalovirus (HCMV) infection can target a specific inflammatory pathway mediated by CCL-21 and CCl-19 chemokines which results in non-canonical NFκB activation and subsequent retinal angiogenesis.

Chapter 3

Investigation on HCMV infection on kidney transplanted patients and the effect of adverse health complications.

3.1 Background: In the preceding chapter, we extensively detailed the pattern of HCMV immunological changes in neonates, with a particular emphasis on viral latency and specific clinical presentations. In our next investigative endeavour, we had to analyse the comprehending the trends of HCMV epidemiology in adult patients who have undergone renal transplantation. HCMV is known to cause end-organ diseases (EODs) in a substantial percentage of transplant recipients, ranging from 20 to 60%. It is important to address the impact of HCMV as it significantly contributes to increased chances of several health complications, which sometimes lead to death in diverse populations, which is favoured by the risk of renal tissue rejection. [1]. It has been documented that HCVM can cause acute infections as well as chronic health complications in renal transplant patients, which leads to drastic deleterious effects where most of the symptoms are observable as fever, diarrhoea, and abdominal pain, but in later stages, several life-threatening symptoms such as leukopenia and other co viral infection like hepatitis, appears which leads to serious health issues. The adverse effect of HCMV associated health complications were found to be directly depending upon the previous exposure to other pathogenic agents and administration of immunosuppressive drugs on both transplant recipient and donor [2]. When evaluating HCMV-associated health adverseness in post kidney transplant patients, it is essential to consider the pre transplanted HCMV infectivity status of the transplanting acceptor and kidney donor. Studies have demonstrated that individuals in the D+/R- and D+/R+ groups face an elevated risk for HCMV-related complications post-transplantation. This underscores the importance of thorough serostatus assessment to effectively manage and minimize the risk of HCMV severity in transplanted patients. [3,4]. Hence, the diligent monitoring of the serostatus of both donors and recipients can play a pivotal role in mitigating the chances of health adverseness and enhancing the overall success rate of transplant procedures.[5]. HCMV infection in post-transplanted cases can result in vascular injury, leading to various complications such as rejection of a renal allograft, thrombotic microangiopathy and atherosclerosis.[6,7]. Moreover, in subsequent instances of human cytomegalovirus (HCMV) association with renal transplant patients following the transplantation, there can be occurrences of bone marrow suppression, leading to decreased production of blood cells, respiratory and gastrointestinal ailments such as diarrhoea and ulcers, and neurological complications including encephalitis and peripheral neuropathy.[8,9,10].To prevent the serious adverseness of HCMV co infection monitoring the patients through the proper medication is a must. It has been observed that prophylactic antiviral therapy can possibly reduce the chances of HCMV infection in transplant patients

[11]. Late-onset HCMV infection refers to the occurrence of other co-pathogenic infections (bacterial or fungal) that are very significant after a short period following a transplant. The correlation between renal rejection and the serious severity of HCMV infection has not been extensively researched. However, it is crucial to effectively manage and vigilantly monitor HCMV infectivity following transplantation. Previous investigations reported that acute HCMV infection can seriously change the immunological parameters of the patients and lead to gradual renal rejection within a few months of transplantation, highlighting the importance of proactive measures in preventing and addressing HCMV infections in transplant recipients. [12,13].

In this chapter, we have meticulously documented a comprehensive report based on the findings of a study focused on 240 patients who underwent kidney transplant procedures and admitted to the Department of Nephrology at SSKM Hospital in Kolkata, India. The report delves into various aspects such as post-transplant outcomes, long-term prognosis, and the impact of different immunosuppressive regimens on patient recovery and quality of life. The goal of the investigation was to acquire knowledge about latent Human Cytomegalovirus infection by analyzing different biochemical and immunological characteristics related to transplanted kidney rejection, such as patient demographics, post-transplant complications, immunosuppressive therapy regimens, viral load measurements, and long-term graft outcomes.

3.2 Methodologies:

Patient Selection

The study aimed to comprehensively analyze the impact of Cytomegalovirus infection following kidney transplanted patients in a five-year follow-up study. At the beginning of the study, a total of 252 patients were selected for renal transplant and subsequently tested positive for Human Cytomegalovirus (HCMV) infection. This investigation was carried out at a specialized hospital and utilized a retrospective data analysis and prospective follow-up methods. The study sought to elucidate the long-term impact of CMV infection on post-transplant outcomes, including graft survival, rejection rates, and overall patient health. The research observed the patients' past HCMV antibody status and other medical indicators, recorded the effects of the post-transplant situation, and carried out subsequent inquiries to establish a connection of HCMV-related renal rejection. Out of the 252 patients who underwent the transplant procedure, 12 individuals were excluded from the study. Among these, 9 patients were excluded due to discontinued follow-up, and 3 patients were excluded due to other health complications that unfortunately resulted in their passing within one year of the transplant. During the 5-year period following transplantation, the remaining 240 patients were regularly monitored for initial and follow-up investigations of HCMV antigenemia and real-time PCR studies for accurately measuring viral load testing for 6-

month intervals. This monitoring was conducted in accordance with ethical guidelines, the study was approved by the institutional ethics committee, aligning with the principles of the 1964 Helsinki Declaration. Furthermore, the study itself received approval from the ethics committee of the IPGME&R, SSKM Kolkata, by initial support from Nephrology Department of , SSKM hospital, Kolkata. All clinical and biochemical features from both study populations along with control groups, were meticulously recorded during each observational period. In addition to clinical data, routine serum biochemical reports, including comprehensive metabolic panels and kidney function tests, were diligently documented for further analysis. The selection criteria for renal rejected patients involved a thorough assessment based on histopathological observation of antibody mediated rejection techniques (ABMR). For patients with negative ABMR reports, further confirmation of renal rejection was sought through renal tissue biopsy, which involved microscopic examination of the kidney tissue to identify signs of rejection at the cellular level.

Sample Collection

5 to 10 mL of freshly collected blood samples were subjected to PBMC isolation from both initial investigation as well as follow up study in an EDTA vial. After the successful isolation of PBMC from the collected whole blood the remaining serum and PBMC stored at -80°C for later investigation.

HCMV Antigenemia assay and antiviral administration:

To determine the number of HCMV-infested peripheral blood mononuclear cells, an HCMV pp65 antigenemia assay was performed by using a Millipore pp65-3247x antigenemia assay kit through which lower matrix protein of HCMV (pp65) can easily be qualitatively measured. Patients were considered positive for human cytomegalovirus antigen (HCMV-Ag) if a single positive HCMV-infested cell was detected in $2.5X10^5$ leukocytic cells in their blood samples. Upon testing positive, these patients underwent weekly monitoring from the initial month of antiviral medication following the operation. Subsequently, they were monitored every three months, and followed-up continued for the next two years during the chronic phase of HCMV infection. This monitoring schedule was designed to closely observe the patient's response to treatment and disease progression over an extended period. After the administration of antiviral therapy, the previously HCMV-positive patients were again monitored through antigenemia assay for another year every three-month interval on the basis of a visible number of viral antigenemic cells. For previously antigenemia-negative patients, the follow-up screening was performed in the same manner.

Positive HCMV-Ag patients were subjected to either orally prescribed antiviral drug or intravenous injection of ganciclovir. The dosage ranged from 0.5-0.75 mg/kg/day and was

adjusted based on the biochemical characters and immunological standpoints of the respective patients. The treatment duration was 20-25 days. The dosage was further adjusted according to the successive crosschecking of the antigenemia report till HCMV antigenemia levels were under the threshold value for the infection. Before the transplant, all individuals were subjected to received antiviral prophylaxis for eliminating the chances of later infection. The therapeutic dose for this prophylactic treatment was 500 mg/kg/day for a duration of 15 days, as determined by clinicians' observation [14].

Quantification of HCMV viral copies for determination of viral load:

The extraction of viral DNA from the lymphocytes, as well as qualitative and quantitative determination of HCMV viral titer, were reported in Chapter 1. Before the transplant and during the initial screening of the HCMV infectivity after the operation, HCMV UL-83 and UL-55 (gB- glycoprotein B) genes were assessed for qualitative and quantitative estimation, whereas in follow-up analysis, the quantitative screening was done using glycoprotein B gene transcript as well as specific gene US-28 gene which is responsible for delayed expression (latent infection) of the virus. The details of PCR protocols are mentioned in Chapter 1.

Biostatistics:

In this research, SPSS version 25.0 was used for conducting the statistical procedures. Continuous variables of different groups were measured using either Student's t-test or Welch's test based on the data distribution. For the categorical features, significance of the groups was measured by Chi-square followed by the confirmatory of Fischer’s exact tests were used to analyze the within the study population. Furthermore, to conduct multivariate analysis and assess the relationship between multiple independent variables and a binary outcome, regression model followed by multiple logistical way with the the statistical significance were calculated by 95% CI.

3.3 Result of the Investigation:

Demographic distribution of the patients:

In this research, post-transplantation patients were categorized based on their HCMV-Ag positivity or negativity and qualitative PCR assay outcomes. Among the selected 240 patients, male and females were 182 and 58 respectively. It has been observed that among them 79 patients (32.91%) were positive whereas 161 (67.08%) patients were found to be HCMV negative. The study samples form a wide range of demographic (rural and Urban) area where no significant variations were observed on HCMV status (p=0.356). The age groups of HCMV infectivity groups were also insignificant with the median age of 46-53 years (p-0.894). After the initial screening of HCMV status the observational standpoints of follow up

studies were conducted for a at least 60-66 months, where the positive patients were minutely monitored for minimum 62 months for observing the disease severity. Among the 240 samples, there were 187 living donors, with 65 (34.75%) testing positive for HCMV and 122 (65.24%) testing negative. Additionally, there were 53 deceased donor samples, with 14 (26.41%) testing positive for HCMV and 39 (73.58%) testing negative. This information provides a comprehensive overview of the HCMV positivity rates among both living and deceased donors in the study. Among the deceased donors it was observed that, 14 tested positive for viral infection (26.41%), while 39 tested negative (73.58%). On the basis of ABO blood compatibility, no significant variations in HCMV positivity were observed (p=1.000). it has been documented that 93 patients received kidneys from any close member of their family, whereas 147 received them from non-blood-related donors. Demographic investigation among the related donors it was found that HCMV positivity was 32.91%, whereas in the case of non-related donors, 67.08% donors were found to be HCMV positive. Patients were divided into four distinctive groups based on the HCMV status of the donor and recipient such as: D-/R-, D-/R+, D+/R+, D+/R-. in the post-transplant scenario, it was observed that the slightly high no of HCMV-positive cases was observed in D+/R- groups (35.44%) in comparison to D+/R+ cases (31.64%) and D-/R+ cases (32.91%). An interesting discovery was made regarding the correlation of HCMV infection and possible renal rejection. Among the 79 cases with HCMV infection, 22 patients experienced rejection within two years of their transplant, with 13 of them having HCMV infection (p=0.004). Additionally, renal rejection was observed within 33 patients after two years of transplantation, and 22 patients among them were found to be HCMV positive even after antiviral therapy (p<0.001). Renal rejected patients were also tested positive for both histopathology report associated with HCMV antibody and tissue punch biopsy. Out of the 13 early renal rejection cases, seven tested positive for antibody responses, and six were confirmed through biopsy. This study on late renal rejection was found to be accurately determined by punch biopsy in comparison with antibody-mediated histopathology. Out of 22 positive cases, biopsy correctly diagnosed 20 patients, while antibody-mediated histopathology only correctly diagnosed a fewer number of cases. This difference was found to be statistically significant with a p-value of 0.027, indicating the strong superiority of biopsy in accurately diagnosing late renal rejection. **(Table 3.1)**

Table 3.1: Status of HCMV infectivity by analyzing the demographic character of patient groups followed by Univariate comparison.

	HCMV infectivity status			**Significance**
	No Viral infection (N=161)	**Viral infection (N=79)**	**Total sample (N=240)**	
Tested individuals				
Age in years (median)	49(47-53)	48(46-51)	49(46-53)	0.894
Male	133 (82.60%)	49 (62.02%)	182 (75.83%)	
Female	28 (17.39%)	30 (37.97%)	58 (24.16%)	
Geographic Location				0.356
Urban	82 (50.93%)	45 (56.96%)	127 (52.91%)	
Rural	79 (49.08%)	34 (43.03%)	113 (47.08%)	
Time for continuous follow-up (months)	62(60-63)	64(66-61)	63(60-66)	
Type of Recipient				0.157
Graft matched donor	67 (41.61%)	26 (32.91%)	93 (38.75%)	
Graft non-matched donor	92 (57.14%)	53 (67.08%)	147 (61.25%)	
Types of Donor				0.244
Alive donor	122 (65.24%)	65 (34.75%)	187 (77.91%)	
Donor previously deceased	39 (73.58%)	14 (26.41%)	53 (22.08%)	
Blood groups reactivity				1.000
Compatible blood group	91 (56.31%)	44(55.69 %)	135 (56.25%)	
Non-compatible blood group	70 (43.47%)	35 (44.30%)	105 (43.75%)	
Pre-transplanted HCMV immunological status				0.164

D+,R+	34 (21.11%)	25 (31.64%)	59 (24.58%)	
D+,R-	51 (31.67%)	28 (35.44%)	79 (32.91%)	
D-,R+	38 (23.60%)	26 (32.91%)	64 (26.66%)	
D-,R-	38 (23.60%)	0 (0%)	38 (15.83%)	
Renal Rejection				
Less than 2 year	9 (5.59%)	13(16.45 %)	22 (9.16%)	0.004
More than 2 year	11 (6.83%)	22 (27.84%)	33(13.75%)	<0.001
Rejection within two years of transplant				0.027
Histopathology (+)	5 (3.10%)	2 (2.53%)	7 (2.91%)	
Histopathology (-)	6 (3.72%)	20 (25.31%)	26 (10.83%)	
Punch biopsy report	6 (3.72%)	20 (25.31%)	26 (10.83%)	
Rejection after two years of transplant				1.000
Histopathology (+)	5 (3.10%)	7 (8.86%)	12 (5%)	
Histopathology (-)	4 (2.48%)	6 (7.59%)	10 (4.16%)	
Punch biopsy report	4 (2.48%)	6 (7.59%)	10 (4.16%)	

Comparative account of clinical and biochemical factors of renal rejected patients with respect to HCMV infection within less than two years of transplant.

The study was carried out to compare the clinical and biochemical parameters in renal transplant patients who tested positive for HCMV and experienced rejection within two years of the transplant with those who did not experience rejection. Since most of the donors in this study belonged to donor-positive groups (in the D+R+ and D+R- groups), the association of HCMV-dependent renal rejection was thoroughly examined. Patients who underwent renal transplants and experienced rejection showed few symptomatic clinical manifestations associated with HCMV infection. However, insignificant correlations were observed within asymptomatic and symptomatic manifestations of HCMV. All patients received antiviral drugs for precautions before the transplant. However, as a diverse group of patients within this cohort, a huge number of cases were experienced with reoccurring HCMV infections,

leading to renal rejection. However, no correlation of HCMV positivity in pre-transplantation scenarios in respect to post-transplant condition was found to be co-related with HCMV-mediated renal rejection. In a comparative study of clinical parameters in HCMV-positive renal rejected and non-renal cases, it was observed that renal-rejected patients exhibited a statistically significant increase in fever (p=0.017) and infections in the urinary tubes (p=0.007) in comparison to controlled HCMV infected patients with no rejection. However, parameters such as hematuria, leukopenia, diabetes, and diarrhoea did not demonstrate significant variations between these two groups. Additionally, the analysis of serum biochemical parameters revealed that SGPT, ALP, and creatinine levels did not show significant differences in early rejection cases between the groups. **(Table 3.2)**

Table 3.2: comparative assessment of different risk factors among HCMV-positive cases with graft rejection within less than two years of transplant through multiple regression model

	Renal rejection of HCMV-positive samples less than Two year (N=13)					
	Univariate test			**Multivariate test**		
Risk Factors	**Odds Ratio**	**P value**	**95% CI [lower-upper]**	**Odds Ratio**	**P value**	**95% CI [lower-upper]**
HCMV clinical manifestation transplant						
Asymptomatic	0.54	0.121	0.24-1.18			
Symptomatic	1.93	0.457	0.81-3.71			
Symptoms						
Pneumonia	0.63	0.213	0.30-1.30			
Fever	2.88	**0.017**	1.01-8.21	7.81	**0.035**	1.16-52.56
Leukopenia	0.72	0.470	0.30-1.73			
Diabetes	0.86	0.768	0.33-2.23			
Hematuria	0.96	0.925	0.44-2.10			
UTI	3.13	**0.007**	1.11-8.79	9.97	**0.017**	1.50-56.21
Diarrhea	1.98	0.095	0.81-4.86			

Biochemical parameters						
Alkaline phosphatase	0.94	0.981	0.56-1.74			
creatinine level	1.23	0.470	0.67-2.25			
SGPT	1.08	0.833	0.51-2.21			
Reoccurred HCMV infection after HCMV prophylaxis						
Recipient positive	1.03	1.000	0.53-1.97			
Donor positive	1.12	0.689	0.72-1.74			

Comparative account of clinical and biochemical factors of renal rejected patients with respect to HCMV infection after two years of transplant.

An analysis was carried out on cases of renal rejection related to HCMV to evaluate clinical symptoms and the relationship between post-transplanted HCMV positive donors/recipients with receving antiviral therapy without rejection and persistent HCMV-infected post- renal rejected cases. In the initial analysis, a significant link was observed that a significant number of HCMV-infected patients with no viable symptoms can lead to renal rejection after two years of transplant($p=0.040$). However, upon further analysis involving multiple factors, this connection was deemed to be not statistically significant. Additionally, the initial test revealed a significant correlation between the recurrence of HCMV infection in late renal rejection cases and recipients who were positive for HCMV before the transplant ($p=0.011$). Nevertheless, this relationship also lost its significance in the multiple regression analysis of the categorical characters. Notably, specific clinical parameters were found to have found to be significantly impactful in cases of late rejection. The presence of blood in the urine (hematuria) showed a significant correlation ($p=0.032$), along with persistent urinary tract infections being identified as a notable risk factor ($p=0.047$). Moreover, the occurrence of diabetes was found to be significant ($p=0.005$) compared to patients who tested positive for HCMV but did not experience rejection. Although a low white blood cell count (leukopenia) was initially considered a significant risk factor, further analysis showed no viable significance in this parameter. In terms of biochemical markers, an elevation of creatinine level in patients' blood was found to be directly associated with HCMV infected renal jected casees which were observed after two years of transplant in respect ot HCMV control no rejected group.($p<0.001$) **(Table 3.3)**

Table 3.3: comparative assessment of different risk factors among HCMV-positive cases with graft rejection within more than two years of transplant through multiple regression model

	Renal rejection of HCMV-positive samples more than Two years (N=22)					
	Univariate test			**Multivariate test**		
Risk Factors	**Odds Ratio**	**P value**	**95% CI [lower-upper]**	**Odds Ratio**	**P value**	**95% CI [lower-upper]**
HCMV clinical manifestation transplant						
Asymptomatic	**1.78**	**0.040**	**0.97-3.25**	2.08	0.060	0.47-11.11
Symptomatic	1.47	0.280	0.80-2.69			
Symptoms						
Pneumonia	0.82	0.562	0.43-1.56			
Fever	0.75	0.362	0.40-1.38			
Leukopenia	2.25	**0.036**	0.97-5.20	3.52	0.193	0.52-23.52
Diabetes	2.70	**0.005**	1.19-6.09	9.33	**0.023**	1.24-70.01
Hematuria	1.92	**0.032**	0.99-3.75	8.30	**0.029**	1.23-55.80
UTI	2.01	**0.047**	0.94-4.22	2.14	0.419	0.33-13.59
Diarrhea	1.37	0.436	060-3.11			
Biochemical parameters						
Alkaline phosphatase	0.67	0.271	0.33-1.36			
creatinine level	2.35	**<0.001**	1.24-4.44	9.43	**0.023**	1.35-65.59
SGPT	0.75	0.441	0.36-1.55			
Reoccurred HCMV infection after HCMV prophylaxis						
Recipient positive	**2.14**	**0.011**	**1.12-4.09**	1.32	0.406	0.49-12.68

Donor positive	1.14	0.381	0.91-1.44			

Quantitative assessment of immunological parameters in respect to viral infectivity for renal rejection:

Through antigenemia assay, it was discovered that there was a notably higher average number of HCMV-positive cells (9 ± 2 cells) transplant rejected cases within two years of transplant, with respect to patients infected with HCMV and were not prone to renal rejection (6 ± 2 cells) ($p = 0.030$). However, Late renal rejection shows fewer positive HCMV cells in leukocytes compared to early rejection, with no significant variations ($p = 0.224$). In the analysis of viral quantified DNA, it was noted that early rejected (within two years) patients showed significantly high viral copies compared to HCMV infected non-rejected patients (p-0.007). Additionally, in cases of late rejection, there was a decrease in viral log copy, but a notifiable difference was still evident, with significantly high HCMV viral copies in renal rejected patients ($p<0.001$). In a study, the quantitative estimation of CD4+ cells were conducted on both the study groups. The findings revealed that in the early cases, no significant variations in CD4+ cell counts were observed ($p=0.384$). However, the rejected cases reported after two years of transplant were found to be with persistent HCMV (Human Cytomegalovirus) infection, and there was a significant reduction in CD4+ cell counts ($p<0.001$) compared to HCMV-infected non-rejected patients. These results suggest a potential association between late renal rejection and reduced CD4+ cell counts in the presence of persistent HCMV infection. In a study of biochemical parameters, it was observed that elevated creatinine concentration in early rejection cases was statistically insignificant among the groups ($p=0.331$). However, a notable significant increase in creatinine concentration (2.62 ± 0.81 ng/dL) was identified in the delayed rejection patients, with a statistically significant difference ($p<0.001$). This finding suggests that creatinine levels may serve as a valuable indicator for distinguishing between different rejection scenario. **(Table 3.4)**

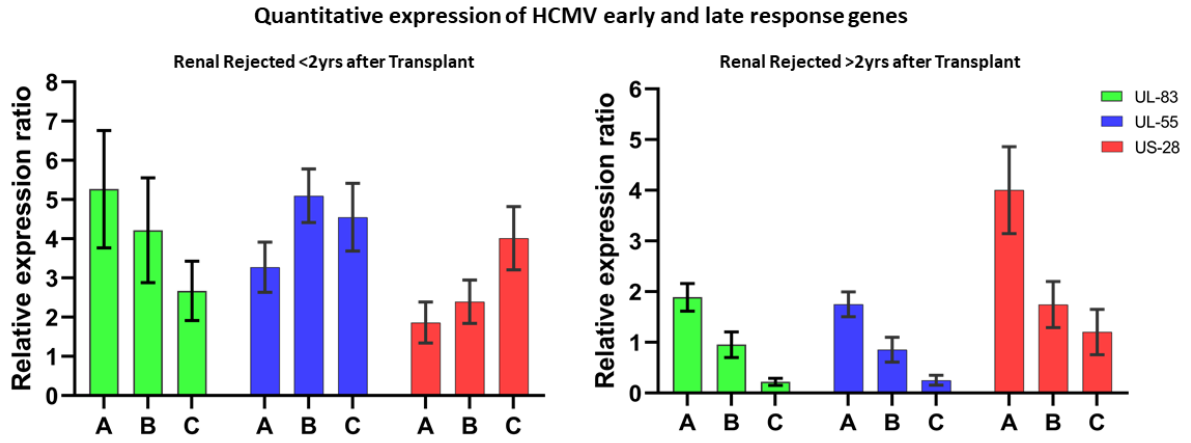
Table 3.4: Assessment of HCMV associated renal rejected cases with respect to different immunological and viral factors through student t-Test

		Early rejected patients (within two years of transplant)				**Late rejected patients (after two years of transplant)**			
		Mean ±SD	**P Value**	**95% CI**		**Mean ±SD**	**P Value**	**95% CI**	
				Upper	**Lower**			**Upper**	**Lower**
Antigenemia (no of	Renal Rejection	9.15±2.41	0.030	-4.194	-0.225	1.71±1.05	0.224	-0.850	0.208

cells/250000 leukocytes)	No Rejection	6.94±2.98				1.39±0.62			
CMV viral load (DNA log copies/µg)	Renal Rejection	7.84±0.89	0.007	-0.509	-0.343	2.96±0.94	<0.001	-1.986	-0.987
	No Rejection	6.34±1.08				1.47±0.71			
CD-4+ Cell count	Renal Rejection	515.68 ±261.9	0.384	-279.20	110.34	460.36 ±252.10	<0.001	121.94	411.48
	No Rejection	430.34 ±261.3				827.24 ±243.78			
Creatinine level (mg/dL)	Renal Rejection	1.57±0.76	0.331	-0.756	-0.267	2.62±0.81	<0.001	-1.794	-0.912
	No Rejection	1.33±0.53				1.26±0.67			

The relative gene expression through m-RNA transcriptional levels of HCMV major immediate early and late response genes showed distinct patterns in both early and late renal rejection patients. The study involved conducting initial observations of rejection, by histopathological observation or tissue biopsies. Subsequently, the patients were closely monitored with follow-up assessments at 3-month intervals for a duration of up to 6 months to track any changes in gene expression profiles and renal rejection status. In early cases of rejection, the expressivity of the UL-83 and UL-55 genes were significantly higher ($p<0.001$) at 5.26, 3.27 and 4.21, 5.09 respectively, compared to the HCMV latent gene US-28 with expressing fold changes of 1.86 and 2.39. In late cases of renal rejection, the relative expression of US-28 (ratio 4.02 and 1.76) was found to be highly significant ($p<0.001$) in comparison to the expression ratios of HCMV early response genes UL-83 and UL-55, which were 1.39, 0.95 and 1.75, 0.85 respectively, at the initial observation.

Image 3. 1: Quantitative HCMV gene expression of early and late renal rejected patients in different time frame[A]. immediate screening after transplant; follow-up checking after antiviral medication after three months [B]; and six months [C]



<u>Comparative assessment of distinguishable clinical parameters of HCMV infected rejected and non-rejected patients:</u>

A distinct statistical study was conducted to compare renal transplant patients who experienced rejection with and without HCMV infection. The objective was to evaluate the impact of different risk factors on the patients' outcomes. The analysis focused on discerning variations in various parameters associated with the patients' conditions. The results revealed that several factors significantly influenced HCMV-induced renal rejection. The study definitively identified haematuria, leukopenia, diabetes, and urinary tract infection (UTI) were the main features strongly correlated with HCMV-associated renal rejection ($p<0.001$). An unequivocal finding revealed the prevalence of elevated creatinine levels in both HCMV-induced and non-induced cases of renal rejection, indicating its significance as a pivotal biochemical indicator for impaired kidney function. These robust findings underscore the crucial role of monitoring these parameters in evaluating and addressing renal rejection. In renal transplant recipients with HCMV infection, marginal elevations were observed in markers such as alkaline phosphatase and SGPT; however, these differences did not reach statistical significance. Conversely, a notable reduction in CD-4+ cell count, crucial for immune function, was evident in these patients. ($p=0.031$). This indicates that HCMV could potentially weaken the host immunological functions, leading to an increased risk of renal rejection. **(Table 3.5)**

Table 3.5 Comparison of specific biochemical parameter of HCMV dependent and independent renal rejection through univariate analysis.

	Total number of renal rejected patients (N=55)		
	Positive HCMV status (n=35)[%]	**Negative HCMV status (n=20) [%]**	**Significance**
Clinical factors for rejection			
Fever	20 (57.14)	8 (40)	0.160
Pneumonia	16 (45.71)	7 (35)	0.476
Hematuria	23 (65.71)	5 (25)	**<0.001**
Leukopenia	24 (68.57)	6 (30)	**<0.001**
Diabetes	26 (74.28)	6 (30)	**<0.001**
Diarrhea	22 (62.85)	11 (55)	0.155
UTI	24 (68.57)	6 (30)	**<0.001**
Biochemical factors for rejection			
High SGPT	18 (51.42)	9 (45)	0.164
High Alkaline phosphate	16 (45.71)	11 (55)	0.339
Elevated Creatinine level	20 (57.14)	13 (65)	0.547
High CD-4+ Cell count	14 (47.17)	28 (70)	**0.031**

3.4 Discussion

Renal rejection comes in two primary forms: acute and chronic. Acute rejection poses a risk in a very short time frame following a transplant, as the body's immune system may launch an attack on the newly transplanted organ.[17]. Patients who have undergone kidney transplants are known to frequently experience cytomegalovirus (HCMV) infection. This viral infection can significantly raise the morbidity rate and reduce the longevity of the transplanted organ, leading to an increased risk of acute renal rejection.[18] Chronic renal rejection, a long-term complication following kidney transplantation, is characterized by gradual deterioration of the transplanted kidney function, resulting from persistent immune-mediated damage.[19] According to a previous study, the influence of HCMV on the onset of chronic rejection is contingent on two potential factors. Firstly, the virus might hinder effective treatment. Secondly, individuals undergoing treatment for acute rejection might experience the increased activity of the dormant HCMV virus.[20] Prior studies have demonstrated that inactive HCMV presents a specific threat to the long-term function of transplanted kidneys. It has been recognized as a standalone major risk factor of graft failure, surpassing the risk of mortality. These findings underscore the notion that dormant viruses can impede adverse impacts on grafted tissue [21]. Our comprehensive investigation revealed that high HCMV viremia during the rejection within two years of transplant significantly influenced, without any discernible symptoms apart from infections in the urinary tract. The findings also indicates that viremia exceeding 500 viral DNA copies per microgram can lead to significant health issues and increase the risk of kidney rejections.[22]. It has been noted

that the use of prophylactic but it's not always guaranteed that administering HCMV drugs to both HCMV-positive living donors and recipients before a transplant will prevent the chances of viral recurrence. This suggests the existence of additional contributing factors that can lead to HCMV recurrence despite prior treatment. The need for more research on HCMV recurrence in transplant cases is crucial to develop better prevention and management strategies. For suspected rejection associated with HCMV infection, a combination of histopathological approaches with HCMV-specific antibodies and tissue punch biopsy can diagnose early rejection. However, in cases of prolonged HCMV infection post-transplantation, punch biopsy is significantly superior in confirming rejection. In our study, we found that patients experiencing early rejection had a significantly higher HCMV viral load (7.84 ± 0.89 log copies) compared to non-rejected patients. Patients with late rejection initially showed similar levels of DNA viremia. Following antiviral therapy, there was a decrease in viremia compared to the early stages. However, we noted a gradual and significant increase in patients experiencing late renal rejection after two years with a persistent minute viral load. [23]. In early cases of renal rejection, we observed significantly high HCMV pp65 antigenemia reports in respect to later delayed HCMV dependent rejection cases. [24] Despite the absence of HCMV-positive leukocytes, latent HCMV infection appeared to play as a critical pawn in delayed rejection. Due to any gold standard established latent viremia threshold designated to date so, here, antiviral therapy was reinitiated when viremia exceeded 500 copies/µg. Among the patients who received reinitiated therapy, 22 out of 79 did not respond to therapy and increasing HCMV viral load was associated with renal rejection. Out of the late renal rejection cases with latent infections, nine patients passed away undergoing further transplant procedures, while the remaining patients were subjected to transplant. Only 7 out of 13 were successfully transplanted and survived, while 6 patients succumbed to various other health complications.

The study demonstrated that during the initial stages of the transplant process with prominent HCMV infection can cause viral-related renal rejection, Within the time span of two years post-transplant the HCMV UL-83 and UL-55 genes showed higher expression ratios compared to the US-28 gene due to acute infectivity but in late delayed rejection cases, the US-28 gene had a substantially higher relative suggesting that latent HCMV infection may play a significant role in increasing the incidence of graft rejection. Previous studies have indicated that a reduction in CD4+ cell counts (less than 500 cells/mm3) was noticed alongside an ongoing cytomegaloviral infection.[25] and consistently elevated creatinine levels have been identified as a crucial risk factor [26]. Our investigative approaches revealed that in early renal rejection, there is no significant decrease in CD4+ count and no elevation in serum creatinine. However, in late rejection, there is a significant reduction in CD4+ counts which were only possible due to persisting viral infection and a significant increase of

creatinine concentration (2.62+0.81 mg/dL) in delayed rejected patients. These findings suggest that the slight decrease in CD4+ count during latent infection may be a significant risk factor, along with HCMV viremia, for late renal rejection. It was also observed that in both early and late renal rejection cases, infections of the urinary tracts were a very much a significant threat associated with HCMV positive individuals at any time post-transplant, whereas in later cases two parameters were significantly determined the progressivity of rejection i.e. diabetes and hematuria leading to further progression of urinary infection.

Our study is the first exploration of HCMV-induced post-transplantation in the Indian sub population. In order to minimise graft loss, novel antiviral treatments must be developed and immunological modulation in rejected patients with latent HCMV infection must be investigated further.

Chapter-4

Investigation of ethnomedicinal sources as a potential HCMV antiviral therapy: emphasizing on wild medicinal mushrooms

4.1 Background: Human cytomegalovirus (HCMV) is a virus with double-stranded DNA, classified as a member of the beta-herpesvirinae family, and has the potential to serious health complications with poor immune system developing individuals, including those with AIDS. HCMV can also lead to serious birth defects in newborns.[1,2]. In immunocompetent individuals, HCMV infection typically does not cause any symptoms or only mild symptoms. However, this virus can seriously increase lymphotic disease in these individuals. [2,3].Currently, there is no approved vaccine for Human Cytomegalovirus (HCMV) infections. To treat HCMV infections, healthcare providers have a limited number of antiviral drugs at their disposal. These include drugs such as ganciclovir, valganciclovir, cidofovir, foscarnet, and the recently approved letermovir. These drugs are used to manage HCMV infections in various patient populations, and their use depends on factors such as the severity of the infection and the patient's medical history.[4]. It has been observed that ganciclovir treatment can initially so primary toxicity but in later stages it develop neutropenia [5,6]. In immune compromised patients ocular infection leading to retinitis can severely develop proliferative clinical manifestations.[7]. The prolonged use of certain drugs has been found to lead to the emergence of drug-resistant strains of Human Cytomegalovirus (HCMV), posing significant challenges for disease management. A recent study has identified mutations in two key proteins, UL54 and UL97, of the HCMV virus as major contributors to causing structural mutation on specific drug interacting region leading to development of resistance strains. Specifically, various nucleotide polymorphisms in the UL54 gene have been associated with the development of ganciclovir and cidofovir-resistant HCMV strains. These findings shed light on the mechanisms underlying drug resistance in HCMV and highlight the importance of continued research in this area for the development of effective management strategies.[8,9]. Due to the limitations of synthetic compounds, natural compounds extracted from medicinal herbs have emerged as a promising solution for the development of new antiviral therapies. In this context, extensive research has been conducted on various mushrooms and their bioactive constituents to explore their potential antiviral properties. These natural compounds have demonstrated the ability to inhibit the activity of specific viral enzymes, which are crucial for the synthesis of viral nucleic acids, as well as for the adsorption and uptake of viruses into mammalian cells. This research suggests that natural compounds derived from medicinal herbs and mushrooms hold significant promise for the development of effective antiviral treatments. [10]. Some bioactive compounds found in *Ganoderma lucidum*, *Coriolus varsicolor*, *Schizophyllum commune*, and *Lentinus edodes* mushrooms have demonstrated antiviral

properties against immunodeficiency virus and herpesviruses.[11]. One of the major terpenoid compounds extracted from Ganoderma lucidum are termed as Ganoderiol F have been found to inhibit the HIV dependent cytopathic effect in MT4 cell line. This compound holds promise for the development of new treatments or therapies for HIV infection.[12]. Ganodermadiol, which is a triterpene compound isolated from Ganoderma lucidum, has demonstrated a potent antiviral response against Herpes simplex virus type-I (HSV-1). This natural compound shows promise as a potential treatment or preventive measure against HSV-1 infections.[13].

Protein-bound polysaccharides extracted from Trametes versicolor, a type of mushroom also known as Turkey Tail, have demonstrated promising antiviral activity against HIV and cytomegalovirus in laboratory studies. This natural compound shows potential in inhibiting the replication of these viruses and could be a valuable area of research for the development of antiviral treatments.[14] Recent research has revealed that berberine (BBR), a bioactive alkaloid derived from plants, and deguelin (DGN), a flavonoid also derived from plants, have demonstrated the ability to inhibit the lytic replication of the human cytomegalovirus (HCMV) in fibroblast cell lines. This finding suggests the potential of these plant-derived compounds in developing treatments for HCMV infections. [15-17]. The purpose of this study was to explore and identify naturally occurring medicinal mushrooms with significant anti-cytomegalovirus properties. This research aimed to investigate the potential antiviral effects of specific mushroom species and their compounds against cytomegalovirus, a common virus that can cause serious complications in immunocompromised individuals and newborns. The study involved analyzing the antiviral properties of various mushroom extracts and compounds to potentially develop new antiviral treatments or supplements.

4.1 Methodologies:

Isolation and identification of Mushroom:

The fruit body of different wild mushrooms were collected from several distinctive areas of North 24 Parganas, South 24 Pargnas, Nadia. During the collection all the distinctive morphological features were noted and chemical spot identification had performed (Kornerup & Wanscher, 1978). The fruit bodies were collected in sterile containers and brought back to the laboratory. The morphology, anatomy of fruit bodies and measurement of reproductive organs will be recorded. The collected mushrooms were identified by the represented gold standard key of Bakshi,(1971) and Pacioni-Philip (1981).For further Molecular identification ,extraction of genomic DNA and amplification of ITS1-5.8S –ITS2 genes of rDNA by PCR technology few amounts of collected samples were preserved in -20°C

Isolation and extraction of mushroom bioactive components for further analysis

During the rainy season, some local mushrooms from natural habitat, Polyporus alveolar and *Phellinus sp*., as well as two edible Basidiomycota mushroom, *Pleurotus ostreatus* and

Lentinus squarrosulus, were meticulously collected from diverse locations across West Bengal, India. The specimens were then carefully transported to the laboratory using aseptic container to ensure their integrity. Upon arrival at the laboratory, the mushrooms underwent thorough morphological identification using the standard taxonomic keys of Basidiomycota, allowing for accurate classification and detailed study.[18,19]. The basidiocarps were cleaned, air-dried, crushed into powder using a Wiley mill, and sieved. As per the gold standard protocol of Mizuno et al., further extraction procedures were done [20,21]. The 10 g of powdered basidiocarp were combined with 200 ml of 80% methanol (in a 1:20 ratio) and then repeatedly refluxed at a temperature of 60°C ± 2°C for 6 hours. The resulting materials were collected and subjected to rotary evaporation at 56°C ± 2°C for 3 hours to eliminate traces of extracting solvent. The partially dried materials were then placed into a fresh autoclaved centrifuge tube and stored at 4°C.

Standardization of susceptible cell line and preparation of virus culture

The fibroblast lung cell line (human) MRC5 (ATCC/CCL/344-USA) was cultured in DMEM in association with L-Glutamine (2 mM), 5% FBS, Penicillin-streptomycin conjugate (100 IU/ml from HiMEDIA). The cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. For the fluorescence expression of EGFP protein, U373-pUL112-113 (clone 1B4) cells, which stably express Enhanced Green Fluorescent Protein (EGFP) in association of inducible promoter of HCMV IE proteins respective promoter UL112-113 of Human Cytomegalovirus (HCMV), were cultured in DMEM (10% FBS) supplemented with G418 (750 µg/ml), a generous gift from Dr. Anna Luganini from the University of Turin, Italy. The cells were also maintained in a humidified atmosphere with 5% CO_2 at 37°C. [22]. The laboratory strain of Human Cytomegalovirus, designated as AD169, was obtained from the American Type Culture Collection (ATCC) under catalog number VR-538. The virus was propagated in MRC5 cell line in a 5% CO2 environment until a pronounced cytopathic effect was observed. Subsequently, the virus was purified through ultracentrifugation of the infected cells and culture filtrate. The resulting viral stock was then carefully preserved at -80°C to maintain its viability for future experimentation and research purposes.

Determination of the cytotoxicity of the mushroom by non polar solvent (methanol) extract.

Before conducting the cytotoxicity test, each semi-dried extract was dissolved in 0.1% dimethyl sulfoxide (DMSO) to improve solubility. For the cytotoxicity assay, human lung fibroblast cells (MRC5 cells) at a density of 5 X 10^4 cells per well and 1B4 cells were seeded in a 96-well plate. The plate was then incubated at 37°C in a 5% CO_2 environment for 24 hours to allow the cells to adhere and grow. Following the incubation period, the cells were gently

washed with 1X phosphate-buffered saline (PBS) to remove any non-adherent cells, debris, and residual media. After washing, various concentrations of the mushroom extract were applied in triplicate to the wells, with a final volume of 100 µl per well, using complete Dulbecco's Modified Eagle Medium (DMEM). The cells were then incubated again at 37°C in a 5% CO_2 environment for 5 days to allow for the assessment of cytotoxic effects.As a negative control, Triton X-100 at a concentration of 0.1% was used. This detailed methodology ensures that the cytotoxicity assay is conducted systematically and with the necessary controls to accurately assess the effects of the mushroom extract on the cells. After incubating for 5 days, 10 microliters of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent from Hi-Media EZcount was added to each well of the cell culture plate. Following a 5-hour incubation period at 37°C, the purple formazan crystals formed as a result of the reduction of MTT by mitochondrial dehydrogenases. These crystals were dissolved using a 1% DMSO (dimethyl sulfoxide) solution. The absorbance was then measured at 570 nanometers using an ELISA reader (SpectraMax M2) to quantify the amount of formazan formed, which is directly proportional to the number of viable cells. Cell viability was determined using the formula [(Absorbance in each dilution – Absorbance of cell-free blank) / Absorbance of untreated control] × 100%, This method provides a reliable assessment of cell viability and proliferation in the experimental conditions.[23].

MTT based antiviral investigation and determination of 50% effective concentration through different dosage.

The antiviral activity of the selected mushrooms was assessed by conducting experiments using the lab strain AD169 on the MRC5 cell line. To initiate the experiment, MRC5 cells were initially seeded at a density of 4 X 10^4 cells per well in a 96-well plate, followed by an incubation period of 24 hours at 37°C. Subsequently, the wells were carefully washed with 1X PBS and then infected with the AD169 strain at a multiplicity of infection (MOI) of 0.5 PFU/cell in serum-free medium for 6 hours at 37°C.

Following the viral inoculation, the medium containing the viral strains was removed, and the cells were treated with varying concentrations of mushroom extracts ranging from 1 mg/ml to 0.1 µg/ml in triplicate. The plates were then placed in an incubator with 5% CO_2 at 37°C for a duration of 5 days. After the completion of the incubation period, the viability of the cells, which is indicative of the effective concentration of the mushroom extracts, was determined using MTT reagents in accordance with the manufacturer's protocol.. 50% end point was measured as: [(Absorbance of infected cells with test compound- Absorbance of HCMV infected cells)/(Absorbance of control blank-Absorbance of HCMV infected cells)] x 100%.

[24]. The concentration of the extracts, which has been determined to be 50% effective, also signifies a 50% reduction in virus-induced cell mortality.

EGFP expression of reporter cells:

For a qualitative EGFP analysis, the reporter cell line 1B4 cell (a specific type of cell line) were seeded at a density of $1x10^5$ cells per well in 12-well plates and incubated in a 5% CO_2 incubator at 37°C for 24 hours to allow the cells to adhere and grow. The following day, the cells were infected with the Human Cytomegalovirus (HCMV) AD 169 strain at a multiplicity of infection (MOI) of 0.5 PFU/cell for 6 hours to establish viral infection. Subsequently, freshly prepared Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and G418 (750 μg/ml) was added to the infected cells, and the cells were treated with the previously determined half-maximal effective concentration (EC_{50}) of *Pleurotus sp* (PE) and *Lentinus sp* (LE) extracts to assess their impact on viral infection and EGFP expression. EGFP expression, indicative of viral infection, was examined 72 hours post-infection (hpi) under an inverted Zeiss fluorescence microscope to visualized the proportion of infected cells and assess the effects of the treatments on viral replication and spread within the cell population.

Assessment of HCMV viral load

Exploring the impact of mushroom extracts on viral replication, both MRC5 and 1B4 cell lines were infected with Human Cytomegalovirus (HCMV) at a Multiplicity of Infection (MOI) of 0.5 PFU/cell. Following virus adsorption for 6 hours at 37°C, the cells were incubated with an effective concentration of the PE (Mushroom Extract PE) and LE (Mushroom Extract LE). After the HCMV-infected cells were treated, they were collected at hours post-infection (h.p.i.), The extraction of viral DNA was done by using a nucleopore viral DNA isolation kit, and the process of viral load was determined by using the HCMV glycoprotein gH gene described in Chapter 1.

Assessment of time-dependent testing of mushroom crude compound

Prior to the experiment, MRC5 cells were seeded in six wells plate with $1.2X10^6$ concentration in each well and incubated for 24 hr to make the well with 80%confluence. Following the incubation period, the cells were infected with the Human Cytomegalovirus (HCMV) strain AD169 at a multiplicity of infection (MOI) of 0.5 plaque-forming units (PFU) per milliliter and were allowed to absorb the virus for 6 hours in serum-free DMEM. Subsequently, the infection media was replaced with freshly prepared DMEM supplemented with 10% fetal bovine serum (FBS). To assess the antiviral activity, the half-maximal effective concentration (EC_{50}) of phosphonoformic acid (PE), letermovir (LE), and ganciclovir (used as a positive

control) were added to the cells. The plates were then incubated at 37°C in a 5% CO_2 environment. At different time intervals post-infection (12, 24, 36, 48, 60, and 72 hours post-infection), the virus-infected cells were harvested, and the total viral DNA was extracted using the Nucleopore DNA extraction kit. The total viral load was estimated using previously described primers specific to the Human Cytomegalovirus glycoproteins gH.. Viral load calculation was done by: 100 -[(viral copies of treated cell / Viral copies of controlled cell) x 100],

4.3 RESULTS

Measurement of cytotoxicity

It was observed that cytotoxicity of *Phellinus sp* (PhE) and *Polyporus sp*. (PoE) were much more in comparison to other mushrooms like *Pleurotous sp.* (PE) and *Lentinus sp*. (LE) . The 50% cytotoxicity (CC_{50}) of PE was 502.12 µg/ml and 822.28 µg/ml respectively on MRC5 and 1B4 cell line. This suggests that PE extract has a relatively lower impact on cell viability compared to PhE and PoE extracts. Furthermore, a much less promising cytotoxicity in LE. were 429.61 µg/ml and 468.07 µg/ml respectively in the MRC5 and 1B4 cell line. This indicates that LE extract also has a moderate cytotoxic effect on the cell lines tested.

In contrast, both the cytotoxic compounds from PhE and PoE showed toxicity in 1B4 cell lines as well. The CC_{50} value of PhE was found to be 39.08 µg/ml and 40.43 µg/ml on the MRC5 as well as 1B4 cell line, indicating a significantly higher cytotoxic impact compared to PE and LE extracts. Similarly, for PoE, the cytotoxicity was found to be at 53.43 µg/ml and 50.54 µg/ml in respective cell lines, indicating a high level of cytotoxicity. Given the less cytotoxic nature of *Pleurotous sp*. and *Lentinus sp*. mushrooms, they were chosen for further study to investigate any potential antiviral activity against HCMV, in contrast to the high toxicity of *Polyporus sp* and *Phellinus sp* extracts. This selection was based on the aim to explore potential antiviral properties while minimizing cytotoxic effects on the cell lines**.. (Table 4.1)**

Table 4.1: Cytotoxicity and antiviral activity of selected mushrooms

	50% cytotoxicity value*		**50% Antiviral activity (EC_{50})***	**Selectivity index (S.I)=CC_{50}/EC_{50}**
Name of the Mushroom	**MRC5****	**1B4****	**MRC5**	**MRC5**
PE	502.12±3.34	822.28 ±1.27	80.39±1.266	6.24
LE	429.61± 1.59	468.07 ± 0.80	69.48±1.68	6.18
PhE	39.08 ± 1.29	40.43 ± 1.06	-	-
PoE	53.43 ±2.36	50.54 ± 1.56	-	-

* *All the experiments were done in triplicate*

** *Mean value of mushroom extract (µg/ml) represented with S.D.M*

Antiviral activity of the compounds

PE and LE's antiviral effects were assessed utilising the MTT test on MRC5 cell lines. PE's EC_{50} value was 80.39 µg/ml, whereas LE's was 69.48 µg/ml.. These two extracts have selectivity indices [(S.I)=CC50/EC50] of 6.24 for PME and 6.18 for LE. No discernible antiviral response was seen in the case of *Phellinus sp*. and *Polyporus sp*. extracts due to extremely high CC_{50} values. Table 4.1 displays the whole analysis.

Alteration of EGFP expression of HCMV in vitro system in presence of different doses of extracted compounds:

After infecting MRC5 cells with the AD169 strain, we employed different quantities of extracts to evaluate the dose-dependent activity of *Pleurotus sp*. and *Lentinus sp*. The findings, as shown in Figure 1(a), show that at 180 µg/ml and 160 µg/ml, respectively, the crude extracts of *Pleurotus sp*. and *Lentinus sp*. both completely suppress HCMV replication. As seen in Figure 1(b), the EC50 values of ganciclovir were found to be 1.301 µg/ml, while the EC_{50} values of PE and LE were found to be 80.39 µg/ml and 69.48 µg/ml, respectively. Moreover, treatment with EC_{50} values equivalent to 80.39 µg/ml and lower dramatically decreased the number of 1B4 cells expressing EGFP and the qualitative assessment of the HCMV-induced cytopathic impact on the MRC5 cell line. **(Figure 4.1).**

Figure 4.1(a): **The antiviral effect of non-polar solvent (methanol) extracts of *Pleurotus* and *Lentinus* species on the MRC5 cell line was shown to be dose-dependent manner; Figure 1(b): - Ganciclovir EC_{50} dosage estimate using the MRC5 cell line.**

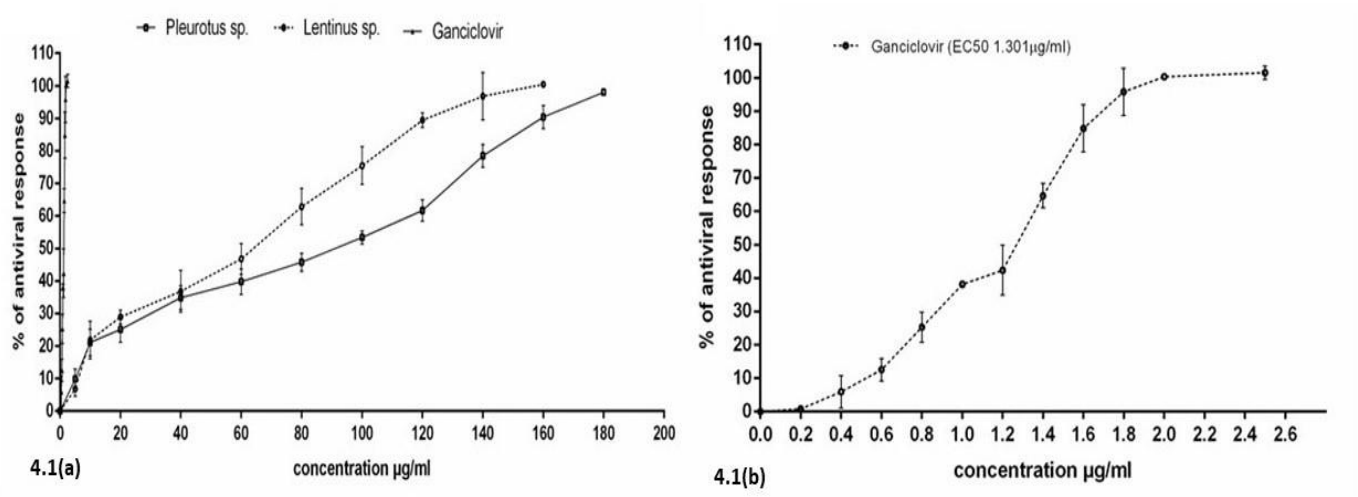
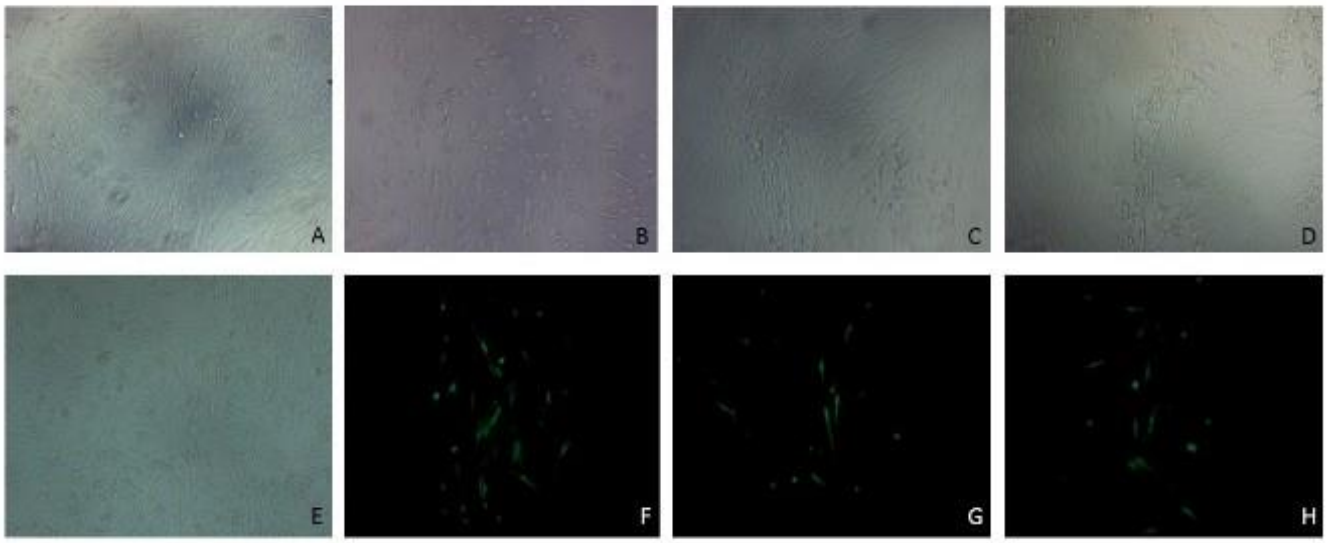


***Figure 4.2*- PE and LE's qualitative antiviral response to AD169-infected MRC5 cell line (A-D) and infected 1B4 cell line (E-H); untreated, uninfected control cell (A, E); untreated, infected control MRC5(B), 1B4(F) expressing EGFP; *Pleurotus sp*. EC_{50} -80.39 µg/ml treated (C, G); *Lentinus sp*. EC_{50} -69.48 µg/ml treated (D, H).**

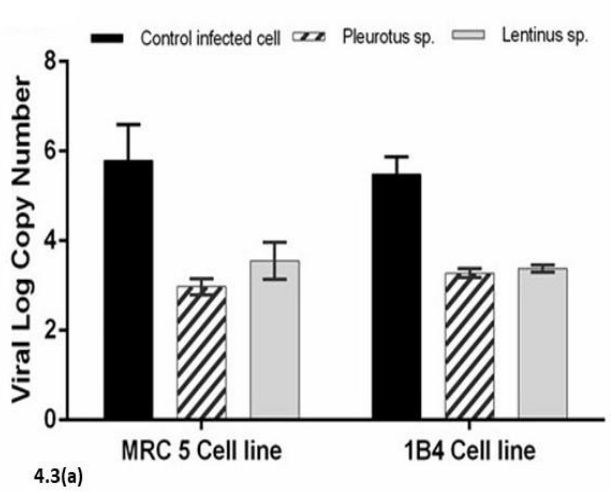


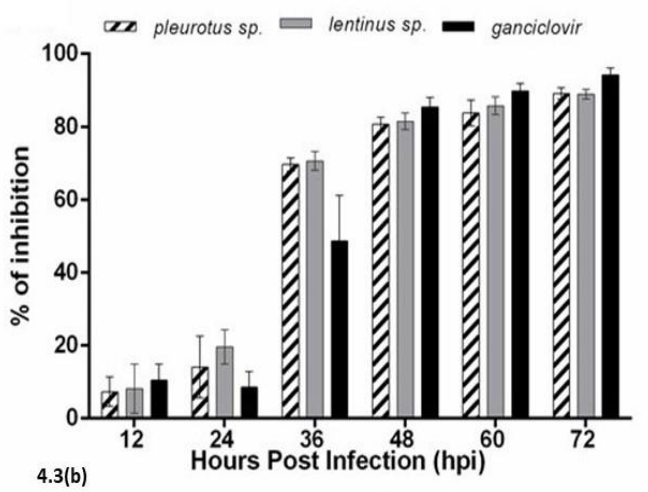
Assessment of HCMV viral load

The antiviral activity of PE (Pomegranate Extract) and LE (Lemon Extract) on both MRC5 (human lung fibroblast) and 1B4 (human astrocytoma) cell lines infected with HCMV (Human Cytomegalovirus) strain AD169 was quantified. The viral load was measured 72 hours post-infection. Viral log copy numbers were determined using a standard curve for the HCMV glycoprotein H gene.

The untreated but infected MRC5 cells and infected 1B4 cells exhibited viral log copy numbers of 5.79 and 5.48, respectively. Upon treatment with PE, a significant decrease in viral load was observed, with log viral loads of 2.97 for MRC5 cells and 3.27 for 1B4 cells. In the case of LE treatment, a slightly less pronounced response was observed on infected MRC5 cells (log viral load of 3.55), while similar responses were noted on infected 1B4 cells (log viral load of 3.38). represented in **Figure:4.3(a).**

Figure 4.3(a): **- Lentinus sp. (LE) and Pleurotus sp. (PE) extracts' quantitative antiviral response to the HCMV viral load. Figure 3(b): - Gancicovir, PE, and LE extracts' time-dependent antiviral response on the MRC5 cell line.**





<u>Antiviral testing on time-dependent checking of extracted compounds of LE and PE</u>

PE and LE's time-dependent responses, when combined with the common medication ganciclovir, demonstrated a noteworthy beneficial reaction. Using the following formula, [100-(Vira log copies of treated cells/Viral log copies in control x100)], reduction of viral load was estimated in every extract treated experimental sets by comparison with untreated infected control cells. In 12 and 24 hours post-injury, the percentage of inhibition for PME-treated cases was 7.30 and 14.04, while for LME-treated cases, it was 8.13 and 19.65. The percentages of viral load inhibition of both LME and PME at 36 hpi were 70.57 and 69.65, respectively. Thus, it is evident from this evidence that the mushroom extracts begin to significantly slow down the spread of viruses at an early initiatory phase of replication. **Figure 4.3(b).**

4.4 Discussion

We have shown in this study that only two wild mushrooms, Pleurotus sp. and Lentinus sp., may have the bioactive constituents which can be act as a promising antiviral agent against HCMV. Out of the four that were analysed for their antiviral activity (*Polyporus alveolari*, *Phellinus sp*., *Pleurotus ostreatus*, and *Lentinus squarrosulus*). The cytotoxic activity demonstrates a unique response contingent on the cell type used. Compared to the MRC5 cell line (502.12 ± 3.34 and 429.61 ± 1.59 µg/ml, respectively), the mushroom extracts from Pleurotus sp. and Lentinus sp. exhibit lower CC50 values (822.28 ± 1.27 and 468.07 ± 0.80 µg/ml, respectively) in the 1B4 cell line.

The findings reveals that glioblastoma and astrocytoma cell lines may tolerate the cytotoxicity of *Pleurotus sp*. mushroom better than the human fibroblast cell line. However, when both cell

lines were treated with Lentinus sp. extract, there was no discernible cytotoxic difference. Upon treatment with *Phelinus sp*. (39.08 ± 1.29 µg/ml in MRC5 and 40.43 ± 1.06 µg/ml in 1B4) and *Polyporus sp.* (53.4 ± 32.36 µg/ml in MRC5 and 50.54 ± 1.56 µg/ml in 1B4 cell line) extracts, comparatively high cytotoxicity was observed on both cell lines. This is due to the high concentration of glycosylated proteins and proteoglycans in both mushrooms, which demonstrate high cytotoxicity and may possess antitumor properties.

[25]. *Phellinus linteus*, a type of medicinal mushroom, contains a β-(1 → 3) linked glycan. This glycan has been found to exhibit anti-metastatic and immunomodulatory properties in cancer cells. Moreover, hispolon, a phenolic compound derived from *Phellinus* species, has demonstrated the ability to trigger programmed cell death in breast cancer cell lines. These findings highlight the potential of compounds derived from *Phellinus linteus* and related species in the development of cancer treatments.[26,27]. Lectins derived from *Polyporus adusta* have demonstrated an anti-proliferative effect on cancer cells.[28]. As previously mentioned, the antiviral response was solely examined using the extract of *Pleurotus sp*. and Lentinus sp. because of the high cytotoxicity of *Phellinus sp.* and *Polyporus* sp. etc. *Pleurotus eryngii* mycelial extract has been found to exhibit antiviral properties against influenza viruses.

[29]. The flavonoid fraction JLS-S001 derived from Lentinus edodes has been found to inhibit the replication of HSV-1 specifically at the late stage of the viral replication cycle.[30]. The EC_{50} values for *Pleurotus sp.* and *Lentinus sp*. were 80.39 ± 1.26 µg/ml and 69.48 ± 1.68 µg/ml, respectively. This suggests that both mushrooms contain unique antiviral compounds. To achieve a full antiviral response, the extracts were administered in a dose-dependent manner. *Pleurotus sp*. exhibited a complete antiviral response at a concentration of 180 µg/ml, while Lentinus sp. extract showed complete antiviral response at 160 µg/ml. The selectivity indexes for both *Pleurotus sp*. and *Lentinus sp*. were 6.24 and 6.18, respectively. HCMV UL-112/113 encodes four phosphoproteins (p34, p43, p50, p84) that enhance the Immediate Early-2 gene-mediated transactivation of early gene promoters.[31,32]. The stable cell line 1B4 transfected with UL-112/113 was utilized for the qualitative screening of antiviral compounds from *Pleurotus sp*. and *Lentinus sp.* extracts through EGFP expression. The number of fluorescent cells observed in plate number 1 infected 1B4 cell line treated with 50% effective concentration of both mushroom extracts was significantly lower (≤ 13 for PE and ≤ 12 for LE) compared to the untreated control cells (≤ 20). Similarly, the number of foci in MRC5 cells was also reduced in the treated cells compared to the infected cells without treatment. We can postulate that the bioactive compounds in the mushroom extracts entered the cells after 6 hours post infection and likely disrupted the efficient transcription of viral genes. To investigate whether these compounds are responsible for interacting with the viral transcriptomes, we conducted quantitative real-time analysis of the glycoprotein H (gH-UL-75) gene. HCMV UL-75 is a late response gene essential for complete infectious virus production and is only expressed through

the activation of IE2 genes associated with UL-79/87/95 proteins. [33]. The MRC5 cell line's log viral copy counts for the untreated, PE-treated, and LE-treated samples were 5.79, 2.97, and 3.55, respectively, according to the quantification of viral load performed using the HCMV glycoprotein H gene as a reference. Similarly, in the 1B4 cell line, the log viral copy counts were 5.48, 3.27, and 3.38 for the untreated, PE-treated, and LE-treated samples, respectively.. Time-dependent antiviral response research showed that both mushroom extracts responded to HCMV replication similarly, with the exception of ganciclovir, which caused the viral load to decrease with time. 48 to 72 hours post-infection (hpi) showed an 80%-90% inhibition, and 36 hpi showed significant reductions in viral load (70%-72%) in both PE and LE as compared to ganciclovir (50%-55%). These results unequivocally show that the crude extracts of both mushrooms successfully prevented the human cytomegalovirus from multiplying in vitro.[34-39]. Our experimental findings indicate that both Punica granatum extract (PE) and *Lippia citriodora* extract (LE) effectively impede the propagation of Human Cytomegalovirus (HCMV). These results suggest that PE and LE have the potential to be developed into alternative natural anti-HCMV compounds. This is a promising discovery that could lead to the development of new treatments for HCMV infections.

Chapter 5

Role of extracellular enzyme laccase on the targeting Human cytomegalovirus replication machinery

5.1 Background: *Human Cytomegalovirus* (HCMV) is a ubiquitous virus belonging to the herpesvirus family. Despite its widespread prevalence, it rarely leads to symptomatic illness in individuals with a healthy immune system. However, it can pose significant risks to individuals with weakened immune systems, such as transplant recipients and people living with HIV/AIDS.[1], It plays a significant role in causing a wide range of serious illnesses in individuals who have received transplants.[2], AIDS patients, and neonatal patients[3][4]. Asymptomatic human cytomegalovirus (HCMV) infection in neonatal patients can lead to hepatosplenomegaly (enlargement of the liver and spleen), cholestasis (decreased bile flow), nephrotic syndrome (kidney disorder characterized by proteinuria, low protein levels in the blood, high cholesterol levels, and hypoalbuminemia (low levels of albumin in the blood) within the first three months of life. In adults with HIV, HCMV can cause diseases including retinitis (inflammation of the retina leading to vision loss), gastrointestinal issues, and pneumonitis (inflammation of the lung tissue), whereas latent HCMV infection in renal transplant patients can lead to late renal rejection, posing a risk to the transplanted kidney. Antiviral drugs currently authorized for treating Human Cytomegalovirus (HCMV) infections work by targeting the viral DNA polymerase (UL-54 gene) activity. These drugs include ganciclovir (GCV), cidofovir (CDV), foscarnet (FOS), and valganciclovir (VGCV). Ganciclovir is a nucleoside analogue of guanosine, cidofovir is a non-cyclic analogue of cytidine monophosphate, and valganciclovir is an L-valyl ester prodrug of ganciclovir. Foscarnet, on the other hand, structurally resembles the pyrophosphate anion and binds to the HCMV polymerase, effectively blocking the pyrophosphate-binding site. This action prevents the incorporation of incoming deoxynucleotide triphosphates (dNTPs) into viral DNA. However, these medications have limitations due to their toxicity, pharmacokinetic challenges, and susceptibility to viral resistance. Extended exposure of human cytomegalovirus (HCMV) to chemical drugs such as ganciclovir and foscarnet frequently leads to the emergence of drug-resistant mutant viruses. These mutants often exhibit mutations in the HCMV UL54 gene, which encodes the viral DNA polymerase, and the UL97 gene, which encodes the viral kinase. These mutations can confer multi-drug resistance, posing significant challenges for combinational therapeutic strategies involving these synthetic drugs. Consequently, there is a critical need to explore and develop new anti-HCMV compounds to effectively combat drug-resistant strains and improve treatment outcomes.

Replicative DNA polymerases, which are enzymes responsible for copying DNA, have the remarkable ability to synthesize long stretches of DNA without detaching from their

templates. This process is facilitated by accessory protein subunits known as processivity factors, which help the polymerases maintain prolonged association with the DNA template.

In the case of the human cytomegalovirus (HCMV) DNA polymerase, the catalytic subunit, encoded by the UL54 gene, is composed of 1242 amino acid residues. This catalytic subunit works in conjunction with an accessory protein, UL44, which consists of 433 amino acid residues. UL44 forms a head-to-head C-shaped homodimer, meaning it is composed of two identical subunits, creating a structure that resembles the letter "C." This unique structure of UL44 facilitates the entry of DNA molecules into the catalytic domain of the HCMV DNA polymerase, allowing for efficient DNA synthesis during viral replication.

In the field of virology, researchers have been exploring various antiviral strategies, one of which involves inhibiting the binding of the HCMV (Human Cytomegalovirus) DNA polymerase accessory protein. This approach shows promise in combating viral infections. Furthermore, while limited research has been conducted on the antiviral properties of mushroom extracts against *Herpesvirideae,* preliminary investigations using a specific extracellular enzyme known as laccase have shown promising results. These results specifically indicate the potential of laccase in inhibiting the entry of the Hepatitis C Virus into peripheral blood cells. This research opens up new avenues for exploring natural antiviral compounds and their mechanisms of action.[5].

In our quest to find alternative antiviral bioactive compounds, our previous research led us to the discovery that bioactive compounds obtained from the crude extract of the edible mushroom *Pleurotus ostreatus* exhibited highly promising antiviral properties. Our findings suggested that these compounds could potentially serve as effective agents against a range of viruses.[6]. The investigation also revealed that the use of crude extracts, specifically from [specify the source, e.g., plant, fungus, or animal], effectively reduced the Human Cytomegalovirus (HCMV) DNA copies in an in-vitro system. This reduction was achieved by inhibiting the activity of the HCMV DNA polymerase. Given that laccase is a proteinaceous structure with potential therapeutic properties, our aim was to explore whether laccase could elicit an antiviral response specifically targeted against the HCMV DNA polymerase.

5.2 Material and Method

Extraction of extracellular laccase enzyme from mushroom *Pleurotus pulmonarius* :

The mycelial strain of *Pleurotus pulmonarius* MTCC 1805, a type of oyster mushroom, was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC) in Chandigarh, India. The strain was cultivated in a laboratory setting using potato dextrose agar (PDA) media, which provided the necessary nutrients for its growth. Subsequently, the mycelium was transferred to a mixture containing 20% potato extract, 2% dextrose, and 0.5% yeast extract for optimal growth conditions over a period of 5 days.

Following the incubation period, the culture supernatant, which is the liquid portion of the culture, was separated from the solid components through centrifugation at 21,000 x g for 30 minutes. The resulting culture filtrate, approximately 1000 ml in volume, was then concentrated through a process known as lyophilisation, which involves freezing the liquid and then removing the ice through sublimation to obtain a concentrated form of the culture filtrate.

Subsequently, 100 ml of the concentrated material underwent precipitation with a gradient of 0–80% ammonium sulphate. This process allowed for the separation of proteins based on their solubility at different levels of ammonium sulphate saturation, enabling the isolation of specific components from the concentrated culture filtrate. ((NH4)2SO4). The resulting protein precipitate was then collected via centrifugation at 80,000 x g for 1 hour at 4°C using a high-speed centrifuge, such as a Beckman Coulter Optima XE-90. The precipitated pellet was dissolved in a 10 mM solution of sodium acetate buffer with a pH of 5.0, which was prepared using high-purity water and analytical grade chemicals. Subsequently, the solution was subjected to dialysis against the same buffer using a dialysis membrane with a molecular weight cut-off suitable for retaining the protein of interest.

Following this, a 5 ml aliquot of the enzyme solution, which was prepared under sterile conditions in a laminar flow hood to minimize contamination, was introduced onto a DEAE-Sephadex (A-50) column measuring 22 x 220 mm. The column had been pre-equilibrated with the sodium acetate buffer, and the sample application was carried out using a peristaltic pump at a flow rate of 1 ml/min. Elution of the enzyme was achieved through the use of a linear gradient ranging from 0 to 0.6 M NaCl, which was prepared using high-purity salt and the sodium acetate buffer. This resulted in the separation of two distinct laccase peaks, which were carefully collected and analysed for purity and activity. After the second peak fractions were obtained, they were concentrated to 1 ml and then dialyzed against 100 mM sodium acetate buffer at pH 5.0 to remove any impurities or unwanted substances. Subsequently, the dialyzed sample was applied to a Bio Gel P-200 column measuring 16 x 650 mm. The active fractions, containing the desired components, were carefully collected and combined. Following this, the combined fractions were concentrated to increase the concentration of the target substances and subsequently dialyzed again using the same 100 mM sodium acetate buffer at pH 5.0 to ensure the purity and stability of the final sample.[7].

Permissive cell line culture and Lab strain virus AD169 culture:

MRC-5 (ATCC CCL-171) cell line was cultured in DMEM with 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/ml) at 37°C and 5% CO_2. AD169 (ATCC VR-538) virus and Towne (VR-977) stock has been prepared and infection has been standardized in MRC-5 cells. In brief, cells were seeded in T75 flask and after 24 hours when cells reached at confluence of 80% media was removed and washed twice with serum free media. Then infection media containing virus were inoculated in the flask and kept overnight. After that infection media

were removed and again washed twice with serum free media and then warm complete media containing 10% FBS were added and kept in 37°C incubator. Every alternate day 5 ml fresh media were added without removing any media. Now cells were observed for Cytopathic Effect (CPE). Once CPE were observed after almost 7 days' media was removed, cell debris were removed by spinning at low speed of 1000g for 5 minutes. Media was processed for ultracentrifugation to concentrate the virus.

Cell Cytotoxicity assay of laccase enzyme:

To screen the cytopathic effect of the enzyme, MRC5 cells were seeded in 96 well plates at the density of 10,000 cells per well. After 24 hrs when cells reach to confluence about 80 % the cells were treated with different amount of serially diluted enzymes. The Triton 0.1% has been used as a negative control. To check the cell cytotoxicity for these compounds they were kept for 48-72 hours keeping untreated wells as vehicle control. The cells were incubated at 37°C in 5% CO2. After 72 hours MTT assay was carried out by EZcount^{TM} MTT Cell Assay kit (Himedia) following the manufacturer's protocol and reading of absorbance was taken at 570 nm. *(The percentage of cell viability has been calculated by the formula described in Chapter 4)*.

Antiviral Response of different concentration of mushroom extract:

Human fibroblast monolayer MRC5 cells were seeded in 96 well plate at a concentration of 10,000 cells per well and kept at 37*C for 24 hrs. After that cells were incubated (8 hrs.) in serum free DMEM with HCMV AD169 virus at 0.5 MOI for the virus absorption. After the incubation, the infection media was removed, and fresh media was added to each well with different concentrations of mushroom extracts (4-12 μM) and the antiviral drug ganciclovir (0.6 – 2.4 μM) in triplicate. 0.1% triton has been used as a negative control. After 7 days of incubation at 37*C in 5% CO_2 viral inhibition rate was carried by MTT test. *(Viral inhibition rate was calculated by the formula described in Chapter 4).* Time-dependent and dose-dependent assays of the following enzyme concentrations were the Gold standard method of Plaque reduction assay [9], and Viral yield reduction assay was performed as per protocol [10].

HCMV DNA quantification in the in vitro system after being treated with variable concentrations of enzyme to analyse the alteration of viral replication

To understand the impact on viral replication machinery, 50% effective concentration dose of the respective enzyme was used on MRC5 cell line as described in Chapter 4 and the cells were harvested from 0,12,24,36,48,60 and 72 hr post infection and subjected to viral genome DNA extraction procedure by Nucleopore DNA Sure mini kit. Extracted DNA was then quantified by HCMV Glycoprotein-H gene by previously described method in Chapter 1. The rate of viral synthesizing DNA was quantified [12].

Expression of cellular metabolic changes depending upon HCMV replication

For understanding the changes in host cellular genes depending upon HCMV replication, quantitative expression of host metabolic phosphofructokinase genes was measured. MRC5 cells were seeded in a 6-well plate in similar way as described above and infecting with different viral strains as well as EC_{50} concentration of laccase enzyme. Total m-RNA was harvested from the 12, 24, 36, 48 and 72 hpi Cellular metabolic transcripts were quantified by $2^{-\Delta\Delta Ct}$ method for relative quantification, for two test genes i.e. phosphofructokinase-1 genes F-5' AGCTGTATTCAGAAGAGGGCAAAG 3' and R-5' CCACCCTGCTGCATGTGA3' as well as pyruvate dehydrogenase genes transcript F-5' GACGAAAAGGAGGTTGTGCTAAAG 3' and 5' CCATTGCCCCCGTAGAAGTT 3' along with cellular β-actin F-5'GCTGTGCTACGTCGCCCTG3' and R-5'GGAGGAGCTGGAAGCAGCC3' as a control.

Preparation of *in silico* modeled structure of laccase and cytomegalovirus DNA polymerase (UL54):

The protein sequence of the laccase enzyme (UniProt ID: O60199_PLEOS; residues 1-533 aa) and HCMV DNA polymerase UL-54 (UniProt ID: B9VXM3; residues 1-1242 aa) was retrieved from the UniProt database. Subsequently, template-based homology modelling was employed to determine the precise structural conformation of these proteins. The crystallographic structure of *Coriolopsis gallica* (PDB ID: 5A7E) served as the template for modelling the conformation of the laccase enzyme, while the structure of DNA polymerase δ of Saccharomyces cerevisiae (PDB ID: 3IAY) was utilized for modelling HCMV DNA polymerase. Furthermore, to investigate the interaction between the major HCMV DNA polymerase-associated protein UL-44 and the laccase enzyme, the crystal structure of UL-44 was obtained from the PDB database (ID: 1T6L).[13] , and dimerization and developing stable conformation[14] During the process of target-specific docking, the amino acid sequence alignment of both proteins was meticulously prepared using the ESPRIPT tool. Subsequently, homology modelling was carried out using MODELLER v 10.4, and the best-modelled structures for both proteins were selected based on MODELLER scoring results and structural geometry. Prior to docking, structural bonding refinement was performed using CHIMERA v1.17.3, and the finalized modelled structures underwent energy minimization using AVOGADRO v1.2.0.

***In silico* docking of laccase enzyme on HCMV DNA polymerase:**

For the first step of flexible protein-protein docking HCMV DNA polymerase and Laccase enzyme was performed in HPEPDOCK 2.0 server (http://huanglab.phys.hust.edu.cn/hpepdock/)[15]. The server utilized structural affinity within the residues of interacting proteins to predict the best 10 models. These models were then

assessed based on relative Root Mean Square Deviation (RMSD) value and docking energy to select the most suitable interacting conformations. Subsequently, each docked complex underwent further analysis in Chimera 1.17.3 to predict the interaction sites. Following this, the best 3D docked structure was chosen for rigid target-specific docking in HADDOCK 2.2.[16] and ClusPro 2.0[17] server. During the subsequent analysis, we further examined the active interacting residues using the PRODIGY [18] server for binding energy prediction.

Dynamics simulation of the *in silico* docked complex in GROMACS dynamics simulation

In order to investigate the conformational changes over time and the mechanism of interaction between the receptor and its ligand, we conducted Molecular Dynamics (MD) Simulation using Gromacs 2024. This simulation allowed us to study the dynamic behaviour of the molecular system at the atomic level, providing valuable insights into the structural changes and the binding dynamics of the receptor-ligand complex[19] . The investigation was conducted on a workstation running Ubuntu 20.04 LTS 64-bit with 16 GB of RAM and an Intel Core i5-7500 CPU processor. Additionally, the workstation was equipped with an 8 GB GTX 1640-Ti GPU processor for enhanced graphical processing. The topology of the receptor and docked molecules was meticulously prepared using the OPLS-AA/L all atom force field to ensure accurate and reliable results.[20]and the solvation of the complex was achieved using the SPC (Simple Point Charge) water model, which is commonly used in molecular dynamics simulations to accurately represent the behaviours of water molecules. This model incorporates parameters to restrain the movement of water molecules, providing an effective way to simulate solvation effects in the system.[21] with cubic periodical boundary conditions. Following the solvation neutralization of the complex, ions were added, and the system underwent energy minimization using the steepest descent algorithm with a Verlet cut-off scheme (less than 1000 KJ/mol/nm). The system was then equilibrated in two phases. First, an NVT ensemble was conducted at 300 K for 5000 picoseconds to stabilize the system's temperature. Subsequently, an NPT ensemble was employed at 1 atm pressure for a 5000 picoseconds NPT simulation. Following equilibration, all systems underwent production Molecular Dynamics (MD) simulation at a stable temperature of 300 K and a fixed pressure of 1 atm. The simulation utilized a time step interval of 2 femtoseconds and implemented the Parrinello–Rahman method for constant pressure simulation. Molecular dynamics simulations were conducted for a duration of 10 nanoseconds. The Hawkdock server was used to calculate the bonding distance between each interacting residue and the free energy.[22].

5.3 Result:

Cytotoxicity report of Laccase enzyme:

To evaluate the cytotoxicity of the Laccase enzyme, MRC5 cells were subjected to a range of concentrations of the enzyme using the MTT assay. The findings revealed that the Laccase enzyme did not demonstrate any cytotoxic effects on the MRC5 cell line, displaying a CC_{50} value of 510.34±0.98 µM. In comparison, cells treated with ganciclovir exhibited a CC50 value of 331.26±1.24 µM. Furthermore, the EC50 concentrations of the Laccase enzyme and ganciclovir were assessed on the MRC5 cell line, as well as on AD169 and Towne-treated cells, using varying concentrations (0.1, 1.0, 10, 20, and 30 µM). The results of the study showed that the half-maximal effective concentration (EC50) dose of Laccase enzyme on AD169 and Towne strains were determined to be 9.46 ± 0.96 µM and 8.58 ± 1.05 µM, respectively. In comparison, in cells treated with ganciclovir, the EC50 dose was found to be 1.86 ± 0.21 µM and 0.95 ± 0.08 µM for AD169 and Towne strains, respectively. These findings provide valuable insights into the differential effects of Laccase enzyme and ganciclovir on the

two strains, highlighting their varying sensitivities to these treatments. **(Table 5.1)**

Table 5.1: Cytotoxicity and antiviral efficacy of Laccase enzyme on HCMV

Test compound	**CC_{50} value (µM)[a]**	**Virus tested**	**EC_{50} value(µM)[b]**	**SI index (CC_{50}/ EC_{50})[c]**
Laccase enzyme	510.34±0.98	AD169	9.46±0.96	53.94
		Towne	8.58±1.05	59.48
Ganciclovir	331.26±1.24	AD169	1.86±0.21	>150
		Towne	0.95±0.08	>250

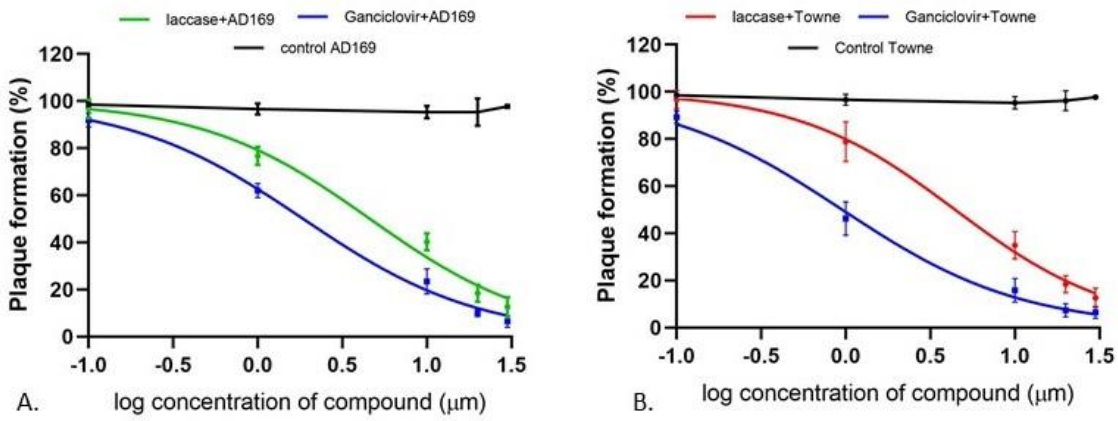
a. *50% endpoint of Cytotoxicity was determined by triplicated experiment calculating Mean value±SD*
b. *Effectivity of the enzyme (50 % end point) was determined by Plaque reduction assay.*
c. *The ratio of CC50 and EC50 was determined as a selectivity index and ganciclovir was used as positive control.*

Antiviral efficacy of tested enzyme on different concentration:

In our comprehensive study, we set out to investigate the extensive antiviral effects of the Laccase enzyme in comparison to ganciclovir. To achieve this, we meticulously conducted a viral plaque reduction assay on the MRC5 cell line, a well-established model for viral infection studies. We meticulously assessed the dose-dependent antiviral responses and rigorously analysed the data using one-way ANOVA followed by Tukey's multiple-comparison tests to ensure statistical robustness. The results of our study revealed compelling evidence of the

antiviral potential of the Laccase enzyme. Specifically, we found that treatment with AD169 led to a noteworthy reduction in plaque formation for both ganciclovir (p-0.019) and the Laccase enzyme (p-0.049) in viral-infected cells. Furthermore, our findings also demonstrated significant reductions in plaque formation in Towne-infected cells treated with ganciclovir (p-0.02) and laccase (p-0.03), highlighting the broad-spectrum antiviral efficacy of the Laccase enzyme. These results provide valuable insights into the potential of the Laccase enzyme as a promising antiviral agent and underscore the importance of further exploration of its therapeutic applications in combating viral infections. **(Figure 5.1)**

Figure 5.1: antiviral efficacy of tested drugs-laccase enzyme (4-12 µM concentration) and positive control drug ganciclovir (0.6-2.4 µM) is represented by log concentration on both AD169 and Towne strains. Data represented by triplicated mean±SD

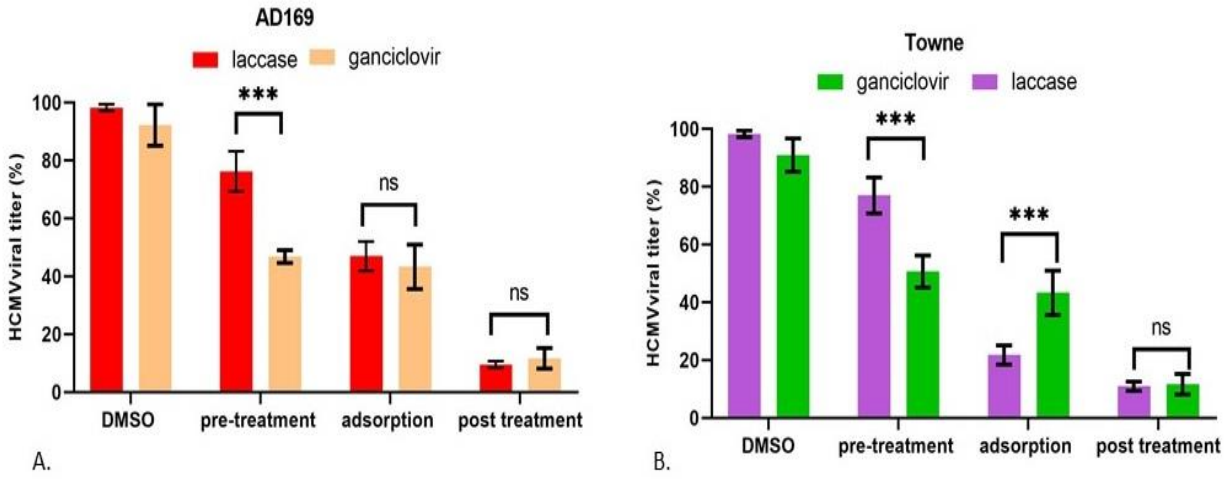


Time dependent antiviral efficacy analysis of laccase enzyme:

In order to conduct a more comprehensive investigation, we delved into the antiviral efficacy of laccase enzymes on various strains of Human Cytomegalovirus (HCMV) infected MRC5 cells at different time points. We utilized a plaque reduction assay to measure the amount of viral titres. This time-dependent assay involved treating the cells with the EC50 concentration of laccase enzyme at different stages: before infection (-2 to -1 hour), during viral absorption (-1 to 0 hours), and post-infection (0 to 72 hours post-infection). Our findings revealed that in each scenario, the cells treated with laccase enzyme exhibited a significant reduction in viral titres compared to cells treated with DMSO. However, we also noted several significant variations in the growth of different viral strains. In the pre-treatment scenario, the antiviral drug ganciclovir was found to significantly reduce the viral titre in AD169 ($p<0.001$) and Towne (<0.001) respectively. This indicates the effectiveness of ganciclovir in reducing viral replication prior to further treatment. In contrast to the reduction in viral titre during the adsorption and post-treatment period, no significant variations were observed between the effects of ganciclovir and laccase treatment ($p=0.557$). This suggests that ganciclovir and

laccase may have similar effects on viral titre during these stages of treatment. However, when comparing the effects of ganciclovir and laccase treatment on Towne-infected cells, a significant reduction in viral titre was observed in the laccase-treated adsorption period ($p<0.001$) compared to ganciclovir-treated cells. This highlights a potential differential impact of laccase treatment specifically in the adsorption period for Towne-infected cells. In the post-treatment scenario, both of these compounds were effectively found to reduce the viral titres in AD169 and Towne treated cells. However, no significant variations were observed between them. This result signifies that laccase not only has the ability to halt viral propagation similar to ganciclovir, but it can also initiate a protective barrier in host cells prior to viral infection. This suggests that laccase could potentially play a dual role in combating viral infections by both inhibiting viral replication and providing a pre-emptive defence mechanism in host cells. (Figure 5.2)

Figure 5.2: Time-dependent antiviral effectivity of Laccase enzyme in comparison with positive control ganciclovir- different time treatments were compared with respect to negative control (DMSO) treated cells viral infected (Both AD169[A] and Towne strains[B]) cells. The triplicated datasets were analysed by one-way ANOVA followed by Dunett's multiple comparison test.

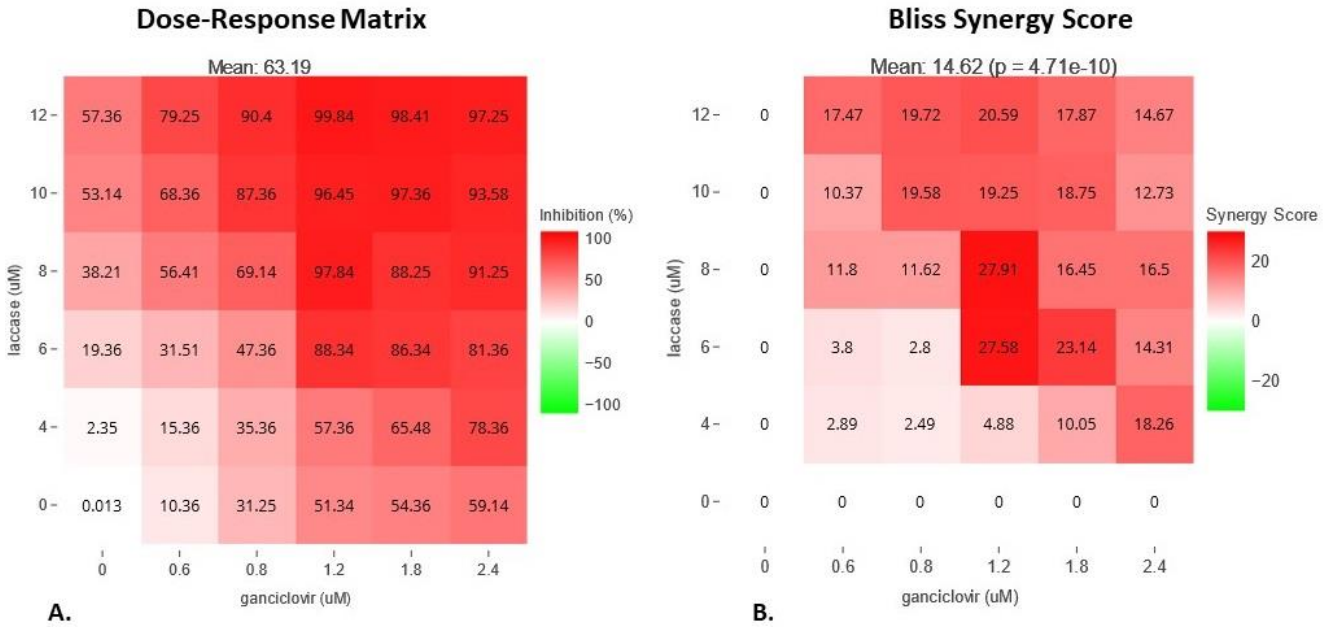


<u>Effect of the combinational effect of different doses of both laccase and ganciclovir on MRC5 cells</u>

Our research has uncovered compelling evidence indicating that both laccase and ganciclovir hold promise in controlling viral proliferation under pre- and post-treatment conditions. The primary aim of our study was to explore the potential synergistic effects of combining these two agents in reducing viral titres. To evaluate the efficacy of laccase in conjunction with ganciclovir, we conducted a comprehensive analysis using a drug combinational therapy approach. Our findings revealed a synergistic effect of laccase when used in combination with

ganciclovir. We treated MRC5 cells with varying concentrations of ganciclovir (ranging from 0.6 to 2.4 µM) and laccase enzymes (ranging from 4 to 12 µM). Subsequently, we performed a viral reduction assay to gain insights into the combined impact of these treatments on viral proliferation. In our study, we found that none of the combinations of both laccase and ganciclovir showed any antagonistic effect, indicating that the mode of action of these two compounds is distinctly different. Through a detailed analysis of the Bliss synergy score algorithm, we observed that the combination of 6 µM and 8 µM laccase concentrations exhibited the maximum synergistic effect when combined with 1.2 µM of ganciclovir. Additionally, moderate synergistic effects were observed when 6 µM of laccase concentration was combined with 1.8 µM of ganciclovir, while slight synergistic effects were observed at higher concentrations of both compounds. **(Figure 5.3**). Furthermore, it was also observed that none of the concentration gradients were reducing the viability of the cells.

Figure 5.3: positive combinational effect (synergy) of laccase enzyme and ganciclovir- [A] dose-responsive matrix on variable concentration of test compounds were measured as % of drug efficacy. [B] Bliss algorithm score pattern of both the drugs to understand the synergistic relationship.



<u>Impact on viral replication machinery and cellular metabolic transcript for controlling replication:</u>

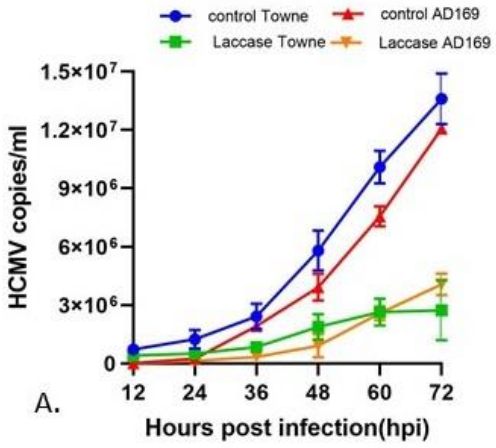
It has been well observed that ganciclovir mainly acts as a nucleotide analogue to inhibit the activity of HCMV DNA polymerase. As it was observed that both these compounds could act synergistically, so we aimed to check the further progression of HCMV inhibition by only laccase enzyme through analysing viral copies and rate of replication in a time-dependent manner.

In order to accurately establish the timeframe for the resolution of newly synthesized viral DNA, MRC-5 cells were infected with a MOI of 0.5 PFU/cell for both strains. This lower infectivity concentration has been consistently utilized in previous experiments to minimize the likelihood of a cell receiving multiple virions and initiating replication with more than one template genome. Consequently, a lower MOI was employed in these experiments to determine the rate of viral DNA synthesis. To determine the change in genome, copy number over time and the rate of nucleotide incorporation, we created a standard curve of HCMV genome copy numbers using purified HCMV ATCC strains (both Towne and AD169). Subsequently, we conducted qPCR with specific primers for HCMV gH genes to quantify the viral DNA copy number at each time point (hpi). The nucleotide incorporation rate was determined by calculating the number of base pairs (bp) synthesized per minute. This was achieved by dividing the total size of the HCMV genome (230,000 bp) by the difference between the logarithm quantity of the final and the initial genome copy number within each post-infection interval. During the early stages of viral infection (12-36 hours post-infection), there is not a significant increase in DNA accumulation compared to later stages of infection (**Figure 5.4A**). However, it is worth noting that there was a noticeable variation in baseline DNA quantity between the AD169 and Towne strains in the control sets. In the absence of any antiviral drugs, the rate of DNA synthesis significantly increased in both AD169 (~226 bp/min) and Towne (~248 bp/min). However, when treated with laccase, the rate of DNA synthesis notably decreased to ~132 bp/min in AD169 and 68 bp/min in Towne. In the subsequent observations during the later stages of post-infection, it was noted that cells treated with laccase exhibited a significantly lower DNA synthesis rate of approximately 105 bp/min at 48 to 60 hpi and 128 bp/min at 60 to 72 hpi during AD169 infection compared to the control sets. In the case of Towne treated with laccase, a much more promising antiviral response was observed where fewer viral copies were synthesized**. (Figure 5.4B).**

The process of viral replication relies on the availability of large quantities of biopolymer subunits and the energy needed for their assembly, which are mainly obtained from the host's respiratory system. Previous studies have demonstrated that HCMV infection can enhance glucose uptake in fibroblasts. The elevated levels of glycolytic intermediates observed during HCMV infection were associated with upregulation of transcript levels of various enzymes that are dependent on respiration [23]. So for the purpose we have tested expression fold of m-RNA transcript of two major respiratory regulatory enzymes from glycolysis and tri-carboxylic acid cycle (TCA) for exact such as phosphofructokinase-1 and pyruvate dehydrogenase respectively. The relative expression of both these m-RNA transcripts were measured on ganciclovir treated as well as laccase treated cells in comparison to untreated (DMSO treated) control sets at 12 to 72 hpi.

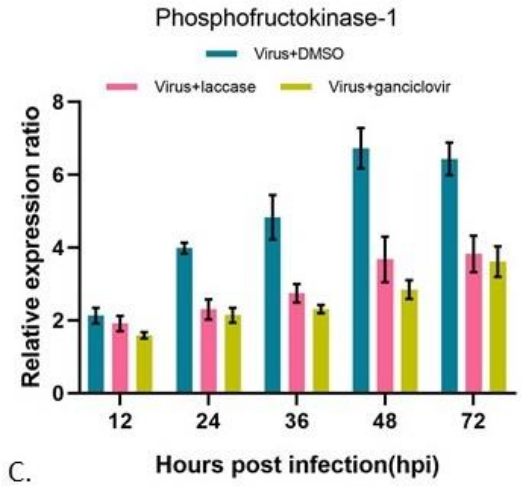
At 12 hours post-infection (hpi), there were no significant changes in the relative expression of phosphofructokinase-1 across all experimental sets. However, in the subsequent 24 to 72 hpi period, the expression of phosphofructokinase-1 was significantly decreased in cells treated with both laccase and ganciclovir compared to control cells (**Figure 5.4C**). During the analysis of pyruvate dehydrogenase relative expression, it was consistently observed that both laccase and ganciclovir-treated cells exhibited a significant reduction in the relative expression of certain enzymes compared to control untreated HCMV infected cells, from the early stages of HCMV infection (12 hpi) to the late stage (up to 72 hpi) **(Figure 5.4D) (Table 5.2)**

Figure 5.4: Replication rate of the virus and cellular respiratory transcript impacted due to alteration by tested enzyme- [A] viral copies of the AD169 and Towne treated cells treated with the presence of EC_{50} dose of enzyme and observed the impact on different post infection time frame (12-72 hpi). [B] The rate of replication observes within each successive intermediate time frame, represented by the synthesizing base pair growth rate per min. [C] relative m-RNA expression of Phosphofructokinase-1 and [D] Pyruvate Dehydrogenase gene transcript on HCMV Towne strain infected MRC5 cells, tested in different hpi (12 to 72)



Rate of Replication				
	Base pair/min			
Hours post infection	AD169		Towne	
	Control	Laccase	Control	Laccase
12-36 hpi	~86	~55	~116	~52
36-48 hpi	~226	~132	~248	~68
48-60 hpi	~313	~105	~423	~106
60-72 hpi	~363	~128	~419	~114

B.



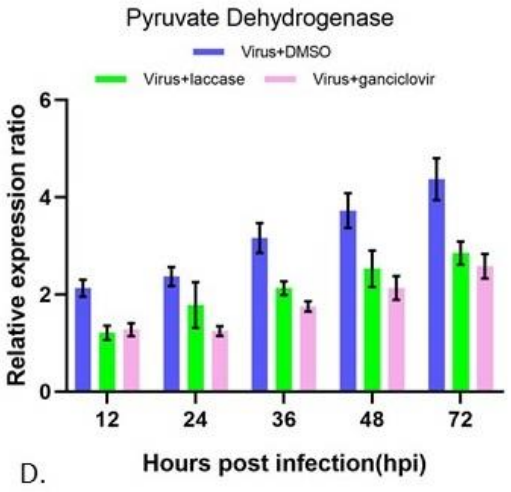


Table 5.2: Expression of host metabolic responsive genes on HCMV infection: A comparative follow-up analysis of relative m-RNA expression ratio of phosphofructokinase-1 and pyruvate dehydrogenase genes analysed by Real time PCR. The Mean+/-SD has been calculated and One-way ANOVA was performed to estimate the significance among the laccase treated and ganciclovir treated HCMV infected MRC5 cells (n-3 in each groups). The expression fold has been measured in comparison to DMSO treated HCMV infected (n=3) taken as negative control group.

Phosphofructokinase-1					
Hours post infection	**Mean ± SD**		**Significance (P-Value)**	**95% confidence interval**	
				Lower	**Upper**
12 hpi	Ganciclovir	1.59+0.54	0.068	-1.155	0.074
	.Laccase	1.91+0.21	0.489	-0.833	0.400
24 hpi	Ganciclovir	2.14+1.84	<0.001	-2.383	-1.304
	.Laccase	2.30+1.68	0.005	-2.435	-0.931
36 hpi	Ganciclovir	2.31+2.52	0.031	-4.508	-0.532
	.Laccase	2.74+2.09	0.032	-3.383	-0.345
48 hpi	Ganciclovir	2.84+3.88	0.004	-5.410	-2.356
	.Laccase	3.67+3.05	0.007	-4.777	-2.356
72 hpi	Ganciclovir	3.61+2.82	0.003	-4.086	-1.561
	.Laccase	3.82+2.61	0.006	-4.001	-1.225
Pyruvate dehydrogenase					
Hours post infection	**Mean ± SD**		**Significance (P-Value)**	**95% confidence interval**	
				Lower	**Upper**
12 hpi	Ganciclovir	1.27+0.85	0.007	0.380	1.319
	.Laccase	1.21+0.91	0.006	0.436	1.397
24 hpi	Ganciclovir	1.24+1.12	0.007	0.583	1.657
	.Laccase	1.78+0.58	0.047	-0.749	1.916
36 hpi	Ganciclovir	1.75+1.40	0.018	0.507	2.306
	.Laccase	2.12+1.03	0.030	0.186	1.880

48 hpi	Ganciclovir	2.13+1.59	0.010	0.644	2.542
	.Laccase	2.52+1.20	0.034	0.133	2.267
72 hpi	Ganciclovir	2.58+1.79	0.014	0.643	2.943
	.Laccase	2.85+1.52	0.024	0.364	2.682

Protein-protein docking interaction study of viral DNA polymerase and Laccase enzyme:

In numerous prior studies, it has been consistently documented that ganciclovir serves as an inhibitor of HCMV DNA polymerase, effectively impeding viral replication. Our previous experiments have demonstrated that laccase can similarly achieve this outcome. So, our objective was to determine whether laccase targets any specific sites on HCMV DNA polymerase that are distinct from the sites targeted by ganciclovir. We utilized the crystal structure of yeast DNA polymerase δ as a template to construct a three-dimensional model of the HCMV UL54 DNA polymerase through homology modelling. This approach was based on the 32% sequence identity between the two polymerases. As laccase is a large proteinaceous structure (525 amino acid) so we had performed flexible docking on HCMV DNA polymerase (1242 amino acid) to check any distinctive binding site. It has been observed that laccase binds in several binding sites of DNA polymerase among them the most promising binding conformation was determined according to best docking score**. (Figure 5.5A)**. further analysis of the docked residues in a target specific docking in ClusPro server it has been determined that mostly laccase binds with the viral polymerase with 8-salt bridge (average bond length 2.74A^0) 13 hydrogen bonding (average bond length 2.71 A^0) and 187 non bonded interaction (with average bond length 3.52 A^0) (**Table 5.3**). Most of the HCMV DNA polymerase binding residues were found to be located within the c-terminal domain of 1004-1242 amino acid regions where majority of salt bridge formation was visible within 1004-1037 residues and 1173-1191 residues (**Figure 5.9A and B**). In previous studies it was reported that c-terminal residues of HCMV DNA polymerase UL54 is mostly required for binding of an essential replication accessory protein UL44[24]. So we had performed a similar molecular docking of HCMV DNA polymerase along with processivity factor UL44 with DNA loop[25] in rigid docking and found a very distinctive supportive result which showed the entry of DNA loop and binding of UL44 molecule to DNA polymerase.(**Figure 5.5B**)

Figure 5.5: Surface-based molecular docked result of viral protein and antiviral enzyme*-[A] molecular docked complex of Viral DNA polymerase (green) being bounded by antiviral test compound laccase (purple) and [B] The binding of replication of accessory protein UL44 (orange) on the similar confirmatory sites of viral protein.*

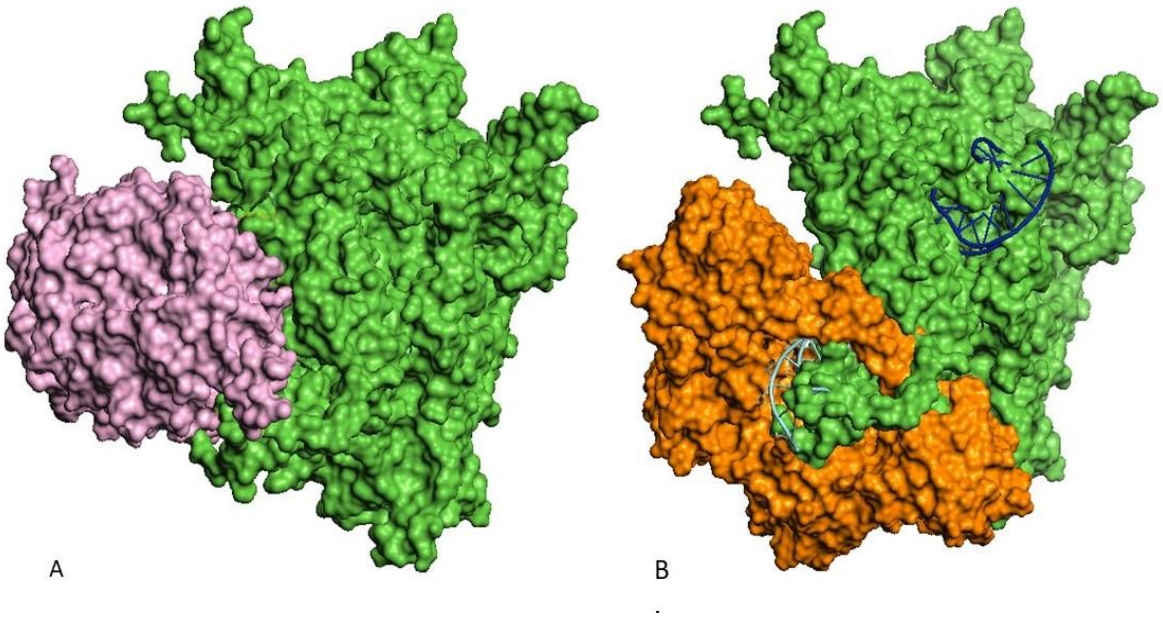


Figure 5.6: Superimposition of HCMV DNA polymerase with template structures*: [A] HCMV DNA polymerase (green) is superimposed on DNA polymerase δ of Saccharomyces cerevisiae (PDB ID-3IAY) (in orange). [B] cartoon representation of HCMV DNA polymerase where the DNA entered(brown) within the replication cleft along with exiting DNA (blue) portion*

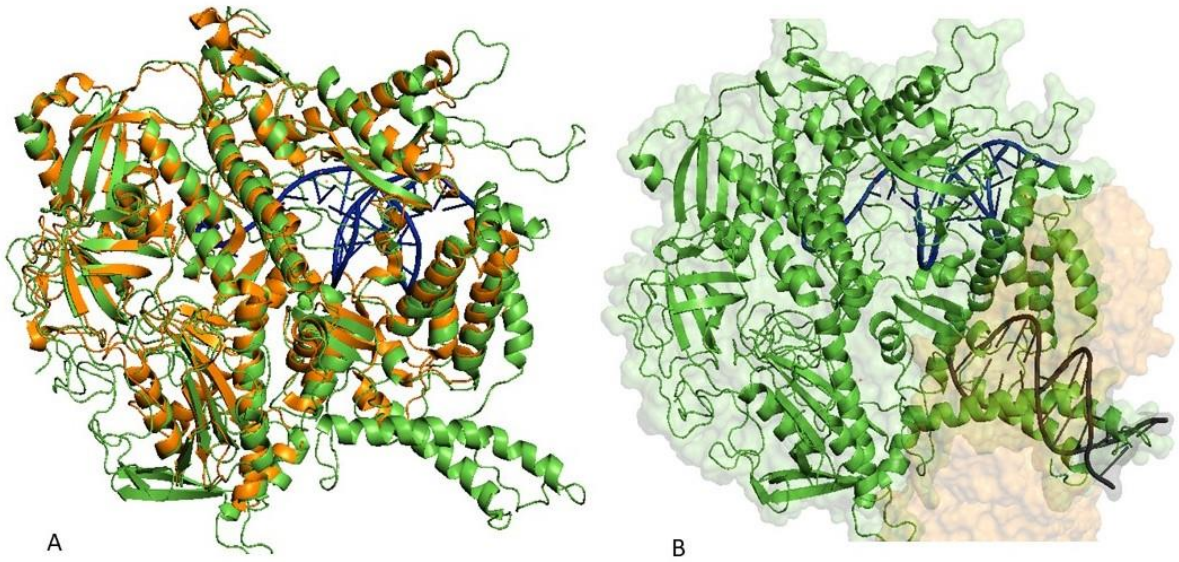


***Figure 5.7**: **Binding of HCMV accessory protein UL44 with UL54-**[A] surface representation of HCMV DNA polymerase UL54 (transparent green) with the UL44 accessory protein (orange) bonded with DNA entering for replication. [B]shifting the view to 900 to represent the "C" shaped clamp structure of UL44*

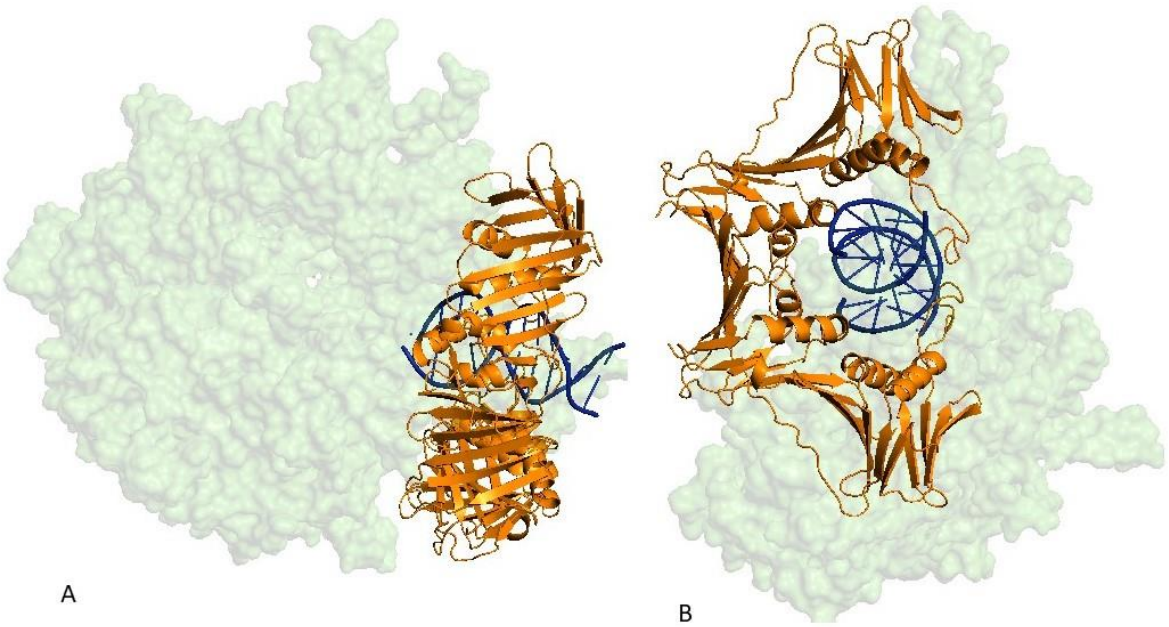


***Figure 5.8: Comparative representation of UL44 and Laccase binding site on HCMV DNA polymerase**. [A] UL44 (orange) bounded with HCMV UL54 (in green) along with entering DNA strand (blue) for replication, [B] laccase enzyme (cyan) binding site on HCMV DNA polymerase.*

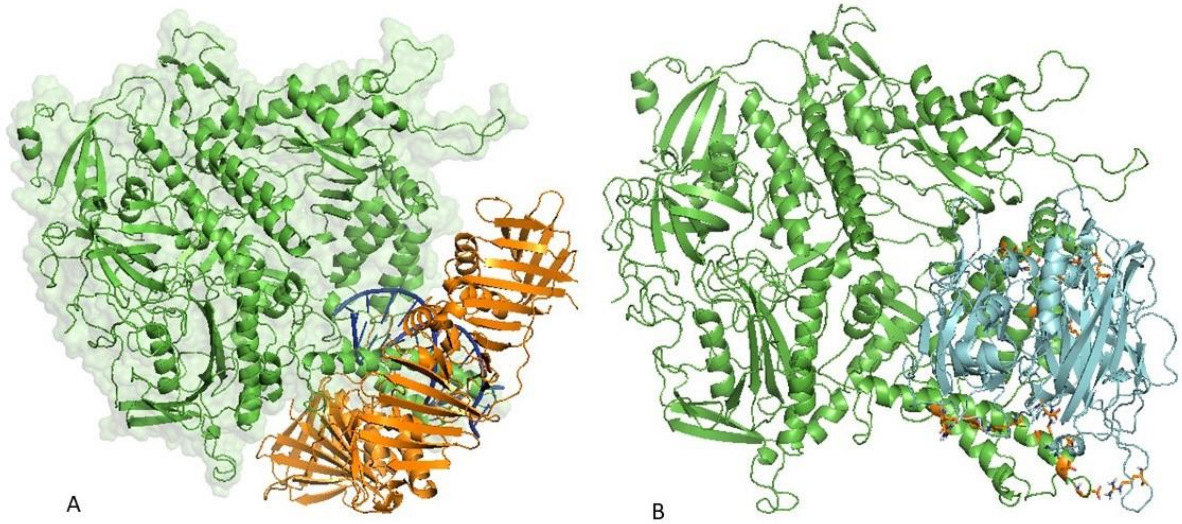


***Table 5.3 Docking result and binding energy of interacting residues of receptor molecule (HCMV DNA polymerase) and ligand molecule (Laccase)**. All the surface interacting amino acid residues of both receptor and ligand are documented with the binding distance and the free binding energy of the interacting residues.*

	Receptor residue	Binding distance	Ligand Residue	Free binding energy
salt bridge				
1.	GLU-1004	2.72	ARG-293	-17.32
2	ASP-1005	2.90	ARG-293	-16.65
3	ARG-1030	2.78	GLU-296	-5.37
4	ARG-1037	2.71	GLU-318	-3.35
5	ASP-1173	2.71	ARG-162	-5.03
6	ARG-1178	2.80	GLU-257	-8.99
7	ASP-1187	2.61	LYS-254	-2.23
8	GLU-1191	2.70	ARG-443	-8.45
Hydrogen bonding				
1	GLU-1007	2.82	GLY-294	-4.47
2	ARG-1034	2.79	PRO-288	-6.06
3	ARG-1034	2.66	THR-235	-2.59
4	ARG-1037	2.70	SER-321	-6.25
5	ARG-1050	2.65	THR-322	-4.93
6	ASP-1173	2.64	ARG-162	-5.03
7	ASP-1173	2.71	ARG-162	-5.03
8	ARG-1178	2.90	GLU-257	-8.99
9	ASP-1187	2.62	LYS-254	-0.71
10	ASP-1187	2.56	LYS-254	-0.71
11	LYS-1188	2.55	ASN-441	-3.79
12	GLU-1191	2.71	ARG-443	-8.45
13	CYS-1242	3.02	LEU-333	-2.90

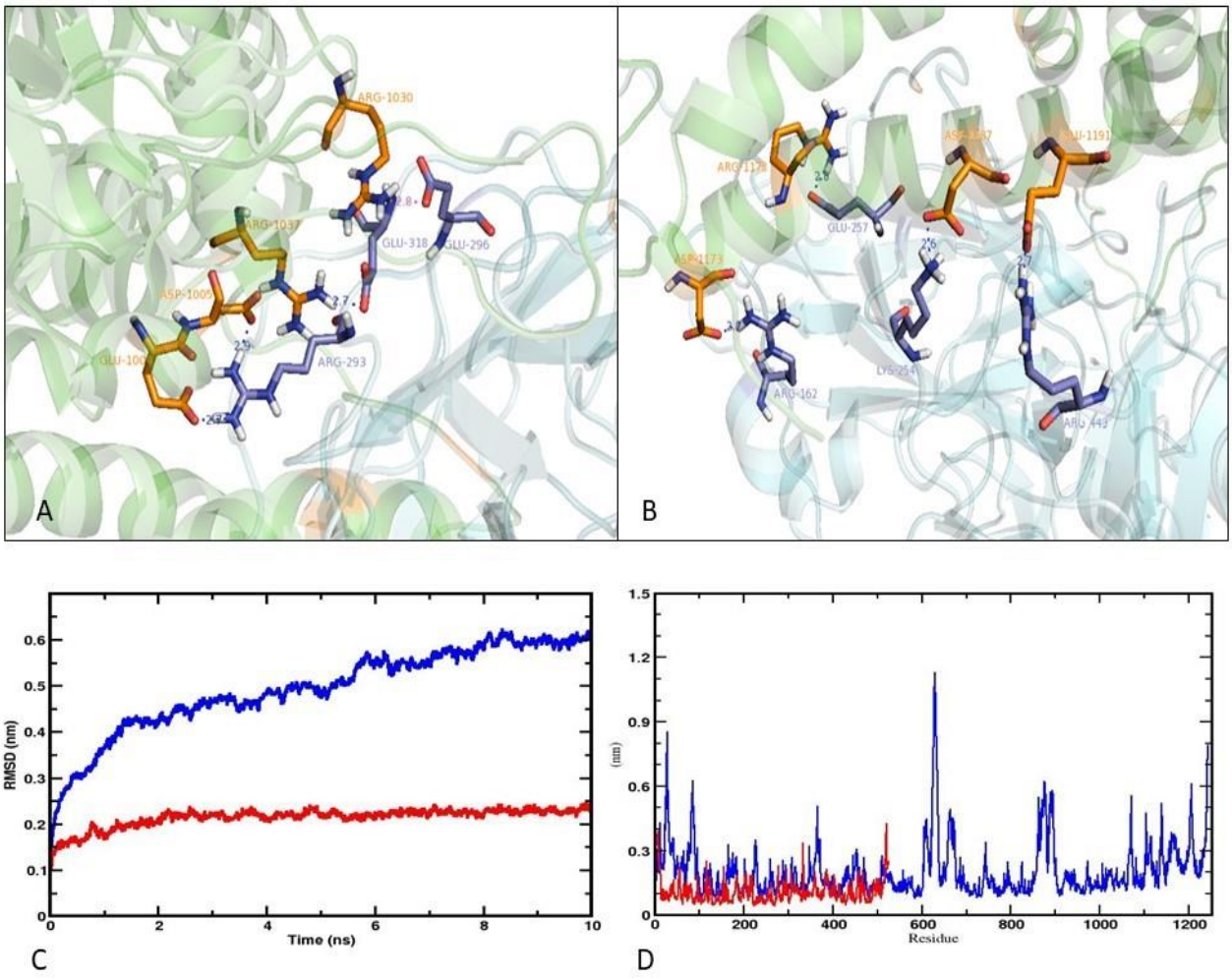
Dynamics simulation of Docked complex:

During the molecular dynamics simulation, the stability of the docked complex was assessed. The total binding energy of the laccase-UL54 complex was determined to be -14.3 kcal mol-1. To track conformational and structural changes of the backbone atoms, RMSD analysis was conducted on the complexes. It was observed that within the docked condition the RMSD value of receptor protein (UL54) initially fluctuated up to 2 ns trajectory with an average RMSD of 0.34 nm, but it shows moderate elevation up to 10ns trajectory showing average RMSD of 0.56 nm. In contrast to ligand (laccase) a slight elevation was observed up to 2 ns trajectory but for the remaining trajectory run no fluctuation was observed with an average RMSD of 0.25 nm.

The data indicates that the receptor molecule, a sizable protein comprising 1242 amino acids, notably lacked a crystallographic structure for HCMV polymerase. Consequently, the experimentally determined structure, established through homology modelling, exhibited some structural inaccuracies during energy minimization of residues 600-770 and 824-979 (**Figure 5.9C**). This was attributed to the absence of a corresponding structure in yeast DNA polymerase[26]. As the MD simulation was performed on the basis of protein-protein interaction so different binding site of laccase on HCMV DNA polymerase (1004-1242 aa) with a stable acceptable range of RMSD value of laccase interaction indicating a good stability of the complex.

We utilized the Root Mean Square Fluctuation (RMSF) to confidently analyse local variations within the protein chain residues and to accurately assess changes in the position of the ligand atoms interactions under specific temperature and pressure conditions. It has been observed that in receptor molecule (UL54) maximum RMS fluctuation was observed within 600-710 aa residues and 834-921 residues which were previously justified due to unavailability of the corresponding conformation due to structural template molecule. The average RMSF of HCMV DNA polymerase and laccase were 0.21 nm and 0.19 nm respectively which were acceptable **(Figure 5.9D)**. Similarly free binding energy of each interacting residues were also measured through Hawkdock server where it was observed that salt bridge interaction of GLU1004 and ASP1005 of HCMV DNA polymerase makes the maximum binding affinity with ARG293 residue of the laccase with the total binding energy of -17.32 Kcal mol^{-1} and -16.65 Kcal mol^{-1} respectively and moderate binding energy of GLU1191-ARG443 , ARG1178-GLU257 , ARG1030-GLU296 and ASP1173-ARG162 with -8.45 Kcal mol^{-1} , -8.99 Kcal mol^{-1}, -5.37 Kcal mol^{-1} and -5.03 Kcal mol^{-1} respectively.

Figure 5.9: Protein-protein docking and dynamics simulation result of antiviral enzyme with the specific target sites of HCMV UL54 (DNA polymerase). Amino acid residues on HCMV DNA polymerase (green) marked in orange are bounded with amino acids of Laccase (Cyan) marked in blue. [A] Residues from 1004-1037 [B]Residues from 1173-1191 on HCMV DNA polymerase. [C] RMSD plot and [D] RMSF plot of. HCMV DNA polymerase (Blue) and Laccase (red)



5.4 Discussion

In our previous research, we discovered that extracts of *Pleurotus* exhibited no toxicity in vitro. Furthermore, in our current study, the extracellular enzyme laccase demonstrated a comparable response to Ganciclovir-treated cells. We evaluated the antiviral effectiveness of laccase alongside ganciclovir and observed a significant reduction in plaque formation compared to HCMV-infected control cells. Ganciclovir is a synthetic drug, so its EC_{50} value was notably lower in comparison to the bioactive compound laccase. A thorough analysis revealed that ganciclovir exhibited highly promising antiviral efficacy against AD169, while laccase demonstrated marginally superior efficacy in Towne-infected cells. In the time-dependent assay, both laccase and ganciclovir displayed a significant reduction in viral titre as compared to the control DMSO-treated HCMV infected cells. Based on the data from both AD169 and Towne pre-treatment scenarios, it was noted that there was a significant reduction in HCMV titre with the EC_{50} dose of ganciclovir in comparison to the laccase. However, during the adsorption period, it was observed that only laccase was capable of significantly reducing the HCMV titre compared to ganciclovir treatment in Towne-infected cells, a result that was deemed insignificant in the context of AD169 treatment under similar conditions. Based on precise observations of post-transplant cases, it is evident that laccase demonstrated a highly promising ability to inhibit viruses, similar to the effects of ganciclovir (**Figure 5.2**). This

suggests that laccase has the potential to be a valuable antiviral agent. In previous studies, laccase has demonstrated the potential to serve as a nutrient supplement in conjunction with citric acid to enhance melanin production in an in vitro system which unveils new dimensions for antibiotic development [27]. Not only that it was also found the laccase has the potency to be a detoxifying compound. Experimental evidence suggested that laccase could be used for detoxification different aflatoxins responsible for causing serious health complications in animal if ingested with contaminated foods[28]. A very promising detoxification was also recently reported of laccase to reduce the cytotoxicity of the anticancer drug doxorubicin from *in vitro* system[29]. Upon investigation, we have discovered that laccase exhibits antiviral responses. We are now exploring the potential synergistic or antagonistic effects that may arise when laccase is treated with ganciclovir. Our research has revealed a synergistic effect between Laccase and ganciclovir. In our study, we conducted a viral reduction assay to assess the combined impact of different concentrations of ganciclovir and laccase. The maximum synergistic response occurred at 6 and 8 μM of laccase combined with 1.2 μM ganciclovir doses **(Figure 5.3**). These concentrations were below the average EC_{50} concentrations of individual compounds when tested against AD169 and Towne.

An investigation was conducted to determine the time-dependent viral inhibition of HCMV, focusing on the quantification of viral copies. The study revealed significant variations in viral DNA quantification (12-72 hpi) between drug-treated and untreated cells. Interestingly, a sudden spike in viral progression was observed after 36 hpi in untreated control conditions. However, when treated with laccase at EC_{50} doses, a distinct reduction in viral copies was consistently observed in successive post-infection stages for both HCMV strains (**Figure 5.4A**). The rate of viral replication was also measured, which distinctively showed that the synthesis of base pairs was much more effectively reduced in Towne-treated cells in comparison to AD169 from 36-72 hp. (**Figure 5.4B**).

Several studies have indicated that the replication of Human Cytomegalovirus (HCMV) is heavily reliant on a range of cellular metabolic bio regulators. Given its slow growth and extensive coding potential, HCMV leverages host mitochondria to satisfy the bioenergetics and biosynthetic demands of its replication [30]. Metabolic analysis of HCMV-infected cells has confirmed heightened glycolytic and pyrimidine nucleotide synthesis and indicated increased activity in the citric acid cycle. HCMV's robust induction of glycolysis leads to elevated glucose-derived citrate production, which is subsequently exported from the mitochondria and utilized to facilitate fatty acid synthesis. The increased levels of glycolytic intermediates observed during HCMV infection correspond to elevated transcript levels of various glycolytic enzymes. One of these enzymes, phosphofructokinase-1 (PFK-1), plays a critical role in regulating glycolytic flux and pyruvate dehydrogenase flux in the TCA cycle, ultimately increasing the production of acetyl Co-A, a key component in initiating lipid biosynthesis. [31].

So we aim to verify the relative expression of the phosphofructokinase-1 and pyruvate dehydrogenase genes in laccase treated HCMV infected cells in comparison to uninfected control cells. During the initial stages of HCMV replication at 12 hpi, there was no significant increase in m-RNA expression observed in drug-treated HCMV infected cells (both ganciclovir and laccase) compared to control infected cells (no drug). However, in the subsequent stages, it was observed that drug-treated cells demonstrated a noticeable reduction in phosphofructokinase-1 expression. The relative expressions of pyruvate dehydrogenase m-RNA also exhibited similar effects, indicating a significant reduction in HCMV replication. This was evidenced by lower expression levels of respiratory system regulatory genes. **(Figure 5.4C and 5.44D)** .

As laccase is a larger protein molecule compare to ganciclovir, so we had to perform surface based flexible docking to check any distinctive binding site of laccase on HCMV DNA polymerase (UL54). We have found that laccase bind to several novel residues on the surface of HCMV polymerase near the C-terminal domains of HCMV DNA polymerase **(Figure 5.9A and 5.9B)**. In a previous detailed investigation, it was reported that the UL44 protein, an important accessory protein involved in HCMV DNA replication, needs to form a dimer at the C-terminal residues of UL54. This dimerization allows UL44 to create a C-shaped clamp structure that functions to hold the entry point of the synthesizing DNA [32],[33] [34]. It was found that there are two nuclear localization signals (NLSs) situated at amino acids 1153–1159 (NLSA) and 1222–1227 (NLSB). These NLSs are of primary important for the UL44 binding site on HCMV DNA polymerase[35]. In our comprehensive investigation, we discovered that laccase binds to the HCMV DNA polymerase at specific residues located within the c-terminal domain spanning amino acids 1004-1242. The majority of salt bridge formations were observed within residues 1004-1037 and 1173-1191. Total 13 hydrogen bond interaction were also noticed in the same region. (**Table 5.3**) within an average bond distance of $2.5A^0$. In the study, it was observed that there were a total of 157 non-bonded interactions in the vicinity of the laccase enzyme, occurring within the 1191 to 1242 amino acid residues of the DNA polymerase. This comprehensive investigation was further bolstered by the acceptable RMSD and RMSF values in molecular dynamics simulation, taking into account the standard solvent and ionic conditions within the bimolecular system.

In summary, our study indicates that repurposing Laccase has the potential to serve as an antiviral drug against HCMV, both on its own and in combination with ganciclovir. This may facilitate the advancement of novel antiviral approaches against this significant viral pathogen by leveraging its dual antimicrobial capabilities. Furthermore, it could enhance our comprehension of how we can manage to exploit HCMV replication by regulating host pathways.

Conclusion of the Study

My PhD research hypothesis was to study the different clinical outcomes of patients, including children with *Human Cytomegalovirus* (HCMV) infection, as well as those with symptomatic or asymptomatic HCMV infection. It has been documented and established first to analyse the impact of HCMV among these patients in the Eastern Indian population. Through extensive research and ongoing molecular diagnostics, provided as a social service, we have employed comprehensive PCR-based diagnostic methods to identify specific HCMV-induced clinical markers, aiming to improve diagnostic accuracy**. (Chapter 1)** Our primary focus has been on neonatal patients, specifically examining the changes in the immunological pathway in those infected with lytic and latent HCMV and experiencing choroidal retinitis. **(Chapter 2)**

It is widely known that during the initial stages of an infection, the first line of immunological defence is carried out by antibodies. If this primary defence fails to protect the immune system, secondary inflammatory molecules begin recruiting different immunological defence systems (such as cytokines, T cells, and macrophages) to combat the pathogens. In the early stages of a pathogenic infection, the host initiates the expression of pro-inflammatory cytokines, which sets off a signalling cascade to activate the host's defence mechanism. In the case of an HCMV infection, the virus induces anti-inflammatory cytokine activity, causing the host's immune system to not recognize the pathogen as a threat. Consequently, the virus can easily invade different host tissues by evading the host's immune system.

Retinitis is a proliferative disease affecting blood vessels. Our detailed investigation has revealed that in all patient groups with retinitis (children), the initiation of NFkB canonical signalling leads to high inflammation and rapid tissue regeneration. In cases of HCMV infection, NFkB non-canonical signalling is activated, initially dampening inflammation for a few days before causing a sharp increase, leading to much more severe clinical complications in HCMV-induced cases compared to normal retinitis. This is the first documented case of HCMV-induced choroidal retinitis in children populations on a global scale.

In renal transplant recipients, HCMV presents a covert threat as it can remain dormant within the recipient for an extended period by evading the host immune. Our observations revealed that some patients experienced renal rejection within 2 years after the operation, while in other cases, renal rejection occurred more than 2 years after the operation. Upon studying the HCMV infectivity status in both groups, we found that patients who experienced early renal rejection (within 2 years) were infected with acute HCMV and exhibited moderate to high clinical complications On the other hand, patients who experienced late renal rejection (more than 2 years) were infected with HCMV within 3-6 months post-transplant and received ganciclovir therapy. Despite prolonged antiviral therapy, the antigenemia reports showed

HCMV-infected cell numbers below the positive cut-off, indicating incomplete HCMV eradication or the transition of HCMV to a dormant state in the patients. After a few years, the dormant HCMV became activated, transitioning from latency to the lytic stage and causing severe renal complications, ultimately leading to renal rejection. **(Chapter 3)**

The risk of latent to lytic switching, coupled with the potential for recurring HCMV infection due to drug resistance, underscores the need to explore new antiviral approaches from ethno medicinal sources for our investigation, we gathered wild mushrooms from their natural habitat and conducted chemical spot identification. We then performed methanolic extraction of the collected fruit bodies to screen for initial evidence of bioactive constituents. In vitro treatment with the methanolic extract of the isolated mushrooms demonstrated significant cell viability for *Pleurotus* sp. and *Lentinus* sp. in both MRC5 and Reporter cell lines (1B4 & 2F7). However, the cytotoxicity of other mushrooms, such as *Polyporus* sp. and *Phellinus* sp., was notably higher compared to *Pleurotus* sp. and *Lentinus* sp. We also assessed the antiviral response of *Pleurotus* sp. and *Lentinus* sp. using standard procedures and found that their extracts could serve as antiviral compounds, exhibiting significant cell viability and reducing viral growth. study of the effects of mushroom crude extracts on the human fibroblast cell line (MRC5) revealed that the *Pleurotus* mushroom extract had very low toxicity, leading us to select this mushroom for antiviral treatment. In comparison, we used Ganciclovir as our control drug and observed that the *Pleurotus* mushroom extract was highly effective in restricting virus growth in the same cell. While synthetic drugs have high antiviral accuracy, their higher toxicity limits their prolonged use. **(Chapter 4)**

After identifying a potential source, our next goal was to identify any active biomolecules with antiviral properties in the mushrooms. We are focusing on mushrooms as a potential source of antiviral compounds due to their rich variety of beneficial bioactive compounds. To isolate these compounds, I cultured *Pleurotus ostreatus* (MTCC-1805), a commonly consumed edible mushroom.

Pleurotus serves as a significant reservoir of bioactive enzymes. Our extraction process has successfully yielded the predominant enzyme, laccase, which has been previously reported to possess antiviral properties in numerous studies. **(Chapter 5).** In our research, it had been observed that the Laccase enzyme effectively inhibits viral propagation by restricting the activity of the Viral DNA polymerase. Our in vitro study involved comparing the expression of various early genes of HCMV and late responsive glycoprotein genes. I found that the presence of Laccase enzyme led to significantly lower expression of these genes. Additionally, when I compared the viral load in cells treated with Laccase to untreated cells, I observed a much lower number of DNA copies in the drug-treated cells, indicating reduced viral DNA

production. This suggests that the DNA Polymerase enzyme might be a suitable target for the action of the Laccase enzyme.

In our analysis of how an enzyme affects the viral DNA polymerase, we employed advanced bioinformatics tools such as Modeller 10.5, ClusPro 2.0, and GROMACS 24.2 software. Our findings suggest that the enzyme may inhibit the viral DNA polymerase by preventing its accessory proteins from binding to it. Through protein-protein docking analysis, we identified that the c-terminal amino acids of the laccase enzyme bind to the catalytic domain of the HCMV DNA polymerase enzyme, thereby preventing the viral DNA polymerase accessory major regulator protein UL-44 from binding. As a result, the viral DNA polymerase becomes non-functional.

The comprehensive investigative findings and inference with encompasses epidemiological perspectives on the current prevalence of HCMV and explores novel approaches to antiviral drug development strategies through the ethnomedicinal gateway. This exploration opens new frontiers in combating drug resistance associated with *Human Cytomegalovirus.*

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List of Publications:

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