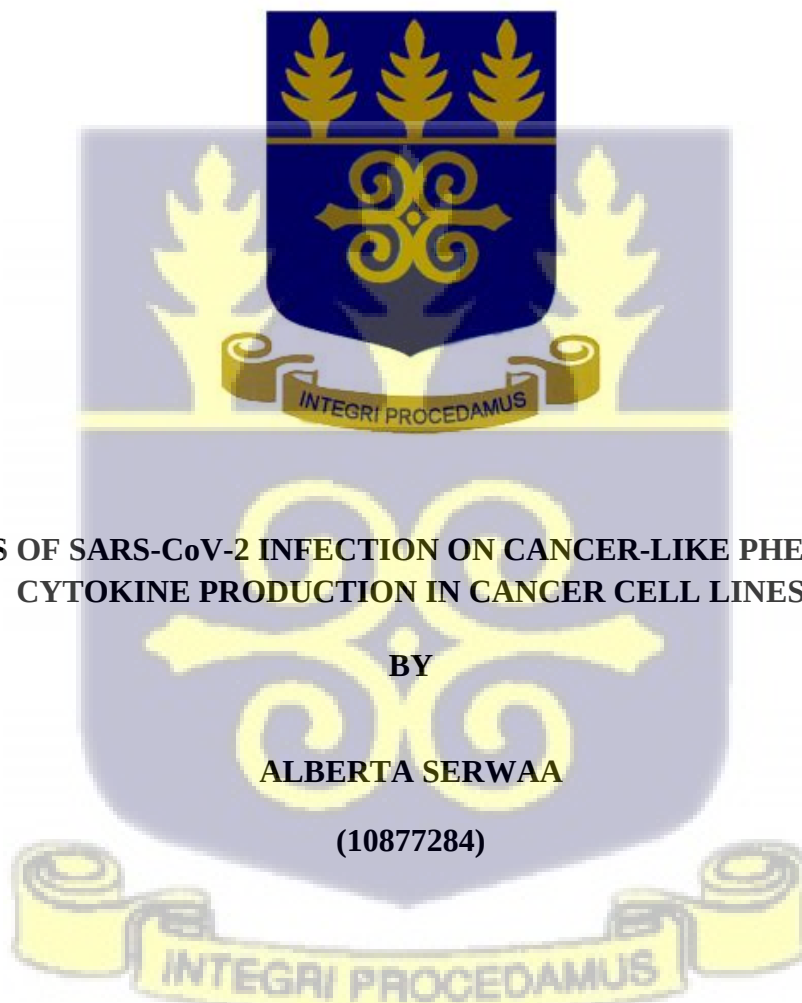


UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

UNIVERSITY OF GHANA - LEGON



**THE EFFECTS OF SARS-CoV-2 INFECTION ON CANCER-LIKE PHENOTYPES AND
CYTOKINE PRODUCTION IN CANCER CELL LINES**

BY

ALBERTA SERWAA

(10877284)

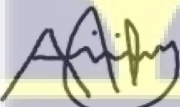
**A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL
FULFILMENT OF THE AWARD OF THE DEGREE OF MASTER OF PHILOSOPHY
IN MOLECULAR BIOLOGY**

DEPARTMENT OF BIOCHEMISTRY, CELL, AND MOLECULAR BIOLOGY

JANUARY 2023

DECLARATION

I, Alberta Serwaa, herewith declare that except for cited references, this MPhil thesis titled “The Effects of SARS-CoV-2 Infection on Cancer-like Phenotypes and Cytokine Production in Cancer Cell Lines” was aptly completed by myself at the Department of Biochemistry, Cell, and Molecular Biology, University of Ghana. I also certify that all results obtained therein are the true reflection of the work completed under the guidance and supervision of Dr. Anastasia Rosebud Aikins and Dr. Peter Kojo Quashie.

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ABSTRACT

The occurrences of cancer and cancer-related mortality are a growing burden worldwide and co-infections with other pathogens such as viruses might contribute to disease pathogenesis. Cancer is one of the leading comorbidities for the recent global pandemic ‘Coronavirus Disease 2019’ (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The infection causes severe respiratory injury, organ failure, and hyper-production of inflammatory cytokines. The causative virus initiates infection by attaching to the human Angiotensin Converting Enzyme 2 (ACE2) receptor through the receptor-binding domain (RBD) within the viral spike protein. Also, successful infection is dependent on the activation of the spike by host proteases like the Transmembrane serine protease 2 (TMPRSS2). Studies have shown that ACE2 and TMPRSS2 are present in healthy cells and may play important roles in regulating cellular function. Other studies have also shown aberrant ACE2 expression in cancer cells. This research sought to study how ACE2 expressed on cancer cells interacts with SARS-CoV-2 spike protein and pseudoviruses (PV’s), and how this interaction affects cancer phenotypic properties and cytokine expression. Gene expression analysis for ACE2 and TMPRSS2 in breast, colorectal, and prostate tumors tissues and normal tissues was carried out using Gene Expression Profiling Interactive Analysis (GEPIA) software. The protein expression levels of ACE2 on seven cancer cell lines (MDA-MB-231, MDA-MB-468, DLD-1, COLO205, HCT-15, 22RV1, and BPH1) were screened using dot blot assay. *In vitro*, analysis of the interaction of Spike and ACE2 was examined by producing SARS-CoV-2 pseudovirus (PV) to infect the cell lines. The transduction efficiency of the PVs was measured by the quantification of luciferase activity. Cancer cell proliferation, viability, migration, and angiogenic markers were analyzed post-infection using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay, cell titer Glo viability

assay, wound healing assay, and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Cytokines expression post-infection was investigated using qRT-PCR. The effects were further confirmed with live virus (LV) infections using MTT and qRT-PCR. Using *in silico* analysis, ACE2 was shown to be expressed highly in colorectal tissues and low in breast and prostate tissues. *In vitro*, ACE2 expression data was consistent with the *in-silico* analysis. However, out of 7 cell lines, a significant PV infection was observed only in 22RV1 (prostate) and DLD-1 (colorectal). PV decreased 22RV1 proliferation but have an inconclusive effect on DLD-1. Proliferation and migration were increased in LV- infected 22RV1 but were decreased in LV- infected DLD-1. Infections increased cytokine levels in 22RV1. Similarly, PV increased cytokine expression for DLD-1 cells. However, LV downregulated IL-1 β and IL-8 in DLD-1. LV infection affected the expression of genes involved in proliferation, migration, angiogenesis, and the expression of cytokines. Although ACE2 expression might not ensure possible infection of SARS-CoV-2. The current findings suggest that infection of 22RV1 and DLD-1 with the virus may affect their cellular properties and gene expression. The infection can upregulate and downregulate the expression of cytokines in cancer contributing to the progression or regression of these cancers. Additionally, PV might not be an appropriate model for the study on the viral-host response partly because LV infections and PV infections have contrasting effects on cancer properties in different cell lines.

DEDICATION

I dedicate this to my late father Mr. Anthony Opoku Mensah and all other persons with cancer who suffered a great deal during this COVID-19 pandemic.



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LIST OF ABBREVIATIONS

22RV1	Human prostatic carcinoma epithelial cell line
ACE2	Angiotensin-converting enzyme 2
BCL2	B-cell lymphoma 2
BRCA	Breast Invasive carcinoma
COAD	Colon adenocarcinoma
CoV	Coronavirus
COVID-19	Coronavirus disease 2019
DLD-1	Colorectal adenocarcinoma cell line
DNA	Deoxyribonucleic Acid
E	Envelope
IL	Interleukin
KI-67	Marker of proliferation
LB	Luria Broth
LV	Live Virus
M	Membrane
MERS-CoV	Middle East respiratory syndrome coronavirus
MMP9	Matrix metalloproteinase 9
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
N	Nucleocapsid
Nsp	Non-structural protein
ORF	Open reading frame

Pp	Poly protein
PRAD	Prostate adenocarcinoma
PV	Pseudovirus
RNA	Ribonucleic acid
q-RT PCR	Quantitative-Reverse transcription polymerase chain reaction
S	Spike
SARS-CoV	Severe acute respiratory coronavirus
SARS-COV-2	Severe acute respiratory coronavirus 2
TCID ₅₀	Median Tissue Culture Infectious Dose
TMPRSS2	Transmembrane serine protease 2
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VIM	Vimentin
WHO	World Health Organization
BSA	Bovine serum albumin
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline-tween 20
BSA-PBS-T	Bovine serum albumin-Phosphate buffer saline-tween 20



CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

As one of the leading causes of death worldwide, cancer continues to be a global burden (Hu & Fu, 2012). The GLOBOCAN 2020 estimates of cancer incidence and mortality show that there were 10.3 million deaths and 19.3 million new cancer incidents worldwide (Sung et al., 2021). In Ghana and some parts of Africa, cancer is a rising and generally unrecognized public health concern (Adam & Koranteng, 2020). Most developing countries, including Ghana, lack regionally pertinent information or cancer data repositories. Despite efforts to overcome this concern, the lack of population-based research data with a suitable representative population remains a challenge (Laryea et al., 2014). Some reports predict that low or medium-development index countries would have the biggest relative rises in cancer incidence by 2040 (Arnold et al., 2022; Wilson et al., 2018). However, there are still large disparities between high and low-income nations

Over the years, research has been undertaken to identify the true etiology of cancer. According to reports, a person is likely to acquire cancer from both hereditary and environmental elements (Garcia-Closas et al., 2014; William Audeh, 2012). The development of some malignancies has been linked to natural elements like hormones. Food additives and medications containing synthetic materials may also promote the growth of cancer. Infectious agents like viruses, bacteria, and parasites may also influence cancer development (Blackadar, 2016). Consequently, it is possible to categorize the emergence and spread of cancer as multifaceted.

Normally, the human immune system mounts an immunological response upon the detection of tumor antigens. These responses include the activation of immune cells and the induction of

inflammatory responses, characterized by the production of various cytokines (Candeias & Gaip, 2016). Malignant cells can, however, develop defense mechanisms to evade the body's adaptive immune response. Examples include producing immunosuppressive substances like cytokines and becoming immune cell resistant (Loose & Wiele, 2009). Additionally, those who have cancer often have altered immune systems, which lowers the body's capacity to defend itself against infections. The mounted resistance to immune response, drugs, and other forms of treatment, all contributes to factors that weaken the immune system. Consequently, this increases the risk of bacterial and viral infections in cancer patients (Steele, 2012). Previous studies have shown that community respiratory viruses are a common cause of respiratory disease in immunocompromised persons with cancer (Whimbey, 1997; Devine & Wingard, 1994). That is, the immunocompromised nature of cancer patients makes them more susceptible to viral infections compared to others. It could thus be inferred that cancer patients are at a higher risk of infection, symptom severity, and even death from SARS-CoV-2 infection.

The etiological cause of the Coronavirus disease 2019 (COVID-19), SARS-CoV-2 is a highly contagious and pathogenic virus that surfaced during the last quarter of 2019 (Hu et al., 2021). The disease was declared a global pandemic by the World Health Organization (WHO) in the first quarter of 2020 after being detected in numerous countries. Research has revealed that the virus initiates infection via attachment to the ACE2 receptor on host cell surfaces using its receptor binding domain within the spike(S) protein (Shang et al., 2020). The process is aided by the proteolytic processing of the spike protein, to allow entry into the host cell. This proteolytic cleavage is brought about by proteases produced by the host. Examples include the endoprotease furin, the lysosomal protease cathepsin L, and the transmembrane protease serine 2 (TMPRSS2) (Hoffmann et al., 2020).

These proteins involved in the successful entry of the virus are expressed in a range of organs and are involved in several biological processes (Cao et al., 2021; Hikmet et al., 2020). In a normal cell, ACE2 expression is crucial in regulating cellular function by generating small proteins that regulate processes such as blood pressure, wound healing, and inflammation (Sriram et al., 2020). Also, the expression of these proteins on cancer cells and their role in cancer progression have been the subject of several studies (Cheng et al., 2021; Dai et al., 2020). The expression of ACE2 on cancer cell surfaces has been shown to have diverse biological functions. Studies reported on the impact of ACE2 in cancer are thus far inconclusive; ACE2 may have both favorable and unfavorable outcomes in cancer development and this differs with different cancer types. One study has shown that the receptor has a beneficial effect in reducing certain cancer properties in different cancer types such as lung cancer, breast cancer, colon cancer, and pancreatic cancer (Xu et al., 2017). The *in vitro* overexpression of the protein in non-small cell lung cancer was shown to reduce the production of vascular endothelial growth factor (VEGF) and inhibit cell growth (Aydiner et al., 2015; Catarata et al., 2020). Similarly, Zhang Q *et al.*, 2019, reported a decrease in breast cancer angiogenesis by ACE2. Nonetheless, it has been shown to enhance the migratory and invasive capabilities of renal adenocarcinomas thereby having a negative effect (Almutlaq et al., 2021; Zheng et al., 2015). Either way, the way cells, particularly cancer cells communicate is through the release of different stimulatory molecules, one of which is broadly referred to as cytokines.

Reports indicate that a hyper-inflammatory condition known as "cytokine storm" is the primary cause of the majority of the severe symptoms that occur after SARS-CoV-2 infection (Song et al., 2020). The term refers to a host-driven severe hyper-inflammatory immune response to cancers and infections, such as those caused by SARS-CoV-2 (Bhaskar et al., 2020). One of COVID-19's

more prevalent comorbidities is cancer, but the immunological import of this is not yet understood. For a SARS-CoV-2-cancer comorbid infection, hyperproduction of cytokines may result in novel cytokine combinations within the tumor microenvironment. This may in turn affect the cancer-like properties of the cancerous cells. Cancerous cells produce and release certain pro-inflammatory cytokines into the tumor microenvironment which can drive cancer pathogenesis (Dehne et al., 2017). However, numerous inflammatory factors may be implicated in mechanisms that promote tumor development (Singh et al., 2019). Cytokines which are produced as a result of infection and infection-related inflammation may serve to reduce cancer development (Maeda & Omata, 2008). Alternatively, these cytokines can be utilized by cancers to promote growth, attenuate apoptosis, and facilitate invasion and metastasis (Lu et al., 2006).

1.2 PROBLEM STATEMENT

One key factor in COVID-19 disease susceptibility, severity, and mortality was associated with comorbidities/persons with underlying medical conditions (Fang et al., 2020; Sanyaolu et al., 2020). COVID-19 comorbidity describes the coexistence of any long-term disease with the COVID-19 disease. The pandemic was shown to have a devastating effect on persons with cancer leading to significantly high mortality risk. The disease's influence on cancer diagnosis, compared with the data before the pandemic observed that the number of deaths caused by breast, colorectal, lung, and esophageal cancer will increase by 7.9–9.6%, 15.3–16.6%, 4.8–5.3%, and 5.8–6.0% in five years, respectively (Maringe et al., 2020). In 2020, most countries suspended routine screening for cancer in response to the COVID-19 pandemic. Also, COVID-19 has been shown to cause dysregulation in immune reactions resulting in a “cytokine storm” (Prompetchara et al., 2020). This poses a critical challenge to cancer patients by increasing poor prognosis (Disis et al., 2020). In cancer patients with COVID-19, the impact of increased health loss varies, making it more

difficult to forecast disease progression and manage illness symptoms. Data on how SARS CoV-2 may influence the growth of cancer cells or its spread however is limited. Also, there is limited data on the effect of SARs-CoV-2 on cytokine production in cancer.

1.3 RATIONALE

Cancer cells expressing the ACE2 receptor for cellular function may be susceptible to SARS-CoV-2 infection. The ACE2 receptor's functionality and signaling may be impacted by this potential infection of cancer cells, which may also have an impact on the phenotypic characteristics of cancer cells. Therefore, it is important to know the relationship between ACE2 levels, viral infectivity, and infection intensity in cancer cells. Again, cytokines are important in the pathophysiology of cancer and have been studied as potential diagnostic and prognostic indicators of cancer. Cytokines can either promote or repress tumor growth when they are generated in response to infections, inflammation, and immune responses. As a result, COVID-mediated cytokine production in cancer cells may stimulate or suppress tumor growth. It is therefore crucial to comprehend the impact of cytokine storms on cancers amid this pandemic. While cancer has proved over time to be a global health concern, co-infection with the new SARS-CoV-2 may contribute to disease pathogenesis and its implications on the worldwide cancer burden and global cancer care are unknown. This in-depth investigation into the plausible interaction and/or infection of the SARS-CoV-2 virus on cancer cells will aid in understanding its impact on cancer characteristics and cytokine generation by cancer cells.

1.4 HYPOTHESES

1. Differential expression of the ACE2 receptor in cancer tissues correlates with the ability to be infected by SARS-CoV-2 virions and pseudoparticles.

2. SARS-CoV-2 interaction with ACE2 receptor on cancer cells alters cancer properties and cytokine production

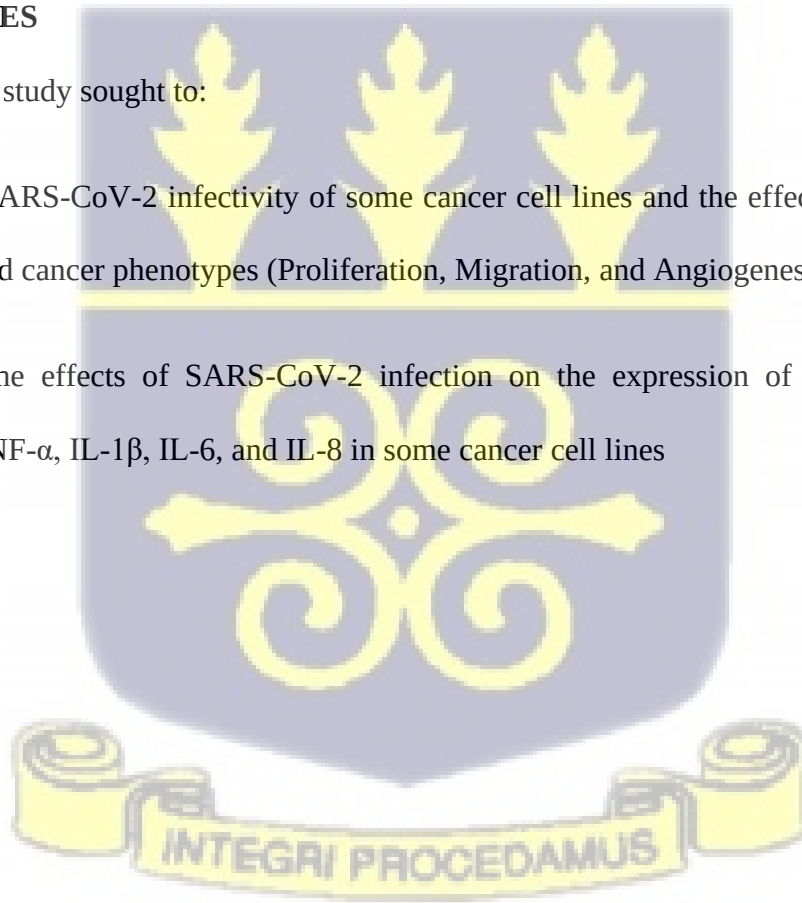
1.5 AIM

To investigate the effect of SARS-CoV-2 infection on cancer-like properties and cytokine production in different cancer cell lines.

1.6 OBJECTIVES

Specifically, the study sought to:

1. Determine SARS-CoV-2 infectivity of some cancer cell lines and the effects of infection on some selected cancer phenotypes (Proliferation, Migration, and Angiogenesis)
2. Determine the effects of SARS-CoV-2 infection on the expression of pro-inflammatory cytokines $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-6 , and IL-8 in some cancer cell lines



CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology of cancer

Cancer is a non-communicable disease that develops as a result of the abnormal growth of cells. This unnatural cell proliferation could lead to cancer cells invading other cell types in the body and spreading their properties (Boutayeb & Boutayeb, 2005; Chaker et al., 2015; Preethi et al., 2021). According to the WHO, the term “cancer” refers to a broad range of illnesses that affect various parts of the body. There are a variety of factors that contribute to its genesis. Mostly, cancer is referred to as a genetic disease brought about by diverse genetic factors (Peto & Houlston, 2001). However, many epidemiological studies have revealed environmental factors, which could be physical, chemical, or biological may be crucial in the disease’s genesis and progression (Montesano & Hall, 2001). A few such environmental factors include infections, radiation, lifestyle choices such as diet, smoking, alcohol, and other occupational exposures (Clapp et al., 2008; Czene et al., 2002).

Data compiled by the WHO implies that cancer is a leading cause of mortality globally, accounting for around 10 million fatalities. Regionally, most cancer cases and related mortality were recorded in Asia, which was 49.3% and 58.3% respectively. Africa recorded about 5.7% cases and 7.2% deaths in 2020. The regional distribution of the number of new cases is shown in Figure 2.1 (Sung et al., 2021).

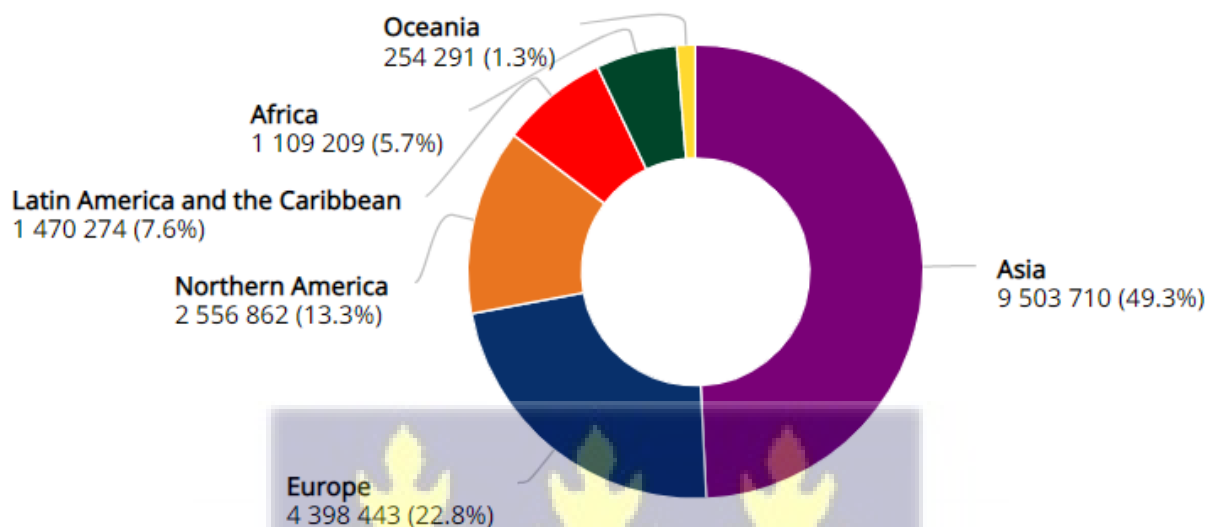


Figure 2.1 Regional distribution of the estimated number of new cases for all cancers (Globocan 2020)

However, as shown in Figure 2.2a below, the breast (11.7%), lung (11.4%), colorectum (10%), prostate (7.3%), stomach (5.6%), and liver (4.7%) were the cancer types with the highest incidence rates worldwide. Breast cancer was highest in women accounting for 24.5% incidence and 15.5% deaths followed by colorectal cancer which was 9.4% and 9.5% cases and death respectively. In men, lung cancer was seen to be the highest with 14.3% cases and 21.5% deaths and this was also followed by prostate cancer with 14.1% and 6.8% cases and death respectively (Sung et al., 2021).

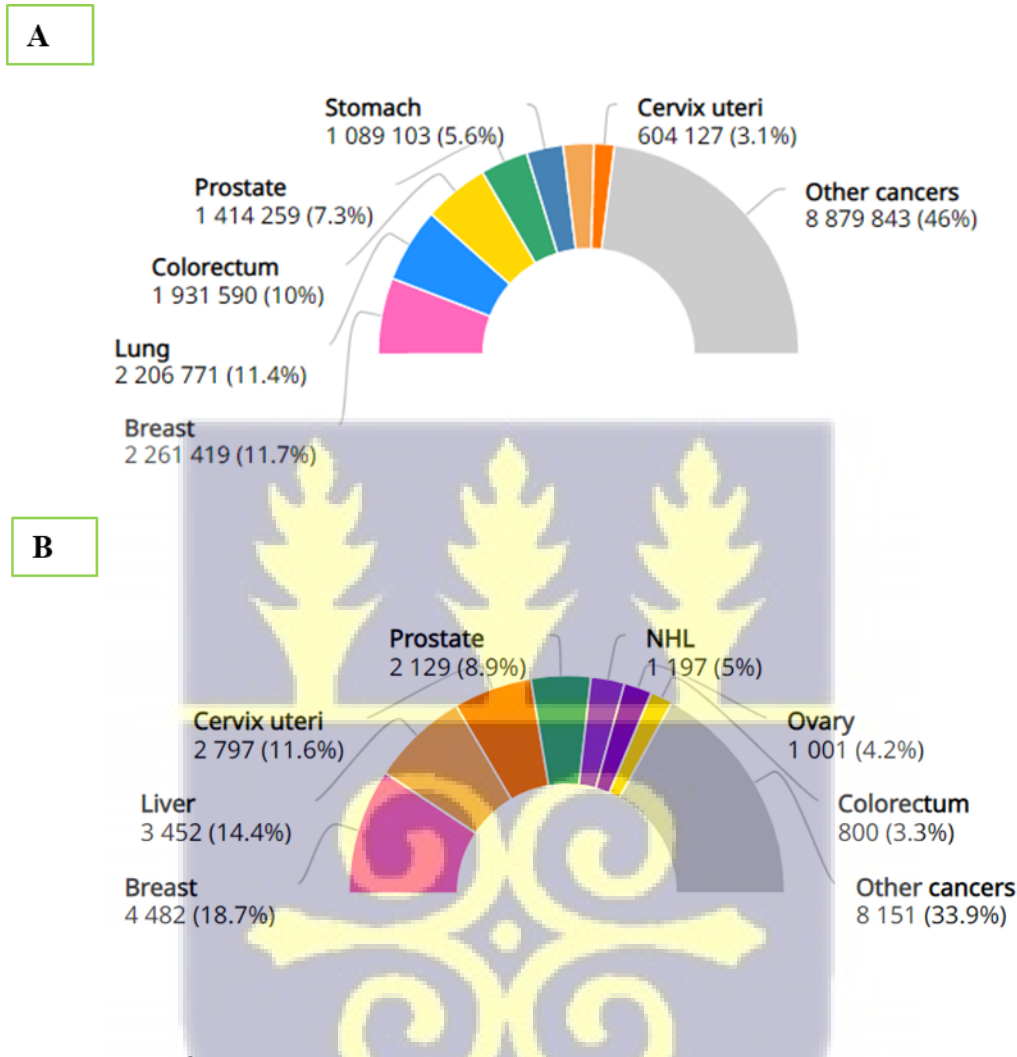


Figure 2.2 Number of (A) new cancer cases worldwide and (B) in Ghana. The data represents both sexes and all ages in 2020 (Globocan 2020)

In Ghana, the case and mortality trends show that there was a total of 24009 new cases and 15802 cancer deaths according to GLOBOCAN 2020 reports. Out of this, cases of breast cancer accounted for 18.7%, liver cancer for 14.4%, uterine cancer for 11.6%, prostate cancer for 8.9%, non-Hodgkin lymphoma for 5%, and other cancers for 41.5%. as shown in figure 2.2b above. Again, breast cancer was the highest among females whilst liver and prostate were the highest cancer cases among men (Siegel et al., 2021).

2.2 Characteristics of cancers (cancer hallmarks)

Normal cells within the human body multiply by a firmly controlled cell division machinery which involves balancing the production and release of growth-promoting signals (Bahassi, 2007). Unlike normal cells, cancerous cells acquire numerous genetic and epigenetic alterations of genes that enable their continuous growth (Liu et al., 2014). This phenomenon is mostly brought about by diverse factors such as exposure of normal cells to infectious particles, chemicals, radiation, and other environmental factors (Czene et al., 2002). These alterations cause the gain of abnormal phenotypic properties which constitute the six biological hallmarks of cancer proposed by Hanahan and Weinberg: (i) increase in cellular proliferation, (ii) resistance to cell death, (iii) evasion of growth suppressors, (iv) enhancement of angiogenesis, (v) activating invasive and metastatic potentials and (vi) enabling replicative mortality (Hanahan & Weinberg, 2011). Studies have uncovered other hallmarks also implicated in cancer progression. These include avoidance of immune destruction, tumor-promoting inflammation, genome instability, and mutations, and lastly, deregulation of cellular energetics (Arakelyan et al., 2023; Pavlova et al., 2022; Pavlova & Thompson, 2016).

2.3 Phenotypic and molecular determinants of cancer progression

2.3.1 Tumor proliferation

Tumor proliferation involves the abnormally rapid growth of cells brought about by the sustenance of signals that promote cell growth. Normal cells regulate the synthesis and the release of growth-promoting signals. However, this control is compromised in cancer cells, enabling the continual proliferation of cancer cells (Nussinov et al., 2017). Many cellular replication genes are overexpressed in cancers making them possible markers to measure cancer progression and diagnosis (Kristiansen, 2018). Some of such include markers of proliferation *KI-67*,

minichromosomal maintenance protein complex (MCM), and proliferating cell nuclear antigen (PCNA) (Cuzick et al., 2011; Juríková et al., 2016). *PCNA* is mostly used as a cell cycle marker that functions importantly in the metabolism of nucleic acids, DNA replication, cell cycle control, and chromatin assembly. The *MCM* serves as a proliferation marker due to its important role in DNA replication. The most common proliferation marker is the *KI-67* (Juríková et al., 2016). It is an important prognostic marker of cell growth and may reflect the aggressiveness of cancers such as breast cancer (Denkert et al., 2015). The *KI-67* gene encodes proteins necessary for proliferation and it is detected in all the phases of the cell cycle process except at the resting stage. High *KI-67* expression levels may indicate rapid cell division and vice versa (Sobecki et al., 2017).

2.3.2 Resistance to Apoptosis

Apoptosis also referred to as programmed cell death is a controlled process of cellular self-destruction that ensures proper regulations of cellular processes such as development, tissue homeostasis, and removal of damaged cells (Chinnasamy et al., 2020). However, cancerous cells mutate and acquire sustained proliferative ability that reduces cell death hence altering the normal balance between cell proliferation and cell death (Evan & Vousden, 2001). Cellular apoptosis can be measured or detected with several assays. This includes dye exclusion test, lactate dehydrogenase leakage assay (LDH), annexin V-FITC assay, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay, DNA fragmentation assay, crystal violet assay, etc. (Chinnasamy et al., 2020). The apoptotic death cascade involves markers such as Bcl-2, *Bcl-2* associated death (*BAD*) promoter, caspases, tumor protein 53, etc. (Smyth et al., 2002), and the measurement of these markers provides useful cancer information. The *Bcl-2* gene can be used as both an apoptotic and proliferation marker. This is because cells that are resisting cell death and actively proliferating highly express the *Bcl-2* (Chinnasamy et al., 2020; Smyth et al., 2002).

2.3.3 Angiogenesis

The process involves the generation of additional blood vessels which enhances the supply of oxygen and other nutrients necessary for tumor growth (Zare et al., 2013). The process may occur as a result of the generation of pro-angiogenic substances by tumor cells through an angiogenic switch (Viallard & Larrivée, 2017; Zhang et al., 2019). An example of a pro-angiogenic factor is *VEGF* which is the key regulator that causes the formation and proliferation of endothelial cells (Zhang et al., 2019).

2.3.4 Cancer cell migration and metastasis

Metastatic malignancies are defined by the invasion and migration of cancer cells. This is caused by the propagation of cancer cells from the primary infection tissue to a secondary organ in the body. The process may occur through migratory processes such as collective cell migration, mesenchymal cell migration, and amoeboid cell migration (Chaffer & Weinberg, 2011; Wu et al., 2021). During this process, the cells gain the ability to change their morphology and their capacity to attach to other cells as well as invade the extracellular matrix (ECM) (Hanahan & Weinberg, 2011). Several other factors contribute to the invasion and metastasis of tumors such as angiogenic factors, proteases (cathepsins, collagenase, and metalloproteases (MMPs)), and adhesion proteins (Chin et al., 2005). The type IV collagenase group *MMP-2* and *MMP-9* are the most reliable prognostic markers out of all the metalloproteases (Chin et al., 2005). *MMP-9* proteolytically digests the ECM molecules to enable cell migration. Also, the epithelial-mesenchymal transition (EMT) is another factor implicated in this process where epithelial cancerous cells acquire mesenchymal properties (Tsai & Yang, 2013). Due to this process, mesenchymal markers like vimentin(*VIM*) express themselves more strongly than epithelial markers like E-cadherin (Qian et al., 2013). *VIM* is known to encode cytoskeletal proteins usually found in mesenchymal tissues

and its higher expression indicates the acquisition of mesenchymal phenotypes through EMT which is indicative of metastasis.

2.3 Infectious agents and cancer

Studies have shown that at least 20% of cancers are associated with infectious pathogens either viral, bacterial, or parasitic infections. It behooves that these infections may play a role in cancers and their progression (De Martel et al., 2012; Yasunaga & Matsuoka, 2018). These infectious organisms may contribute to the onset of cancer or the progression of an existing tumor. This is due to their ability to reside within the tumor microenvironment and operate as carcinogens or create oncoproteins that accelerate the course of cancer (Ahmed & Seddon, 2022; Banerjee et al., 2015). The infectious pathogens within the body may also activate the immune response and cause inflammations that could contribute to promoting cancer (Macleod & Mansbridge, 2015). Nonetheless, some of these pathogens tend to enable antitumor immunity and immune surveillance which is advantageous to cancer regression (Pope et al., 2017; Xuan et al., 2014).

2.3.1 Viruses and Cancer

Certain cancers have been shown to have viral etiology. These may include human immunodeficiency virus (HIV), human papillomavirus (HPV), Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), human T-lymphotropic virus 1 (HTLV-1), hepatitis B & C virus, among others and their effect can either be direct or indirect (Morales-sánchez & Fuentes-pananá, 2014). These viruses may have the potential to initiate cancer by expressing viral oncogenes, viral latency, or through prolonged infection and inflammation leading to cancer-causing mutations in healthy cells (Mitchell et al., 2015; Moore & Chang, 2010; Polansky & Schwab, n.d.). On the other hand, some viruses may tend to influence the existing tumor microenvironments primarily via modulating cytokine production (Mesri et al., 2014).

Studies reveal that comorbid viral infections in cancer may increase the progression of cancer (Panigrahi & Ambs, n.d.; Yasunaga & Matsuoka, 2018). Viral infections may tend to be persistent to cause the activation of chronic inflammation that results in tissue and DNA damage. This implies that novel persistent viruses capable of causing severe inflammatory responses such as SARS-CoV-2 could contribute to cancer progression (Porta et al., 2011; Yasunaga & Matsuoka, 2018). Again, together with the inflammatory pathways, certain mechanisms may be adapted in viral infections to aid cancer progression. Some of these viruses are involved in interleukin (IL) mediated cellular proliferation and survival, ROS oxidative stress, and VEGF-mediated angiogenesis (Yasunaga & Matsuoka, 2018). However, not all viral infections cause carcinogenesis and cancer progression. Owing to their replication cycle, some could be beneficial in tumor regression which has led to recent research into therapeutic viruses (Miest & Cattaneo, 2013).

2.4 Coronaviruses (CoVs)

Coronaviruses belong to a family of viruses that cause infections within the respiratory tract and intestines. They have an enormous host range that is inclusive of mammals and birds (Kim et al., 2020). They are named for the way they appear on electron micrographs; enveloped viral particles with club-like structures on their surfaces, giving the appearance of a crown or corona (Sofi et al., 2020; Xian et al., 2020). They form the biggest group of RNA viruses that are a member of the order Nodovirales. Coronaviridae constitutes one of the four families under this order. The family is classified into four main genera based on their genome structures and phylogeny. That is, Alphacoronaviruses, Betacoronaviruses, Gammacoronaviruses, and Deltacoronaviruses. They constitute about thirty-eight known species (Deep et al., 2020; Wu et al., 2020; Sofi et al., 2020). In general, coronaviruses have been linked to both minor and serious illnesses (Burrell et al., 2017).

Alpha and beta CoVs usually cause infection in mammals while the Gamma and Delta CoVs have birds as their hosts. Currently, there are only seven coronaviruses known to cause infections in humans (Deep et al., 2020; Zhu et al., 2020). Of these, those that cause mild infections include the human Coronavirus 229E (HCoV-229E), human coronavirus OC43 (HCoV OC43), human Coronavirus NL63 (HCoV-NL63), and human Coronavirus HKU1 (HCoV-HKU1). They are mostly implicated in respiratory diseases such as the common cold and are used in coronavirus research (Fung & Liu, 2019). The genomes of coronaviruses range in size from 27 to 32 kilobase pairs (kb). They contain the codes for the four structural proteins envelope, membrane, spike, and nucleocapsids together with additional accessory proteins that determine virulence (Burrell et al., 2017).

Extremely dangerous coronavirus strains include Middle East respiratory syndrome coronavirus (MERS-CoV) and Severe acute respiratory syndrome Coronavirus (SARS-CoV) (Babarinsa et al., 2021). They are known to cause MERS and SARS respectively. The first coronavirus known to cause serious illness in people was SARS-CoV. This highly pathogenic virus emerged in 2002, infecting around 8000 individuals and accounting for the deaths of more than 700 individuals (Pal et al., 2020). The MERS-CoV caused an outbreak in 2009 and has so far infected 2519 individuals. It caused the deaths of 866 individuals and is still responsible for sporadic cases yearly (Alyami et al., 2020). The most recent pathogenic strain that can cause life-threatening illnesses is SARS-CoV-2, which is what is causing the present global pandemic (Deep et al., 2020).

2.5 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

Severe acute respiratory virus 2 (SARS-CoV-2), is an enveloped, positive sense single-stranded RNA (+ssRNA) virus of the genus Betacoronavirus (Jackson et al., 2022). The outbreak was originally discovered in Wuhan, China (Khan et al., 2020). Infection with the virus causes COVID-

19, a serious respiratory infection in humans that is extremely contagious (Hsu et al., 2020; Rudrapal et al., 2020). This disease has spread around the world infecting several hundreds of millions of people, killing millions, and evolving many viral variants along the way. Variants of this virus have been detected around the globe, some with higher transmissibility (Zhang et al., 2020).

2.5.1 SARS-CoV-2 virion morphology and genome organization

Electron microscopy of SARS-CoV-2 revealed that the virus particle is spherical and has a size of about 100 to 150 nm (Jackson et al., 2022; Kumar et al., 2019). As shown in Figure 2.3a, viral proteins include four structural proteins: spike (S), nucleocapsid (N), membrane (M), and envelope (E) (Santos et al., 2020). The membrane, envelope, and spike glycoprotein are rooted in the host-derived lipid bilayer. The bilayer encapsulates the nucleocapsid, which has the viral RNA (Kumar et al., 2019). The spike glycoprotein (S protein) has a molecular weight of 180-200 kDa after N-glycosylation (Casalino et al., 2020; Walls et al., 2020). It is a homotrimeric polypeptide that is embedded within the membrane of the virus to give it the appearance of a crown (Deep et al., 2020). The protein has 3 domains; an extracellular N-terminal which interacts with host proteins, a transmembrane domain which anchors it to the lipid bilayer, and an intracellular C-terminal segment which is usually short. The protein undergoes structural rearrangement upon interaction with the host receptor to facilitate viral entry (Walls et al., 2020; Zhou et al., 2020). The genome of SARS-CoV-2 is approximately 30 kb and contains 15 open reading frames (ORFs) encoding 27 proteins (Baruah et al., 2020). The 5' end which is about 20kb consists of the ORF1a and ORF1b and encodes polyproteins that encode 11 and 5 non-structural proteins (nsp), respectively. These 16 non-structural proteins form part of the replicase and transcriptase complex and are

conserved in coronaviruses of the same family (Alsobaie, 2021; Michel et al., 2020). A diagram showing the genome organization is shown in Figure 2.3B

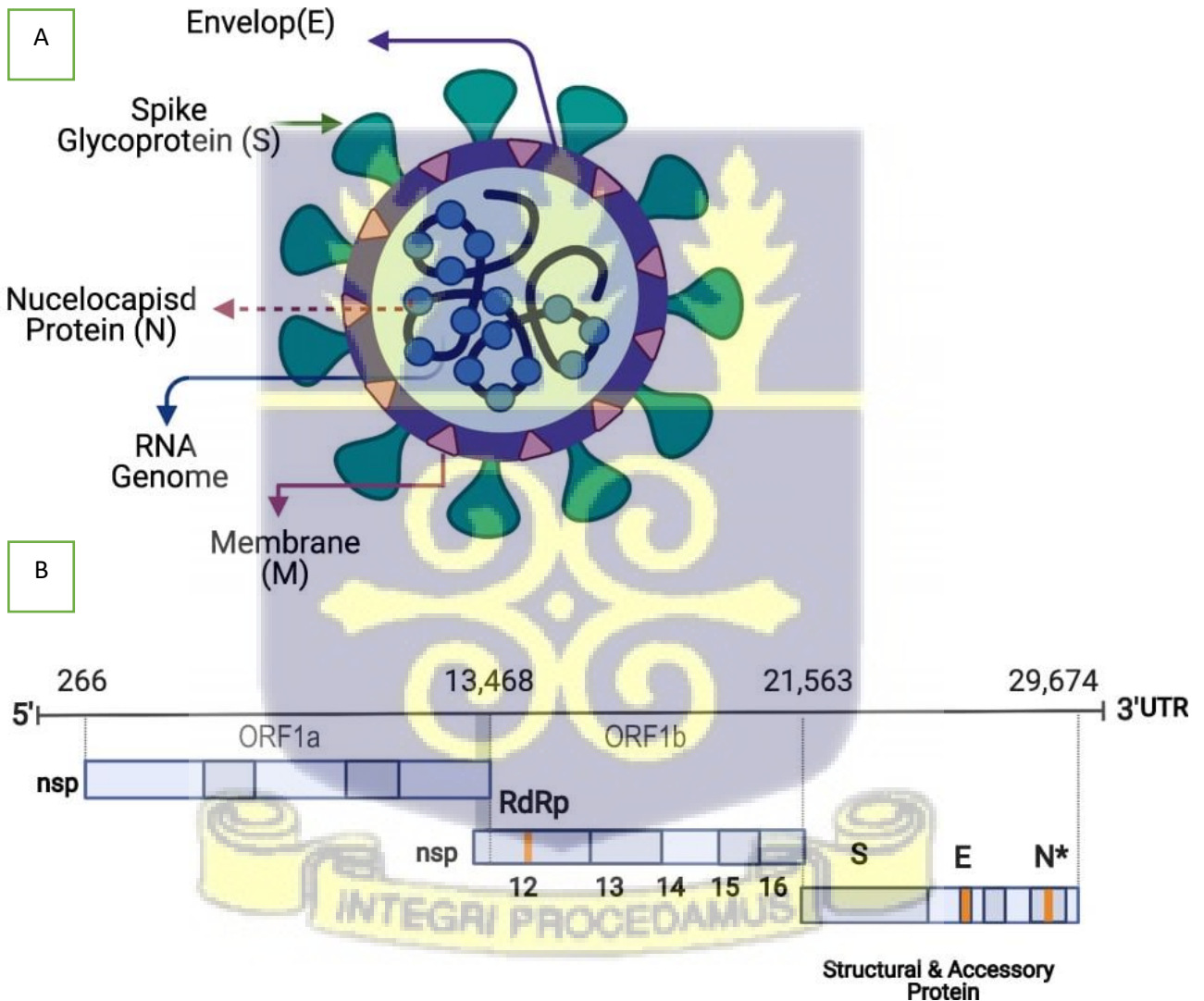


Figure 2.3: SARS-CoV-2 virion structure (A) and genome organization(B) (Alsobaie, 2021)

2.5.2 SARS-CoV-2 entry mechanism and replication cycle

The SARS-CoV-2 replication cycle in the host begins when there is a successful infection of the host cells via specific interactions between the virus S glycoprotein and human ACE2 receptor and

some proteases (Baggen et al., 2021; Peng et al., 2021). The S-protein is made of two domains, 1 and 2 which are separated by a protease cleavage site (Gobeil et al., 2021). The first domain interacts with the host receptor ACE2 and the second mediates viral fusion with the host membrane (Belouzard et al., 2012; Huang et al., 2020). Interaction of the S1 domain and ACE2 causes a conformational change in the S protein that leads to the exposure of the protease site for cleavage. This cleavage is done by host proteases like transmembrane serine protease 2 (TMPRSS2), cysteine protease cathepsin B (CatB), and cathepsin L (CatL) (Hoffmann et al., 2020). These interactions facilitate the entry of the virus into the host cell, replicates, and subsequently triggers the immune system (Zhang et al., 2021). Viral replication occurs in the cytoplasm, which begins with viral gene expression. The virus recruits host machinery such as ribosomes for the translation of its ORF1a and ORF1b to produce polyproteins pp1a and pp1ab (Malone et al., 2022; Q. Zhang et al., 2021). The polyproteins are processed and cleaved by viral proteases to produce 16 non-structural proteins that assemble into the replication-transcription complexes (RTCs). The RTCs produce a new set of genomic RNAs with ORFs which can either serve as a template for further RNA synthesis or encode the structural proteins spike, membrane, envelope, and nucleocapsid as well as additional accessory proteins to be packaged into new virions (Malone et al., 2022). At the end of viral replication, newly produced virions are coated with nucleocapsid proteins and obtain a lipid bilayer that contains all the necessary viral proteins by budding off through the ER-Golgi compartment (Malone et al., 2022). A graphical depiction of the replication cycle is shown in Figure 2.4

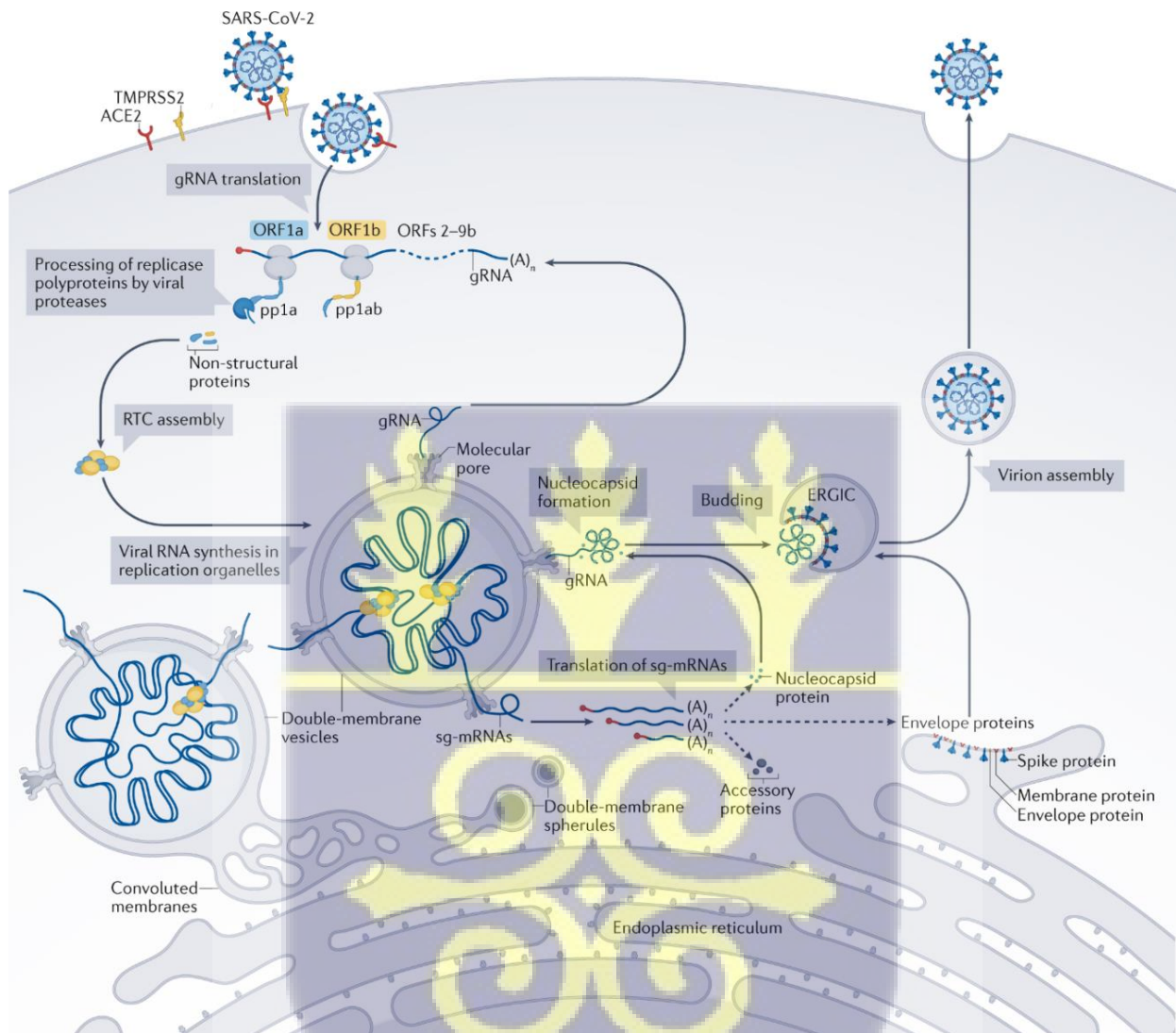


Figure 2.4: SARS-CoV-2 infection cycle (Malone et al., 2022)

2.6 Angiotensin-converting enzyme 2 (ACE2)

ACE2 is an enzyme that belongs to the renin-angiotensin system (RAS) that plays a significant part in cellular functions (Zhang et al., 2019). The enzyme has been established as the canonical receptor for SARS-CoV-2 infection. That is, only cells expressing *ACE2* enable efficient infection and replication of the virus. The receptor was already identified as the receptor that facilitated infection following the 2003 SARS- CoV epidemic (Prabakaran et al., 2004; Bian & Li, 2021). A study showed that the inhibition of *ACE2* receptor expression prevented viral entry (Chen et al.,

2021). There is a necessary interaction that forms a salt bridge between the Lys417 of the S protein and Asp30 on ACE2 to create a spike-ACE2 complex. However, there can be variations within the receptor which can have a possible impact on the functionality of the receptor. This variation can also affect susceptibility and severity of infection (Hoffmann et al., 2020; Hou et al., 2020; Veeramachaneni et al., 2021)

2.6.1 Expression and function of ACE2 in tissues

The enzyme has a crucial role in the renin-angiotensin system. This system's primary role is to maintain electrolyte homeostasis and regulate blood pressure (Tikellis et al., 2012). The enzyme is a membrane-bound carboxypeptidase that catalyzes the hydrolysis of a vasoconstrictor, angiotensin II to vasodilator, angiotensin 1-7 to counterbalance the activity of ACE (Kuba et al., 2013; Menon et al., 2007). ACE2 is expressed in various tissues throughout the human body (Tang et al., 2021). It is expressed in increasing levels in the kidney, testis, small intestine, and heart tissue. But oddly, the level of gene or protein expression seen in the lungs is rather low in comparison to other organs (Ryu & Shin, 2021; Tang et al., 2021).

2.6.2 Expression and function of ACE2 in cancers

Depending on the tumor type, ACE2 may either function to promote tumor progression by enhancing one of the many properties of cancer or inhibit such properties which in turn becomes beneficial in inhibiting tumor progression. ACE2 has been reported to inhibit certain cancer properties such as growth and survival, metastasis, and angiogenesis among others in numerous cancers including cancer of the breast, colon, prostate, lungs, kidney, etc. (Zhang et al., 2019). ACE2 is an indicator of a better prognosis in cancers like breast cancer and hepatocellular carcinoma (Zhang et al., 2019).

Transmembrane serine protease 2 (TMPRSS2)

Transmembrane serine protease 2 (TMPRSS2) is a membrane-bound serine protease that has been implicated in the entry of the SARS-CoV-2 virus. As was previously mentioned, the enzyme cleaves the S protein after binding to ACE2 which is necessary to ensure entry (Stopsack et al., 2020). However, the ability of the virus to utilize alternative endosomal proteases such as cathepsin L makes the function of TMPRSS2 in SARS-CoV-2 infection debatable (Thunders & Delahunt, 2020). It plays a role in pathological processes like tumor cell invasion and apoptosis as well as physiological functions like digestion, blood clotting, tissue modeling, and inflammatory response (Guo et al., 2020). TMPRSS2 is recognized to be involved in cell invasion and metastasis in cancers like prostate cancer. According to research, TMPRSS2 suppression reduced prostate cancer cell invasion and metastasis (Ko et al., 2020). Their expression on cancer cells may contribute to the susceptibility of the cell to SARS-CoV-2 infection

2.7 Coronavirus disease 2019 (COVID-19)

Coronavirus disease refers generally to any disease emanating from coronavirus infection. Coronaviruses are an enormous group of viruses implicated in diseases like mild colds or severe pneumonia (Al-quteimat & Amer, 2020). The 2019 infectious coronavirus disease, however, is caused by SARS-CoV-2 and is transferrable from person to person (Acter et al., 2020). According to WHO, all infected persons, either symptomatic, pre-symptomatic, or asymptomatic could cause transmission of the virus. From November 2019- February 2020, there were increasing cases and deaths occurring in China and the disease was fast spreading, reaching up to about 25 countries. In March 2020, the disease was deemed a public health emergency of global concern by the WHO (Landman et al., 2020), and later that month as a pandemic disease. As of January 13, 2023, data from the WHO dashboard indicated that there have been 661,545,258 confirmed cases globally

and about 6,700,519 deaths on the global front. In Ghana, the total number of confirmed cases was 171,070 between 3 January 2020 to January 13 2023 with 1461 deaths (WHO).

2.7.1 Symptoms, diagnosis, and treatment

The symptoms of COVID-19 may range in severity from those that are asymptomatic or moderate to those that are serious enough to cause death. After exposure to the virus, the onset of symptoms may begin between 2-14 days but mostly after 5 days due to their incubation period (Acter et al., 2020). Without a known diagnosis, a symptomatic person according to the CDC may present with at least two or three of the following signs. That is cough, fever, fatigue, muscle pain, loss of smell, taste disorder, headache, sore throat, nausea, vomiting, diarrhea, congestion, difficulty breathing, confusion, and chest pain among others. Reports on the intense form of the disease are acute respiratory distress syndrome (ARDS) and severe respiratory injury with pneumonia (Al-quteimat & Amer, 2020). Asymptomatic cases, however, are individuals who test positive for the virus and show no sign or symptoms of the disease. These individuals were mostly identified through contact tracing and voluntary testing. However, these individuals could be silent transmitters who spread the virus to other persons for extended periods (Oran & Topol, 2020). Again, due to the asymptomatic nature, the percentage of infected people might not be reflected in the total number of reported cases. It is therefore important to widen the testing programs to include asymptomatic individuals to reduce the silent spreading of the virus and also help with the accuracy of reported cases.

The diagnosis of the disease relies mainly on the clinical manifestation of the disease. However, symptoms of COVID-19 patients are not peculiar to the condition. Hence, there is an inefficiency in the use of clinical presentation as a stand-alone diagnostic tool. The accepted 'gold standard' in the determination of the presence of the virus is real-time quantitative polymerase chain reaction

(RT-qPCR). It is a nucleic acid detection test in which primers and probes are used in the amplification of the ORF1b and N gene of SARS-CoV-2 (Chan et al., 2020; Hu et al., 2020; Tian et al., 2020). High throughput sequencing can be used as a nucleic acid detection method, however, due to the cost and limited equipment, RT-qPCR is the most acceptable. Other forms of diagnosis employed may include chest CT scans characterized by ground-glass opacity and/or double-side infiltrates in the lungs, antibody detection by lateral flow immunoassay and enzyme-linked immunosorbent assay (ELISA) (Biemond et al., 2020).

In the initial stages, there was no specific treatment and medication or approved vaccines, hence common clinical management practices were used to curb infections. Most of the treatments offered were based on common symptoms presented by patients (Al-quteimat & Amer, 2020). These included pain management, fever management, and administering antibiotics. Other broad-spectrum antiviral drugs such as remdesivir and drugs such as chloroquine, azithromycin, doxycycline, zinc tablets, and tocilizumab were used for managing the condition (Geleris et al., 2020; J. Liu et al., 2020; Skipper et al., 2020). Novel strategies used in the management of the condition include the use of convalescent plasma, complement inhibitors, anti-SARS-CoV-2 antibody cocktails, and soluble ACE2 among others (Stone et al., 2020; Xu et al., 2020).

In recent times, there is the availability of vaccines produced to help fight the disease. The vaccines produced were either DNA-based, RNA-based, or protein-based (Soo et al., 2021). Within six to nine months into 2021, there were several vaccine candidates but about 18 of those were approved to be used as an emergency response to curb the spread of the disease (Ndwandwe & Wiysonge, 2021). Some of the commonly known ones such as jcovden (Johnson and Johnson), spikevax (Moderna), comirnaty (Pfizer), vaxzevria (AstraZeneca), sputnik V (Gamaleya), and Serum Institute of India's Covisheild were among those that were approved to be used in Ghana. These

were delivered either as a single-dose therapy or a double-dose therapy. As of January 6, 2023, data from the WHO dashboard indicated that there have been 13,107,022,929 vaccine doses administered worldwide. In Ghana, a report on vaccination dates 11th December 2022 with a total of 21,400,939 doses administered (WHO). That is, more than half of the Ghanaian population have received the vaccines with about 37% receiving at least 1 dose and 28% fully vaccinated.

2.7.2 Immune Response

Upon SARS-CoV-2 infection, pathogen-associated molecular patterns (PAMPs), which initiate the innate immune response, as well as damage-associated molecular patterns (DAMPs) induced by host cell damage are released (Zhou et al., 2020). PAMPs and DAMPs interact with some pathogen-recognition receptors (PRRs) in cells like alveolar macrophages. This results in a wave of inflammation-mediated secretion of chemokines and cytokines into the bloodstream (Ferreira et al., 2021). A clinical data characteristic analysis of death cases in Wuhan, China revealed elevated levels of IL-1, IL-6, IL-7, IL-8, IL-9, IL-10, growth factors, blood growth factors, etc., when compared to healthy individuals (Ferreira et al., 2021). These molecules act as a chemoattractant to mobilize other immune cells like Natural killer cells, T cells, B cells, neutrophils, macrophages, plasmacytoid and myeloid dendritic cells, and plasma cells to the site of infection (Huang et al., 2020). The immune response generated against SARS-CoV-2 is robust and also diverse across different groups as shown in

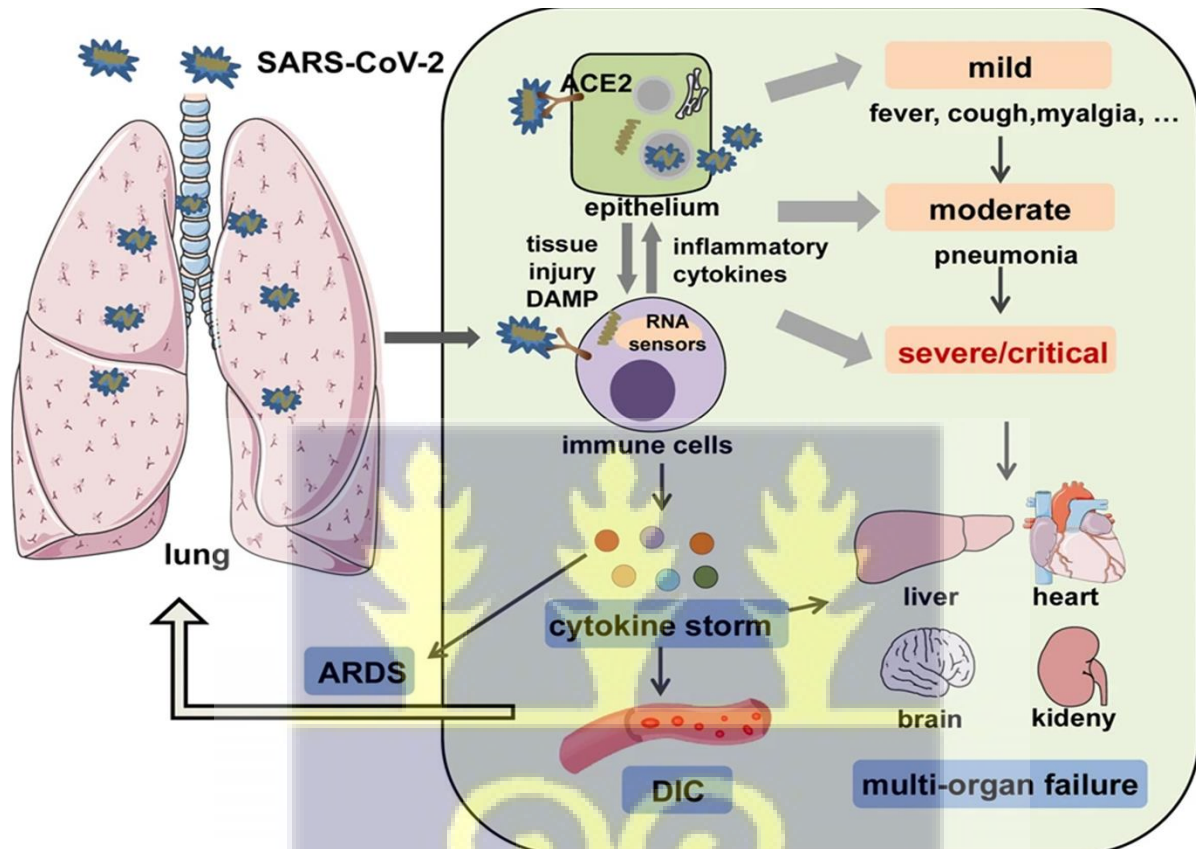


Figure 2.5

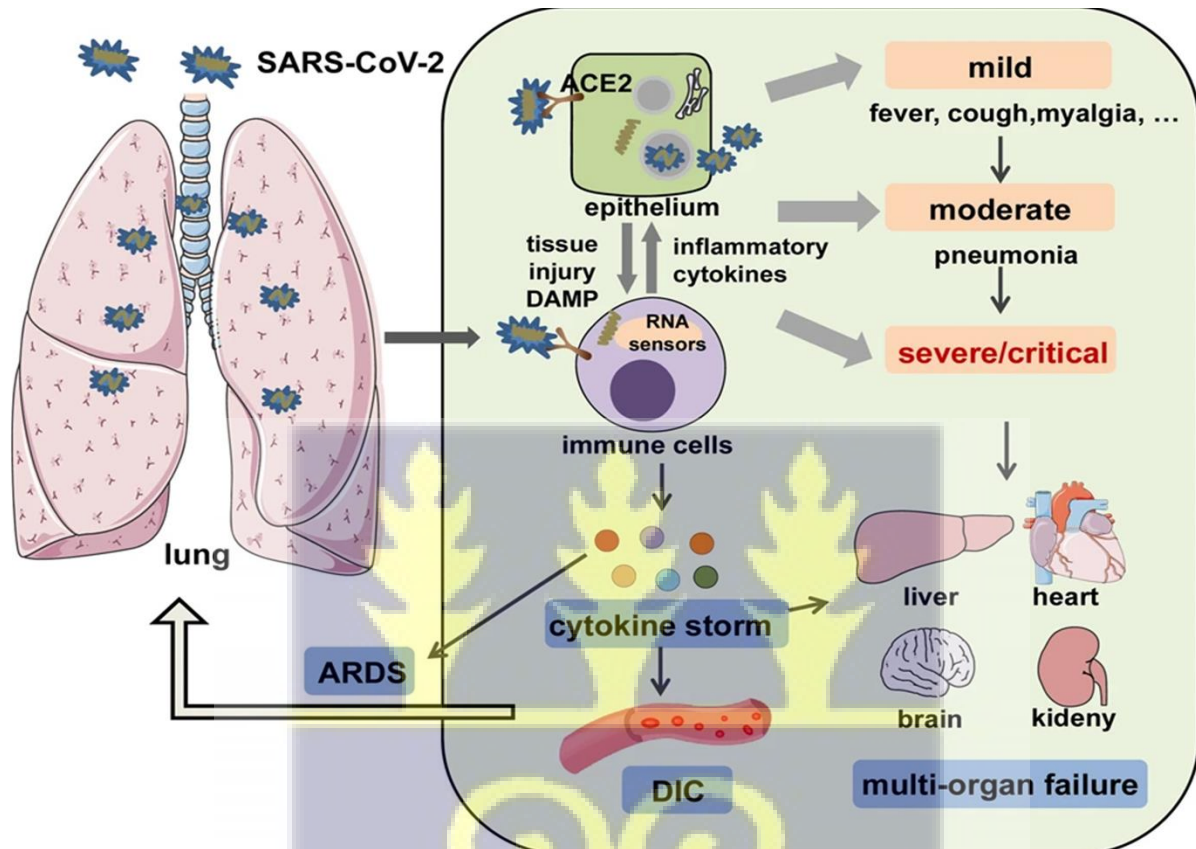


Figure 2.5: SARS-CoV-2 infections immune response and cytokine storm (Yang et al, 2021)

2.7.3 Factors that determine COVID-19 severity

Viruses utilize cell surface receptors to access the cell interior, where their replication process takes place (Wu et al., 2021). Receptors are crucial for the pathogen to enter host cells, and they may also control how a disease develops (Dimitrov, 2000). Studies have identified ACE2, and TMPRSS2, as receptors that interact with spike protein to mediate its entry, and blockage to the receptors does interrupt host cell invasions (Itoyama et al., 2005). Studies have suggested that genetic variation and the level of receptor expression may affect the severity and susceptibility of diseases (Hoffmann et al., 2020a; Kam et al., 2009; Shang, Wan, et al., 2020; Shang, Ye, et al., 2020). There were reports that older people and obesity may contribute to COVID-19 severity

(Wolff et al., 2021). Additionally, some reports indicated that persons with a fatally severe form of the illness also had pre-existing medical issues (Fang et al., 2020; Sanyaolu et al., 2020). Among these comorbidities, some of the most common ones included HIV infection, Hepatitis B infection, respiratory diseases, renal diseases, hypertension, diabetes, cardiovascular disorders, asthma (Oduro-Mensah et al., 2021), and cancers (Guan et al., 2020; Kamyshnyi et al., 2020; Klein, 2020; Sanyaolu et al., 2020).

2.8 Impact of COVID-19 and cancer comorbidity

Comorbid COVID-19 instances are those who have the virus but are also dealing with another illness. This could be a viral, bacterial, fungal, and parasite infection, or non-communicable conditions including cancer, diabetes, heart or lung disease (Fang et al., 2020). Patients living with any form of malignancy have a lowered immune defense which increases their risk of opportunistic infections (Al-quteimat & Amer, 2020; Liang et al., 2020). Cancers added to the severity of the COVID-19 condition, according to research on the relationship between comorbidities and disease severity (Fang et al., 2020). COVID-19-cancer coinfections were linked to an increase in mortality, admittance into the intensive care unit, and dependence on ventilators, as opposed to individuals who only had SARS-CoV-2 infection (Landman et al., 2020). Additionally, normal healthcare checks for cancer patients were no longer being performed due to the strain on healthcare facilities caused by the virus's rapid spread. Treatment therapies were also being delayed which could negatively impact the prognosis for cancer (Al-quteimat & Amer, 2020). There were also records on the linkage between chemotherapy and infection severity. Patients undergoing cancer treatments tended to have COVID-19 illness that was more severe than usual (Landman et al., 2020; Zhang et al., 2020)

Several studies have reported on the relationship between cancer and COVID-19 coinfection. There have also been reviews addressing the possible effects that infection may have on cancer patients and the progression of their condition. Sanyaolu et al., 2020 reported in their study that about 1.5% of a total of 1789 recruited in the study had a form of malignancy. Additionally, a study conducted in China in the early phases of the pandemic revealed about 1.3% of cases were cancer coinfections and among them, lung cancer was the highest, at about 28%. They described their findings to be alarming because it was higher than the overall cancer incidence in the Chinese community (Landman et al., 2020). Other types of cancers discovered to be linked to COVID-19 disease included esophageal cancers, and breast cancers (Zhang et al., 2020). Reports are showing that compared to other types of malignancy, patients with lung cancer coinfection had the least severe complications with a probability of reduced lung volume (Zhang et al., 2020). Nonetheless, the direct effect on the cancer cells and the tumor microenvironment remains unclear.

2.8.1 Impact of COVID-19-related immune response on cancer

As a way of understanding disease physiology and how it affects cancer, studies have predicted the possible pathways that may come to play to either promote or reduce cancer progression as a response to SARS-CoV-2 infection. As ACE2 interacts with the spike to initiate infection, there could be a deleterious effect on the renin-angiotensin system due to changes in its pathways (Verdecchia et al., 2020). This can affect cancer progression in diverse ways. As illustrated in Figure 2.6 below, successful entry could downregulate ACE2 and consequently cause an imbalance as well as elevated bradykinin levels. Either way, downstream processes may lead to reduced anti-proliferation action, angiogenesis, P13K augmentation, and activation of the mitogen-activated protein kinase (MAPK) pathway: all of which could contribute to cancer progression. Reports show there was an increase in cytokine levels observed in the plasma of

severe COVID-19 patients (Bülow et al., 2021; Kovacs-kasa et al., n.d.). This COVID-19-induced hyperproduction of cytokines could also contribute to cancer progression in a person with cancer. The produced cytokines may activate inflammation-mediated cancer progression through the phosphorylation of certain tumor proteins and the activation of pathways such as EMT (Saha & Anirvan, 2020; Yun et al., 2019). However, there is a need for more and further analysis into how these changes and inflammation may affect the progression of cancer.



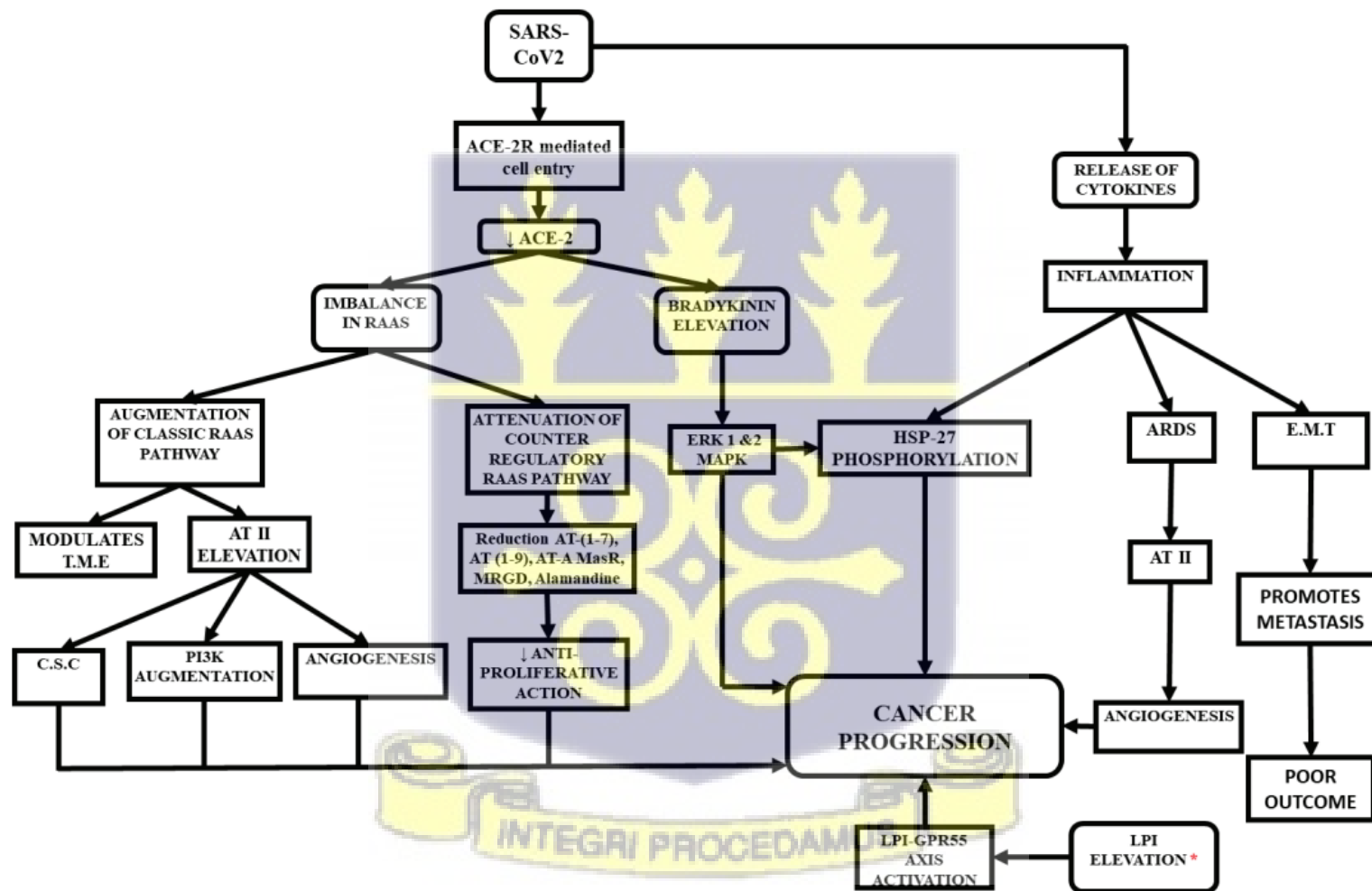


Figure 2.6: Possible impacts of COVID-19 on cancer progression (Saha & Anirvan, 2020)

2.8.2 Impact of COVID-19-related inflammation on cancer

As has been discussed above, COVID-19 disease is linked to an increase in the inflammatory response. When the first line of immune response is activated in response to viral particles and replication, there is an increased inflammatory response that results in a cytokine storm. This could cause the activation of certain proteins that are involved in pathways that promote cancer progression (Hu, et al., 2021; Saha & Anirvan, 2020). The mechanism that brings about this continuous and increased cytokine production may involve the rapid activation of T helper 1 (Th1) cells. The T helper 1 (Th1) cells are known to be involved in the release of IL-6. This interleukin can further activate immune cells to enable other cytokines to be released in higher quantities (Hu, et al., 2021). Continuous and increased cytokine production could also be brought about by ACE2-mediated activation of NF- κ B whose signaling could lead to the release of IL-6 and TNF- α . Again, IL-6 signaling through STAT3 can cause the further release of IL-6 and IL-8 and other chemokines like VEGF (Hu, et al., 2021; Tang et al., 2020)

Studies have reported on the various types of cytokines implicated in the COVID-19-related cytokine storm. It has been shown that there were elevated levels of IL-1 β , IL-6, IL-8, IL-7, IL-10, IL-12, IP-10, and TNF- α among others with IL-6 being the most implicated (Fara et al., 2020; Hu, et al., 2021; Soy et al., 2020; Tang et al., 2020). Within the tumor microenvironment, cytokines may act as drivers of tumorigenesis or as inhibitors of tumors. IL-6 has been known to be involved in signaling that promotes cancer progression and survival (Turnquist et al., 2020). Also, the binding of IL-6 to its receptor could activate pathways and proteins implicated in EMT. IL-1 β has been shown to interact with other cytokines to initiate a cascade of events that results in promoting

EMT. $\text{TNF-}\alpha$ within the tumor microenvironment can cause tumor proliferation as well as metastasis through EMT activation (Heydarian et al., 2021)



CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design

The study was an empirical study that involved the use of SARS-CoV-2 PV produced in the Virology laboratory at the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP). There was also the use of SARS-CoV-2 viral particles isolated at the Noguchi Memorial Institute for Medical Research (NMIMR) from respiratory samples of a patient infected with the wild-type SARS-CoV-2 strain. Cancer cell lines were originally acquired from American Type Culture Collection (ATCC).

3.2 *In silico* gene expression analysis

The differential gene expression of ACE2 and TMPRSS2 was analyzed using Gene Expression Profiling Interactive Analysis (GEPIA) software. The program performs gene expression analysis based on 9736 tumors from The Cancer Genome Atlas (TCGA) and 8587 normal samples from The Genotype-Tissue Expression (GTEx) databases, using standard RNA sequencing data. Briefly, ACE2 and TMPRSS2 gene expression analysis was carried out utilizing the defined sample selections BRCA (Breast invasive carcinoma), COAD (Colon adenocarcinoma), and PRAD (Prostate adenocarcinoma). The expression profiles were presented as box plots comparing the tumor tissues to the normal tissues of the selected samples.

3.3 *In vitro* analysis for ACE2 expression in breast, colorectal, and prostate cancer cell lines

3.3.1 Cell Culture

Every cancer cell line utilized in the study was grown and maintained in a culture medium that was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-strep).

The cells were kept at 37 °C while being exposed to 5% carbon dioxide. Weekly subcultures of cells were performed, and some were kept at -80°C. The list of the various cancer cell lines, their cell types, and their culture medium are shown in Table 1 below.

Table 1: List of cancer cell lines

Cancer Type			Cell Lines	Culture Media
Breast (BRCA)	Invasive Carcinoma		MDA-MB-231	DMEM
			MDA-MB-468	
Prostate (PRAD)	Adenocarcinoma		22RV1	RPMI
			BPH-1	
Colon (COAD)	Adenocarcinoma		DLD-1	RPMI
			COLO205	
			HCT-15	
Controls			HELA- Negative	DMEM
			HEK293T- Positive	

3.3.2 Dot blot

A dot blot analysis was used to determine the level of ACE2 on the cancer cell lines. Briefly, cells were trypsinized and centrifuged at 1000 rpm for 5 minutes. The resuspended pellets were transferred into Eppendorf tubes. Cells were lysed following a 30-minute incubation on ice with 200ul phosphate lysis buffer containing 0.1% imidazole, 0.5% triton X, and a 1X protease inhibitor and then vortexed. 3ul of cell lysates were spotted on nitrocellulose membrane and left to dry for 30 minutes at room temperature. After that, the membrane was blocked for 1 h on the bench with

3% bovine serum albumin (BSA) in 1X PBS-T. The membrane was incubated with the primary antibody (1 in 1000 dilution with 1% BSA-PBS-T) for 1h and 30 minutes before being rinsed thrice with 1X PBS-T for 10 minutes each. The membrane was then incubated with the secondary antibody (1 in 10000 dilutions with 1% BSA-PBS-T) for 1 h and washed five times with 1X PBS-T for 10 minutes each. The detection substrates were added (1:1 dilution of Super Signal West Pico Plus Stable Peroxide and Super Signal West Pico Plus Luminol Enhancer) and viewed using the Amersham™ Imager 600 (GE Life Sciences, UK).

3.4 Pseudovirus production and infection

3.4.1 Bacterial transformation

All bacterial cultures (XL-10 competent cells) were prepared using LB broth and agar by the manufacturer's directions. Briefly, 25g of LB powder was weighed and added to 1000 ml of distilled water. The mixture was stirred gently to allow for a uniform mixture and autoclaved at 121°C and 15psi for 15 minutes before use. For the LB agar, 15g of agar and 25g of LB powder were weighed into 1000 ml of distilled water and stirred gently to mix. The medium was then autoclaved at autoclaving conditions and allowed to cool before pouring under a flame. All autoclaved broth and agar were stored at 4°C.

For the transformation process, the DNA to be used for the transformation was prepared to a working concentration of 10ng/μl. Briefly, 50μl XL-10 competent cells (Stratagene, USA) were aliquoted in a pre-chilled Eppendorf tube and stored on ice. After that, 5μl of the DNA was then pipetted into the tube containing the competent cell and incubated on ice for 30 minutes. Competent cells only were used as a negative control for contamination. After the incubation, the samples were heat-shocked at 42°C for 30 seconds in a water bath and immediately transferred

back to the ice. 100µl of pre-warmed LB broth containing 50mg/ml of ampicillin was added to the tubes. The tubes were shaken at 180 rpm while being incubated for 1h at 37 °C. 30µl of the transformed cells were spread uniformly on the agar plate infused with 50mg/ml of ampicillin. The plates were placed at 37°C for 16 to 18hs.

3.4.2 DNA extraction

Plasmid extractions were carried out using the QIAprep® Spin Miniprep Kit (QIAGEN, Germany) after single colonies from transformed cells were cultured in antibiotic LB broth at 37°C for up to 16hs. Briefly, a 6-minute centrifugation at 5000 rpm was performed on overnight bacteria cultures. After the supernatant was discarded, the pelleted cells were resuspended in 250µl of buffer P1 and then put into a 1.5 ml microcentrifuge tube with labels. 250µl of buffer P2 was added to each tube, and well mixed by inversion till a blue solution was visible. Then, 350µl of buffer N3 was added and thoroughly mixed till the solution became colorless. After that, the mixture was centrifuged for 10 minutes at 13,000 rpm. The supernatant was transferred into QIAprep 2.0 spin column and centrifuged at 13,000 rpm for 1 minute and the filtrate in the collection tubes was discarded. The spin column was washed with 500µl of buffer PB by centrifuging it for 1 minute at 13,000 rpm. The filtrate in the collection tubes was then discarded. Another round of washing was done using 750µl of Buffer PB. To remove the remaining wash buffer, the spin column was centrifuged a second time for 1 minute. The spin column was then transferred into a fresh 1.5 ml microcentrifuge tube and filled with 50µl pre-warmed buffer EB. This was centrifuged for 1 minute to elute the DNA after being incubated at room temperature for 1 minute. The extracted DNA was quantified using Nanodrop (Thermo Fisher Scientific, USA) and kept at 4°C for storage.

3.4.3 Pseudovirus production

SARS-CoV-2 PV was produced using the calcium phosphate precipitation method as described by Chen, 2011 with few modifications. Using the 3-plasmid system, HEK 293T cells were co-transfected with pcDNA 3.1 SARS-CoV-2 S, firefly luciferase-expressing plasmid PCSFLW, and a lentiviral packaging plasmid p8.91. Briefly, the transfection reagent was prepared as followed; 2M CaCl_2 was prepared using CaCl_2 and distilled water, and the solution was filtered under aseptic conditions using a $0.2\mu\text{m}$ syringe driven filter and stored at room temperature. HBS at 2X was prepared with 50mM HEPES, 280mM NaCl, and 1.5mM Na_2HPO_4 . The solution was filtered, aliquoted in microcentrifuge tubes, and stored at -20°C .

About 3×10^5 cells per well were seeded in 6 well plates, with 2ml complete media, and incubated for 24 hours at 37°C with 5% CO_2 . At 3 hours pre-transfection, cells were replenished with fresh media. For each transfection, two separate tubes A and B were prepared. Solution A was made up of $100\mu\text{l}$ of 2X HBS and solution B was made up of $12.2\mu\text{l}$ of CaCl_2 , about $4\mu\text{g}$ of S DNA, $4\mu\text{g}$ of PCSFLW, and $4\mu\text{g}$ of p8.91. The solution was topped up to $100\mu\text{l}$ using nuclease-free water. Solution B was added to solution A gently and kept at ambient temperature for 30 minutes. The resulting mixture was gently applied to cells and swirled gently to ensure uniform distribution. Following that, cells were cultured for 24hs at 37°C with 5% CO_2 . The media was changed after 24hs. Media containing SARS-CoV-2 PV were harvested after 48 and 72hs. The PV was filtered using a $0.45\mu\text{m}$ syringe-driven filter to remove cellular debris. Harvested viruses were aliquoted and stored at -80°C until use. Mock PV stock was made by transfecting HEK 293T cells with the firefly luciferase-expressing plasmid PCSFLW and a lentiviral packaging plasmid p8.91 only.

3.4.4 Pseudovirus luciferase assay

Briefly, cells were seeded and incubated for 24h at 37°C with 5% CO₂. About 300µl of PV was added to each well and cultured for 48h at 37°C with 5% CO₂. The luciferase activity was determined using the One-Step™ Luciferase Assay System (BPS Bioscience, USA) as per the manufacturer's instructions with few changes. The luciferase reagent buffer (A) was thawed to room temperature. The working solution was prepared by making a 1:100 dilution of the luciferase reagent substrate (B) and luciferase reagent buffer (A). Media was discarded and a volume of 200µl of the working solution was added to each well and kept for 15 minutes while rocking gently. After the incubation, the solution was mixed gently by pipetting up and down and about 100µl of the solution in each well was aliquoted into 96 well white plates in duplicates. Luminescence was then determined using the GloMax®-Multi detection system (Promega, USA). Cells only and cells with the mock virus were used to determine the background signal.

3.5 Cell proliferation assay

Roche cell proliferation kit 1 (MTT) (Sigma-Aldarich, USA) was used to examine infection effects on cell growth following the manufacturer's protocol with few modifications. Briefly, cells were harvested and about 1×10^4 per well for 22RV1 and 5×10^3 per well for DLD-1 cells were seeded in a tissue-culture grade 96-well plate and incubated overnight. Cells were then replicately infected with 100µl of PV and incubated for 48h and 72h. Mock viruses and medium alone were used as controls while incubating cells. After each incubation period, 10µl of the MTT labeling reagent was added to each well and incubated for 4h. Afterward, 100µl of the solubilization buffer was added to each well and incubated overnight. The plate was then read with the Varioskan LUX microplate reader (Thermo Fisher, USA) at an absorbance of 570nm. All incubations were done at 37°C with 5% CO₂.

3.6 Cell viability assay

Cell viability was assessed using the Promega CellTiter-Glo® luminescent cell viability assay following the manufacturer's instructions with a few modifications. Briefly, cells were seeded into tissue-culture grade 24-well plate at 1.5×10^5 cells per well and incubated at 37°C with 5% CO₂ for not more than 24h. The cells were then infected with 100µl of PV and incubated for 72h. Cells were incubated with media only and mock virus as controls. All assays were performed in triplicates. A volume of CellTiter-Glo® reagent equal to the volume of the cell culture medium present in each well was added and mixed gently on an orbital shaker for 2 minutes to lyse the cells. The plate was then incubated in the dark for 10 minutes at room temperature. The luminescence was read using the GloMax®-Multi detection system (Promega, USA).

3.7 Wound healing assay

Cellular migration analysis was done using a wound-healing assay with silicone culture inserts (Ibidi GmbH, Munich, Germany) as described by Pijuan et al., 2019 with modifications. Briefly, cells were harvested and about 1×10^4 per well of silicone culture insert for 22RV1 and 5×10^3 per silicone insert well for DLD-1 cells were seeded in a tissue-culture grade 12-well plate and incubated at 37°C with 5% CO₂ overnight to allow the cells to attach to create about 90-95% confluent monolayer. The silicone culture inserts were removed gently and aseptically with sterile forceps. The plate was then washed gently thrice with 1ml sterile 1X PBS to remove non-attached cells. The cells were then infected with 100µl of PV with media only and mock virus as controls to a media volume of 2ml and incubated at 37°C with 5% CO₂. Assays were performed in triplicates for each experimental condition. Images were taken with an Optikalview light microscope (OPTIKA® Ponteranica, Italy) several times at a specific time interval for not more

than 72h. Image analysis was done with ImageJ software to determine the percent (%) of the wound area.

3.8 Gene expression analysis

3.8.1 RNA extraction

Each well in a 6-well plate was seeded with 3×10^5 cells. The cells were infected with PV, with media only, or mock virus, in triplicate, and incubated at 37°C with 5% CO₂ overnight. Total RNA from infected and non-infected cells was extracted using Zymo *Quick-RNA*[™] Miniprep Plus Kit per the manufacturer's protocol. Briefly, cells were lysed with 600µl of RNA lysis buffer and transferred into the yellow Spin-Away[™] column placed in a collection tube, and centrifuged at 13000g for 30 seconds. An equal volume of absolute ethanol was added to flow-through, mixed thoroughly, and transferred into the green Zymo-Spin[™] column in a collection tube. DNase 1 treatment was performed by first washing the column with 400µl of RNA wash buffer. For each treatment, 5µl of DNase 1 and 75µl of DNA digestion buffer were mixed in a fresh nuclease-free tube. The mixture was then added directly to the column matrix and incubated at room temperature for 15 minutes. After DNase 1 treatment, 400µl of RNA prep buffer was added, centrifuged at 13000 g for 30 seconds and the flow-through was discarded. The column was washed twice with RNA wash buffer first with 700µl for 30 seconds and 400µl for 2 minutes at 13000g each. 50µl of DNase/RNase-Free Water was added and centrifuged at 13000g for 30 seconds to elute RNA. RNA was quantified using nanodrop and stored at -80°C.

3.8.2. RT-qPCR assay

RT-qPCR primers were designed using the IDT- PRIMERBLAST platform. Primers were diluted with 1X TAE buffer as directed by manufacturers to a stock concentration of 100mM and further diluted to a working concentration of 10Mm.

Table 2: List of primers for gene expression analysis

Gene	Forward primer sequence 5'>3'	Reverse primer sequence 5'>3'
VEGF	CTCTACCTCCACCATGCCAAGT	TCGATTGGATGGCAGTAGCTG
BCL2	TGCACCTGACGCCCTTCAC	AGACAGCCAGGAGAAATCAAACAG
KI-67	AATTCAGACTCCATGTGCCTGAG	CTTGACACACACATTGTCCTCAGC
MMP9	ACGCACGACGTCTTCCAGTA	CCACCTGGTTCAACTCACTCC
Vimentin (VIM)	TCTCTGAGGCTGCCAACCG	CGAAGGTGACGAGCCATTTC
TNF- α	AGTGACAAGCCTGTAGCCC	GCAATGATCCCAAAGTAGACC
IL-1 β	GCACGATGCACCTGTACGAT	CACCAAGCTTTTTTGCTGTGAGT
IL-6	CAATGAGGAGACTTGCCTGGTGA	TGGCATTGTGGTTGGGTCAG
IL-8	CACCGGAAGGAACCATCTCACT	TCAGCCCTCTTCAAAAACCTTCTCC
GAPDH	TGCACCACCAACTGCTTA	GGATGCAGGGATGATGTTC

The RT-qPCR assay was performed using the Luna Universal OneStep RT-qPCR kit (New England Biolabs, UK). For each reaction, 20µl PCR mix was made with 1µg of RNA, 0.8µl each of forward and reverse primers, 1µl of high-fidelity enzyme mix, 10µl of the reaction mix, and nuclease-free water.

Table 3: RT-qPCR cycling condition for gene expression analysis

Condition	Temp (°C)	Time	Number of cycles
Reverse transcription	55	10 minutes	1
Initial denaturation	95	1 minute	1
Denaturation	95	30 seconds	40
Annealing	56-62	30 seconds	
Extension	60	30 seconds	

3.9 Live virus infection

To confirm the effect of the PV on the cell lines, live virus infections were performed for all assays. Briefly, laboratory stocks of live SARS-CoV-2 virus (LV) isolated from respiratory samples of COVID-19 patients were obtained from the global immunology and immune sequencing for epidemic response (GIISER) project. For all the live virus assays, cells were infected with a viral multiplicity of infection (MOI) of 0.02 based on a TCID₅₀ titration of the virus stock done at the time of viral isolation. All infections were done under appropriate biosafety conditions.

3.10 Statistical Analysis

Raw data were obtained and stored in Excel and data analysis was performed using GraphPad Prism version 9.1.2 (GraphPad Software, San Diego, USA). The One-way ANOVA was used to compare the difference between all groups of the pseudotyped virus infectivity on cancer cells. The paired t-test was used to compare the difference between all groups for LV infection. Descriptive statistics were presented as mean with standard deviation and percentages. Statistical significance was determined at a p-value that is less than or equal to 0.05.



CHAPTER FOUR

4.0 RESULTS

4.1. ACE2 and TMPRSS2 genes are expressed differentially in normal and tumor tissues

To determine the difference in expression levels of ACE2 and TMPRSS2, the gene expression profiles of some selected tumor samples and paired normal tissues were obtained from the GEPIA database. ACE2 is expressed widely across various normal and tumor tissues and the expression levels differ based on the tissue type. ACE2 is downregulated in normal breast and prostate tissues. Also, the expression in breast cancer tissue, as well as prostate cancer tissues, was downregulated indicating that the receptor is generally not localized. Nonetheless, comparing the normal tissues to cancerous tissues, the level of ACE2 in breast and prostate tumors was lower compared to the normal tissues with no significant difference (Figure 4.1a). The level of ACE2 expression in colorectal tumors was significantly higher than that in normal tissue (Figure 4.1a).

Similarly, TMPRSS2 is expressed in varying levels across different tissue types. However, TMPRSS2 was observed to be expressed in a highly significant manner in prostate tumor tissues compared to its paired normal tissues. On the other hand, expression was significantly high in normal breast and colorectal tissues compared to their paired tumor samples. Generally, among the selected samples, TMPRSS2 seems to be more localized in prostate tissues (Figure 4.1b).

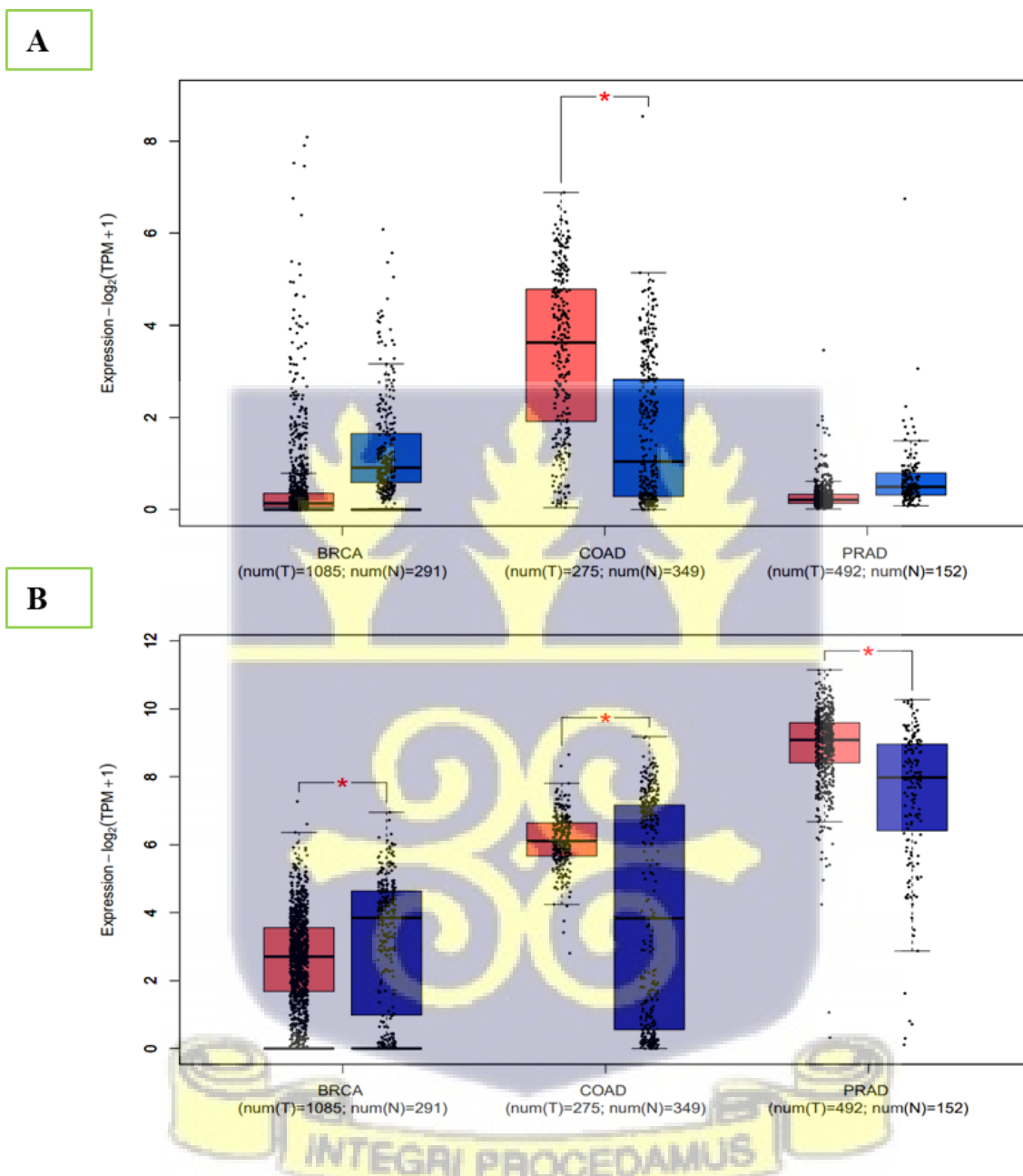


Figure 4.1: Relative expression levels of (A) ACE2 and (B) TMPRSS2 in selected tumors and corresponding normal tissues.

The expression cut-off was set as 1 from the $\log_2$ fold change (Log_2FC) with a p-value cut of 0.01. Data are presented as a fold change of log expression of ACE2 transcription per million (LogTPM). (BRCA= breast invasive adenocarcinoma, COAD= colon adenocarcinoma, PRAD= prostate adenocarcinoma, T=tumor (red), N= normal (blue), num=number)

4.2 *In vitro* analysis of ACE2 expression in some cancer cell lines

In vitro analysis for levels of ACE2 in seven cancer cell lines MDA-MB-231, MDA-MB-468, DLD-1, COLO205, HCT-15, 22RV1, and BPH1 was performed using a dot blot analysis (appendix 1). The assay is a simple direct blotting technique that allows for the analysis of the absence or presence of a protein. As a positive control, HEK29T cell lines transfected to stably overexpress ACE2 were employed, whereas HELA cell lines served as a negative control. The finding suggests that all of the cell lines expressed ACE2 in some amount. However, the data was quantified (Davarinejad, 2018) to check the varying levels of the expression for each cell line using the image j version 1.53t (image j software, National Institute of Health, USA). There was low ACE2 expression in the breast cancer cell lines MDA-MB-231 and MDA-MB-468 (Figure 4.2). The expression was also seen to be low in the prostate cancer cell lines 22RV1 and BPH1. However, it was shown that the expression of ACE2 was elevated in the DLD-1, COLO205, and HCT-15 cells. This correlated with the upregulation of ACE2 expression in colorectal cancer observed from the gene expression analysis from GEPIA shown in Figure 4.1a above.



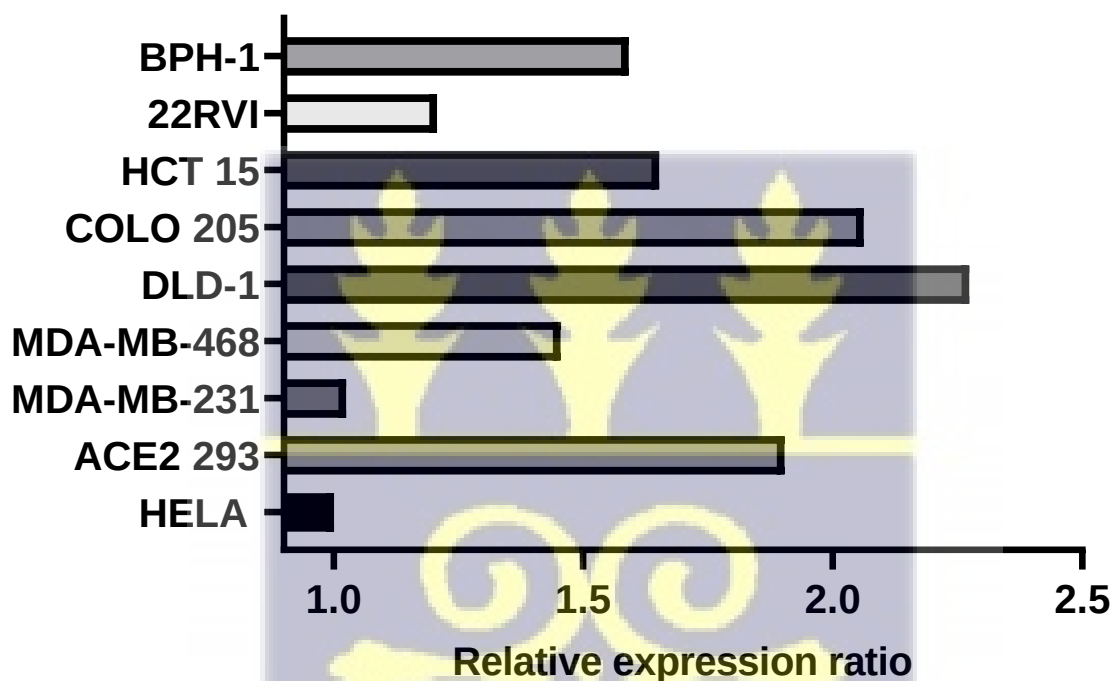
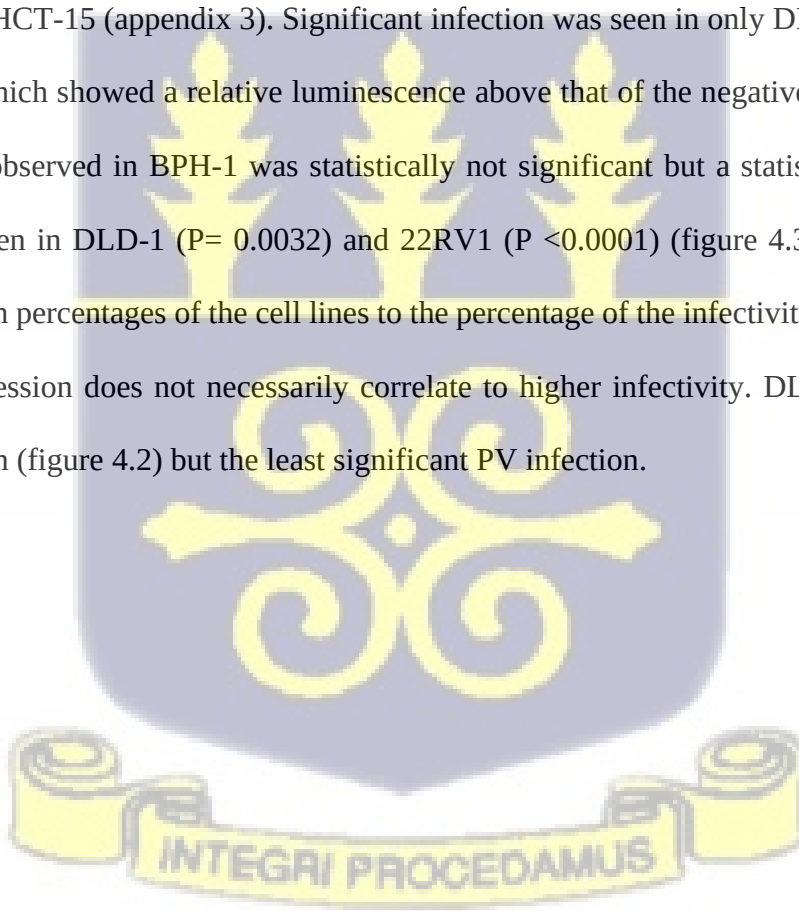


Figure 4.2: Protein expression quantification of ACE2 in cancer cell lines.

ACE2 is expressed highly in DLD-1 among the selected cancer cell lines. The data represents expression levels as a ratio of each protein spot relative to the loading control.

4.3 SARS-CoV-2 PV infection of cancer cell lines

To access the infectivity of the SARS-CoV-2 PV on cancer cell lines, cultured cancer cells MDA-MB-231, MDA-MB-468, DLD-1, COLO205, HCT-15, 22RV1 and BPH1 (HELA and ACE2-HEK293T as negative and positive controls respectively) were infected with PV and mock PV (transfection control). There was little to no infectivity in MDA-MB-231, MDA-MB-468, COLO205, and HCT-15 (appendix 3). Significant infection was seen in only DLD-1, BPH-1, and 22RV1, all of which showed a relative luminescence above that of the negative control (fig 4.3). The infectivity observed in BPH-1 was statistically not significant but a statistically significant infection was seen in DLD-1 ($P = 0.0032$) and 22RV1 ($P < 0.0001$) (figure 4.3). Comparing the ACE2 expression percentages of the cell lines to the percentage of the infectivity of the PV shows that higher expression does not necessarily correlate to higher infectivity. DLD-1 had a higher ACE2 expression (figure 4.2) but the least significant PV infection.



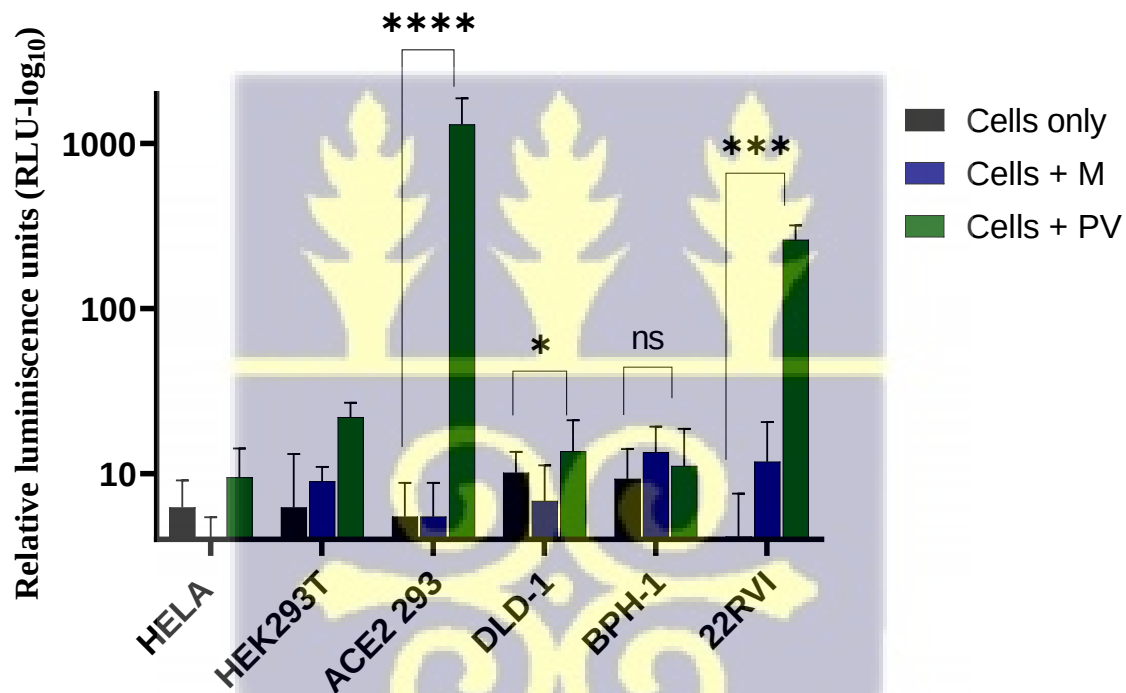


Figure 4.3: Infection analysis of cell lines with PV. A significant infection was observed in DLD-1 and 22RV1 only.

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with one-way ANOVA multiple comparisons

4.4 Phenotypic and molecular analysis of PV-infected 22RV1 cell line

4.4.1 Effects on the proliferation and viability of 22RV1

To explore the PV effect on 22RV1 proliferation, MTT analysis was performed. Cells were infected with PV for 48 and 72 hours. There was a significant decrease in the percentage growth of 22RV1 by the PV for both 48 hours incubation ($p < 0.0001$) and 72 hours incubation ($p < 0.0001$). This demonstrates that the SARS-CoV-2 PV's and ACE2 interaction on 22RV1 cells suppresses the cell line's capacity to proliferate in a time-dependent way (Figure 4.4a).

Based on this result, an analysis of the effects of the PV produced on the viability of the cells using the CellTiter-Glo luminous cell viability assay was made. The analysis was used to assess the possible cytotoxic nature of the PV by analyzing the total number of viable cells 72 h post-infection. The findings indicate that SARS-CoV-2 PV reduces the total number of viable 22RV1 cells post-infection as shown in Figure 4.4b.

Confirmation of the antiproliferative and cytotoxic effect of the PV on 22RV1 was performed by detecting the expression levels of proliferation marker KI-67 and anti-apoptotic marker BCL2 using qRT-PCR. The relative gene expression level of KI-67 post-PV-infection was reduced significantly ($P = 0.0395$) compared to the control in 22RV1 cell lines (Figure 4.4c). This analysis correlates to the results observed for the proliferative phenotypic effect of PV using MTT. The relative gene expression levels for BCL2 between the control and PV-infected cells were not significantly different (Figure 4.4d).

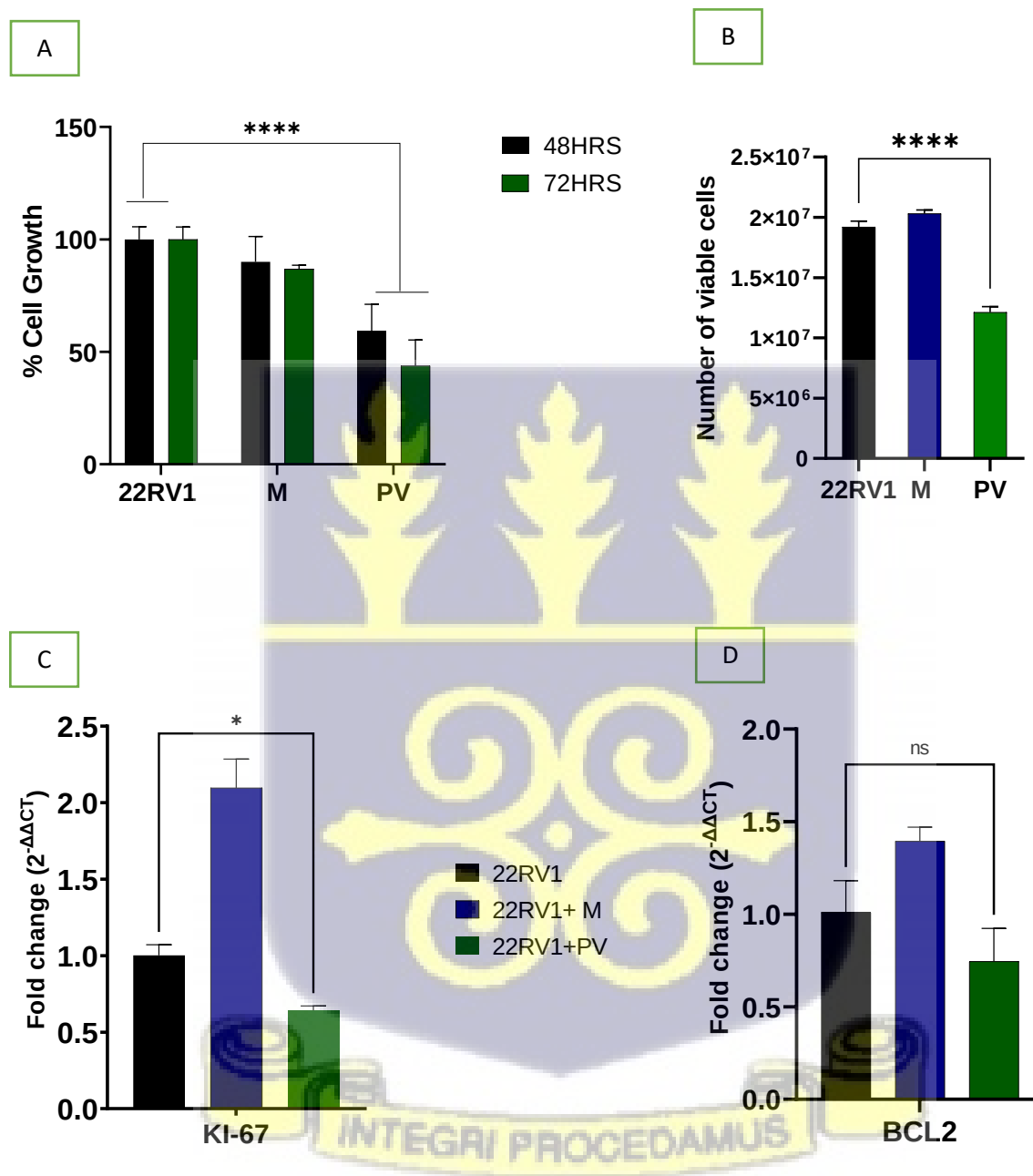


Figure 4.4: PV reduces proliferation and viability of 22RV1.

(A-B) PV infection significantly reduced % growth and cell viability of 22RV1 cell lines

(C-D) Markers of proliferation were also reduced by PV infection in 22RV1 cell lines.

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with one-way ANOVA multiple comparisons

4.4.2 Effects of PV on the migration of 22RV1 cell lines

To explore the effect of PV on cellular migration, the gene expression levels of vimentin and membrane metalloproteinase 9 (MMP9) were analyzed pre and post-infection. Cells were infected with PV for 72 hours and RNA was extracted for analysis using qRT-PCR. *VIM* was non-significantly upregulated in PV-infected 22RV1 cells (Figure 4.5a). Also, PV caused non-significant upregulation of the expression of MMP9 in infected 22RV1 cells than the control. The data suggest that the PV infection in 22RV1 does not necessarily drive the production of molecules involved in the breakdown of the ECM and hence may not contribute to the migratory properties of this cell line.

Based on this result, the migratory effect of the PV was further explored using wound healing analysis. Cells were seeded and allowed to form a monolayer. These monolayers were then infected with PV with images taken at time intervals for not more than 5 days. This is because the 22RV1 cell line is a non-invasive cell line and has a doubling time of about 48 h hence has a low growth rate. Analysis of images shows that 22RV1 showed no visible change in the wound area between infected and non-infected cell lines even after five days of incubation (appendix 4). The result from the analysis of the percent wound area shows that SARS-CoV-2 PV inhibited 22RV1 cell migration, however, this inhibition was statistically non-significant as shown in Figure 4.5c. Nonetheless, the data confirms PV infection of 22RV1 does not affect the migratory properties of the cell line.

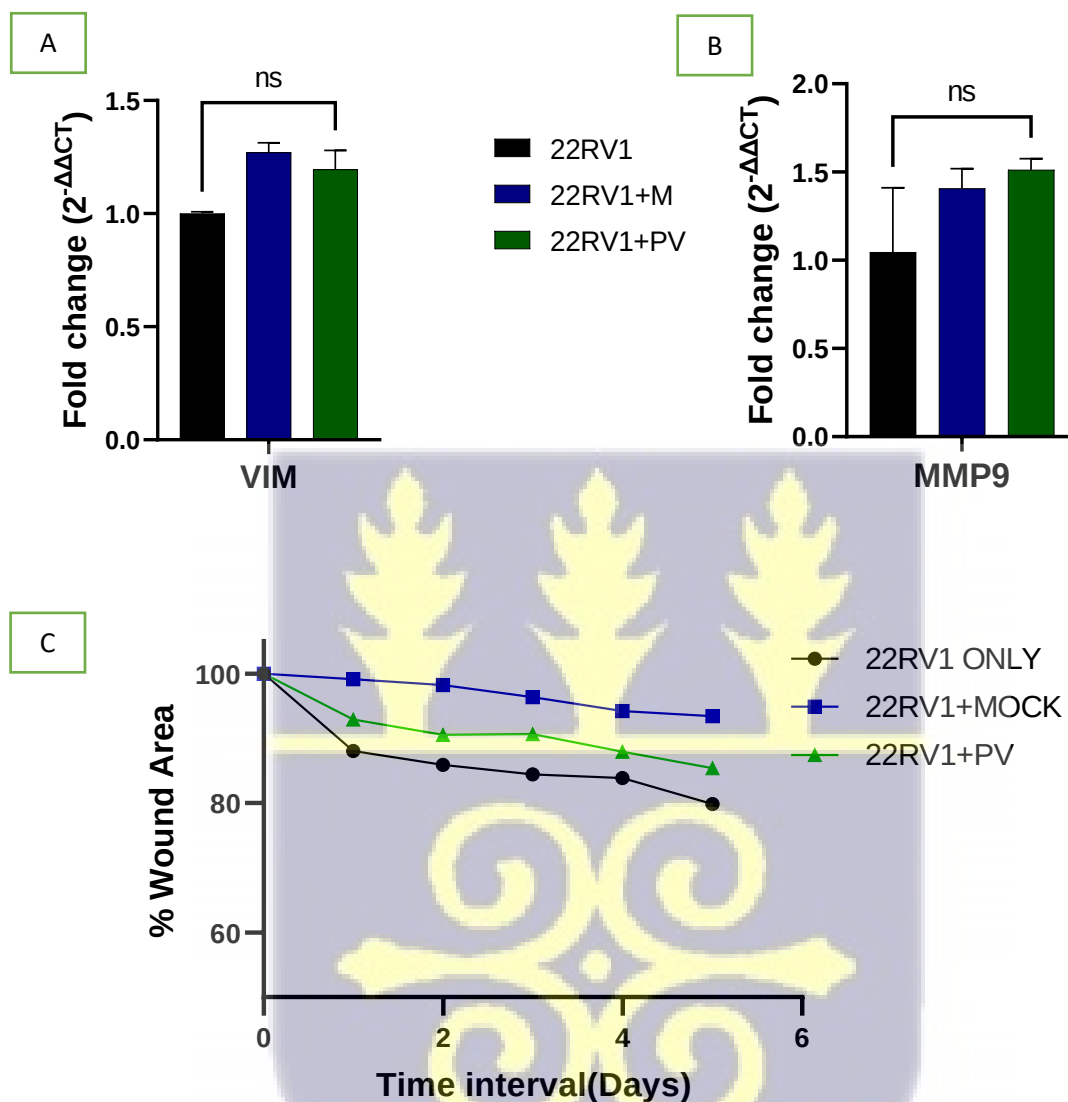


Figure 4.5: Effects of infection on the migration of 22RV1 cells.

(A-B) RT-qPCR analysis showed a non-significant increase in VIM and MMP9 for PV but a significant increase in VIM and a decrease in MMP9 for LV.

(C) Non-significant inhibition of % wound area for PV-infected cells.

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with one-way ANOVA multiple comparisons

4.4.3 Effects on the expression of angiogenic growth factor VEGF in 22RV1

To evaluate the effect of the PV on tumor angiogenesis, qRT-PCR was performed to analyze the expression of the angiogenic growth factor VEGF. From the results, infection with PV has an increasing effect on the expression of VEGF in 22RV1 cells. However, the upregulation of the gene was not statistically significant in cells as shown in Figure 4.6.

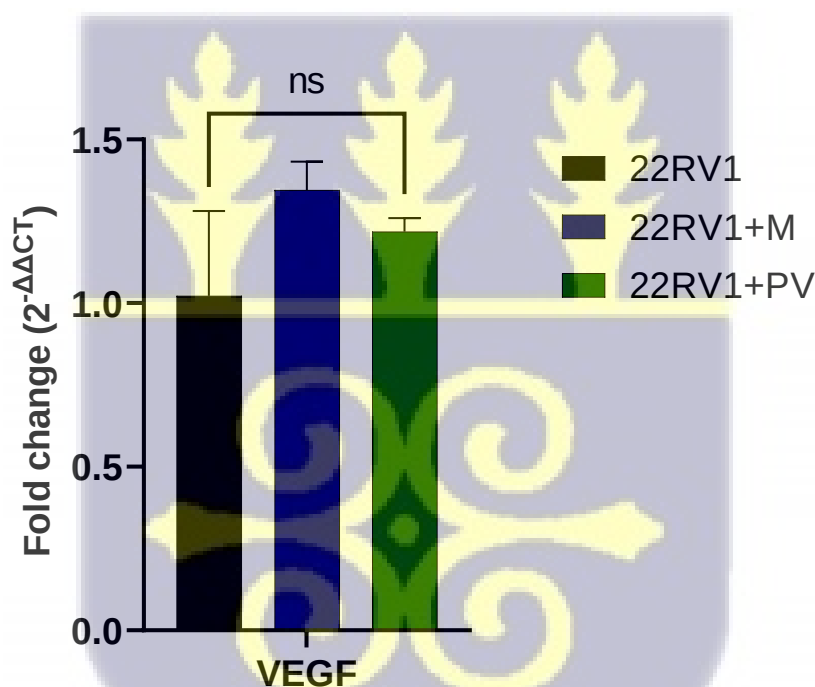


Figure 4.6: Infection causes the upregulation of VEGF in 22RV1.

RT-qPCR analysis of infected 22RV1 cells shows no significant change in VEGF expression in cells infected with PV.

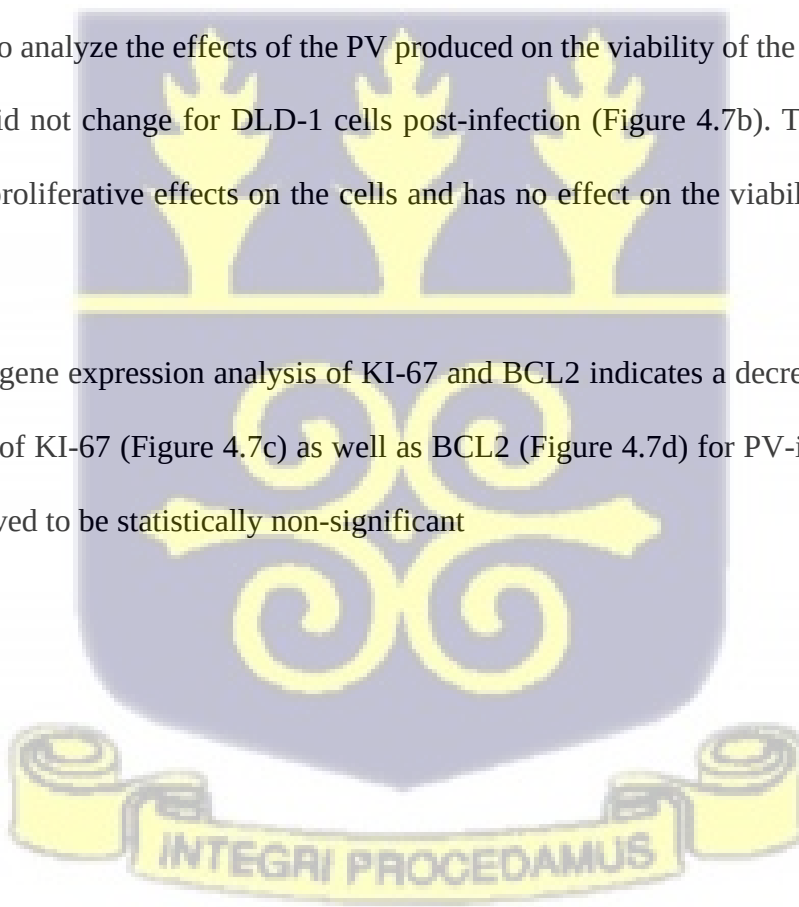
The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with one-way ANOVA multiple comparisons

4.5 Phenotypic and molecular effects of PV-infected DLD-1 cell lines

4.5.1 Effects on the proliferation and viability of DLD-1

MTT analysis was used to access the proliferative effect of PV of DLD-1 cell lines. The data showed that the PV caused an increase in the percentage growth of the cells and this increase was significant only at 72h post-infection ($p=0.0389$). Again, CellTiter-Glo luminescent cell viability assay was done to analyze the effects of the PV produced on the viability of the cells. The number of viable cells did not change for DLD-1 cells post-infection (Figure 4.7b). The results suggest that PV has no proliferative effects on the cells and has no effect on the viability of DLD-1 cell lines.

The data for the gene expression analysis of KI-67 and BCL2 indicates a decrease in the relative gene expression of KI-67 (Figure 4.7c) as well as BCL2 (Figure 4.7d) for PV-infected cells. The effect was observed to be statistically non-significant



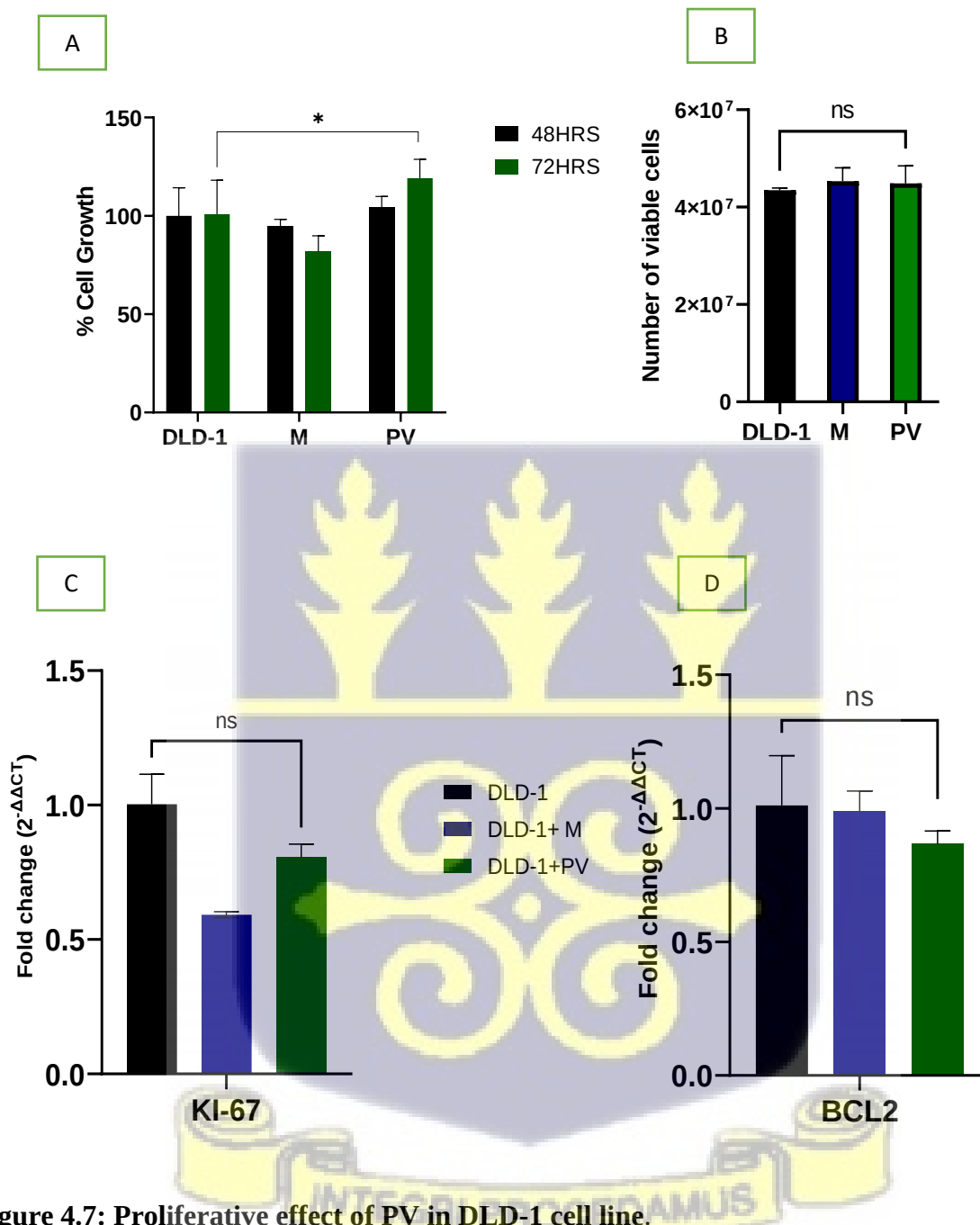


Figure 4.7: Proliferative effect of PV in DLD-1 cell line.

(A) PV significantly increased % growth only 72 h post-infection.

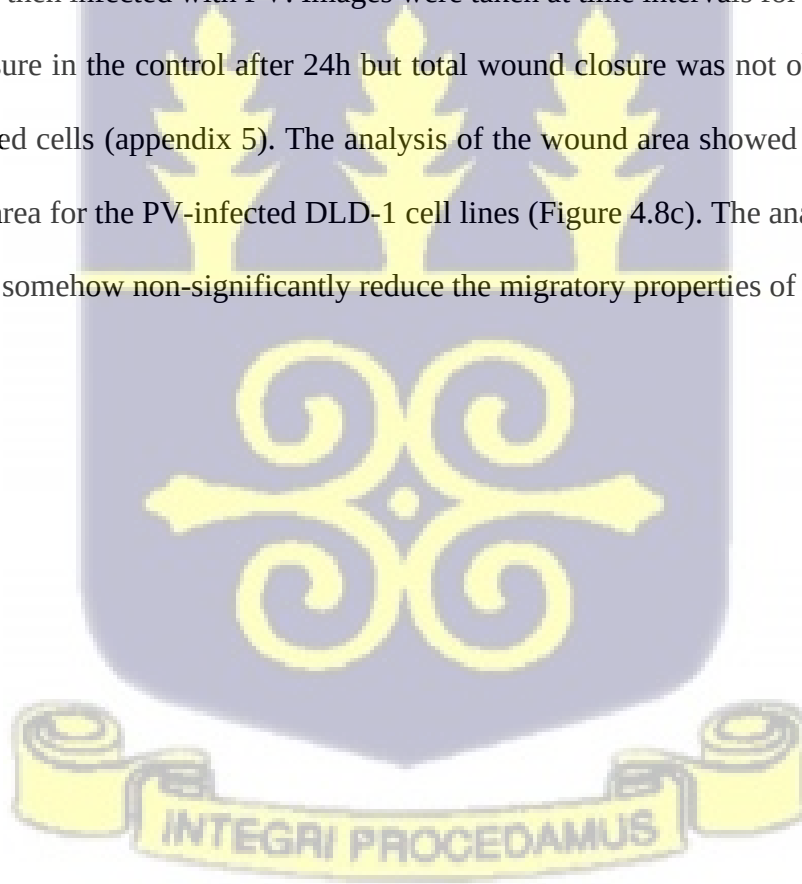
(B) PV does not affect DLD-1 viability

(C&D) KI-67 and BCL2 were non-significantly downregulated in PV-infected cells.

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with one-way ANOVA multiple comparisons

4.4.2 Effects of infection on the migration of DLD-1 cell lines

There was a non-significant increase in the expression of *VIM* when cells were infected with PV (Figure 4.8a). On the contrary, the relative gene expression of *MMP9* increased significantly when DLD-1 cells were infected with PV ($p=0.0186$) (Figure 4.8b). However, the migratory effect of the PV was further explored using wound healing analysis. Cells were seeded and allowed to form a monolayer and then infected with PV. Images were taken at time intervals for 2 days. There was total wound closure in the control after 24h but total wound closure was not observed in the PV and mock-infected cells (appendix 5). The analysis of the wound area showed a slight inhibition in the %wound area for the PV-infected DLD-1 cell lines (Figure 4.8c). The analysis is indicative that the PV may somehow non-significantly reduce the migratory properties of the cell lines.



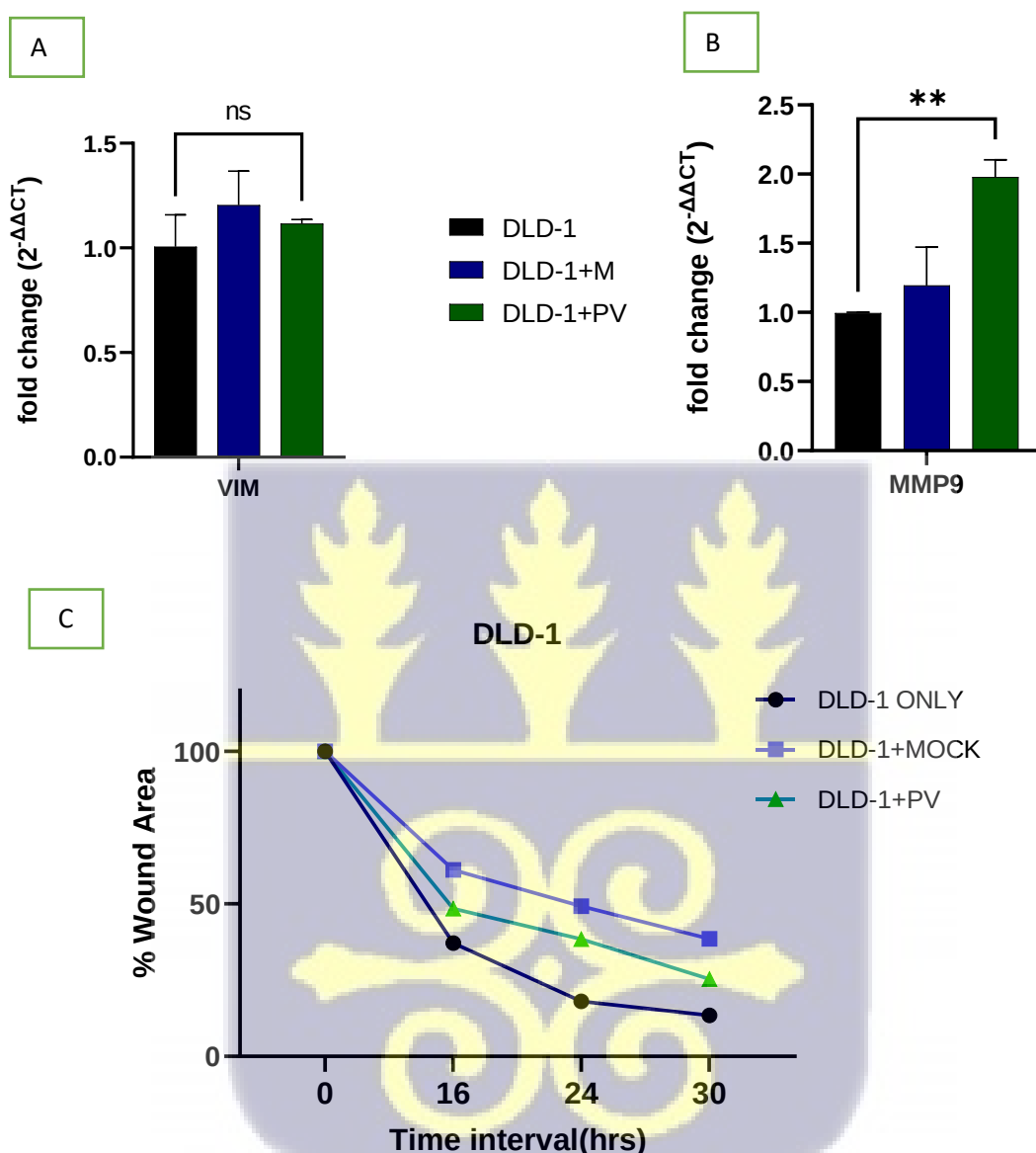


Figure 4.8: Effects of infection on the migration of DLD-1 cells.

(A&B) RT-qPCR analysis of PV-infected cells showed no significant change in VIM but a significant increase in MMP9 ($p=0.0186$). (C) Non-significant inhibition of % wound area for PV infection

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with one-way ANOVA multiple comparisons

4.4.3 Effects of infection on the expression of angiogenic growth factor VEGF in DLD-1

From the results, when DLD-1 cells were infected with PV, there was a non-significant increase in the expression levels of VEGF compared to the control.

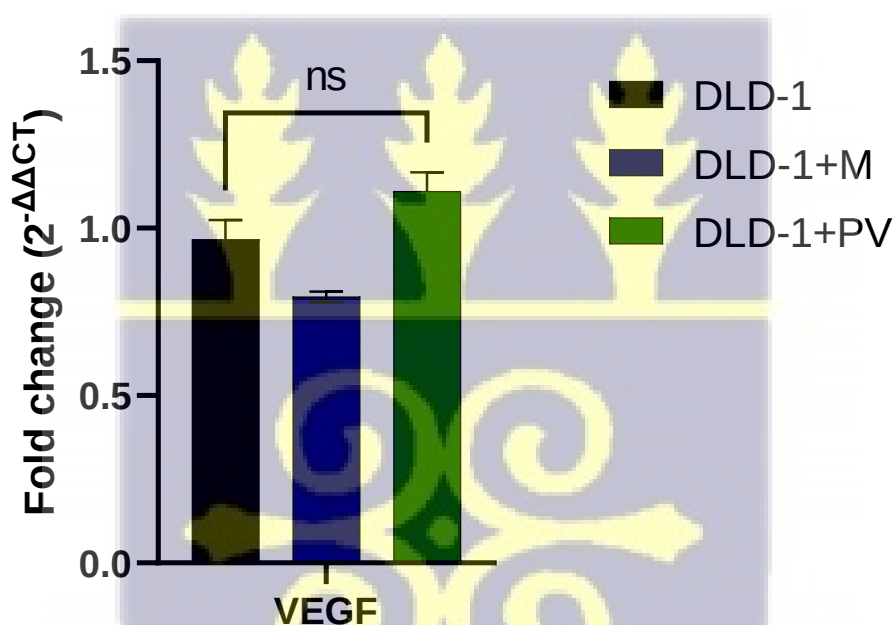


Figure 4.9: Effects of infection on angiogenic growth factor VEGF.

RT-qPCR analysis of PV-infected 22RV1 cells indicated no significant change in VEGF expression.

The data is a representation of the Mean $\pm$ SD of independent experiments and significance levels were determined with one-way ANOVA multiple comparisons

4.5 Cytokine profiling of PV-infected cancer cell lines

4.5.1 Effects of infection on the expression of TNF- α , IL-1 β , IL-6, and IL-8 in 22RV1

To evaluate the effect of PV infection on cytokine expression and production, qRT-PCR was performed to analyze the expression levels of TNF- α , IL-1 β , IL-6, and IL-8. The results show that infection with PV increases the expression of these cytokines compared to the control (Figure 4.10). The increased gene expression levels of IL-1 β and IL-8 were observed not to be significant ($p>0.05$). Nonetheless, statistically significant expression was seen in TNF- α ($p<0.0014$) and IL-6 ($p=0.0008$).



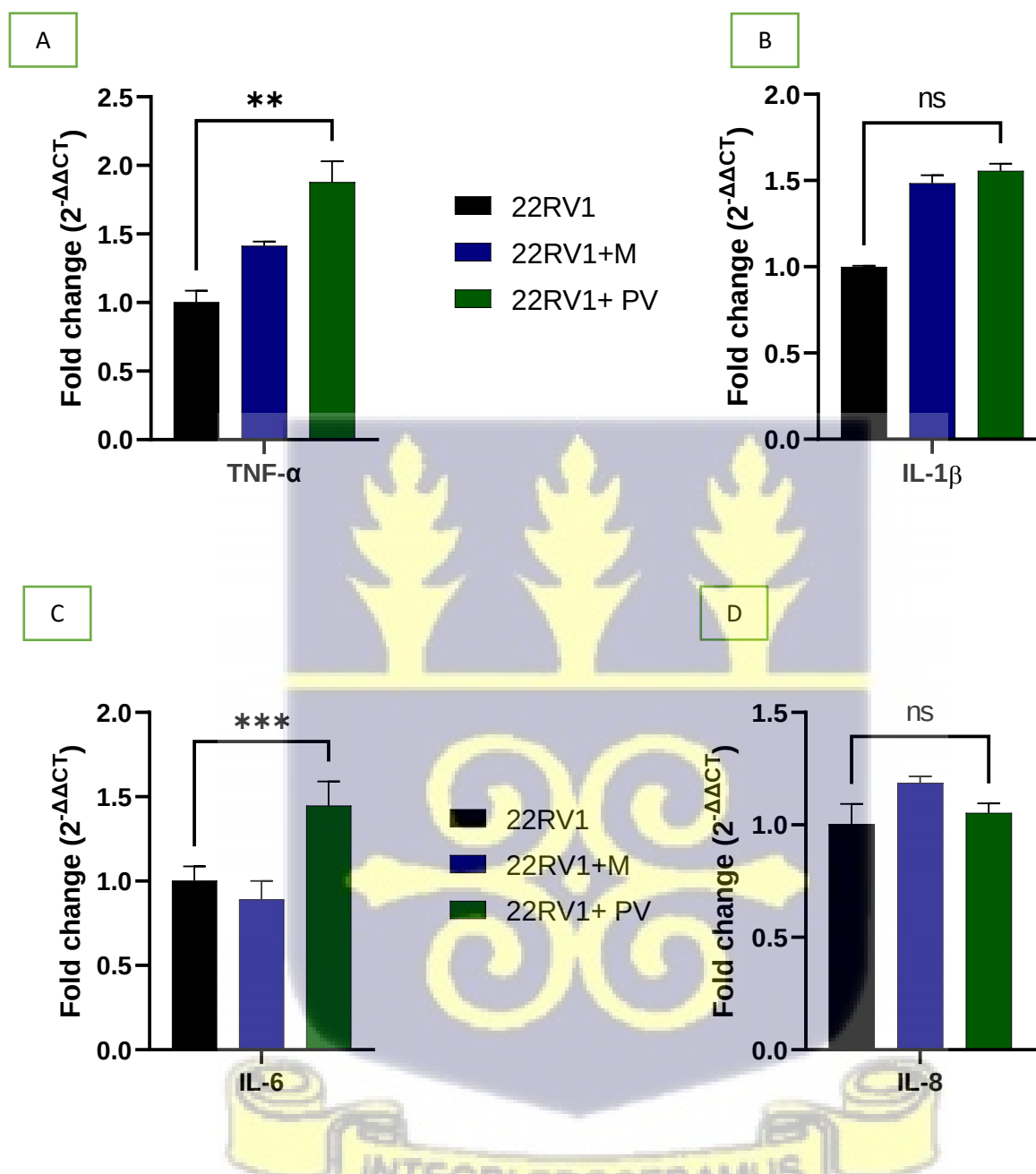


Figure 4.10: Infection increases the mRNA expression levels of some cytokines.

(A-D) RT-qPCR analysis of PV-infected 22RV1 cells showed a significant increase in the expression of TNF- α and IL-6.

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with one-way ANOVA multiple comparisons

4.5.2 Effects of infection on the expression of IL-1 β , IL-6 and IL-8 in DLD-1

The effect of PV infection on TNF- α , IL-1 β , IL-6, and IL-8 regulation and production in DLD-1 cell lines was also analyzed. The gene expression levels of TNF- α were observed to be decreased but non-significantly (Figure 4.11a). Also, the results show that infection with PV increases the expression of IL-6, IL-8, and IL-1 β significantly compared to their controls with a p-value of 0.0003, 0.0005, and 0.0410 respectively (Figure 4.11).



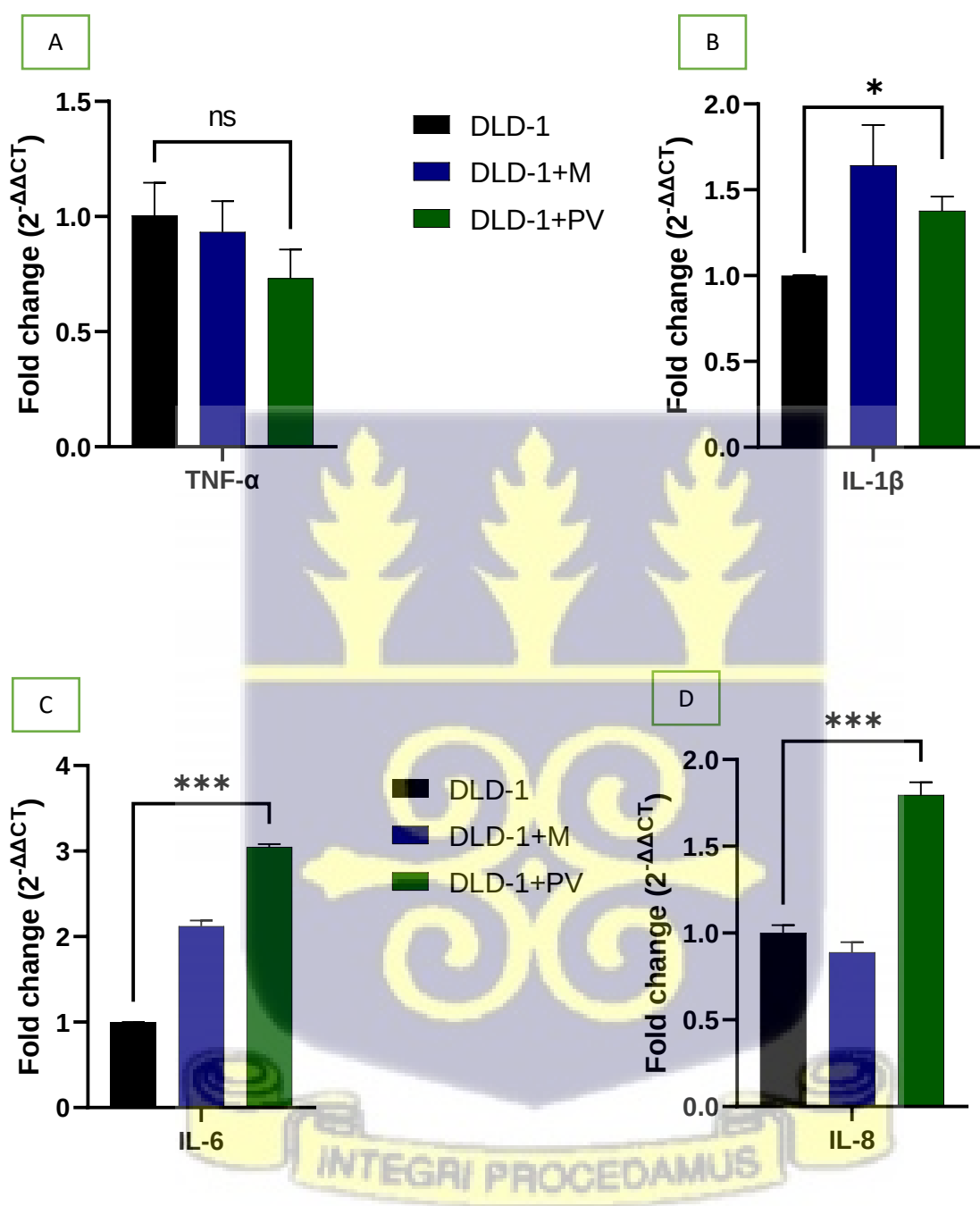


Figure 4.11: Effects of infection on expression of TNF- α , IL-1 β , IL-6, and IL-8.

(A-D) RT-qPCR analysis of PV-infected DLD-1 cells showed a significant increase in selected cytokines except for TNF- α .

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with one-way ANOVA multiple comparisons

4.6 Live virus (LV) infection effect on cancer cell phenotype and gene expression

For all data presented above, the mock which was the PV control showed some effect which indicates that the cells responded to the presence of mock and caused some sort of signaling that affected the properties of the cells. The mock (pseudovirus control) contains a lentiviral backbone for packaging the viral particle and a luciferase gene for determining successful infection without the spike plasmid. Although results from the luciferase assay indicate an absence of pseudo viral particles (appendix 2), it was observed in subsequent analysis to affect the phenotypic properties and gene expression of the cells.

Due to this active response of the mock, LV was used to infect the cell lines, and the phenotypic and molecular effects were analyzed using MTT assay and qRT-PCR.

4.6.1 LV Effects on the proliferation of 22RV1

The LV caused a significant increase in % cell growth for cells infected for 72h ($p=0.0399$). The proliferative effect of the LV-infected 22RV1 was further analyzed by detecting the expression levels of proliferation marker KI-67 and anti-apoptotic marker BCL2 using qRT-PCR. Contrary to what was observed for PV infection, there was a significant increase ($p=0.0011$) in KI-67 expression when the cell was infected with LV. This analysis correlates to the results observed for the proliferative phenotypic effect of LV using MTT.

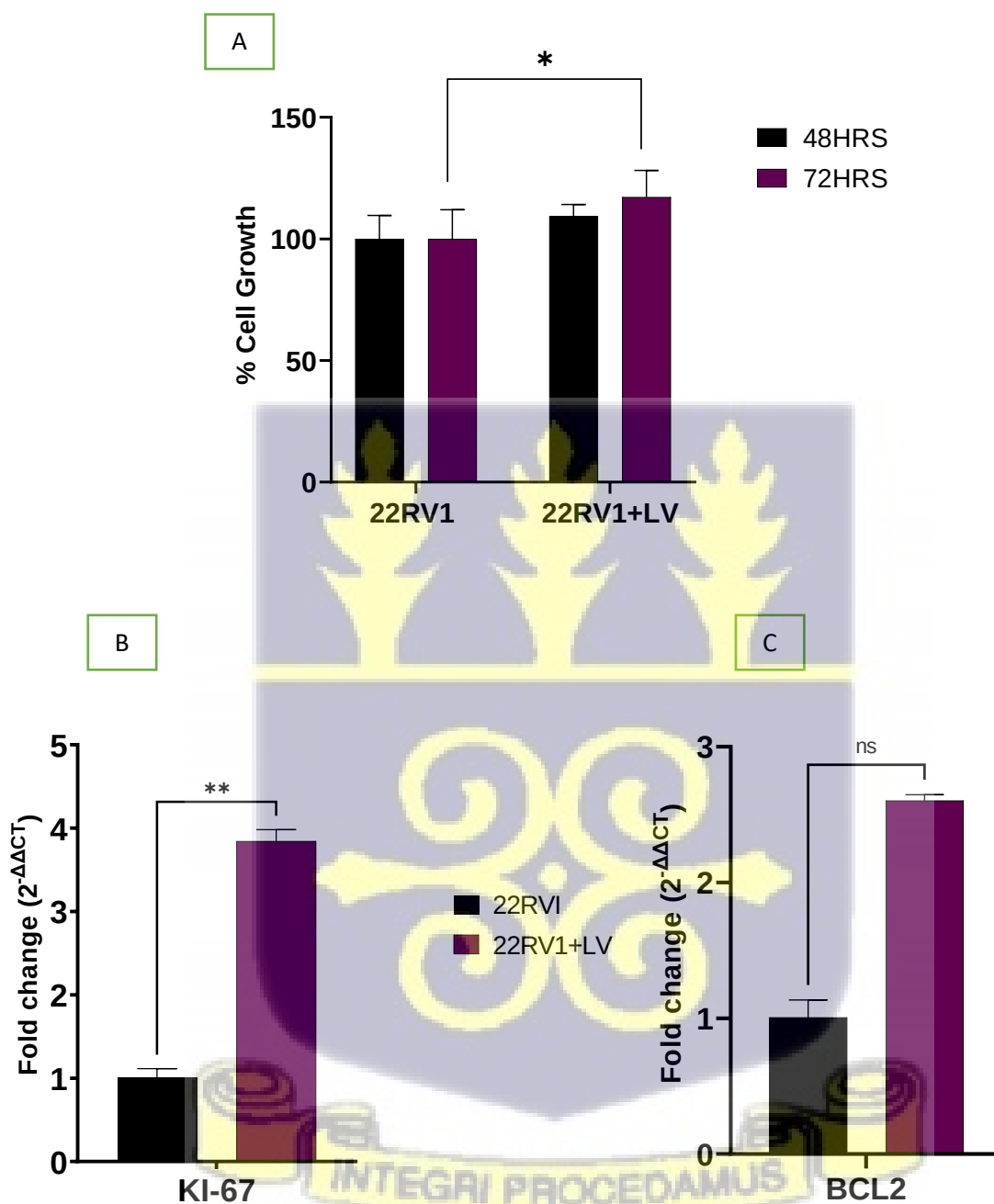


Figure 4.12: LV increases proliferation of 22RV1.

(A) LV significantly increases the % growth of 22RV1. **(B-C)** Markers of proliferation were increased by LV infection. The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with paired t-test and one-way ANOVA multiple comparisons

4.6.2 LV effects on the migration of 22RV1 cell lines

Pre- and post-LV infection VIM and MMP9 gene expression levels were examined. In LV-infected 22RV1 cells, there was a negligible upregulation of the EMT marker VIM (Figure 4.13a). However, LV significantly reduced the expression of MMP9 ($p=0.0393$) as shown in Figure 4.13b.

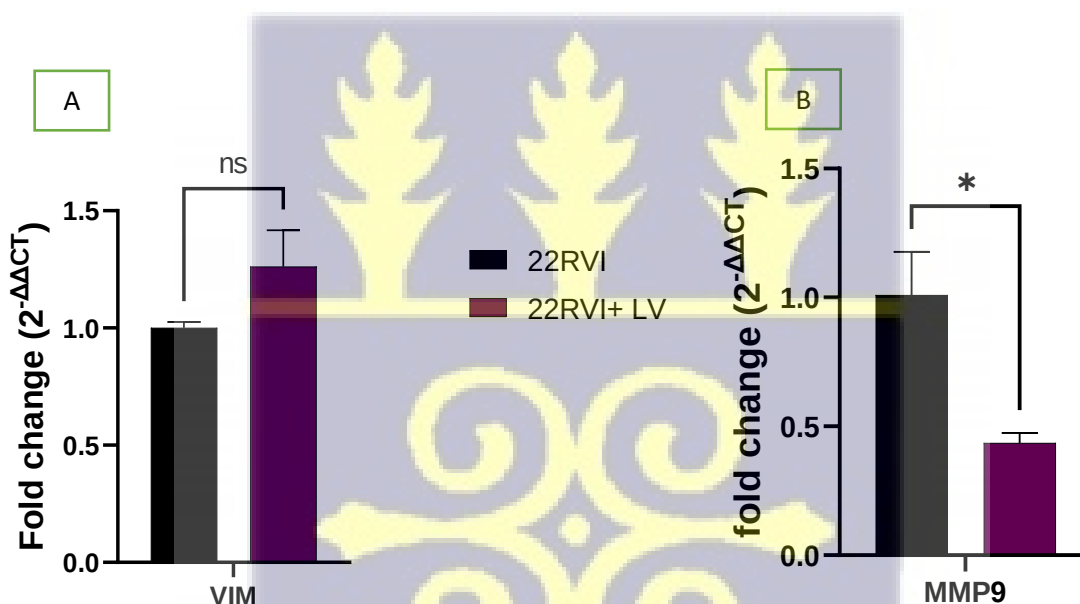


Figure 4.13: Effects of infection on the migration of 22RV1 cells.

(A-B) RT-qPCR analysis showed a non-significant increase in VIM but a significant decrease in MMP9 for LV.

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with paired t-test

4.6.3 LV Effects on the expression of angiogenic growth factor VEGF in 22RV1

The expression of the VEGF marker increased in response to LV infection. Figure 4.14, however, illustrates that the overexpression of the gene was not significant.

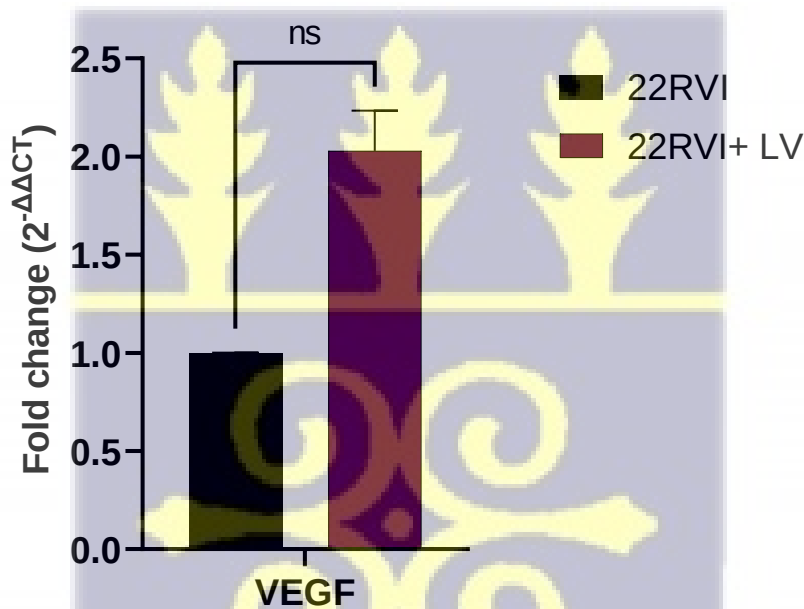


Figure 4.14: Infection causes the upregulation of VEGF in 22RV1.

RT-qPCR analysis of infected 22RV1 cells shows a significant increase in expression in cell lines infected with LV.

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with paired t-test

4.7 Effects of LV infection in DLD-1 cell lines

4.7.1 Effects on the proliferation and viability of DLD-1

The data showed that the LV caused a significant decrease in % cell growth 72h post-infection as illustrated in Figure 4.15a. Similarly, gene expression analysis of KI-67 and BCL2 showed a decrease in the relative gene expression of KI-67 (Figure 4.15b) as well as BCL2 (Figure 4.15c). The decreased expression level observed in KI-67 was non-significant. However, that of BCL2 was significant with a p-value of 0.0465 (Figure 4.15c)



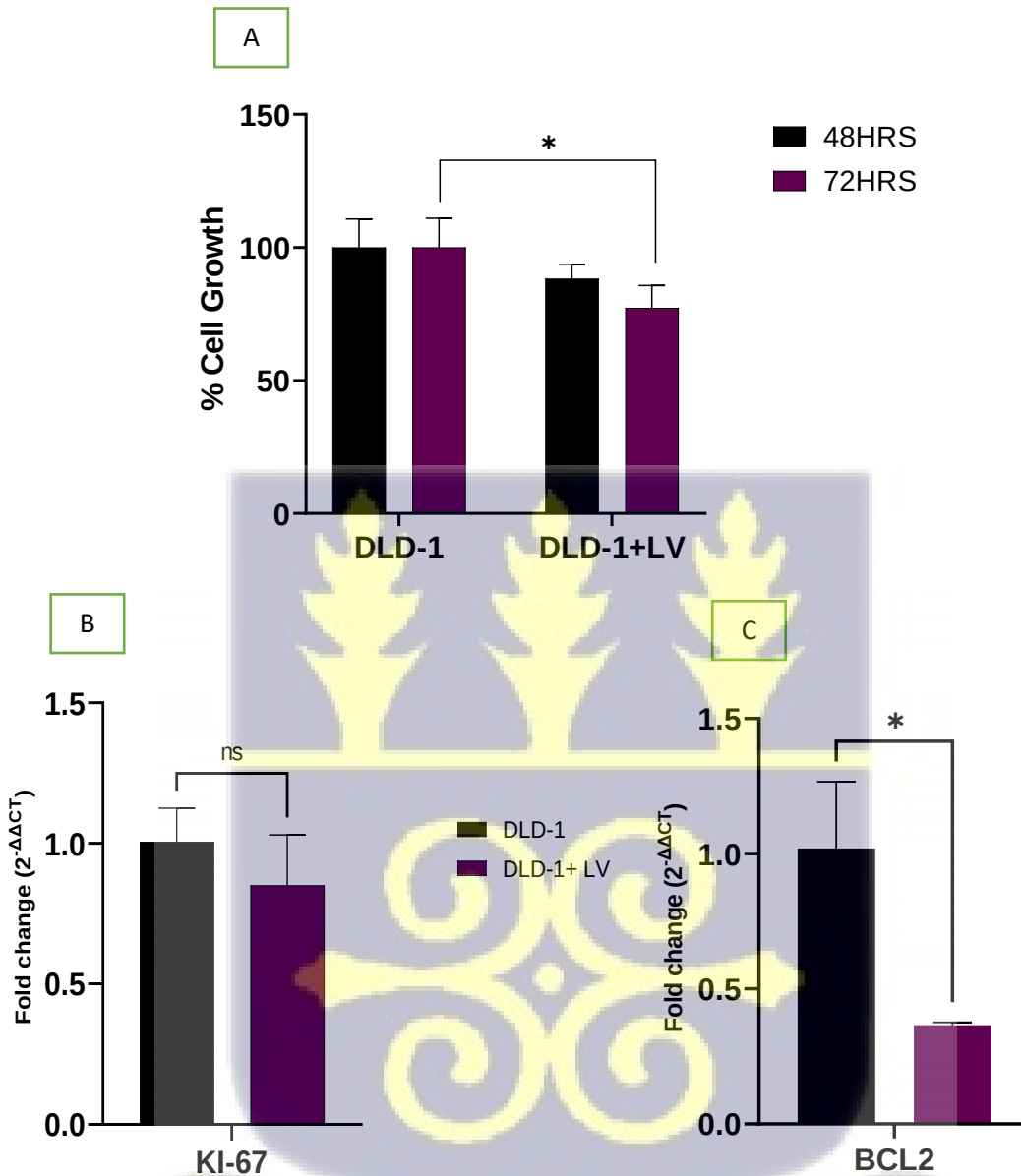


Figure 4.15: Proliferative effect of LV in DLD-1 cell line.

(A) LV decreases DLD-1 proliferation.

(B&C) Expression levels of KI-67 and BCL2 were downregulated

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with paired t-test and one-way ANOVA multiple comparisons

4.7.2 LV Effects on the migration of DLD-1 cell lines

There was a significant downregulation of VIM in the LV-infected DLD-1 cells. However, expression of MMP9 decreased non-significantly when cells were infected with LV. This suggests LV may reduce the migratory properties of DLD-1 through the downregulation of certain genes like VIM and MMP9.

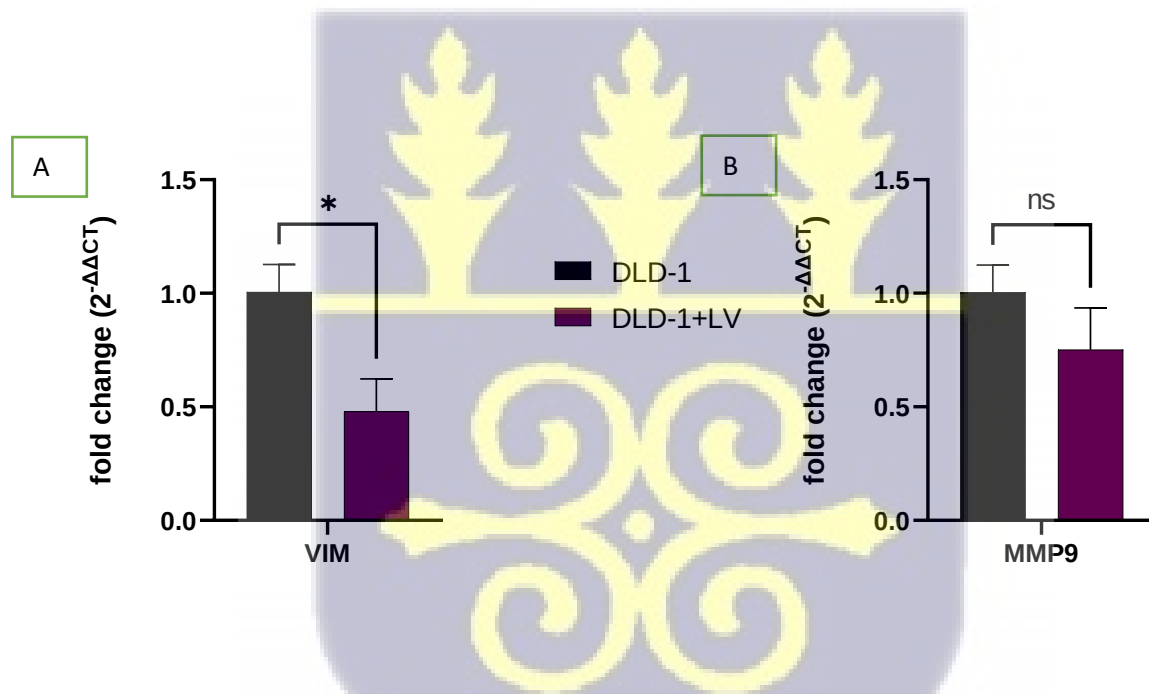


Figure 4.16: LV reduces migration markers of DLD-1 cells.

(A&B) LV downregulates the expression of both VIM and MMP9. The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with paired t-test

4.7.3 Effects of infection on the expression of angiogenic growth factor VEGF in DLD-1

The data show that infection with the LV significantly causes the downregulation of the angiogenic marker VEGF with a p-value of 0.0210.

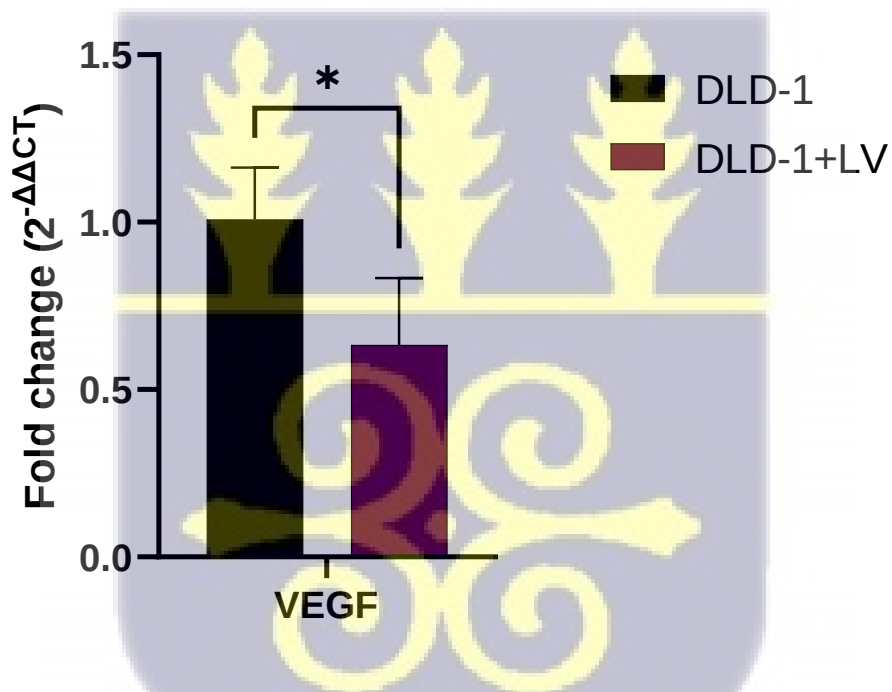


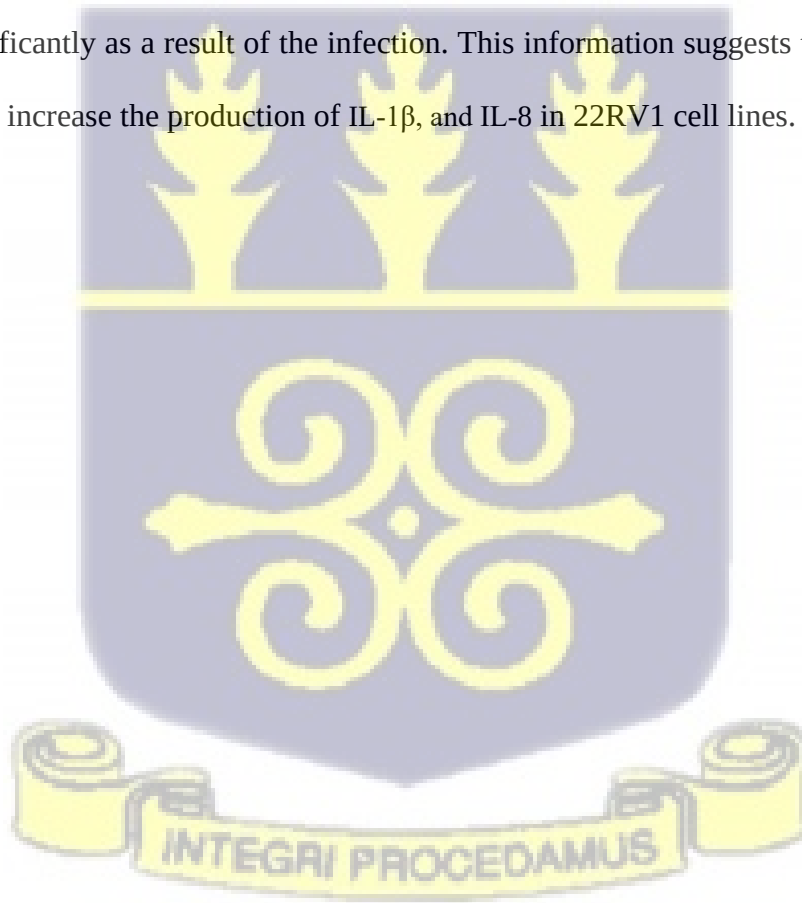
Figure 4.17: Effects of LV infection on angiogenic growth factor VEGF.

RT-qPCR analysis of cells showed a significant decrease in VEGF expression when infected with LV. The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with paired t-test

4.8 Cytokine profiling of LV-infected cancer cell lines

4.8.1 Effects of infection on the expression of TNF- α , IL-1 β , IL-6, and IL-8 in 22RV1

According to the data, the live virus elevated the selected cytokines' mRNA expression levels in 22RV1 cells. The most highly expressed gene, with a p-value of 0.0103, was IL-1 β . Additionally, the p-value for the significant rise in IL-8 was 0.0483. TNF- and IL-6 expression, however, did not change significantly as a result of the infection. This information suggests that SARS-CoV-2 can significantly increase the production of IL-1 β , and IL-8 in 22RV1 cell lines.



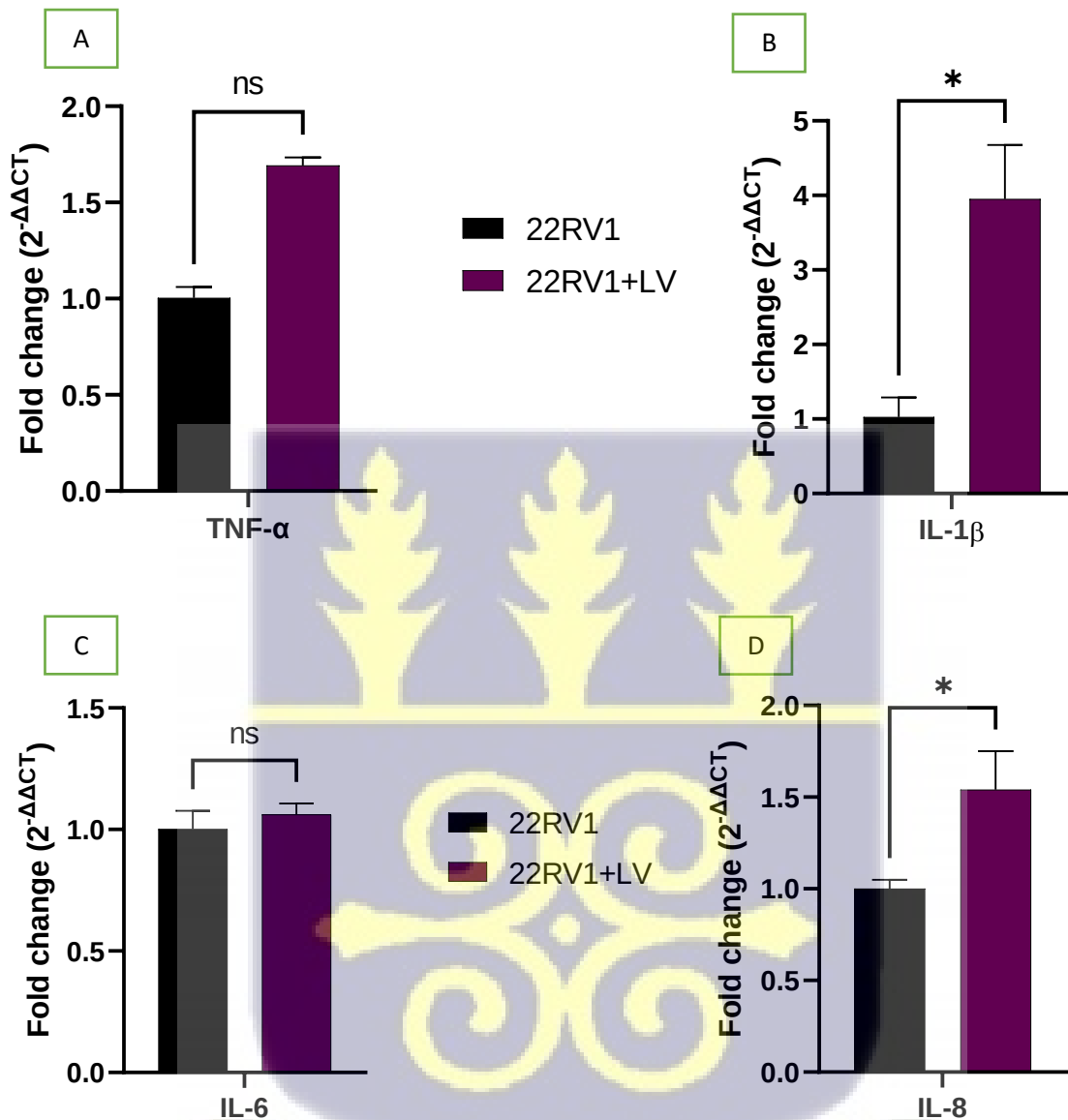


Figure 4.18: Infection increases the mRNA expression levels of some cytokines.

(A-D) LV infection upregulated the expression of TNF- α , IL-1 β , IL-6, and IL-8.

The data is a representation of the Mean $\pm$ SD of experimental groups and significance levels were obtained with paired t-test

4.8.2 Effects of infection on the expression of IL-1 β , IL-6 and IL-8 in DLD-1

TNF- α and IL-6 were upregulated by LV infection, although IL-1 β and IL-8 expression was downregulated (Figure 4.19). According to the statistics, TNF- α significantly increased, increasing fourfold. Only TNF- α and IL-1 β showed a significant expression fold change, with p-values of 0.0295 and 0.0310, respectively. However, it was shown that the changes in IL-6 and IL-8 levels were not statistically significant ($p>0.05$).



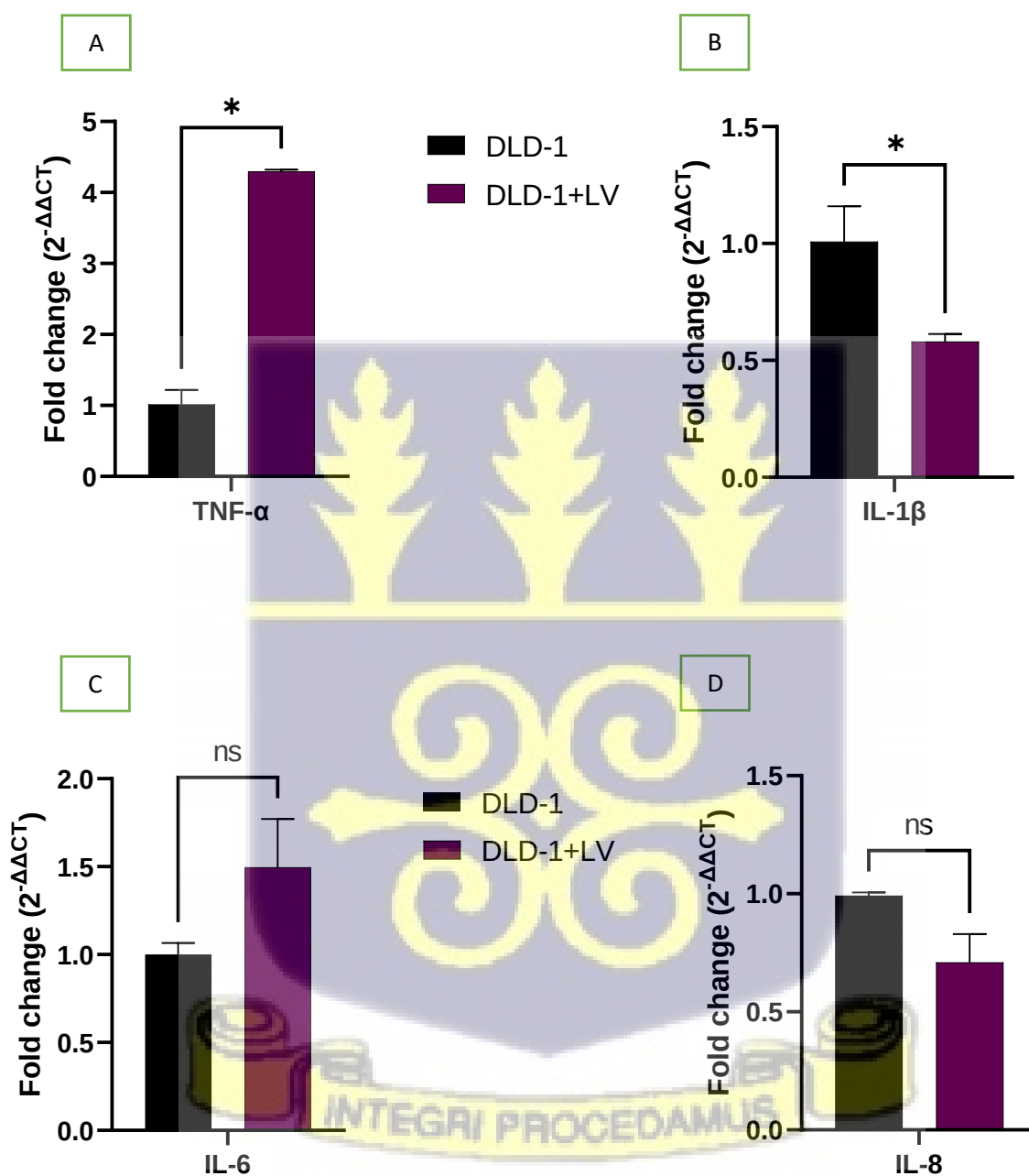


Figure 4.19: Effects of infection on expression of TNF-α, IL-1β, IL-6, and IL-8.

(A-D) RT-qPCR analysis of LV-infected DLD-1 cells showed increased expression levels of TNF-α and IL-6 but decreasing expression levels of IL-1β and IL-8. The data is a representation of the Mean ± SD of experimental groups and significance levels were obtained with paired t-test

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

5.1 DISCUSSION

Several studies have shown that risk factors that predispose a person to develop severe COVID-19 disease include people living with chronic comorbidities such as cancer (Rashedi et al., 2020; Rod et al., 2020). This study investigated the infectivity of the SARS-CoV-2 PV on cancer cells. The study also highlights the effects of the infection on cancer-like properties and cytokine expression in cancer cell lines

The key enzyme ACE2 is expressed in diverse tumors or cancerous tissues. Data from the initial *in silico* analysis is indicative that, increased ACE2 expression may confer a better prognosis for breast and prostate cancer and that decreased expression could contribute to a poor prognosis. This is in line with previous studies where ACE2 was reported to inhibit the metastasis of prostate cancer (Zhang et al., 2019). Again, the overexpression of ACE2 was found to exert inhibitory properties against breast cancer angiogenesis, proliferation, and metastasis both *in vitro* and *in vivo* by suppressing the VEGF/VEGFR2/ERK pathway (Zhang et al., 2019). This shows that ACE2 may slow the growth of breast cancer and act as a possible tumor suppressor. The level of ACE2 in colorectal tumors, however, was considerably higher than in healthy tissues. It can be inferred that the development of the cancerous nature of colorectal tissues correlates to the increased expression of the ACE2 receptor. Studies have revealed that ACE2 overexpression was positively correlated with immune cell infiltration rather than patient survival outcomes (Ahmadi et al., 2020) thereby indicating a possible impact on the prognosis of colorectal cancer.

Data from dot blot analysis indicates ACE2 was expressed in varying amounts across all the selected cancer cell lines. Quantitative analysis of blots was represented as a ratio of each protein spot relative to the loading control. The ACE2 receptor was found not to be expressed in HELA cells (Y. Nie et al., 2004). Hence, it is mostly used as a control cell line for ACE2 expression or as a recombinant cell line stably overexpressing ACE2 (El-Shennawy et al., 2020; Matoba et al., 2016). From the analysis, the breast cancer cell lines exhibited lower ACE2 expression, with MDA-MB-231 having the lowest almost as similar to the negative control. Since overexpression of ACE2 in breast cancer reportedly has cancer-inhibitory properties, it is least expressed in highly metastatic MDA-MB-231. This result shows that the cell lines express ACE2 and that the expression pattern follows the initial *in silico* analysis performed.

5.1.1 SARS-CoV-2 PV infectivity of cancer cells

Analysis of the infectivity of the PV produced showed that there was significant RLU (above 10000) recorded when transfected cell lines (ACE2-HEK293T) were infected as opposed to non-transfected cell lines which gave RLU below 5. This is consistent with other studies involving SAR-CoV-2 neutralization and indicates that the generated PV can infect permissive cells that are expressing ACE2 receptors (Crawford et al., 2020; Nie et al., 2020). Nonetheless, PV infection of the cancer cell lines showed infectivity in 22RV1 and DLD-1 cell lines out of the selected cancer cell lines. A study by Darvish-Damavandi et al, 2020 showed that intestine and colorectal cancers are susceptible tissues to SARS-CoV-2 by studying cell entry genes (Darvish-Damavandi et al., 2020). Also, other studies showed that the higher ACE2 expression in colorectal cancer promoted SARS-CoV-2 PV infection compared to normal colon epithelial cells (Li et al., 2021). However, the data indicates that not all cancer cells expressing the ACE2 receptor may be susceptible to the SARS-CoV-2 pseudo viral particle infection. This can be due to alterations or mutations leading

to variations within the gene sequences of *ACE2* on the different cancer cell types. That is, possible variations in the *ACE2* gene could affect its interaction with the spike protein. Some studies have predicted the role of genetic variation and the expression level of the receptors on disease severity and susceptibility (Bakhshandeh et al., 2021; Hoffmann et al., 2020; Hussain et al., 2020; Shukla et al., 2021). It can also be inferred that higher *ACE2* expression levels in cancer cell lines may not correlate with spike infectivity. One possible explanation for these findings is the expression levels of host proteases such as *TMPRSS2* that enable the entry of the virus into its host. 22RV1 is a prostate cancer cell line and these cells are known to have increased *TMPRSS2* expression (Mollica & Rizzo, 2020). Also, based on the expression profile from the human protein atlas (HPA), *TMPRSS2* is expressed highly in prostate adenocarcinomas.

5.1.2 Effects of infections on cancer-like properties of cell lines

Since 22RV1 and DLD-1 cell lines were the only cell lines that showed significant infection with the PV, these two cell lines were used to analyze the subsequent effect of the infection on cancer properties and cytokine expression.

The results show that the interaction of the PV with the *ACE2* receptor on 22RV1 cells reduced proliferation and viability. This was comparable with a recent study where the SARS-CoV-2 spike inhibited growth but rather promoted apoptosis of the LNCaP prostate cell line (Johnson et al., 2022). However, PV infection may not drive the production of molecules involved in the breakdown of the ECM and the acquisition of mesenchymal phenotypes through EMT. This means it may not contribute to the migratory properties of this cell line. For DLD-1, SARS-CoV-2 PV mostly showed very low to non-significant effects on all cancer phenotypes analyzed. Although

results seem inclusive, it will be beneficial to look into the expression of other markers that are recruited in these mechanisms since a pool of genes facilitates these processes.

Mock PV infections, however, showed some effect in all assays conducted with PV instead of the expected neutral effect. The mock contains a luciferase gene and a lentiviral packaging backbone that encodes certain proteins such as Gag-pol, tat, nef, rev, etc. Although not much has been reported concerning how these packaging proteins may affect cancer properties, a report by Stewart et al., 1999 indicated a possible cell cycle arrest and apoptotic effect in tumors by HIV Vpr. Some reports also show that HIV-related proteins are implicated in the progression of cervical cancer. Nonetheless, the cancer cell lines used for the study responded to the presence of mock and caused some sort of signaling that affected the properties of the cells. Hence the need to utilize the live virus (LV) as a way of establishing the actual effect on the cells.

LV infections induced cell growth and upregulated proliferation markers in 22RV1 cells a result seen to be antithetical to what was observed for the PV infection. Also, *VIM* was significantly upregulated and *MMP9* was significantly downregulated which does not show exactly how LV may be implicated in 22RV1 migratory properties. However, in a non-invasive prostate adenocarcinoma such as that of the 22RV1 cell line, interaction with the SARS-CoV-2 virus may contribute to poor prognosis of the disease since it could cause an increase in proliferation and *VEGF* expression. This data however is in contrast with reports by Johnson et al., 2022 of SARS-CoV-2 spike interaction promoting apoptosis of prostate cancer cell line. Another study reported the ability of SARS-CoV-2 pseudovirus to inhibit the growth of prostate cancer cell lines in a time-dependent manner and also affected the expression of some genes (McKay et al., 2022). However, this discrepancy may result from the use of a live virus and not a pseudo-viral particle. Also, for the LV-infected DLD-1 cells, data was in contrast to PV-infected DLD-1 cells and shows that

SARS-CoV-2 and colorectal cancer coinfection may have a better prognosis of colorectal cancers. From the initial *in silico* and *in vitro* expression analysis, ACE2 was expressed highly in COAD tumors. Indicating that ACE2 expression may correlate positively to the aggressiveness of colorectal cancers. Therefore, interaction with the spike RBD may cause negative feedback that downregulates the ACE2 receptor. This possible negative feedback may affect how it contributes to colorectal cancer aggressiveness and infer a better prognosis. This result is comparable to reports by Ottaiano et al, 2021 on the possible reduction of tumor burden in three metastatic colon cancer patients during SAR-CoV-2 infection.

5.1.3 Effects of infections on the expression TNF- α , IL-1 β , IL-6, and IL-8 of cancer cell lines

Findings on cytokine expression align with reports that indicate SARS-CoV-2 infection may cause a hyper-inflammatory response (Song et al., 2020). For LV infections, there was a significant increase in the expression levels of IL-1 β and IL-8 in 22RV1 cell lines. However, there was a non-significant change for TNF- α and IL-6. TNF- α is a possible inducer of apoptosis in prostate cancer (Maolake et al., 2018). However, a study by Chakravarty et al, 2020 demonstrated that cytokines such as IL-1, IL-6, and IL-8 can cause an acceleration in the progression of an existing prostate tumor (Chakravarty et al., 2020). This indicates that SARS-CoV-2 infection may contribute to prostate cancer progression through the release of such cytokines.

Nonetheless, findings from LV-infected DLD-1 showed an increase in TNF- α and IL-6 but a decreased expression in IL-1 β and IL-8. Study shows that epithelial cells found within the colonic mucosa usually release cytokines such as IL-1, IL-6, and IL-8. TNF expression in colorectal cancers is mostly associated with better prognosis (Csiszar et al., 2004). Hence, the increase in TNF- α expression might be implicated in the decreasing phenotypic effect for LV-infection DLD-

1 cells. IL-6, IL-1 β , and IL-8 all of which are known to be implicated in tumor progression were either downregulated or non-significantly upregulated in the case of IL-6. The data suggest that SARS-CoV-2 infection may downregulate tumor-promoting cytokines such as IL-1 β and IL-8 and upregulate the expression of cytokines key to survival in colorectal cancer to contribute to a better prognosis.

In general, live virus infection decreased the proliferative, migratory markers and the expression of angiogenic growth factor VEGF in DLD-1 colorectal cells. Although there are limited reports on the effects of infection on colorectal cancer cell lines, the study aligns with other studies that have reported on the possible anti-tumor effect of SARS-CoV-2 infection (Barh et al., 2021; Ottaiano et al., 2021).

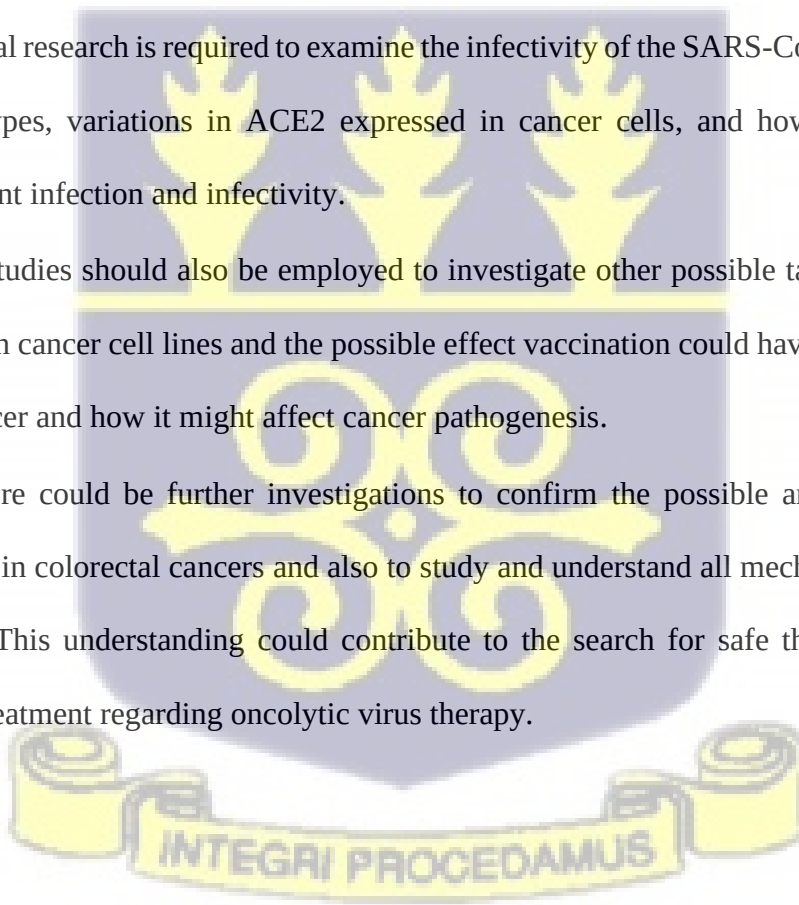
5.2 CONCLUSION

This study substantiates that different cancer cell lines express different levels of the ACE2 protein and that the SARS-CoV-2 virus infects permissive cells expressing the ACE2 receptors. From the study, it can be concluded that SARS-CoV-2 spike interaction with ACE2 on cancer cells may affect the properties of cancer. However, the effect may vary depending on the cancer type and could either be beneficial or not to the cancer cell. The research demonstrates that cancer cells may react differently to various genetic components of the virus. As a result, given that vaccines are created using either genetic material or a protein from the virus, cancer cells may react to their administration. Again, not only are prostate cancer and colon cancer patients at a higher risk of developing severe forms of the SARS-CoV-2 infection but the presence of the virus within the tumor microenvironment and interaction with the ACE2 receptors may activate pathways that are involved in cancer cell proliferation, migration, and angiogenesis. Similarly, the infection can also

activate pathways that are involved in cancer cells producing and releasing cytokines that contribute to cancer pathogenesis. This data supports the hypothesis that possible interaction may alter ACE2 function in the cancer cell line and also affect how cancer cells regulate the production of cytokines.

5.3 RECOMMENDATIONS

- Additional research is required to examine the infectivity of the SARS-CoV-2 virus in other cancer types, variations in ACE2 expressed in cancer cells, and how they will affect subsequent infection and infectivity.
- Further studies should also be employed to investigate other possible targets of the spike protein on cancer cell lines and the possible effect vaccination could have on people living with cancer and how it might affect cancer pathogenesis.
- Also, there could be further investigations to confirm the possible antitumor effect of infection in colorectal cancers and also to study and understand all mechanisms that come to play. This understanding could contribute to the search for safe therapies for colon cancer treatment regarding oncolytic virus therapy.



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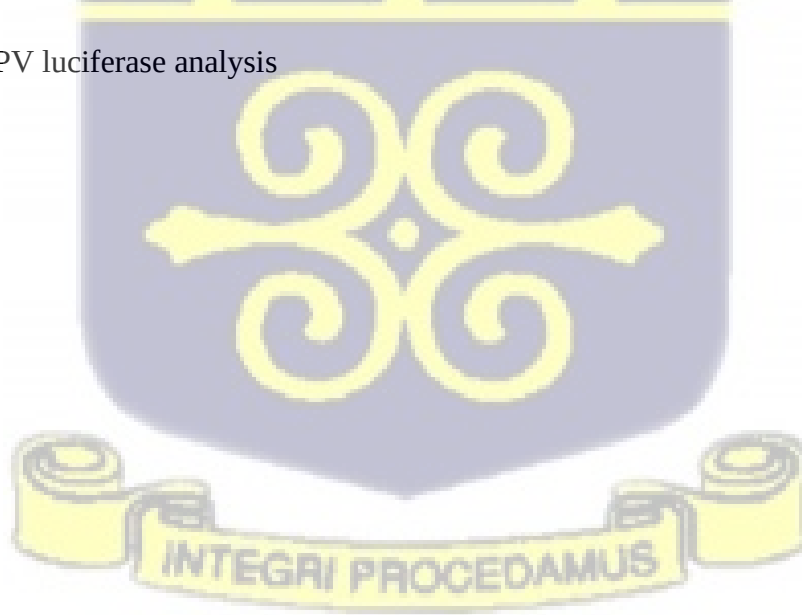


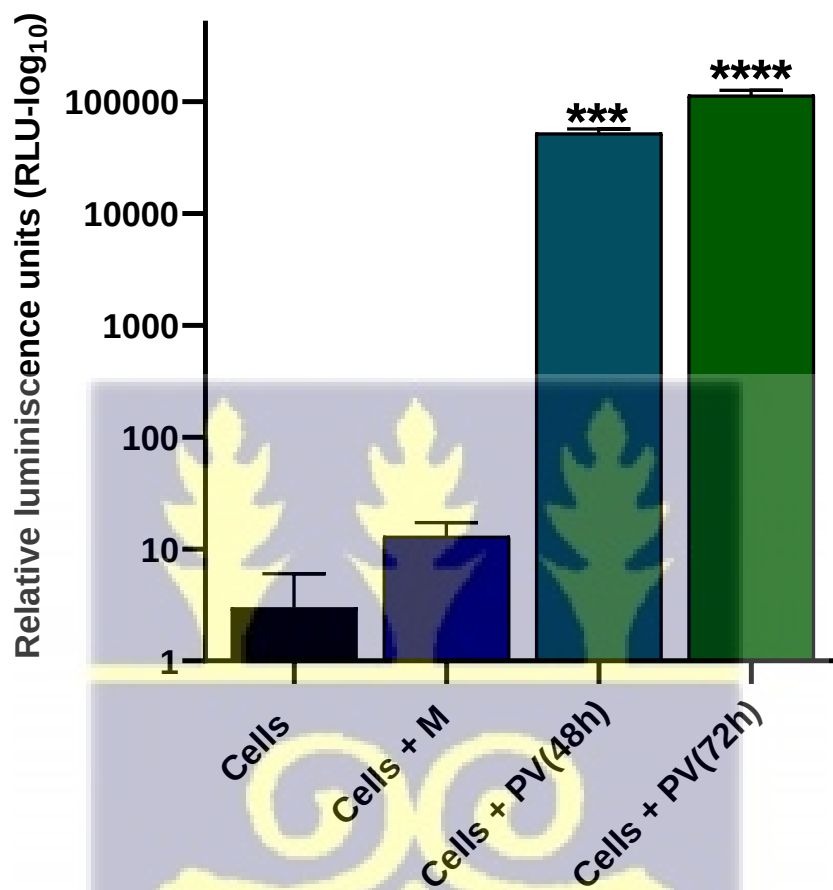
APPENDIXES

APPENDIX 1: Dot blot image for ACE2 expression



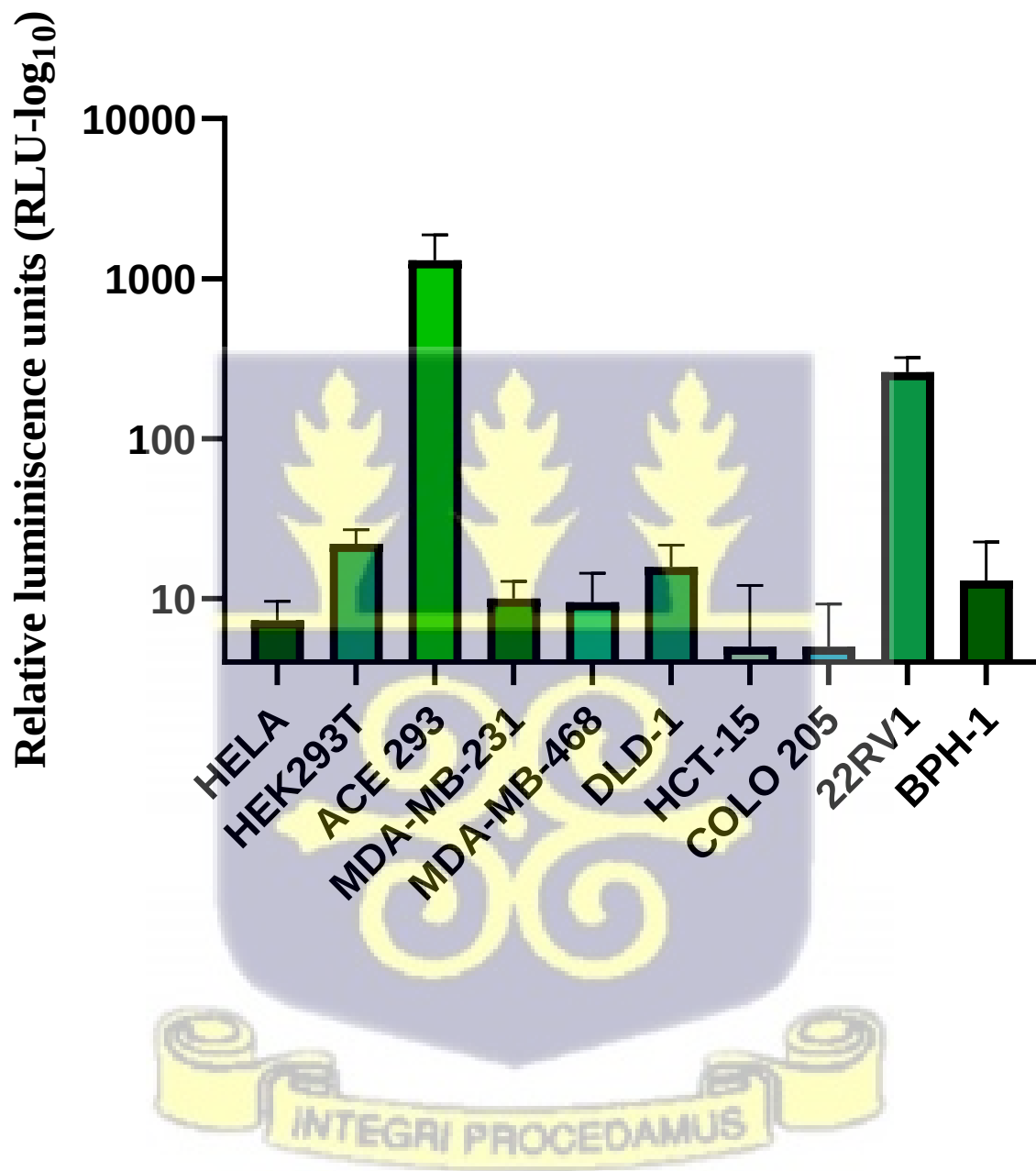
APPENDIX 2: PV luciferase analysis



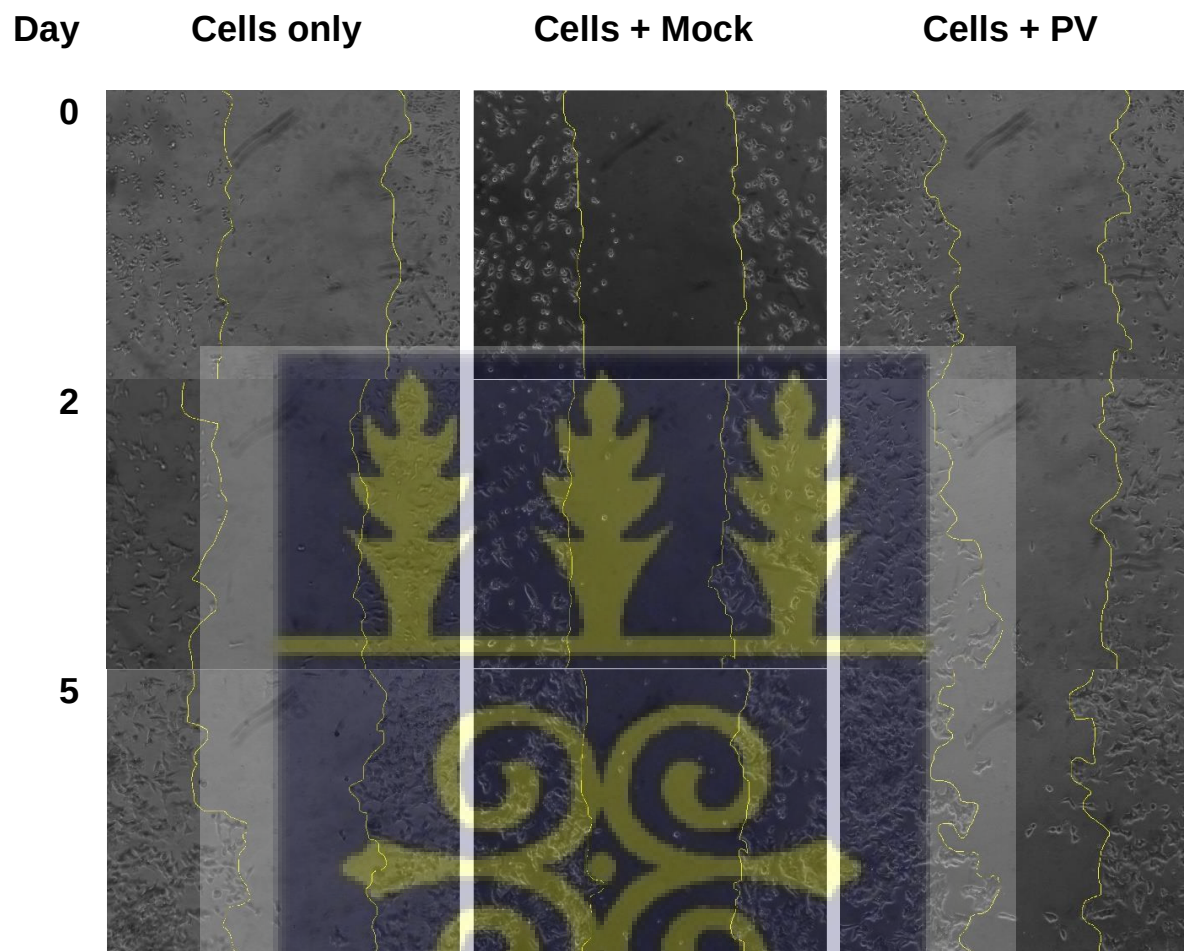


APPENDIX 3: SARS-CoV-2 PV infectivity of cancer cell lines





APPENDIX 4: Wound healing images for PV-infected 22RV1 cells



APPENDIX 5: Wound healing images for PV-infected DLD-1 cells

