THE EXPRESSION LEVEL OF FAM26F IN PATIENTS INFECTED WITH SARS-CoV-2, ITS TREND DURING THE PROGRESSION OF THE DISEASE

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A thesis submitted in partial fulfillment of the requirement for the degree of

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DEDICATED TO

Me, myself, and I!

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LIST OF ABBREVIATIONS

APC	Antigen-Presenting Cell
ART	Antiretroviral Treatment
BCR	B Cell Receptor
Ca_hom_mod	Calcium Homeostasis Modulator
Ca^{+2}	Calcium
ECL	Enhanced Chemiluminescence
ER	Endoplasmic Reticulum
FAM26F	Family with Sequence Similarity 26, Member F
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GC	Glucocorticoids
GSH	Glutathione
H_2O_2	Hydrogen Peroxide
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
HMM	Hidden Markov Model
HO^-	Hydroxyl Radicals
IFN	Interferon
Ig	Immunoglobin
IL	Interleukin
INAM	IRF-3–dependent NK-Activating Molecule
IP3	Inositol1,4,5-trisphosphate
IPA	Ingenuity Pathway Analysis
LPS	Lipo Polysaccharide
MA	Micro Array
mDC	Myeloid Dendritic Cell
MS	Mass Spectrometer
MSA	Multiple Sequence Alignment

MTS	3-(4,5–dimethylthiazol–2–yl)-5-(3-carboxymethoxyphenyl)-2-(-4-sulfophenyl)-2H-Tetrazolium
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Centre of Bio-Informatics
NK	Natural Killer
PAGE	Poly-Acrylamide Gel Electrophoresis
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PDB	Protein Data Bank
PDCs	Plasmacytoid DCs
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
PMS	Phenazine Methyl Sulphate
PRR	Pattern Recognition Receptors
PVDF	Polyvinylidene Difluoride
QRT-PCR	Quantitative-Reverse Transcriptase Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RBC	Red Blood Cell
RLR	RIG-I-Like Receptor
ROS	Reactive Oxygen Species
RT	Room Temperature
RyR	Ryanodine Receptor
S.D	Standard Deviation
SDS	Sodium Dodecyl Sulphate
Ser	Serine
SIV	Simian Immunodeficiency Virus
TCR	T Cell Receptor
TGN	Trans Golgi Network
Thr	Threonine
TLR	Toll-Like Receptor

TMD	Transmembrane Domain
TNF	Tumor Necrosis Factor
Trx	Thioredoxin
Tyr	Tyrosine
VMD	Visual Molecular Dynamics

ABSTRACT

Family with sequence similarity 26, member F (FAM26F) is a significant modulator of innate immune system that plays an essential role in wide and varied immune responses; however, the association between FAM26F expression and COVID-19 infection is not yet known. The current study investigated the differential expression of FAM26F during COVID-19 infection. The investigation also examined how the expression levels of FAM26F change as the infection worsens. Using Real-time qPCR, the expression of FAM26F and marker genes was evaluated. Our results indicate that FAM26f is significantly downregulated in COVID-19-infected patients compared to the control group ($t = 3.138$, $df = 59$, $P = 0.0027$). Higher expression was observed in COVID-19 recovered cases, most likely because of decreased infection and enhanced immunity. The expression level of FAM26F was negatively correlated with the absolute neutrophil count (Pearson $r=-0.383$, $P = 0.0470$), which may be a result of ROS burst, which deregulates Ca+2 levels and consequently decreases FAM26F expression. Our research is the first to demonstrate an association between FAM26F and COVID-19 infection. It is possible to improve COVID-19 clearance or containment by reversing ROS production and Ca2+ deregulation with calcium modulators and antioxidants, most likely by modifying the expression of FAM26F, and this possibility merits further study.

INTRODUCTION

Immunity pertains to the fundamental capacity of a host to hold out against attack of pathogens, which otherwise would have an adverse impact on host's ability to survive. To provide immunity against foreign antigens including bacteria, viruses, parasites, cancer cells, and fungus, the immune system is made up of a variety of cells, cellular components, or substances that mediate immunity, as well as complex pathways. In addition to the physical and chemical defenses against infection, the immune system can be split into two groups: innate immunity and adaptive immunity. Proteins are the focal elements responsible for directing convoluted immune networks and producing distinctive malleable inflammatory responses. One of the recently discovered immune player is the FAM26F (Family with sequence similarity 26), which has an ability to effect intricate immunological responses. In 2010, scientists discovered a molecule residing on the cell surface, derived from TLR signals, and was found to regulate mDC–NK contact conciliated Natural Killer cell activation. Due to its ability to activate NK cells, FAM26F has the potential to be employed as a therapy for malignancies that are sensitive to NK cells (Ebihara et al., 2010a).

In humans, the calcium modulatory protein FAM26F is found on chromosome 6. It is made up of 1141 bp of gene and 315 amino acid protein having a molecular weight of 34.258kD. Fam26F contains a Calcium modulatory domain (Ca hom mod) that is important in the maintenance of calcium concentrations in the cytosol and is a pore-forming cation channel that aids in the movement of macromolecules, calcium ions, and micromolecules inside, outside, or within a cell (Malik et al., 2020). FAM26F contains an immunoglobin-like fold composed of sheets paired with antiparallel strands connected by a single di-sulfide bridge that envelopes a hydrophobic inner core. The immunoglobulin folds or domains communicate with one another via beta-sheets (Figure 1.1).

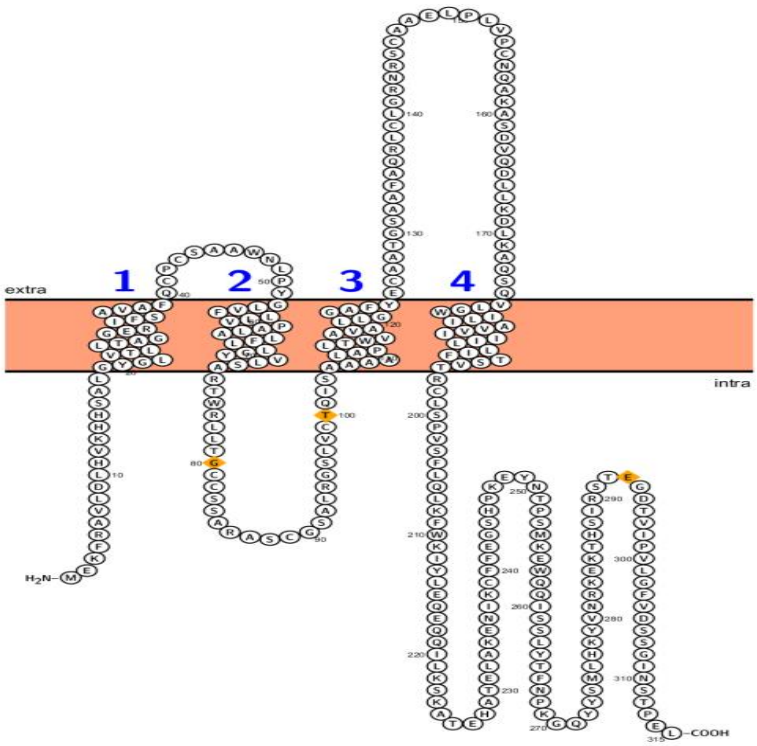

Figure 1.1: Structure of FAM26F - An immunoglobin-like fold made up of sheets paired with antiparallel strands linked by a single di-sulfide bridge that surrounds a hydrophobic inner core. Beta-sheets allow immunoglobulin folds or domains to communicate with one another (Potapov et al., 2004).

The production of interferon-gamma (IFN-g) by Natural Killer (NK) cells is promoted by inducing the expression of FAM26F on the surface of immune cells, hence FAM26F has the potential to be utilized in immunotherapy (Kasamatsu et al., 2014). Pre-infection levels of FAM26F were found in SIV-infected Rhesus macaques to correlate with the overall viral load during the acute phase of infection, establishing FAM26F as one of the earliest prognostic markers that can provide details about the forte and speed of the antiviral immune response (Javed et al., 2016). Inside the cell, FAM26F exhibits homeostatic role by upholding the level of calcium and was showed to negatively correlate with Thioredoxin level; a study revealed that various immune responses are

either activated or suppressed by interplay of FAM26F with CD30R or Thioredoxin with CD30R (Malik et al., 2020). According to a recent study on the relationship between FAM26F and HBV infection, as its level lowers in HBV infection compared to control, FAM26F expression can be a main predictive marker for infection in HBV patients (Jabeen et al., 2021).

The control of intracellular Ca2+ signaling, which is connected to ROS regulation in turn, is one of the strategies viruses use to generate a favorable cellular environment due to the varied nature of a Ca2+ signal. Fascinatingly, FAM26F is also known as Calcium Homeostasis Modulator Protein 6 (CALHM6) because it has a conserved Calcium Homeostasis Modulator (Ca hom-mod) domain. Recently, it was discovered that FAM26F shares functional similarities with calcium-binding proteins, leading to the hypothesis that cytosolic calcium disturbances control the expression of FAM26F (Casciano et al., 2017). Additionally, among the 371 genes with variable expression that were functionally connected to oxidative stress and inflammation, FAM26F was found to be one of the best classifiers (Defamie et al., 2008). In the placental transcriptome of Villitis of Unknown Etiology (VUE), FAM26F was similarly found to be considerably up regulated alongside several chemokines, MHC class I, and MHC class II molecules, where its expression increased in relation to the severity of the inflammatory process (Brito et al., 2005).

Following an epidemic of a respiratory illness with no known cause in Wuhan City, Hubei Province, China, in December 2019, a new type of beta coronavirus genus known as severe acute respiratory syndrome coronavirus 2 was found (SARS-CoV-2). 1 On February 11 of that year, the World Health Organization (WHO) designated the sickness as Coronavirus Disease 2019 (Covid-19). As of November 4, it has been connected to more than 628 million confirmed cases, more than 6.57 million fatalities, and high contagiousness, infectivity, and asymptomatic incubation periods (Cascella et al., 2022) . Around the world, there is considerable research on its

pathogenesis. The SARS-CoV-2-caused COVID-19 is a complicated illness in which the interactions between the virus and its target cells, the immune system's activity, and the body's overall reaction to these events are linked. Severe COVID-19 infection results in exuberant endothelial inflammatory responses, vascular thrombosis, and an unbalanced and uncontrolled cytokine response (known as a cytokine storm) (Montazersaheb et al., 2022). The uneven host response to the infection is likely what causes the pathological changes in the organs and tissues, such as the increased activation of immunological, endothelial, and platelet cells. Most likely, the oxidative stress brought on by cell activation may have a significant impact on the etiology of COVID-19 (Vollbracht & Kraft, 2022). The COVID-19 disease spectrum is varied, and age and the existence of co-morbid conditions affect how the disease manifests. Early suspicion, diagnosis, monitoring, and recognition of problems, as well as patient care and disposition, all depend heavily on biomarkers. Each of these elements individually may have significant effects on the administrative structure and the healthcare system, directly affecting patient care. It goes without saying that clinical assessment will be crucial at every stage, and bedside decision-making will need to significantly incorporate biomarkers. Instead of using a single biomarker, we could choose to use a biomarker panel. We cannot disregard difficulties with availability and price. The large amount of data that is constantly being added to the COVID-19 literature makes it impossible for doctors to compile and critically analyze it to extract information that is practically beneficial for patients. It is crucial to have national or regional policies that adapt the information accessible to the needs of the local community.

Here, we suggest that COVID-19 and FAM26F expression are related because it is possible that the COVID-induced inflammation has certain effects on the synthesis of FAM26F. FAM26F is also regarded as a crucial immunological signaling cascade participant, activator, and regulator of

the innate immune response. Therefore, the current study investigated how FAM26F is differentially expressed during COVID-19 infection. The investigation also probed into how FAM26F's expression levels alter as the infection worsens.

OBJECTIVES

1. Mortality Rate of COVID-19 across various age groups and genders.
2. High Neutrophil and low Lymphocyte count, a hallmark of COVID-19
3. Expression of FAM26F in infected vs control groups
4. Trends in expression level of FAM26F as the disease progresses

LITERATURE REVIEW

2.1 FAM26F

The FAM26F (Family with Sequence Similarity 26) is a recently identified immune player with the capacity to trigger complex immunological reactions. An on-cell surface molecule generated from TLR signals was reported to control mDC-NK contact conciliated Natural Killer cell activation in 2010, according to research. FAM26F has the potential to be used as a therapeutic for cancers that respond well to NK cells because of its capacity to stimulate NK cells (Ebihara et al., 2010a). On chromosome 6 in humans, the calcium-modulating protein FAM26F can be present. It is composed of a 315 amino acid protein with a molecular weight of 34.258 kD and a gene of 1141 base pairs. The pore-forming cation channel Fam26F comprises a calcium modulatory domain (Ca hom mod) that is crucial for maintaining calcium levels in the cytosol and facilitates the flow of macromolecules, calcium ions, and micromolecules inside, outside, or within a cell (Kasamatsu et al., 2014). A hydrophobic inner core is encased by an immunoglobin-like fold in FAM26F that is made up of sheets paired with antiparallel strands joined by a single di-sulfide bridge. Beta-sheets are how the immunoglobulin folds or domains communicate with one another. FAM26F has the potential to be used in immunotherapy because it encourages the expression of FAM26F on the surface of immune cells, which in turn helps Natural Killer (NK) cells produce interferon-gamma (IFN-g) (Kasamatsu et al., 2014). FAM26F has been identified as one of the earliest prognostic markers that can provide information about the strength and speed of the antiviral immune response. Pre-infection levels of FAM26F were found in SIV-infected Rhesus macaques to correlate with the overall viral load during the acute phase of infection (Javed et al., 2016). A study demonstrated that different immune responses are either activated or repressed by interaction of

FAM26F with CD30R or Thioredoxin with CD30R, which maintains the level of calcium inside the cell and was shown to negatively correlate with Thioredoxin level (Malik et al., 2020). FAM26F expression may be a key predictive marker for infection in HBV patients, according to a recent study on the link between FAM26F and HBV infection. As its level is lower in HBV infection compared to control (Jabeen et al., 2021).

Due to the complex structure of a Ca2+ signal, one method used by viruses to create a beneficial cellular environment is the control of intracellular Ca2+ signaling, which is linked to ROS regulation in turn. Fascinatingly, because FAM26F has a conserved Calcium Homeostasis Modulator (Ca hom-mod) domain, it is also known as Calcium Homeostasis Modulator Protein 6 (CALHM6). Recently, it was found that FAM26F functions similarly to calcium-binding proteins, which gave rise to the theory that changes in cytosolic calcium regulate the production of FAM26F. (Casciano et al., 2017). FAM26F was also discovered to be one of the top classifiers among the 371 genes with variable expression that were functionally linked to oxidative stress and inflammation (Defamie et al., 2008). Similar to other chemokines, MHC class I, and MHC class II molecules, FAM26F was discovered to be significantly up regulated in the placental transcriptome of Villitis of Unknown Etiology (VUE), where its expression increased in response to the severity of the inflammatory process (Brito et al., 2005)

2.2 FAM26F AS PROGNOSTIC BIOMARKER

Protracted non-progressors of the simian immunodeficiency virus (SIV) as well as some uninfected macaques can decrease viral replication in vitro without destroying the infected cells. By using transcriptional profiling, (Javed et al., 2016) sought to pinpoint the variables causing non-cytolytic viral suppression and explore their possible effects on SIV replication. FAM26F RNA levels separated CD8+ cells of controllers and non-controllers, according to the results of

microarray tests and additional validation with cells from infected and uninfected macaques (P=0.001). FAM26F was expressed in both CD4+ T-cells and B-cells, though. In vitro IFN- treatment led to an average 40-fold increase in FAM26F expression in lymphocytes, and ex vivo FAM26F RNA levels in peripheral blood mononuclear cells associated with plasma IFN-but not with IFN-. Even though individual expression levels varied up to tenfold, baseline FAM26F expression seemed to be consistent for months. Investigating FAM26F's role in SIV infection indicated that it was elevated after infection (P = 0.0008), but that, unlike MX1 and CXCL10, it did not directly correlate with viral burden. However, during the acute and post-acute phases of infection, pre-infection levels of FAM26F were inversely correlated with overall plasma viral load (AUC) (e.g., AUC weeks post infection 0-8; no AIDS vaccine: P0.0001, Spearman rank correlation coefficient (rs)=0.89, n=16; immunized with an AIDS vaccine: P=0.033, rs=0.43; n=25). Prior to infection, FAM26F transcript levels can reveal details regarding the intensity and rate of the immune system's antiviral response. The authors concluded that FAM26f expression served as one of the early prognostic markers and may be used in conjunction with MHC-typing to predict illness development prior to SIV infection.

Researchers wanted to learn more about how HBV infection affected the differential expression of FAM26F in vivo in HBV-infected people and in vitro in HepAD38 and HepG2 cell lines (Jabeen et al., 2021). Also assessed were the impact of antioxidants and calcium inhibitors on the control of FAM26F expression. The expression of FAM26F and the well-known HBV infection indicators IRF3 and IFN- were both measured at the same time. Real-time qPCR and western blot were used to assess the expression of FAM26F and marker genes. According to their findings, IRF3 and IFN- followed the same trend as FAM26F in terms of differential expression. According to the in vitro investigation, FAM26F expression was dramatically reduced in both HBV-infected cell lines as

compared to control cells that were not infected with the virus. N-acetyl-L-cysteine (NAC), EGTA-AM, BAPTA-AM, and Ru360 treatment of cells dramatically increased the expression of FAM26F in both cell lines. In addition, all HBV-infected groups in the in vivo investigation showed significantly lower FAM26F expression than controls ($p = 0.0007$). The expression was higher in the HBV recovered cases, most likely because of these people's increased immunity and decreased infection. Their research is the first to demonstrate a link between FAM26F and HBV infection. It was suggested that FAM26F expression might serve as an early indicator of HBV infection and is therefore deserving of further study.

2.3 FAM26F OXIDATIVE STRESS

Through extracellular Ca2+ influx and Ca2+ release from intracellular stores (Endoplasmic Reticulum (ER) and Golgi), which ultimately mediates the release of significant amounts of ROS, calcium homeostasis plays a key role in the activation of immune system cells (Nunes et al., 2013). By producing ROS, the host cell is then able to effectively activate IRF-3 via RIG-I and the downstream antiviral genes (Soucy-Faulkner et al., 2010). Due to its calcium homeostasis modulator domain, FAM26F, also known as Calcium homeostasis modulator protein 6 (CALHM6), has recently come to be recognized as a potent innate immunity modulator (Malik et al., 2017). FAM26F is functionally related to calcium-binding proteins, specifically to Thioredoxin, and it has been suggested that cytosolic calcium disturbances control the expression of FAM26F (Malik et al., 2020). Like this, it has been demonstrated that HBV expression is associated with alterations in calcium homeostasis and an increase in ROS levels (Waris et al., 2001). It was hypothesized that FAM26F might be involved in Ca2+ and ROS regulation following HBV infection because of the Ca2+ modulation it causes and its interaction with Thioredoxin. Therefore, (Jabeen et al., 2021) exhibited how FAM26F expression is regulated in vitro, in vivo,

and in HBV-infected individuals as well as how ROS and calcium inhibitors affect this regulation. IRF3, and IFN- were also investigated concurrently with FAM26F for their expression regulation due to the importance of IRF3 and IFN- in mediating antiviral response and their reported involvement in regulating and enhancing FAM26F expression (Ebihara et al., 2010a; Zhang et al., 2010). Their findings showed that when compared to control cells that were not infected with HBV, the expression of FAM26F, IRF3, and IFN- was significantly down-regulated in both HBV replicating (HepAD38) and HBV induced (HepG2) cells. IRF3, and IFN-, whose expression has decreased, can be attributed to the various ways that HBV has evolved to evade the host immune system by interrupting IFN-inducing cascades (Bai et al., 2015; Khan et al., 2016; Wan et al., 2017). It's interesting to note that both IRF3 and IFN- are necessary for promoting the expression of FAM26F. When NK cells were activated by FAM26F, it was discovered that this process required the activation of TICAM-1 and IRF-3 because TICAM-1 or IRF3 knockout mDC was unable to fully induce NK cytotoxicity (Ebihara et al., 2010a). FAM26F was found to be a responsive gene that was induced by more than one cytokine (IFN-, IFN-, and IL-10) in a different study looking into the diverse macrophage activation in response to cytokines (Zhang et al., 2010). Given that IRF3 and IFN- are FAM26F's upstream players and that viral infection impairs their expression, it was also discovered that FAM26F expression decreased after HBV infection. This decrease in FAM26F expression, which is comparable to the decline in IRF3 and IFN- expression, suggests that FAM26F may also be used as a marker for HBV infection.

Hepatocytes can suffer oxidative damage from HBV, which eventually results in the development of liver disease (Ren et al., 2016). HBV can also cause excessive oxidative stress and ROS production (Bolukbas et al., 2005; Hu et al., 2011). Both the HepAD38 and HepG2 cell lines were treated with the antioxidant NAC in the current study to counteract the negative effects of ROS,

which increased the expression of FAM26F, IRF3, and IFN. This suggests that NAC therapy reduced HBV replication in some way, increasing the virus-induced inhibitory effect of these antiviral immune proteins. This is in line with earlier research that found similar outcomes when ROS were suppressed in HBV-infected cells.

In both the HepAD38 and HepG2 cell lines, they further assessed the impact of calcium chelators on the expression of our target proteins. In both cell lines, exposure to EGTA-AM and BAPTA-AM increased the expression of FAM26F, IFN-, and IRF3. It was hypothesized that calcium chelation would reduce HBV replication and subsequently weaken its grip on the antiviral immune pathways, leading to increased expression of FAM26F, IRF3, and IFN-. HBx acts on stored cytosolic calcium as a fundamental activity for HBV replication (Bouchard et al., 2001). This idea is supported by the findings of (Jabeen et al., 2021). Since compounds that promote cytoplasmic calcium mobilization or accumulation can even take the place of HBx in specifically promoting HBV DNA replication through a variety of pathways, the significance of using calcium chelators for inhibiting HBV replication has long been reported (Bouchard et al., 2003). For instance, Pyk2 activation by HBx, a crucial stimulator of HBV DNA replication, was prevented by chelating cytosolic calcium with BAPTA-AM (Bouchard et al., 2001). Cyclosporine A and Cyclosporine H, two cyclosporine derivatives that inhibit cytosolic calcium signaling, reduced HBV replication as well (Bouchard et al., 2003). Reduced HBx-mediated HBV replication is the result of blocking store-operated calcium entry (SOCE) (Yang & Bouchard, 2012). Another study found that the intracellular calcium chelator BAPTA-AM can prevent the replication of wild-type HBV in cells (Bouchard et al., 2001).

The primary transporter involved in the uptake of Ca2+ into mitochondria, mitochondrial calcium uniporter (mCU) (Matlib et al., 1998) is specifically and powerfully inhibited by ru360. As was

previously mentioned, altered mitochondrial Ca2+ uptake is necessary to increase the cytosolic calcium plateau, which is necessary for HBV replication. This Ca2+ uptake by the mitochondria dampens Ca2+-mediated inhibition of additional Ca2+ release from the ER and/or Ca2+ entry through the SOC channel, extending Ca2+ entry into the cytosol and increasing cytosolic calcium levels (Hajnóczky et al., 1995; Parekh, 2003; Waris et al., 2001). Both HepAD38 and HepG2 cell lines were treated with Ru360 to block this mechanism and examine the effects that followed. Both the expression of the FAM26F mRNA and the protein were significantly upregulated, according to the results. IRF3 and IFN- expression was also increased, though not by as much. The significant increase in the expression of FAM26F suggests that Ru360 has a high potential for preventing HBV infection by inhibiting mitochondrial Ca2+ uptake. Additionally, it suggests that different pathways are involved in controlling the expression of FAM26F. According to earlier research, HBV can also be inhibited by preventing the mitochondrial calcium uptake system. For instance, blocking the HBx-induced rise in cytosolic calcium levels by inhibiting the mitochondrial permeability transition pore (MPTP) (Ren et al., 2016)

FAM26F significantly decreased in all the HBV-infected groups compared to controls during the in vivo experiments. However, HBV recovered cases had the highest levels of FAM26F expression, which was consistent with in vitro findings and demonstrated the significance of FAM26F as a vital immune modulator and antiviral agent.

2.4 COVID-19

A pneumonia outbreak that started in the Chinese city of Wuhan in December 2019 quickly expanded throughout the world and presented a significant public health issue. Official confirmation that the outbreak in Wuhan, China, is being caused by the new coronavirus 2019-nCoV came on January 9, 2020 (Wu et al., 2020). Later, the sickness was given the name

Coronavirus Disease-2019 or COVID-19 by the World Health Organization (WHO) and the novel coronavirus was given the name Severe Respiratory Disease Syndrome-Coronavirus-2 (SARS-CoV-2) by the International Committee on Taxonomy. Coronaviruses (CoVs) are reported to be the cause of several respiratory, renal, gastrointestinal, and neurological problems in both people and animals. SARS-CoV was discovered to be the cause of an epidemic in 2002–2003 that originated in China and the Asia–Pacific area, impacted 8000 persons in 37 countries, and had a 10% fatality rate. Fever, dyspnea, a dry cough, and hypoxemia were frequently seen in SARS-CoV infected individuals (Cheng et al., 2007; Ksiazek et al., 2003). Although the SARS-CoV-2 virus and the SARS-CoV virus have 79.6% sequence similarity, SARS-CoV-2 is determined to be more virulent (Shang et al., 2020). SARS-CoV-2 can eventually result in mortality in extreme instances and can cause anything from acute pneumonia and minor respiratory problems to multiple organ failure. In the pathophysiology of COVID-19, the immune system is crucial. Widespread tissue damage is caused by SARS-induction CoV-2's of an uncontrolled innate immune response and impairment of adaptive immunological responses. For COVID-19, there is currently no viable treatment.

2.5 Mortality Rate of COVID-19 in Age Groups

One of the causes for the greater death rate in the elderly population has been theorized to be a hyperinflammatory immune response (Chen et al., 2020). A study showed that older persons have increased prostaglandin D2 expression levels, which hinders respiratory DCs' migration to draining lymph nodes and, consequently, the generation of T cell responses (Zhao et al., 2011). Age-related alterations in lymph nodes have an impact on naive T and B cell development and maintenance. According to a recent study, T cell activation, proliferation, and differentiation are all diminished with advancing age (Nikolich-Zugich et al., 2020). Therefore, it is conceivable to

propose that age-related immune system dysfunction increases the severity of COVID-19 in elderly individuals.

Additionally, a recent study has revealed that whereas those over the age of 60 are particularly susceptible to household transmission, those under the age of 20 are less sensitive (Jing et al., 2020). Unexpectedly, it has been found that children's immune systems play a critical role in protecting them from COVID-19. Children's lower levels of the proinflammatory cytokines TNF- and IL-6 are seen, which further lessens neutrophil infiltration and lung damage. Children also have lower amounts of proinflammatory cytokines and higher levels of immunomodulatory cytokines like IL-10 and IL-13. Additionally, children's lung epithelium, known as TMPRSS2, expresses less ACE2 and has a superior ability to heal lung infections (Lingappan et al., 2020).

2.6 COVID-19 & Immunopathogenesis

Innate immune cells that detect the molecular patterns linked to the pathogens are activated by viral infection, stimulating the host immune system. The adaptive immune system is triggered when the innate immune responses fail to destroy the virus. Interleukin-6 (IL-6), interferon (IFN-), interferon-inducible protein-10 (IP-10) and monocyte chemoattractant protein-1 (MCP-1) are cytokines or chemokines that are secreted in response to the induction of innate and adaptive immune cells. These cytokines and chemokines encourage the migration of neutrophils and monocytes/macrophages from the circulation to the infection site. To get rid of the viral infection, these cells emit the cytotoxic chemicals. Normally, this reaction can get rid of the virus, but occasionally the immune system is dysregulated, which causes the immunological homeostasis to be upset (Dandekar & Perlman, 2005). When SARS-CoV-2 is infected, a strong inflammatory response to the virus triggers excessive production of pro-inflammatory cytokines, which encourages lung pathogenesis and respiratory failure. Pro-inflammatory cytokines cause an

unregulated buildup of neutrophils, monocytes, and macrophages at the infection site. Reactive oxygen species (ROS), which are produced by these cells, cause tissue damage and cell death (Dandekar & Perlman, 2005)

The overproduction of proinflammatory cytokines is one of the main causes of lung pathogenesis in COVID-19 patients. The worst results are seen when cytokine levels are increased early (Lucas et al., 2020). Patients with COVID-19 who have dysregulated immune systems produce a cytokine storm that encourages lung inflammation. IFN-, IL-1, IL-6, IL-8, IL-12, transforming growth factor (TGF), and chemokines C-C motif ligand 2 (CCL2), CXCL9, and CXCL10 levels are elevated in severe COVID-19 patients (Mehta et al., 2020). Additionally, COVID-19 patients displayed increased plasma concentrations of IL-2, IL-7, IL-10, GM-CSF, macrophage inflammatory protein 1 (MIP1-), tumour necrosis factor (TNF), and MCP-1 (Huang et al., 2020). Those in the non-survivor group were found to have higher levels of IL-6 during the length of the clinical course than patients in the survivor group (Zhou et al., 2020). Cytokine storm can violently attack the body in severe situations, resulting in ARDS, multiple organ failure, and ultimately death (Jyoti & Devi, 2013). A cytokine storm causes an uncontrolled flood of immune cells, particularly neutrophils and monocytes, into the lungs. These cells release a variety of inflammatory cytokines and chemokines, including IL-1, IL-6, TNF-, CCL2, CCL7, and CCL12, which worsen the condition. According to earlier research, IL-1 enhanced pyroptosis, which is the term for non-programmed cell death following pathogenic infection (Xu et al., 2020). Unchecked cell infiltrations encourage lung injury due to excessive toxic chemical secretion, such as proteases and ROS. Additionally, it results in alveolar injury, pulmonary edoema, the production of hyaline membranes, and pneumocyte desquamation, all of which are the initial symptoms of ARDS (Risitano et al., 2020; Tian et al., 2020).

2.7 Biomarkers observed in COVID-19

The COVID-19 disease spectrum is varied, and age and the existence of co-morbid conditions affect how the disease manifests. To preserve the most lives possible, prompt diagnosis and hospitalization, risk assessment, efficient use of intensive care services, proper therapy selection, monitoring, and timely discharge are all crucial. Clinical evaluation is essential, but laboratory markers, or biomarkers, can offer extra, unbiased information that has a big impact on these aspects of patient care (Samprathi & Jayashree, 2021) . Lymphocytopenia, a defining feature of COVID-19, is caused by a variety of factors, including I direct viral invasion and lysis because lymphocytes express the ACE2 receptor on their surface (ii) lymphocyte apoptosis brought on by interleukins (iii), decreased lymphocyte turnover because of the atrophy of lymphoid organs brought on by the "cytokine storm," and (iv) decreased lymphocyte proliferation because of lactic (Terpos et al., 2020) . There have been reports of both thrombocytopenia and thrombocytosis. However, serious bleeding and thrombocytopenia are not common (Amgalan & Othman, 2020). It has been demonstrated that thrombocytopenia is associated with higher mortality risk and other coagulation parameters (Bao et al., 2020). Overall, it is evident that severe COVID 19 disease is linked to significantly higher levels of leukocytes, neutrophils, infection biomarkers (like CRP, PCT, and ferritin), cytokines (like IL-2R, IL-6, IL-8, and IL-10), and lower lymphocyte counts (Hou et al., 2020).

MATERIALS AND METHODS

3.1 Study Subjects

Mardan Medical Complex was targeted as the sampling location being the central hospital at the epicenter of disease outbreak in the nearby localities. The study was approved by the Ethical Review Board of Mardan Medical Complex and Institutional Review Board (IRB) of Atta Ur Rahman School of Applied Biosciences (ASAB), NUST, Islamabad, Pakistan. Informed consent was taken from all the participants. Clinical data on the patients, such as their age, gender, oxygen saturation level, lymphocytes count, treatment regime and platelets count were collected as well. We also observed if these patients survived or died. Blood samples were collected in EDTA tubes from 45 patients hospitalized in the ICU on par of severe COVID-19 infection were included in the study as the experimental group. 20 healthy individuals, with no implication of deteriorating health, were sampled as healthy controls. The blood samples were processed immediately after collection.

3.2 Follow Ups

To evaluate the expression of FAM26f during hospitalization which lead to either one of the two outcomes, either patients form the illness recovered or died. Hence blood samples were collected at intervals of 48 hours till the patients departed from the ICU. 18 patients were followed up following the set criteria. The sampling scheme is illustrated in Figure 3.1

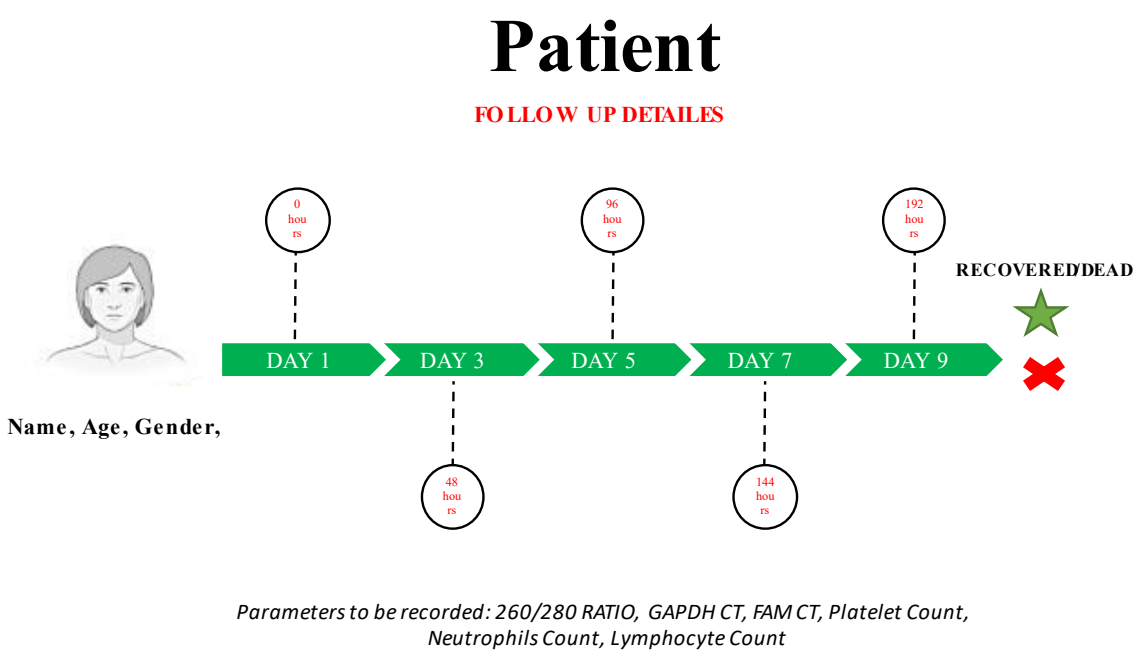

Figure 3.1: The patient was followed up after every 48 hours, the clinical parameters were penned down only with the first collection of samples only. The follow ups only stopped if the patient either died or had recovered.

3.3 RNA Extraction

RNA extraction of all COVID-positive samples was initiated at Abdul Wali Khan University Mardan. RNA extraction was performed using RBC lysis buffer pH 7.3 (17.98 g NH_4Cl, 2g $KHCO_3$, 400 µL 0.5 M EDTA in 200 mL total volume) and Solarbio Triquick Reagent R1100. For this purpose, 5 mL blood was mixed with 25 mL RBC lysis buffer and incubated at room temperature for 10 minutes. Mixture was centrifuged at 600 rcf for 10 minutes and supernatant was discarded. Pellet was resuspended in 1 mL lysis buffer and incubated at room temperature for 5 minutes. After this, centrifuge was performed at 3000 rpm for 2 minutes, supernatant was aspirated, and pellet of white blood cells (WBCs) was resuspended in 1 mL PBS. Again, centrifuge was performed at 3000 rpm for 2 minutes, supernatant was discarded, and the final pellet was

resuspended in 1 mL Solarbio Triquick Reagent R1100. The successfully extracted WBCs with added Solarbio Triquick Reagent R1100 were stored at -80 ℃ and transported to ASAB, NUST Islamabad after 3 days for further processing.

The received samples were thawed properly and mixed with 200 µL chloroform and vortexed for 30 seconds. Then, this mixture was incubated at -20 °C for 3 minutes and centrifuged at 12000 rcf for 10 minutes at 4 °C. Aqueous phase was meticulously transferred to new microcentrifuge tube and 500 µL of isopropanol was added. This mixture was inverted several times and then incubated at -20 °C for 10 minutes. Afterwards, this mixture was centrifuged at 12000 rcf for 10 minutes at 4 °C. Supernatant was discarded and pellet was washed with 1 mL 75% ethanol. Centrifuge was performed at 12000 rcf for 2 minutes at 4 °C. Supernatant was discarded and final RNA pellet was dried in sterile conditions at 4°C for 10 minutes before final suspension in nuclease free water. Presence of RNA was confirmed first using Berthold Detection Systems GmbH, Pforzheim (A 260/280 ratio of nearly 1.8 to 2 indicated RNA purity) and then denaturing gel electrophoresis performed at 90 V, 90 mA for 50 minutes (Figure 3.2).

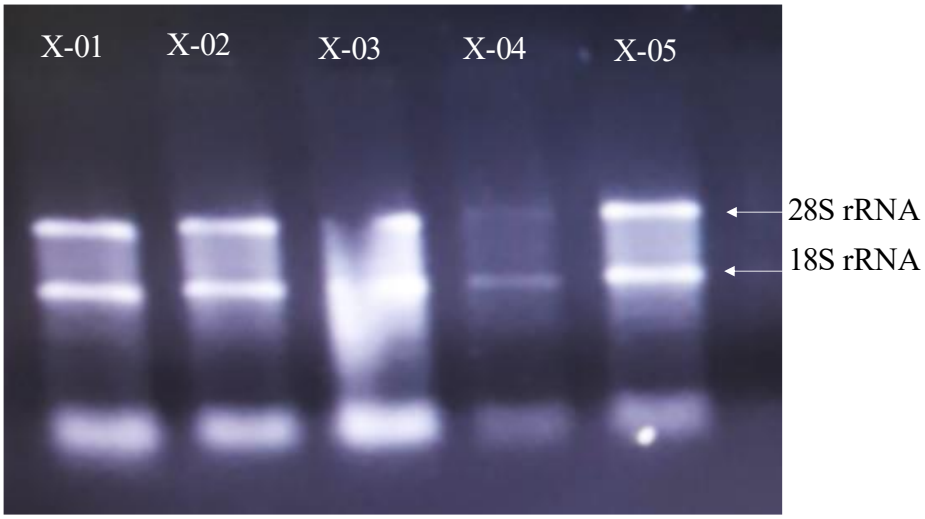

Figure 3.2: Image showing RNA denaturing gel to determine the integrity and purity of the extracted RNA from COVID (+) patients. The distinct bands indicate 28S and 18S RNA, indicative of indicative of integral and pure RNA.

3.4 cDNA Synthesis

Extracted RNA was quantified using nanodrop and 1 µg of RNA was used for cDNA synthesis using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit K1622. For this purpose, 1 µg of RNA is mixed with 1 µL random hexamer in total volume of 12 µL. This mixture was incubated at 65 °C for 5 minutes. Then, 4 µL 5X buffer, 1 µL ribolock, 2 µL 10 mM dNTPs, and 1 µL RT enzyme were added in this mixture. Temperature timeline for this reaction was 25 °C for 5 minutes, followed by 42 °C for 60 minutes, and finally 70 °C for 5 minutes. Synthesized cDNA was then diluted in 1:1 ratio. The synthesized CDNA was confirmed for DNA contamination by using GAPDH primers, nRTC (No Reverse-Tresncriptase Control) and NTC (No Template Control). The aim of this PCR was to confirm the purity of the extracted RNA.

Table 3.2: Reagents and their quantities used for cDNA synthesis

Component	Volume
RNA	1/concentration of RNA in ul
Random Hexamers	1ul
Total volume	12ul

This mixture was micro-centrifuged for 15 sec and heated at 65°C for 5 minutes in a PCR machine. After this incubation step, the PCR tube was then transferred to an ice cooler. The remaining ingredients were added in a specified volume in the tube.

Component	Volume
5X RT Reaction Buffer	4ul
10mM dntps	2ul
Ribolock	1ul
Revert aid enzyme	1ul

3.5 PCR test for cDNA quality

As an optional step, a cDNA quality test was performed after cDNA synthesis to verify the appropriate synthesis of the cDNA from each sample. A GAPDH primer set was used. In this PCR protocol, 5 ng of cDNA was used as a template with the following PCR cycling conditions: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 62 °C for 40 s, and 72 °C for 1 min, with the final elongation step at 72 °C for 5 min. Each primer was used at a concentration of 500 nM in 2× PCR premix reagent (Quest Taq PCR Mix, Catalog #: QM13532, BioQuest, USA). The amplicons were subjected to electrophoresis in a 2% agarose gel at 130 V for 20 min and visualized under UV light.

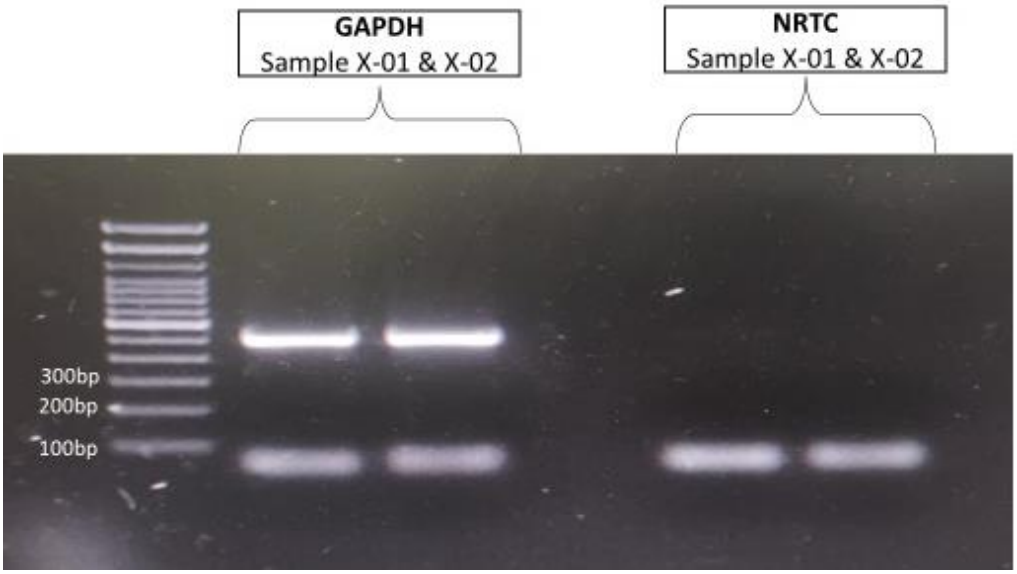

Figure 3.3: **Representative gel image of the test of cDNA purity.** A single sample was amplified using GAPDH primers along with a no reverse transcription control to ensure that the extracted RNA does not have contaminating DNA.

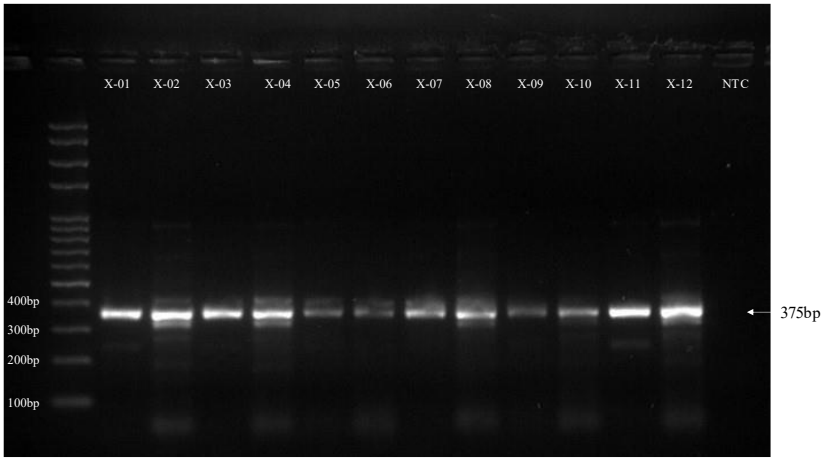

Figure 3.4: **Representative gel image of the test of cDNA purity**. A single sample was amplified using GAPDH primers along with a no reverse transcription control to ensure that the extracted RNA does not have contaminating DNA.

3.6 Real-time PCR analysis

House-keeping gene was first finalized using real-time PCR for further gene expression analysis of uninfected and infected samples. Real-time PCR was then performed on each sample for C_t value determination of house-keeping gene (GAPDH) and *FAM26f* in a series of duplicate reactions. Real-time PCR was performed using Solis BioDyne 5X HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) in the final concentration of 1X, 5 pmol of forward and reverse primers, 250 ng of cDNA in a reaction volume of 20 µL. Real-time PCR was performed at 95 °C initial activation for 12 minutes, followed by 40 cycles of 95 °C denaturation for 15 seconds, annealing at 60 °C for 20 seconds, and extension at 72 °C for 20 seconds (Figure 3.5). Fluorescence for EvaGreen was captured at extension stage.

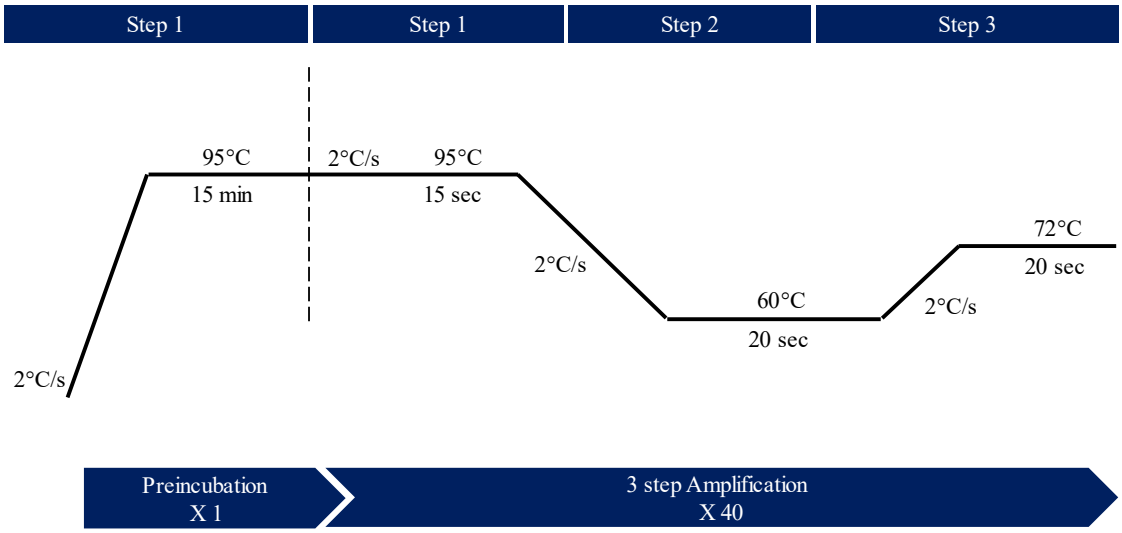

Figure 3.5: Schematics of QPCR analysis; 95 °C initial activation for 12 minutes, followed by 40 cycles of 95 °C denaturation for 15 seconds, annealing at 60 °C for 20 seconds, and extension at 72 °C for 20 seconds.

Gene copy number and fold change for *FAM26f* were calculated for each sample after real-time PCR. For copy number, following formulae were used:

$$\Delta C_t = C_{t_{(target)}} - C_{t_{(house\ keeping)}}$$

$$Copy\ number = 100 \times 2^{-\Delta C_t}$$

These formulae provide us with a relative quantification that for 100 copies of house-keeping genes how many copies of target gene (*FAM26f*) will be present.

For fold change calculation, average of ΔC_t values for infected and uninfected groups were calculated respectively for both genes. Then, following formulae were applied:

$$\Delta\Delta C_t = \Delta C_{t_{(infected)}} - \Delta C_{t_{(uninfected)}}$$

$$Fold\ change = 2^{-\Delta\Delta C_t}$$

3.7 Statistical analysis

Statistical analysis was carried out using Graph-Pad Prism 6.0 software (Graph-Pad Software, San Diego, CA USA) where $p < 0.005$ was considered significant. Unpaired t-test at 95% confidence interval was used to compare the sample groups. Linear correlations were identified using Pearson correlation

RESULTS

4.1 Mortality Rate

The mortality rates were calculated based on age group, gender, absolute neutrophil, and lymphocytes count. 3 patients lied in the age group 20-40, 19 patients in age group 40-60, 16 patients in age group 60-80 and 4 patients in age group 80-100 with mortality rate 66.66%. 68.42%, 75% and 100% respectively. In 14 males and 28 females, we observed equal mortality rate i.e., 71.42%. Only 8 patients were found to have absolute neutrophil count in the normal range (2.0 to 7.99 x 10^9/L). The rest had higher or extremely high levels of neutrophils; 22 patients in the range (8.0 to 13.99 x 10^9/L), 9 patients in the range (14.0-19.99 x 10^9/L) and 3 patients in the range (20.0-24.99 x 10^9/L), with respective mortality rates of 75%, 63.63%, 77.77% and 100% respectively. Similarly, 23 patients had low lymphocyte count between (0 and 0.99 x 10^9/L), 18 patients had lymphocyte count between (1.0-1.99 x 10^9/L) with mortality rate 69.56% and 72% respectively, while only one patient with a lymphocyte count between 2.0-2.99 x 10^9/L and couldn't survive (Figure 4.1).

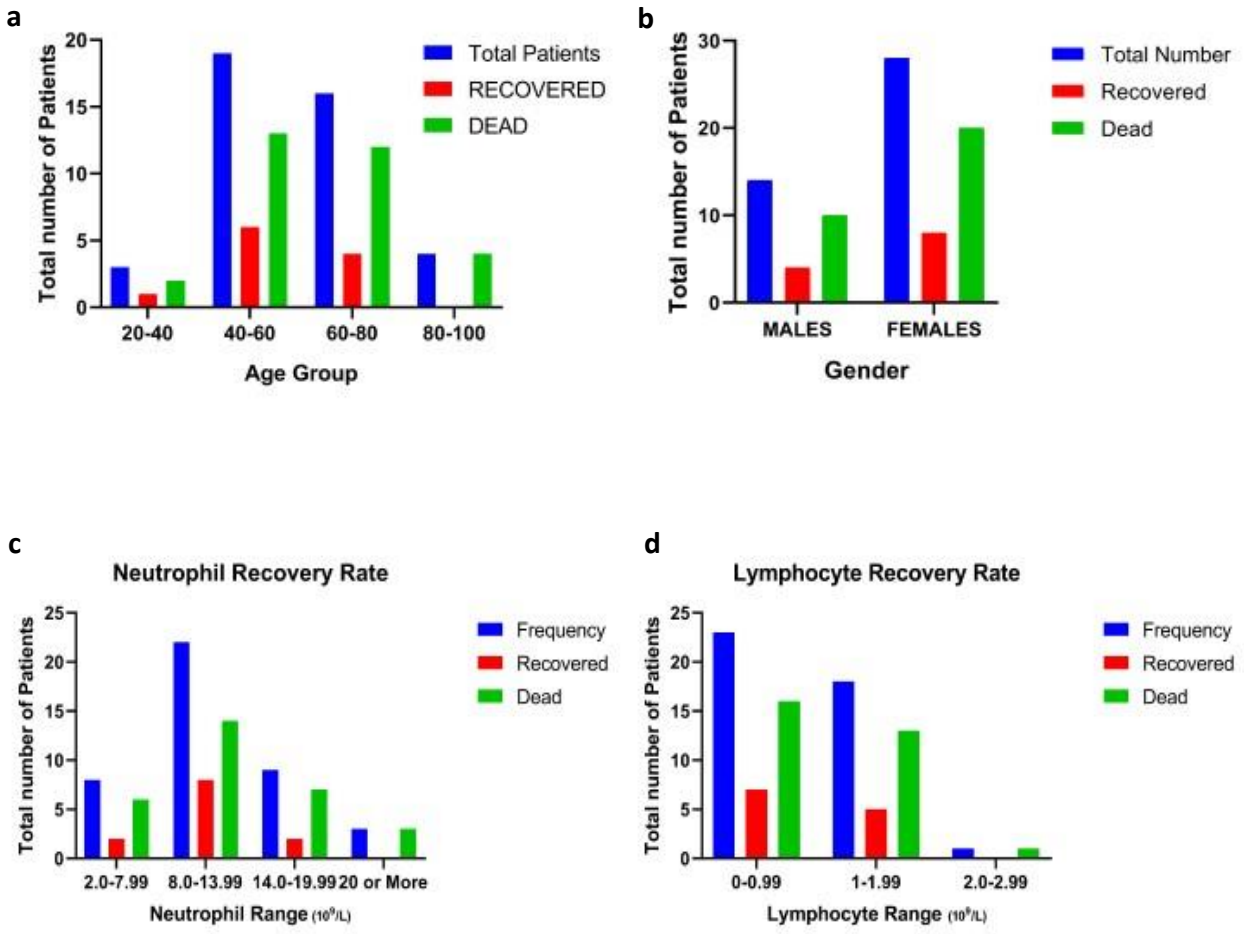

Figure 4.1: Bar Charts representing mortality rates **a**. Across various age groups; 3 patients in age group 20-40, 19 patients in age group 40-60, 16 patients in age group 60-80 and 4 patients in age group 80-100 with mortality rate 66.66%. 68.42%, 75% and 100% respectively. **b.** Across male and female gender; 14 males and 28 females with equal mortality rate i.e., 71.42%. **c.** Across various neutrophil ranges; 8 patients in the normal range (2.0 to 7.99 x 10^9/L), 22 patients in the high range (8.0 to 13.99 x 10^9/L), 9 patients in the range (14.0-19.99 x 10^9/L) and 3 patients in the range (20.0-24.99 x 10^9/L), with respective mortality rates of 75%, 63.63%, 77.77% and 100% respectively. **d.** Across various lymphocyte ranges; 23 patients had low lymphocyte count between (0 and 0.99 x 10^9/L), 18 patients had lymphocyte count between (1.0-1.99 x 10^9/L) with mortality rate 69.56% and 72% respectively, while only one patient with a lymphocyte count between 2.0-2.99 x 10^9/L died.

4.2 Differential Expression of FAM26F

The Expression of FAM26F was analyzed by RT-PCR in COVID (+) (n=42) and COVID (-) patients (n=19). FAM26f is significantly downregulated in patients infected with COVID-19 (t=3.138, df=59, P = 0.0027) as compared to the control group (Figure 4.2). The results demonstrate that the expression of FAM26f is significantly lower in infected patients.

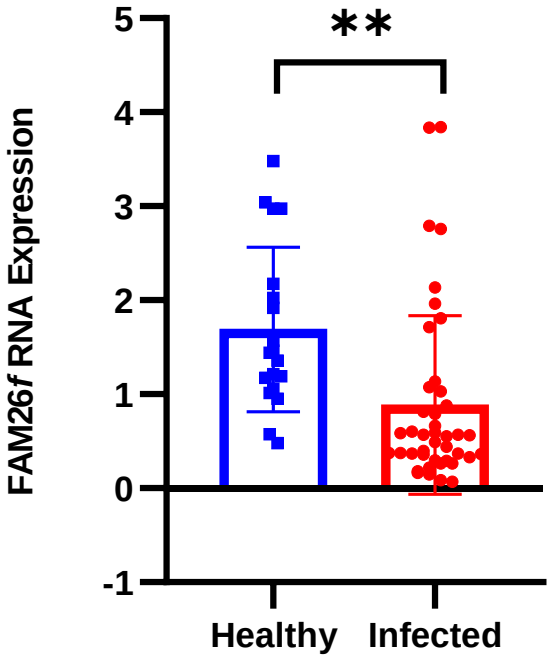

Figure 4.2: Expression of FAM26f in COVID (+) (n=42) and COVID (-) patients (n=19). FAM26f is significantly downregulated in patients infected with COVID-19 (t=3.138, df=59, P = 0.0027) as compared to the control group.

4.3 Follow Ups

18 patients either recovered or died during the follow ups: 8 patients recovered/died on day 3, 6 patients recovered/died on day 5, 3 patients died on day 7 and 1 patient survived and was discharged on day 9 of hospitalization (Table 1). The expression of FAM25f was analyzed during

the follow ups and no significant increase or decrease in its expression level was observed (Figure 4.3-4.7). Only 5 out of 18 patients had a positive outcome i.e., survival (Figure 4.7); four patients had increased levels of FAM26f expression whereas one patient who recovered had decrease levels of FAM26f expression.

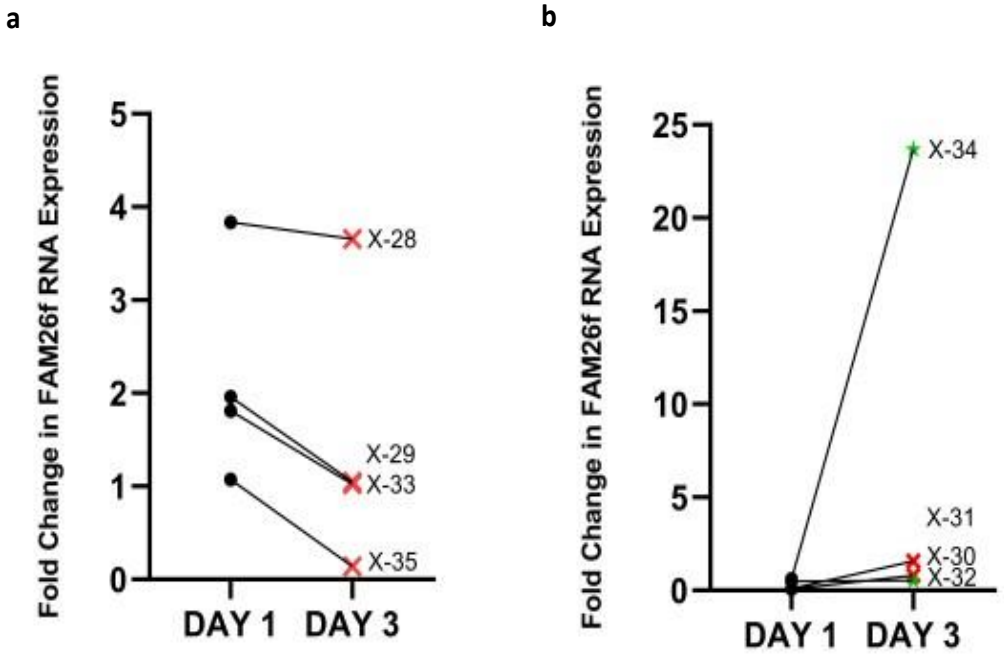

Figure 4.3: Fold change in FAM26f mRNA level on Day 3 of hospitalization (n=8), ★ represents recovery and **x** represents death. **(a)** On Day 3, the expression of FAM26f in PBMCs of X-28, X-29, X-33, and X-35 (0.79 to 0.57, 0.15 to 2.59, 0.29 to 1.99 and 0.16 to 1.08) decreased on Day 3 and the patients couldn't survive. **(b)** The expression of FAM26f in PBMCs of X-32 and X-34 increased (0.49 to 0.53, 0.60 to 23.66) and the patients survived. The expression of FAM26f in PBMCs of X-30 and X-31 increased (0.07 to 0.80, 0.08 to 1.56) but the patients couldn't survive.

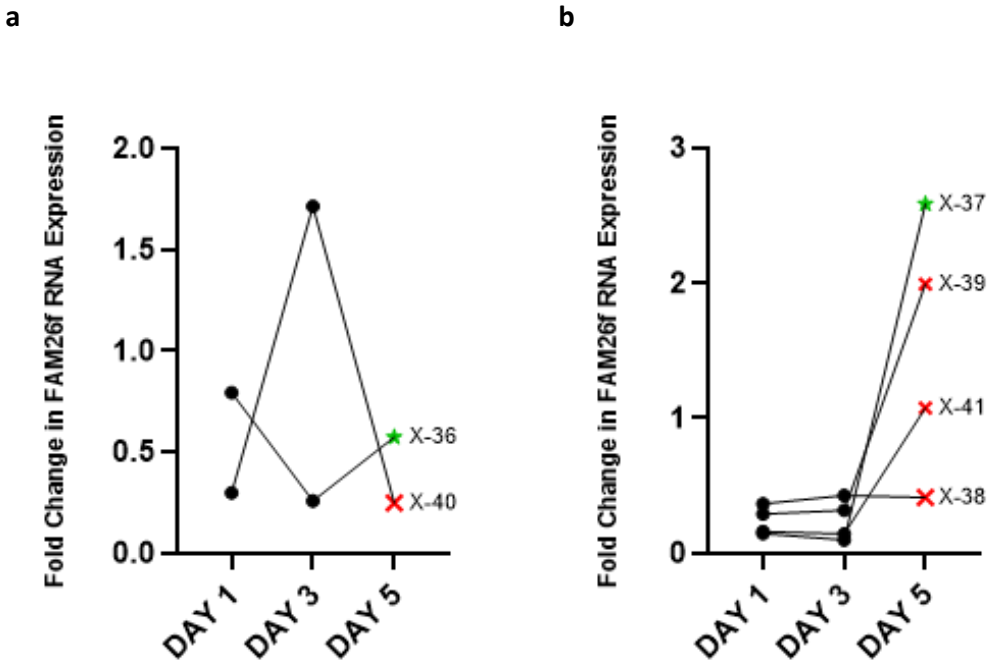

Figure 4.4: Fold change in FAM26f mRNA level on Day 3 and Day 5 of hospitalization (n=6), ★ represents recovery and **x** represents death. **(a)** The expression of FAM26f in PBMCs of X-36 and X-40 decreased (0.79 to 0.57, 0.29 to 0.24) and the patient X-36 survived, and X-40 couldn't. **(b)** On Day 5, the expression of FAM26f in PBMCs of X-37, X-38, X-39, and X-41 increased (0.14 to 2.5, 0.36 to 0.41, 0.29 to 1.99 and 0.16 to 1.07) on Day 5, only X-37 survived.

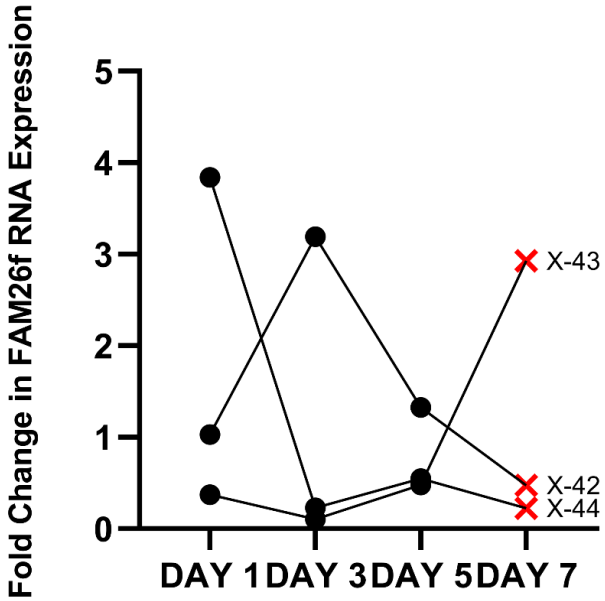

Figure 4.5: Fold change in FAM26f mRNA level on Day 3, Day 5 and Day 7 of hospitalization (n=3). On Day 7, the expression of FAM26f in PBMCs of X-42 and X-44 decreased to the lowest whereas it increased in X-43 but none of the patients could survive. **x** represents death.

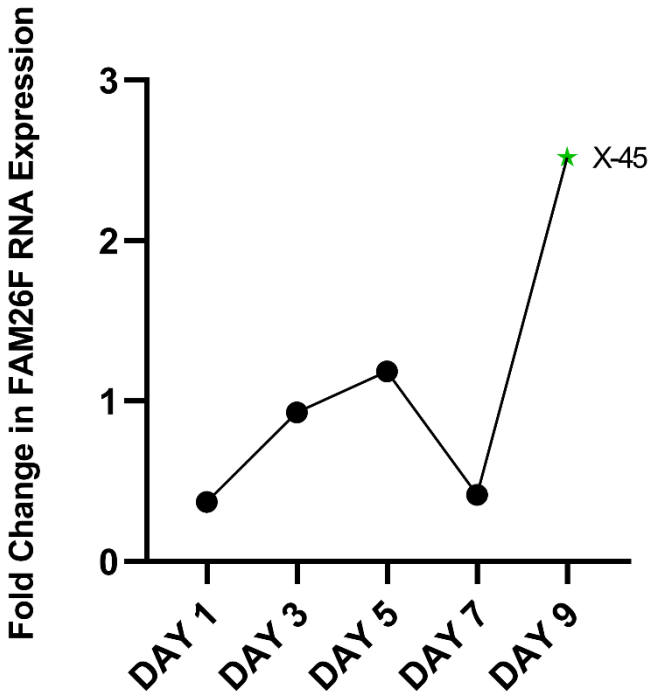

Figure 4.6: Fold change in FAM26f mRNA level on Day 9 of hospitalization (n=1), ★ represents recovery. On Day 9th, the expression of FAM26f in PBMCs of X-45 increased and the patient survived. The expression of FAM26f in PBMCs of X-36 and X-37 increased but the patients couldn't survive.

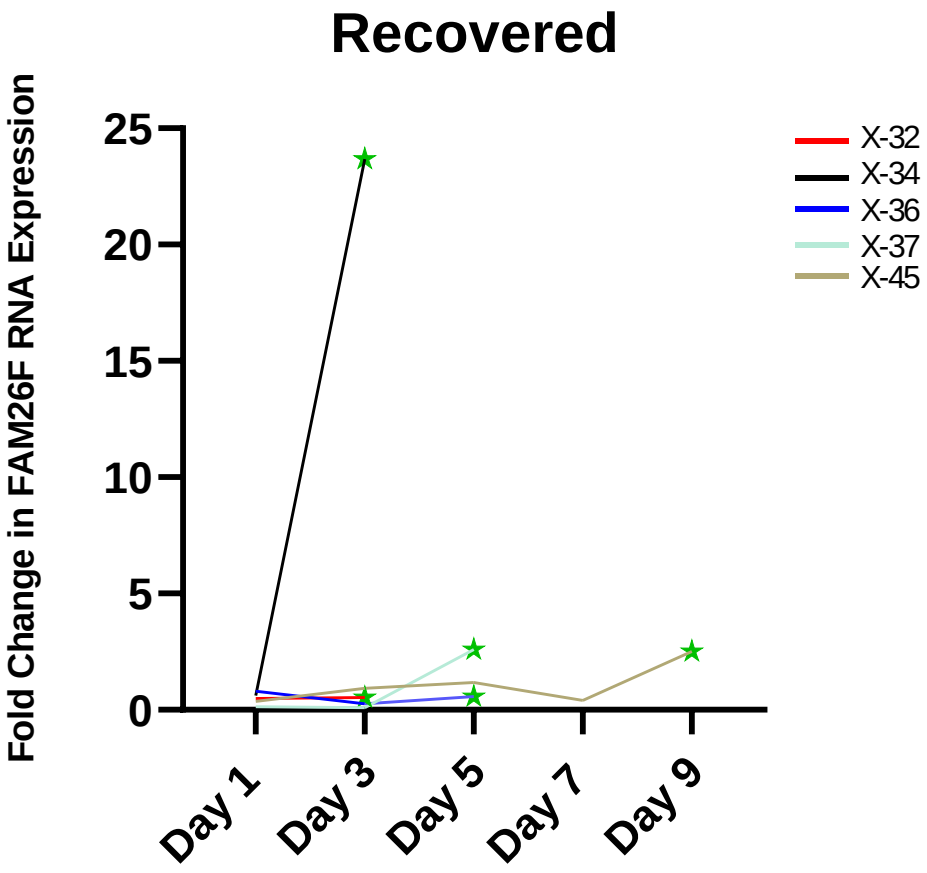

Figure 4.7: Fold change in FAM26F mRNA level in PBMCs of patients who could survive (n=5), ★ represents recovery. X-32 and X-34 survived with an increased expression on Day 3 (0.49 to 0.53 and 0.60 to 23.66). X-36 survived with an overall decreased expression on Day 5 (0.79 to 0.57) and X-37 survived with increased expression of FAM26F in PBMCs of patients (0.14 to 2.58). X-45 was hospitalized for 10 days with maximum expression of FAM26f on Day 9 (0.37 to 2.51).

Table 4.1. Fold Change in mRNA level of FAM26f in PBMCs of COVID-19 (+) Patients.

SAMPLE	DAY 1	DAY 3	DAY 5	DAY 7	DAY 9	DAY 11
X-28	3.836674	3.657496	X			
X-29	1.961362	1.046706	X			
X-30	0.071687	0.802657	X			
X-31	0.083496	1.5625	X			
X-32	0.493067	0.531763	*			
X-33	1.808578	1.023745	X			
X-34	0.605356	23.66784	*			

X-35	1.072781	0.138107	X			
X-36	0.793805	0.25879	0.573496	*		
X-37	0.145779	0.099226	2.58624	*		
X-38	0.366747	0.428051	0.414618	X		
X-39	0.290147	0.319715	1.994264	X		
X-40	0.299547	1.713393	0.24911	X		
X-41	0.162426	0.143473	1.075387	X		
X-42	1.030151	3.192875	1.327633	0.474624	x	
X-43	0.374193	0.10198	0.477263	2.92788	x	
X-44	3.839334	0.231143	0.546334	0.222497	x	
X-45	0.370066	0.929068	1.185796	0.416635	2.517263	*

* presents the patients who recovered from COVID-19 and x present the patients who died because of the disease.

4.4 Correlation of FAM26f with Absolute Neutrophil Count

We also investigated whether FAM26F RNA levels could be related to absolute neutrophil count. FAM26f gene expression significantly correlates with the neutrophil count (10^9/L) (Pearson r=-0.383, P = 0.0470) as depicted in Figure 4.8.

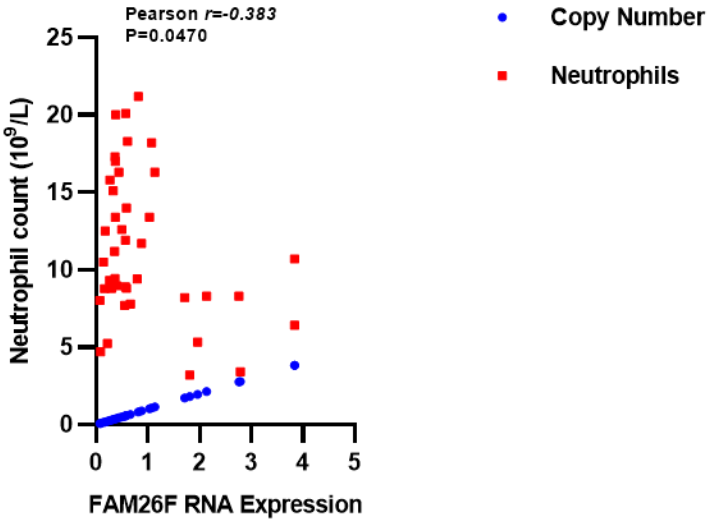

Figure 4.8: FAM26f gene expression negatively correlates with the neutrophil count (10^9/L) (n=42). Significance and Pearson *r* are revealed in the figure.

DISCUSSION

In December of 2019, the first case of COVID-19 was reported in Wuhan, the capital city of the central Hubei Province of the People's Republic of China. A substantial percentage of vendors and dealers from the Huanan seafood wholesale market contracted pneumonia of unknown origin, followed by a slew of cases throughout the city of Wuhan. China announced at the beginning of January 2020 that this outbreak is caused by a novel coronavirus pneumonia that belongs to the coronavirus family and has been designated 2019-nCov by Chinese researchers. The Chinese government and the World Health Organization (WHO) began working on it. China announced the first COVID-19-related death of a 61-year-old seafood market worker. On January 30, 2020, the WHO declared a medical emergency and issued a high alert to authorities to manage cross-border movements. In the meantime, on February 11, the World Health Organization (WHO) dubbed a new coronavirus disease COVID-19 (WHO, 2020) and began pressuring governments to take control measures, such as a travel ban across international borders. WHO declared the disease to be pandemic in March 2020, when the virus had spread to 114 countries. The Federal Ministry of Health confirmed the first COVID-19 case in Karachi on February 26, 2020, and another case was confirmed in Islamabad on the same day. The province Sindh was the focal point, as the highest number of cases, 20 in just 15 days, were reported there. (NCOC, 2020).

The first objective of this investigation was to compare the mortality rates of older adults with those of their younger counterparts. Initial data indicate that older adults are uniquely vulnerable. Case series have revealed that age is an independent predictor of mortality (Zhou et al., 2020). As reported by (Bonanad et al., 2020) mortality was 1.1% in patients aged 50 years and increased exponentially after this age. As anticipated, patients aged 80 years or more had the highest

mortality rate. All age groups had significantly higher mortality rates compared to what was immediately younger. Patients aged 60 to 69 years had the greatest mortality risk increase compared to those aged 50 to 59 years (OR 3.13, 95% CI 2.63 to 3.76). The same trend was observed in the current investigation with the mortality rate greatest in patients aged 80 or more. None of the patients in the age group (80-100) could survive. Patients aged 60-80 had the highest mortality rate (75%). Similarly, the immediately younger age group (40-60) showed a mortality rate of (68.42%), followed by age group (20-40) in which a slightly lesser mortality rate (66.67%) was reported. These findings commensurate with the results of (Henkens et al., 2022) where the highest mortality was recorded in patients who were aged 70 or more (76%). Globally, male patients with comorbidities have a higher mortality risk, according to COVID-19 mortality data that is disaggregated by gender. Emerging global research indicates that male gender plays a significant role in the increased risk of COVID-19 mortality among adult patients. Males tend to have lower serum IgG antibody production, significantly reduced CD4+ T cell reserves, and lower circulating ACE2 expression compared to females, which may explain this association. Male sex is also associated with increased disease severity upon hospital admission, higher ICU admission rates, and elevated clinical markers including lymphopenia and inflammatory indexes. For e.g. The objective of (Kelada et al., 2020)'s systematic review was to determine the effect of gender on the risk of mortality from COVID-19 in adult patients. The search of ten databases revealed that males have a higher mortality risk than females. In eight studies, male gender was significantly associated with an increased mortality risk from COVID-19. After adjusting for confounding variables, a single study found no significant correlation between male gender and mortality (Asfahan et al., 2020). According to our findings males and females both had a mortality rate of 71.42 %, which corresponded with the findings of the research by (Asfahan et al., 2020). Our study

has a major limitation that the population size is small (n=42), so to fully evaluate sex-differences in the mortality risk from COVID-19, further studies will need to be undertaken globally.

Among the distinctive characteristics of the severe COVID-19 clinical syndrome is an abnormally high neutrophil-lymphocyte ratio for a viral infection. Neutrophils play an important role in viral clearance by producing NETs and IFN. In patients with severe COVID-19, the neutrophil count rises while the lymphocyte count decreases. Four meta-analyses have previously validated this pattern in severe COVID-19 infection (Chan & Rout, 2020; Ghahramani et al., 2020; Lagunas-Rangel, 2020; Lan et al., 2020), however, the biological basis for the association between neutrophilia and severe COVID-19 infection is currently unknown. In the current investigation, 8 out of 42 patients had neutrophil concentrations in the normal range (2.0 to 7.99 x 109/L), 22 patients were in the high range (8.0 to 13.99 x 109/L), and the mortality rate was lowest (63.63%) in this range. The remaining patients fell within the extreme ranges; 9 patients fell within the range (14.0-19.99 x 109/L) and 3 patients fell within the range (20.0-24.99 x 109/L), with respective mortality rates of 77.77% and 100%. Similarly, 23 patients had a low lymphocyte count between (0 and 0.99 x 109/L). Such patients had the lowest mortality rate (69.56%). 18 patients with a lymphocyte count between (1.0-1.99 x 109/L) had a 72% mortality rate, while only one patient with a lymphocyte count between 2.0-2.99 x 109/L died. Neutrophils are indispensable mediators of the innate immune response to acute infection and inflammation28,29. Nevertheless, their high functional capacity may contribute to tissue damage in severely ill patients. As reported by (Veenith et al., 2022) it is possible that neutrophilia's high ROS production contributes to the characteristic tissue damage profile of patients with severe COVID-19 infection. In fact, it has been suggested that neutrophil infiltration contributes to pulmonary complications and that ROS release contributes to both cytokine storm and blood clot formation in SARS-CoV-2 infection. As

indicated by our findings, understanding the role of neutrophils and lymphocytes in the pathogenesis of severe COVID-19 could lead to the identification of key therapeutic targets and/or biomarkers for the early identification of patients who may benefit from immunomodulatory agents to control hyperinflammation and reduce mortality rates.

Calcium homeostasis plays a crucial role in the activation of immune system cells through extracellular Ca2+ influx and Ca2+ release from Golgi and Endoplasmic Reticulum (ER), which subsequently arbitrates the release of significant amounts of ROS (Nunes et al., 2013). The host cell can effectively activate IRF-3 by generating ROS through RIG-I and the downstream antiviral genes (Soucy-Faulkner et al., 2010). FAM26F has recently come to be known as a potent innate immunity modulator due to its calcium homeostasis modulator domain (Ebihara et al., 2010). According to a report, Thioredoxin and other calcium-binding proteins share functional relationships with FAM26F, and it has been proposed that cytosolic calcium imbalances regulate the expression of FAM26F (Zhang et al., 2010). It's reported that COVID-19 leads to hypoxia in which case have been found to have higher intracellular Ca2+ concentrations. (Danta, 2021). The fate of immune cells and their abilities could be impacted by hypoxia, which could also stimulate the cytokine storm and inflammatory pathways. Hyperinflammation and activated cytokine sending signals are responsible for the severity and death of COVID-19 patients. (Serebrovska et al., 2020), the massive mortality rate observed in our study can be attributed to this. Because of the Ca2+ modulation it causes and the interactions it has with Thioredoxin, it has been hypothesized that FAM26F may play a role in the regulation of Ca2+ and ROS after COVID-19 infection. However, there is no available research conducted or statistics in this regard. To better understand how COVID-19 infection affects patients' FAM26F expression, the current study was created. According to our research, FAM26f is significantly downregulated in COVID-19-infected

patients when compared to the control group (t=3.138, df=59, P = 0.0027). The fact that COVID recovered cases had the highest levels of FAM26F expression, however, indicated the significance of this protein as a vital immune modulator and antiviral agent. It is possible to improve COVID-19 clearance or containment by reversing ROS production and Ca2+ deregulation utilizing calcium modulators and antioxidants, most likely by modifying the expression of FAM26F. Here, we suggest that COVID-19 and FAM26F expression are related because it is possible that the COVID-19 inflammation has some effects on the synthesis of FAM26F. This study is the first to demonstrate a relationship between FAM26F and COVID-19. Overall, our study's findings demonstrate the importance of FAM26F as an innate immune modulator, and it is suggested that FAM26F expression may serve as an early indicator of COVID-19 infection. The expression level of FAM26F negatively correlated with the absolute neutrophil count (Pearson r=-0.383, P = 0.0470), which might be attributed to ROS burst which deregulates Ca+2 levels and hence decreases the expression of FAM26F.

CONCLUSION AND FUTURE PROSPECTS

The current study is the first to definitively link FAM26F to COVID-19. It is hypothesized that FAM26F expression could serve as an early predictive marker for COVID-19, and our findings provide further evidence for the significance of FAM26F as an innate immune modulator. Our study shows that FAM26F is a significant molecule with conspicuous intrinsic value for further research, whether it is involved directly during the regulation of viral replication or eventually through the immune dissent.

REFERNECES

Amgalan, A., & Othman, M. (2020). Hemostatic laboratory derangements in COVID-19 with a focus on platelet count. *Platelets*, *31*(6), 740–745. https://doi.org/10.1080/09537104.2020.1768523

Asfahan, S., Deokar, K., Dutt, N., Niwas, R., Jain, P., & Agarwal, M. (2020). Extrapolation of mortality in COVID-19: Exploring the role of age, sex, co-morbidities and health-care related occupation. *Monaldi Archives for Chest Disease*, *90*(2), Article 2. https://doi.org/10.4081/monaldi.2020.1325

Bai, L., Zhang, W., Tan, L., Yang, H., Ge, M., Zhu, C., Zhang, R., Cao, Y., Chen, J., Luo, Z., Ho, W., Liu, F., Wu, K., & Wu, J. (2015). Hepatitis B virus hijacks CTHRC1 to evade host immunity and maintain replication. *Journal of Molecular Cell Biology*, *7*(6), 543–556. https://doi.org/10.1093/jmcb/mjv048

Bao, C., Tao, X., Cui, W., Yi, B., Pan, T., Young, K. H., & Qian, W. (2020). SARS-CoV-2 induced thrombocytopenia as an important biomarker significantly correlated with abnormal coagulation function, increased intravascular blood clot risk and mortality in COVID-19 patients. *Experimental Hematology & Oncology*, *9*, 16. https://doi.org/10.1186/s40164-020-00172-4

Bolukbas, C., Bolukbas, F. F., Horoz, M., Aslan, M., Celik, H., & Erel, O. (2005). Increased oxidative stress associated with the severity of the liver disease in various forms of hepatitis B virus infection. *BMC Infectious Diseases*, *5*(1), 95. https://doi.org/10.1186/1471-2334-5-95

Bonanad, C., García-Blas, S., Tarazona-Santabalbina, F., Sanchis, J., Bertomeu-González, V., Fácila, L., Ariza, A., Núñez, J., & Cordero, A. (2020). The Effect of Age on Mortality in Patients With COVID-19: A Meta-Analysis With 611,583 Subjects. *Journal of the American Medical Directors Association*, *21*(7), 915–918. https://doi.org/10.1016/j.jamda.2020.05.045

Bouchard, M. J., Puro, R. J., Wang, L., & Schneider, R. J. (2003). Activation and Inhibition of Cellular Calcium and Tyrosine Kinase Signaling Pathways Identify Targets of the HBx Protein Involved in Hepatitis B Virus Replication. *Journal of Virology*, *77*(14), 7713–7719. https://doi.org/10.1128/JVI.77.14.7713-7719.2003

Bouchard, M. J., Wang, L.-H., & Schneider, R. J. (2001). Calcium Signaling by HBx Protein in Hepatitis B Virus DNA Replication. *Science*, *294*(5550), 2376–2378. https://doi.org/10.1126/science.294.5550.2376

Brito, H., Juliano, P., Altemani, C., & Altemani, A. (2005). Is the immunohistochemical study of the inflammatory infiltrate helpful in distinguishing villitis of unknown etiology from non-specific infection villitis? *Placenta*, *26*(10), 839–841. https://doi.org/10.1016/j.placenta.2004.10.012

Cascella, M., Rajnik, M., Aleem, A., Dulebohn, S. C., & Di Napoli, R. (2022). Features, Evaluation, and Treatment of Coronavirus (COVID-19). In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK554776/

Casciano, J. C., Duchemin, N. J., Lamontagne, R. J., Steel, L. F., & Bouchard, M. J. (2017). Hepatitis B virus modulates store-operated calcium entry to enhance viral replication in primary hepatocytes. *PloS One*, *12*(2), e0168328. https://doi.org/10.1371/journal.pone.0168328

Chan, A. S., & Rout, A. (2020). Use of Neutrophil-to-Lymphocyte and Platelet-to-Lymphocyte Ratios in COVID-19. *Journal of Clinical Medicine Research*, *12*(7), 448–453. https://doi.org/10.14740/jocmr.v12i7.4240

Chen, J., Kelley, W. J., & Goldstein, D. R. (2020). Role of Aging and the Immune Response to Respiratory Viral Infections: Potential Implications for COVID-19. *The Journal of Immunology*, *205*(2), 313–320. https://doi.org/10.4049/jimmunol.2000380

Cheng, V. C. C., Lau, S. K. P., Woo, P. C. Y., & Yuen, K. Y. (2007). Severe Acute Respiratory Syndrome Coronavirus as an Agent of Emerging and Reemerging Infection. *Clinical Microbiology Reviews*, *20*(4), 660–694. https://doi.org/10.1128/CMR.00023-07

Dandekar, A. A., & Perlman, S. (2005). Immunopathogenesis of coronavirus infections: Implications for SARS. *Nature Reviews Immunology*, *5*(12), Article 12. https://doi.org/10.1038/nri1732

Danta, C. C. (2021). SARS-CoV-2, Hypoxia, and Calcium Signaling: The Consequences and Therapeutic Options. *ACS Pharmacology & Translational Science*, *4*(1), 400–402. https://doi.org/10.1021/acsptsci.0c00219

Defamie, V., Cursio, R., Brigand, K. L., Moreilhon, C., Saint-Paul, M.-C., Laurens, M., Crenesse, D., Cardinaud, B., Auberger, P., Gugenheim, J., Barbry, P., & Mari, B. (2008). Gene Expression Profiling of Human Liver Transplants Identifies an Early Transcriptional Signature Associated with Initial Poor Graft Function. *American Journal of Transplantation*, *8*(6), 1221–1236. https://doi.org/10.1111/j.1600-6143.2008.02249.x

Ebihara, T., Azuma, M., Oshiumi, H., Kasamatsu, J., Iwabuchi, K., Matsumoto, K., Saito, H., Taniguchi, T., Matsumoto, M., & Seya, T. (2010a). Identification of a polyI:C-inducible membrane protein that participates in dendritic cell–mediated natural killer cell activation. *Journal of Experimental Medicine*, *207*(12), 2675–2687. https://doi.org/10.1084/jem.20091573

Ebihara, T., Azuma, M., Oshiumi, H., Kasamatsu, J., Iwabuchi, K., Matsumoto, K., Saito, H., Taniguchi, T., Matsumoto, M., & Seya, T. (2010b). Identification of a polyI:C-inducible membrane protein that participates in dendritic cell–mediated natural killer cell activation. *Journal of Experimental Medicine*, *207*(12), 2675–2687. https://doi.org/10.1084/jem.20091573

Ghahramani, S., Tabrizi, R., Lankarani, K. B., Kashani, S. M. A., Rezaei, S., Zeidi, N., Akbari, M., Heydari, S. T., Akbari, H., Nowrouzi-Sohrabi, P., & Ahmadizar, F. (2020). Laboratory features of severe vs.

non-severe COVID-19 patients in Asian populations: A systematic review and meta-analysis. *European Journal of Medical Research*, *25*(1), 30. https://doi.org/10.1186/s40001-020-00432-3

Hajnóczky, G., Robb-Gaspers, L. D., Seitz, M. B., & Thomas, A. P. (1995). Decoding of cytosolic calcium oscillations in the mitochondria. *Cell*, *82*(3), 415–424. https://doi.org/10.1016/0092-8674(95)90430-1

Henkens, M. T. H. M., Raafs, A. G., Verdonschot, J. A. J., Linschoten, M., van Smeden, M., Wang, P., van der Hooft, B. H. M., Tieleman, R., Janssen, M. L. F., ter Bekke, R. M. A., Hazebroek, M. R., van der Horst, I. C. C., Asselbergs, F. W., Magdelijns, F. J. H., Heymans, S. R. B., Al-Ali, A. K., Al-Muhanna, F. A., Al-Windy, N. Y. Y., Almubarak, Y. A., … on behalf of the CAPACITY-COVID collaborative consortium. (2022). Age is the main determinant of COVID-19 related in-hospital mortality with minimal impact of pre-existing comorbidities, a retrospective cohort study. *BMC Geriatrics*, *22*(1), 184. https://doi.org/10.1186/s12877-021-02673-1

Hou, H., Zhang, B., Huang, H., Luo, Y., Wu, S., Tang, G., Liu, W., Mao, L., Mao, L., Wang, F., & Sun, Z. (2020). Using IL-2R/lymphocytes for predicting the clinical progression of patients with COVID-19. *Clinical and Experimental Immunology*, *201*(1), 76–84. https://doi.org/10.1111/cei.13450

Hu, L., Chen, L., Yang, G., Li, L., Sun, H., Chang, Y., Tu, Q., Wu, M., & Wang, H. (2011). HBx Sensitizes Cells to Oxidative Stress-induced Apoptosis by Accelerating the Loss of Mcl-1 Protein via Caspase-3 Cascade. *Molecular Cancer*, *10*(1), 43. https://doi.org/10.1186/1476-4598-10-43

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X., Cheng, Z., Yu, T., Xia, J., Wei, Y., Wu, W., Xie, X., Yin, W., Li, H., Liu, M., … Cao, B. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *The Lancet*, *395*(10223), 497–506. https://doi.org/10.1016/S0140-6736(20)30183-5

Jabeen, K., Malik, U., Mansoor, S., Shahzad, S., Zahid, S., & Javed, A. (2021). Effect of oxidative stress and calcium deregulation on FAM26F (CALHM6) expression during hepatitis B virus infection. *BMC Infectious Diseases*, *21*(1), 228. https://doi.org/10.1186/s12879-021-05888-0

Javed, A., Leuchte, N., Salinas, G., Opitz, L., Stahl-Hennig, C., Sopper, S., & Sauermann, U. (2016). Pre-infection transcript levels of FAM26F in peripheral blood mononuclear cells inform about overall plasma viral load in acute and post-acute phase after simian immunodeficiency virus infection. *The Journal of General Virology*, *97*(12), 3400–3412. https://doi.org/10.1099/jgv.0.000632

Jing, Q.-L., Liu, M.-J., Zhang, Z.-B., Fang, L.-Q., Yuan, J., Zhang, A.-R., Dean, N. E., Luo, L., Ma, M.-M., Longini, I., Kenah, E., Lu, Y., Ma, Y., Jalali, N., Yang, Z.-C., & Yang, Y. (2020). Household secondary attack rate of COVID-19 and associated determinants in Guangzhou, China: A retrospective cohort study. *The Lancet Infectious Diseases*, *20*(10), 1141–1150. https://doi.org/10.1016/S1473-3099(20)30471-0

Jyoti, B., & Devi, P. (2013). Detection of human immunodeficiency virus using oral mucosal transudate by rapid test. *Indian Journal of Sexually Transmitted Diseases and AIDS*, *34*(2), 95. https://doi.org/10.4103/0253-7184.120539

Kasamatsu, J., Azuma, M., Oshiumi, H., Morioka, Y., Okabe, M., Ebihara, T., Matsumoto, M., & Seya, T. (2014). INAM Plays a Critical Role in IFN-γ Production by NK Cells Interacting with Polyinosinic-Polycytidylic Acid–Stimulated Accessory Cells. *The Journal of Immunology*, *193*(10), 5199–5207. https://doi.org/10.4049/jimmunol.1400924

Kelada, M., Anto, A., Dave, K., & Saleh, S. N. (n.d.). The Role of Sex in the Risk of Mortality From COVID-19 Amongst Adult Patients: A Systematic Review. *Cureus*, *12*(8), e10114. https://doi.org/10.7759/cureus.10114

Khan, M., Syed, G. H., Kim, S.-J., & Siddiqui, A. (2016). Hepatitis B Virus-Induced Parkin-Dependent Recruitment of Linear Ubiquitin Assembly Complex (LUBAC) to Mitochondria and Attenuation of

Innate Immunity. *PLOS Pathogens*, *12*(6), e1005693. https://doi.org/10.1371/journal.ppat.1005693

Ksiazek, T. G., Erdman, D., Goldsmith, C. S., Zaki, S. R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J. A., Lim, W., Rollin, P. E., Dowell, S. F., Ling, A.-E., Humphrey, C. D., Shieh, W.-J., Guarner, J., Paddock, C. D., Rota, P., Fields, B., … Anderson, L. J. (2003). A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome. *New England Journal of Medicine*, *348*(20), 1953–1966. https://doi.org/10.1056/NEJMoa030781

Lagunas-Rangel, F. A. (2020). Neutrophil-to-lymphocyte ratio and lymphocyte-to-C-reactive protein ratio in patients with severe coronavirus disease 2019 (COVID-19): A meta-analysis. *Journal of Medical Virology*, *92*(10), 1733–1734. https://doi.org/10.1002/jmv.25819

Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., Zhang, Q., Shi, X., Wang, Q., Zhang, L., & Wang, X. (2020). Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature*, *581*(7807), Article 7807. https://doi.org/10.1038/s41586-020-2180-5

Lingappan, K., Karmouty-Quintana, H., Davies, J., Akkanti, B., & Harting, M. T. (2020). Understanding the age divide in COVID-19: Why are children overwhelmingly spared? *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *319*(1), L39–L44. https://doi.org/10.1152/ajplung.00183.2020

Lucas, C., Wong, P., Klein, J., Castro, T. B. R., Silva, J., Sundaram, M., Ellingson, M. K., Mao, T., Oh, J. E., Israelow, B., Takahashi, T., Tokuyama, M., Lu, P., Venkataraman, A., Park, A., Mohanty, S., Wang, H., Wyllie, A. L., Vogels, C. B. F., … Iwasaki, A. (2020). Longitudinal analyses reveal immunological misfiring in severe COVID-19. *Nature*, *584*(7821), Article 7821. https://doi.org/10.1038/s41586-020-2588-y

Malik, U., Javed, A., Ali, A., & Asghar, K. (2017). Structural and functional annotation of human FAM26F: A multifaceted protein having a critical role in the immune system. *Gene*, *597*, 66–75. https://doi.org/10.1016/j.gene.2016.10.029

Malik, U., Zafar, S., Younas, N., Zerr, I., & Javed, A. (2020). Unveiling the Physical and Functional Niches of FAM26F by Analyzing Its Subcellular Localization and Novel Interacting Partners. *ACS Omega*, *5*(35), 22008–22020. https://doi.org/10.1021/acsomega.0c01249

Matlib, M. A., Zhou, Z., Knight, S., Ahmed, S., Choi, K. M., Krause-Bauer, J., Phillips, R., Altschuld, R., Katsube, Y., Sperelakis, N., & Bers, D. M. (1998). Oxygen-bridged Dinuclear Ruthenium Amine Complex Specifically Inhibits Ca2+ Uptake into Mitochondria in Vitroand in Situ in Single Cardiac Myocytes *. *Journal of Biological Chemistry*, *273*(17), 10223–10231. https://doi.org/10.1074/jbc.273.17.10223

Mehta, P., McAuley, D. F., Brown, M., Sanchez, E., Tattersall, R. S., & Manson, J. J. (2020). COVID-19: Consider cytokine storm syndromes and immunosuppression. *The Lancet*, *395*(10229), 1033–1034. https://doi.org/10.1016/S0140-6736(20)30628-0

Montazersaheb, S., Hosseiniyan Khatibi, S. M., Hejazi, M. S., Tarhriz, V., Farjami, A., Ghasemian Sorbeni, F., Farahzadi, R., & Ghasemnejad, T. (2022). COVID-19 infection: An overview on cytokine storm and related interventions. *Virology Journal*, *19*(1), 92. https://doi.org/10.1186/s12985-022-01814-1

Nikolich-Zugich, J., Knox, K. S., Rios, C. T., Natt, B., Bhattacharya, D., & Fain, M. J. (2020). SARS-CoV-2 and COVID-19 in older adults: What we may expect regarding pathogenesis, immune responses, and outcomes. *GeroScience*, *42*(2), 505–514. https://doi.org/10.1007/s11357-020-00186-0

Nunes, P., Demaurex, N., & Dinauer, M. C. (2013). Regulation of the NADPH Oxidase and Associated Ion Fluxes During Phagocytosis. *Traffic*, *14*(11), 1118–1131. https://doi.org/10.1111/tra.12115

Parekh, A. B. (2003). The Wellcome Prize Lecture. *The Journal of Physiology*, *547*(2), 333–348. https://doi.org/10.1113/jphysiol.2002.034140

Potapov, V., Sobolev, V., Edelman, M., Kister, A., & Gelfand, I. (2004). Protein–Protein Recognition: Juxtaposition of Domain and Interface Cores in Immunoglobulins and Other Sandwich-like Proteins. *Journal of Molecular Biology*, *342*(2), 665–679. https://doi.org/10.1016/j.jmb.2004.06.072

Ren, J.-H., Chen, X., Zhou, L., Tao, N.-N., Zhou, H.-Z., Liu, B., Li, W.-Y., Huang, A.-L., & Chen, J. (2016). Protective Role of Sirtuin3 (SIRT3) in Oxidative Stress Mediated by Hepatitis B Virus X Protein Expression. *PLOS ONE*, *11*(3), e0150961. https://doi.org/10.1371/journal.pone.0150961

Risitano, A. M., Mastellos, D. C., Huber-Lang, M., Yancopoulou, D., Garlanda, C., Ciceri, F., & Lambris, J. D. (2020). Complement as a target in COVID-19? *Nature Reviews Immunology*, *20*(6), Article 6. https://doi.org/10.1038/s41577-020-0320-7

Samprathi, M., & Jayashree, M. (2021). Biomarkers in COVID-19: An Up-To-Date Review. *Frontiers in Pediatrics*, *8*. https://www.frontiersin.org/articles/10.3389/fped.2020.607647

Serebrovska, Z. O., Chong, E. Y., Serebrovska, T. V., Tumanovska, L. V., & Xi, L. (2020). Hypoxia, HIF-1α, and COVID-19: From pathogenic factors to potential therapeutic targets. *Acta Pharmacologica Sinica*, *41*(12), Article 12. https://doi.org/10.1038/s41401-020-00554-8

Shang, J., Ye, G., Shi, K., Wan, Y., Luo, C., Aihara, H., Geng, Q., Auerbach, A., & Li, F. (2020). Structural basis of receptor recognition by SARS-CoV-2. *Nature*, *581*(7807), Article 7807. https://doi.org/10.1038/s41586-020-2179-y

Soucy-Faulkner, A., Mukawera, E., Fink, K., Martel, A., Jouan, L., Nzengue, Y., Lamarre, D., Velde, C. V., & Grandvaux, N. (2010). Requirement of NOX2 and Reactive Oxygen Species for Efficient RIG-I-Mediated Antiviral Response through Regulation of MAVS Expression. *PLOS Pathogens*, *6*(6), e1000930. https://doi.org/10.1371/journal.ppat.1000930

Terpos, E., Ntanasis-Stathopoulos, I., Elalamy, I., Kastritis, E., Sergentanis, T. N., Politou, M., Psaltopoulou, T., Gerotziafas, G., & Dimopoulos, M. A. (2020). Hematological findings and complications of COVID-19. *American Journal of Hematology*, *95*(7), 834–847. https://doi.org/10.1002/ajh.25829

Tian, S., Hu, W., Niu, L., Liu, H., Xu, H., & Xiao, S.-Y. (2020). Pulmonary Pathology of Early-Phase 2019 Novel Coronavirus (COVID-19) Pneumonia in Two Patients With Lung Cancer. *Journal of Thoracic Oncology*, *15*(5), 700–704. https://doi.org/10.1016/j.jtho.2020.02.010

Veenith, T., Martin, H., Le Breuilly, M., Whitehouse, T., Gao-Smith, F., Duggal, N., Lord, J. M., Mian, R., Sarphie, D., & Moss, P. (2022). High generation of reactive oxygen species from neutrophils in patients with severe COVID-19. *Scientific Reports*, *12*(1), Article 1. https://doi.org/10.1038/s41598-022-13825-7

Vollbracht, C., & Kraft, K. (2022). Oxidative Stress and Hyper-Inflammation as Major Drivers of Severe COVID-19 and Long COVID: Implications for the Benefit of High-Dose Intravenous Vitamin C. *Frontiers in Pharmacology*, *13*, 899198. https://doi.org/10.3389/fphar.2022.899198

Wan, Y., Cao, W., Han, T., Ren, S., Feng, J., Chen, T., Wang, J., Broering, R., Lu, M., & Zhu, Y. (2017). Inducible Rubicon facilitates viral replication by antagonizing interferon production. *Cellular & Molecular Immunology*, *14*(7), Article 7. https://doi.org/10.1038/cmi.2017.1

Waris, G., Huh, K.-W., & Siddiqui, A. (2001). Mitochondrially Associated Hepatitis B Virus X Protein Constitutively Activates Transcription Factors STAT-3 and NF-κB via Oxidative Stress. *Molecular and Cellular Biology*, *21*(22), 7721–7730. https://doi.org/10.1128/MCB.21.22.7721-7730.2001

Wu, J. T., Leung, K., Bushman, M., Kishore, N., Niehus, R., de Salazar, P. M., Cowling, B. J., Lipsitch, M., & Leung, G. M. (2020). Estimating clinical severity of COVID-19 from the transmission dynamics in Wuhan, China. *Nature Medicine*, *26*(4), Article 4. https://doi.org/10.1038/s41591-020-0822-7

Xu, Z., Shi, L., Wang, Y., Zhang, J., Huang, L., Zhang, C., Liu, S., Zhao, P., Liu, H., Zhu, L., Tai, Y., Bai, C., Gao, T., Song, J., Xia, P., Dong, J., Zhao, J., & Wang, F.-S. (2020). Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *The Lancet Respiratory Medicine*, *8*(4), 420–422. https://doi.org/10.1016/S2213-2600(20)30076-X

Yang, B., & Bouchard, M. J. (2012). The Hepatitis B Virus X Protein Elevates Cytosolic Calcium Signals by Modulating Mitochondrial Calcium Uptake. *Journal of Virology*, *86*(1), 313–327. https://doi.org/10.1128/JVI.06442-11

Zhang, S., Kim, C. C., Batra, S., McKerrow, J. H., & Loke, P. (2010). Delineation of Diverse Macrophage Activation Programs in Response to Intracellular Parasites and Cytokines. *PLOS Neglected Tropical Diseases*, *4*(3), e648. https://doi.org/10.1371/journal.pntd.0000648

Zhao, J., Zhao, J., Legge, K., & Perlman, S. (2011). Age-related increases in PGD_2 expression impair respiratory DC migration, resulting in diminished T cell responses upon respiratory virus infection in mice. *The Journal of Clinical Investigation*, *121*(12), 4921–4930. https://doi.org/10.1172/JCI59777

Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B., Gu, X., Guan, L., Wei, Y., Li, H., Wu, X., Xu, J., Tu, S., Zhang, Y., Chen, H., & Cao, B. (2020). Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. *The Lancet*, *395*(10229), 1054–1062. https://doi.org/10.1016/S0140-6736(20)30566-3

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