Next Generation Sequencing of SARS-CoV-2 Spike Gene from COVID-19 Patients with Characterization of Some Laboratory Parameters

A Thesis

Submitted to the Council of the Erbil Technical Health and Medical College at Erbil Polytechnic University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Laboratory Technology

By

Asmaa Ameen Ghareeb

B.Sc. Medical Laboratory Technology

Supervised by:

Assistant Professor Dr. Sazan Moffaq Abdulaziz

Erbil, Kurdistan

April 2023 Shawal 1444 Gulan 2723

DECLARATION

I declare that the Master of Science (M.Sc.) thesis entitled: **"Next generation Sequencing of SARS-CoV-2 Spike Gene From COVID-19 Patients with Characterization of Some Laboratory Parameters"** is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Signature:

Student Name: Asmaa Ameen Ghareeb

Date:

SUPERVISOR CERTIFICATE

This thesis has been written under my supervision and has been submitted for the award of the degree of Master of Science in Medical Laboratory Technology with my approval as supervisor.

Assistant Professor

Dr. Sazan Moffaq Abdulaziz

Signature Name

Date

I confirm that all requirements have been fulfilled.

Signature:

Name: Assistant Professor Dr. Najat Jabbar Ahmed

Head of the Department of Medical Laboratory Technology

Date**:**

I confirm that all requirements have been fulfilled.

Postgraduate Office

Signature:

Name: Assistant Professor Ahmed Ab. Jalal Ab. Jabbar

Date:

Examining Committee Certification

We certify that we have read this thesis: **Next Generation Sequencing of SARS-CoV-2 Spike Gene From COVID-19 Patients with Characterization of Some Laboratory Parameters** and as an examining committee examined the student (Asmaa Ameen Ghareeb) in its content and what related to it. We approve that it meets the standards of a thesis for the degree of Master of Science in Medical Laboratory Technology.

Signature
Name: Dr. Salah Tofik Jalal Balaky
Assistant Professor
Member
Date: /June/2023

Signature
Name: Dr. Najat Jabbar Ahmed
Assistant Professor
Member
Date: /June/2023

Signature
Name: Dr. Sazan Moffaq Abdulaziz
Assistant Professor
Supervisor/Member
Date: /June/2023

Signature
Name: Dr. Mahmoud Y.M. Taha
Professor
Chairman
Date: /June/2023

Signature
Name: Dr. Jawdat Jaafar Khattab
Professor
Dean of the College of Erbil Health and Medical Technical
Date: /June/2023

Acknowledgments

I would like to take this opportunity to first and foremost thank ALLAH. Who gave me strength to perform my project successfully. I would like to express my heartfelt thanks and appreciation to my supervisors Assistant Professor Dr. Sazan Moffaq, the more supportive and considerate supervisor I could not have asked for.

Special thanks to my great husband Mr. Saman al Salihi for all his support and patience, thank you for being a constant source of support and encouragement in achieving my goals, and my wonderful family, you are the real force behind my success. I would also like to thank Dr. Jangi salihi; thank you for your helpful advice and support.

I want to thank Erbil Polytechnic University and the members of the Department of MLT specially Assist Prof. Dr. Najat Jabbar for his help and for letting me work in their labs and research center while I did my research. I would like to express my sincere gratitude to Special thanks to all member of Erbil COVID-19 project especially Mr. Brwa Husamaldeen Noori for their helping in sample collections and diagnosis, Dr. Dlshad Abdullah Hasan, Mrs. sharmeen for helping me in several conditions during the work. My special thanks with bouquet to Dr. Ismail Mustafa Maulood for helping in analyzing data. Also, my thanks are directed to Dr. Rozhgar and Mr. Mohammad Omar for their helping in re-check the alignments of molecular sequencing data.

Finally, I would like to express my respect and heart full thanks to all my dear friends and classmates for their heartening and support.

SUMMARY

Coronavirus disease-2019 (COVID-19) created a worldwide health problem in late 2019. It was caused by Sever Acute Respiratory Syndrome 2 (SARS-CoV-2), an enveloped RNA virus. The clinical presentation of the disease was found to be variable ranging from mild, moderate to severe. A number of comorbidities such as obesity, diabetes mellitus, organ disorders and age were reported to be associated with morbidity and mortality rates.

The aim of this study was to investigate the association of some laboratory parameters with SARS-CoV-2 infection in Erbil City/Iraq, and to study the circulating Variant of concerns (VOCs) among the infected population through Next Generation Sequencing (NGS) and analysis of the Spike (S) gene.

Throat and nasopharyngeal swabs and blood specimens were collected from suspected cases visited the Central laboratory or admitted to the three COVID-19 specific hospitals in Erbil City/Iraq. The infection was confirmed in 104 patients following RNA extraction and identification by real-time RT-PCR. Then, patients were clinically categorized into mild (n=40), moderate (n=32) and severe (n=32). Blood specimens were also collected from 34 healthy controls. All necessary clinical and demographic information were recorded. Hematological parameters such as lymphocyte count and % and platelet count and other biomarkers (CRP and D-dimer) were measured. Finally, RNA extracts from 15 mild and severe cases were sent to Ankara/Turkey for full S gene sequencing using NGS technique.

Age was significantly associated with COVID-19 (P value= 0.000), in which sever infections were common in extreme ages. No relation was found between ABO, Rh and gender with COVID-19 (P value= 0.41, 0.47 and 0.96

respectively). Death rate was high among sever patients (17(53.1%) as a consequence of multiple comorbidities. Oxygen saturation (SpO2) depressed more significantly in severe and moderate groups than in mild groups. Severe and moderate groups exhibit significantly higher CRP, D-dimer, and lymphocyte% ($P<0.05$) compared to control group. All the studied biomarkers were significantly higher in non-survivors than in survivors ($P<0.001$). There was a highly substantial positive correlation between D-dimer and CRP ($r= 0.69$, P value= 0.000), while a significant negative correlation was observed with other laboratory biomarkers.

The NGS and analysis of the S gene identified two SARS-CoV-2 variants; 13 Delta (B.1.617.2) and 2 Omicron (B.1.1.529). Variants were identified according to the WHO specification of each VOC. On the whole, different mutation classes have been observed including nonsynonymous that constituted the most abundant type of mutation, synonymous, non-frameshift deletions and non-frameshift insertion. L452R, T478K and P681R amino acid variations in spike protein were detected in all Delta isolates and were variant specific. On the other hand, Omicron variants appear with unusual number of mutations (35 mutations). D614G variation was conserved in both variants.

Gender, ABO, and Rh were not associated with COVID-19, but age and a number of comorbidities were significantly associated with disease severity. All studied laboratory biomarkers were associated with mortality. Delta variants showed variations in S gene mutation, whereas both Omicron variants were totally the same. No specific mutations were found to be associated with severity and mortality of COVID-19.

List of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviations	Meaning
%	Percent
+ssRNA	Positive single strain ribo nucleic acid
6-HB	six-helical bundle
ACE2	angiotensin-converting enzyme 2
ARDS	Acute respiratory distress syndrome
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index.
CBC	Complete blood count
CD4+	Cluster differentiation4+
CD8+	Cluster differentiation8+
cDNA	Complementary DNA
CH	Central helix
COPD	Chronic obstructive pulmonary disease
COVID-19	Corona virus disease 2019
CoVs	Coronavirus
CRP	C-reactive protein
CSG	Coronaviridae Study Group
CT	C-Terminal domain
CT	Cytoplasmic tail
CT scan	computed tomography scanning
Ct value	Threshold Cycle number value
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
E	Envelope
EDTA	Ethylene-Diamine-Tetra acetic Acid
FP	Fusion peptide

HCF	Health Care Facilities
SARS-CoV-2	Human corona virus-19
HEPA	High-efficiency particular air
HR1 and HR2	heptad-repeat regions 1, 2
ICU	Intensive care units.
IFN	Interferon
IFN-I	Type I interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-6	Interleukin-6
IQR	Interquartile range
kb	Kilo bite
kDa	Kilodalton
M	Membrane
MERS-CoV	Middle-East Respiratory Syndrome Coronavirus
Mpro	main-protease
mRNA	Messenger ribo nucleic acid
N	Nucleocapsid
NAb	Neutralizing antibody
NaCl	Sodium chloride
NCBI	National Center for Biotechnology information.
NETs	Neutrophil traps
ng/µl	Nanogram/Microliter
NGS	Next Generation sequencing
NK	Natural killer cell
nm	Nanometer
NOD-like receptors	Nucleotide-binding oligomerization dmain -like receptors.

Nsps	nonstructural proteins
NTD	N-Terminal domain
ORFs	Open reading frames
PAMPs	pathogen-associated molecular patterns
paO2/FiO2	Partial pressure of arterial oxygen to fraction of inspired oxygen ratio.
PCR	Polymerase chain reactions
pDC	plasmacytoid dendritic cells
PLpro	papain-like protease
pp	polyprotein
PPE	personal protective equipment
RBD	Receptor-binding domain
RBM	receptor-binding motif
Rh factor	Rhesus factor
RIG-I-like receptors	Retinoic acid-inducible gene I-like receptors
RNA	Ribonucleic acid
rRT-PCR	real-time reverse transcription polymerase chain reaction
S gene	Spike gene
S protein	Spike protein
S1 and S2	Spike subunits 1, 2
SARS-CoV-1	Sever Acute Respiratory Syndrome Coronavirus-1
SARS-CoV2	Sever Acute Respiratory syndrome-2
SD	Standard deviation
SD1 and SD2	Subdomains 1, 2
SEM	standard error of mean.
SNV	Single nucleotide variations
SpO2	Oxygen saturation

SPSS	Statistical Package for Social Sciences
TB	Tuberculosis
TM	Transmembrane domain
TMPRSS2	Transmembrane serine protease2
TMPRSS4	Transmembrane serine protease 4
TNF-α	Tumor necrosis factor-Alpha
TRIS	Tris(hydroxymethyl)-aminomethane.
U.S.A.	United state of America.
UNIPRO	Unified Protocol
VIC	Virus internal control
VOCs	Variants of concern
VOIs	Variants of interest
VUM	Variant under monitoring
WHO	World Health Organization
β-CoVs	Betacoronaviruses
µl	Microliter
$\chi 2$	Pearson Chi-square test

CHAPTER ONE
INTRODUCTION

INTRODUCTION

Coronavirus disease 2019 (COVID-19), that arose from Sever Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), caused a global health crisis in Wuhan-China late in the year 2019 (Guan et al., 2020, Wu and McGoogan, 2020). SARS-CoV-2 belongs to the family of coronaviruses. This renders vulnerable a number of viruses, like SARS-CoV-1, MERS-CoV, and SARS-CoV-2 (Saberiyan et al., 2022).

The clinical presentation of the disease has found to be variable ranging from mild, moderate, sever and critical (Hozhabri et al., 2020). Initial COVID-19 symptoms were often mild, and in some cases the infection went asymptomatic. Acute respiratory distress syndrome (ARDS) and other multi-organ problems developed rapidly, as a result of the rapid viral replication and cytokine storm (Chen et al., 2020). The significant mortality rate associated with COVID-19 was previously attributed to a cytokine storm, an inflammatory immunological response that results in organ failure (Wang and Ma, 2008 a, Ciceri et al., 2020). In severe cases, high levels of interleukin-6 have been reported (Hu et al., 2021), which was undoubtedly induced the liver to release CRP (Sproston and Ashworth, 2018). Inflammatory proteins CRP, IL-6, D-dimer, and ferritin were shown to have higher concentrations in patients who passed away compared to those who survived (Ruan et al., 2020). Decreased lymphocyte and platelet counts have also been associated with severity (Palladino, 2021).

The clinical symptoms reported in SARS-CoV-2 infection were fever, cough, asthenia, dyspnea, sore throat, headache, erythromelalgia, and diarrhea (Young et al., 2020, Wang et al., 2020a).

Common demographic factors associated with disease susceptibility and severity were age, gender, ABO blood groups, Rh factor and family history (Gérard et al., 2020, Zaidi et al., 2020). In addition, a number of comorbidities have been well documented in this issue such as obesity, diabetes mellitus (DM), organ disorders, pregnancy and smoking (Park, 2020, Bermejo-Martin et al., 2020, Zhou et al., 2020).

The World Health Organization (WHO) declared in late 2020 the virus variants and attracted worldwide attention. Since then, many genetic variants of SARS-CoV-2 have been detected. The variants of concern (VOCs) have received global attention and studied extensively. These included the following: Omicron (B.1.1.529), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), Alpha (B.1.1.7) and their subtypes (Alizon et al., 2021, Brown et al., 2021). Although these variants had the same origin, but they were different in pathogenesis, severity of disease and mortality rate (Guan et al., 2020).

Virus spike protein S is a trimeric homologous protein situated on the surface of the virus encoded by the S gene and facilitates viral attachment to host cell receptors (Benton et al., 2020). Variation in number, class, and position of nucleotides in S gene has been reported and generated different VOC mentioned above. These mutations have been studied extensively and some of which has been related to replication rate, infectivity and transmission, affinity to receptors, unusual activation of the immune system, escape from neutralizing antibodies and even vaccive effectiveness (Domingo and Perales, 2019).

As a result of the unstability of the virus genome (similar to other RNA viruses), periodic sequencing of the virus genome, especilaly the S gene, is

necessary to understand disease pathogenesis (Thomson et al.,2020). One of the recently applied methods of sequencing in this field is Next Generation sequencing (NGS). This method has created a significant paradigm shift in the clinical diagnostic sector by allowing for the simultaneous occurrence of several sequencing responses. The NGS technique made it possible to sequence an entire genome down to the base pair level with little time, effort, and error (Collins et al., 2004).

The main aim of the study was to investigate the association of some laboratory parameters in hospitalized and non-hospitalized patients with SARS-CoV-2 infection in Erbil City/Iraq, and to study the circulating VOCs among the infected population through NGS and analysis of the S gene.

Objectives of the study

1. To study the exact SARS-CoV-2 variants circulating among Erbil population during the study period.
2. To investigate the association of a number of laboratory markes with disease severity and mortility rate.
3. To detect the number, classes and rate of mutations in the S gene using NGS technique.

CHAPTER TWO
LITERATURE REVEIW

LITERATURE REVIEW

2. 1. Historical Background

Coronaviruses belong to the subfamily Coronavirinae, which is part of the family Coronaviridae, four genera make up the subfamily Coronavirnae: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus* (Brian et al., 2005).

In November 2002, in southern China Sever Acute Respiratory Syndrome Coronavirus (SARS-CoV-1) first appeared in Guangdong, across 8000 cases and approximately 774 fatalities were recorded in 37 countries between 2002 and 2003 (Chan-Yeung et al., 2003; Peiris et al., 2004). Middle-East Respiratory Syndrome Coronavirus (MERS-CoV), an emergent coronavirus strain that causes severe respiratory disease in humans, was first identified in Saudi Arabia in late 2012 (Zaki et al., 2012). MERS-CoV was responsible for 2494 patients and 858 mortalities (Lee et al., 2017).

In the mid-December-2019 a new virus emerged and caused infection in Hubei capital of Wuhan/ China (WHO, 2020). Investigators and researchers started to identify the virus's genesis by studying the genetic sequence of the virus as a result on 12 January 2020, the genetic sequence of the virus publicly become available in the GenBank (accession no. MN908947.2). This virus was later given the designation Sever Acute Respiratory syndrome (SARS-CoV-2) by the International Committee on Virus Taxonomy because of its resemblance in their symptoms and genomic organization to the SARS virus, later the name changed to human corona virus 19 (SARS-CoV-2). Reported cases from mid-December 2019 to January 2020 all indicated that the virus

has an animal origin, whose origins can be traced back to China's Huanan Bulk Seafood Market, where different types of seafood are sold (Zhu et al., 2020).

All of the available arguments suggested that the genetic sequence of SARS-CoV-2 resembled the coronavirus that isolated from the bat population especially the bat from the Rhinolophus genus (bat-SLCoVZC45 and bat-SL-CoVZXC21) (Wu et al., 2020, Wang et al., 2020b). Genetic analysis of the SARS-CoV-2 represented genetic resemble 50% with MERS-CoV and 80% with SARS-CoV-1 (Lu et al., 2020).

2. 2. Taxonomy

The coronaviruses belong to the family Coronaviridae and are split into 4 genera based on their genomic features, namely *alpha-coronavirus*, *betacoronavirus*, *gammacoronavirus*, and *deltacoronavirus.* Alpha and betacoronaviruses mostly infect mammals, gamma infects avians, and deltacoronavirus infects both avian and mammal hosts (Monchatre-Leroy et al., 2017).

SARS-CoV-2 belongs to beta coronaviruses that resulted in an epidemic widespread death due to causing severe acute respiratory syndrome (SARS-CoV-2, formerly) as well as mild infections. (Tang et al., 2022). The following is the taxonomy of SARS-CoV-2 according to Coronaviridae Study Group (CSG) of the International Committee on Taxonomy of viruses (CSG, 2020).

Realm: Riboviria
Kingdom: Orthornavirae
Phylum: Pisuviricota

Class: Pisoniviricetes

Order: Nidovirales

Family: Coronaviridae

subfamily: Coronavirinae

Genus: Betacoronavirus

Subgenus: Sarbecovirus

Species: Severe acute respiratory syndrome-related coronavirus

Strain: Severe acute respiratory syndrome coronavirus

2. 3. Structure and Genome Organization

SARS-CoV-2 is an enveloped RNA virus. The genome is non-segmented single-stranded positive-sense RNA (+ss RNA) about (27-32 kb) in size that is considered as the largest RNA virus (Akira et al., 2006). The genome encodes four structural proteins; spike (S), nucleocapsid (N), membrane (M), envelope (E), and 16 nonstructural proteins (nsp1-16) (Nasab et al., 2020).

Spike glycoprotein (S) mediates virus's entrance into host cells. Homotrimers are generated by the transmembrane spike glycoproteins and projected from the surface of the virus. The S is crucial for the entrance of the virus to the cells so it is a target of antiviral agents. S protein contains 2 subunits, the S1 and S2. The S1 subunit is made up of the single sequence, N-terminal domain (NTD), subdomains 1&2 (SD1&SD2) and the receptor binding domain (RBD). S1 subunits bind to the host cell receptor (Walls et al., 2020). The S2 subunit, on the other hand, is made up of a number of different parts, such as a fusion peptide (FP), S2' protease cleavage site (S2'), two

repeating heptad regions (HR1 and HR2), a central helix (CH), a transmembrane domain (TM), a connector domain (CD), and a cytoplasmic tail (CT). S2 facilitates host cell and viral membrane fusion, which is its primary function. Protease cleavage occurs between the S1 and S2 subunits; the host protease clave S cleaves at the S2 location, activating the protein necessary for fusion of host cell and viral membranes (Walls et al., 2020; Wrapp et al., 2020). Coronaviruses different types use specific domains in the S1 subunit to recognize different receptors for entry to host cells. In SARS-CoV-1 and hCoV-2, they recognize angiotensin-converting enzyme 2 (ACE2) on host cells by the RBD to enter host cells (figures 2.1 and 2.2) (Walls et al., 2020).

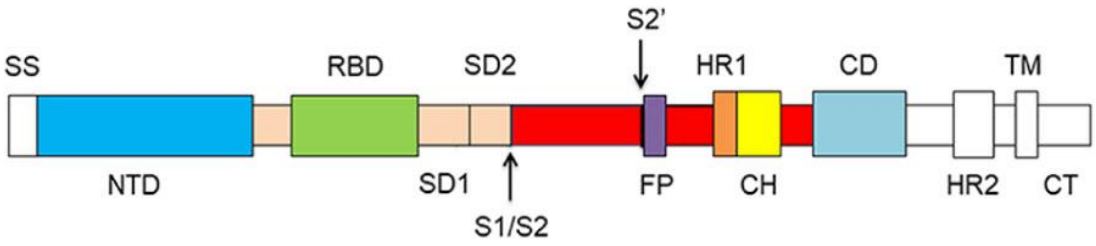

Figure 2. 1: An illustration depicts the structure of the SARS-CoV-2 S protein. The protease cleavage sites are marked by arrows (Walls et al., 2020).

Both HR1 and HR2, collectively known as the hCoV-2 "fusion core region," comprise the six-helical bundle (6-HB), which is crucial for membrane fusion and is dominated by S of hCoV-2, and hence is an attractive medical target (Xia et al., 2020). HR1 is situated at the C-terminus of a hydrophobic FP, whereas HR2 is situated at the N-terminus of the TM domain

(Robson et al., 2020). The difference between the 6-HB of SARS-CoV-1 and hCoV-2 can stabilize the 6-HB conformation of hCoV-2 and facilitate the interaction between HR1 and HR2, leading to an increase in hCoV-2's infectiousness (Xia et al., 2020).

The Receptor Binding Domain (RBD), A part in S of hCoV-2 that recognizes specifically ACE2 receptor. RBD is an important target for Abs and antiviral drugs (Letko et al., 2020). hCoV-2 RBD contains 2 stractural domains:the external subdomain and the core, the core consists of 5 beta sheets and receptor-binding motif (RBM) located between them, RBM contains most binding sites for SARS-CoV-2 and ACE2, the surface of RBM is slightly concave inward to make room for ACE2(Wang et al., 2020c, Lan et al., 2020).

Envelope protein (E) is the most tiny structural protein and has a significant impact on pathogenesis , virus assembly, and release (Nieto-Torres et al., 2014). Membrane protein (M) is in close proximity to other viral structural proteins, such as the nucleocapsid, facilitating viral particle molecular assembly and maybe taking involvement in pathogenesis (Neuman et al., 2011). Nucleocapsid protein (N) is one of the main structural proteins of the virus and is involved in the transcription and replication of viral RNA as well as the packaging of the encapsulated genome into virions (Hurst et al., 2009). The length of the SARS-CoV-2 genomic sequences ranges from 29.8 kb to 29.9 kb, with 12 ORFs encoding 27 proteins (Chan et al., 2020, Wu et al., 2020a).

Open reading frame (ORF) 6 gene is a feature shared by all sarboviruses, including SARS-CoV-2 and SARS-CoV and no orthologues have been found in other Betacoronaviruses, such as MERS-CoV. The protein encoded by the

SARS-CoV-2. The 61 amino acids of the ORF6 gene is located in the endoplasmic reticulum and membrane of vesicles such as autophagosomes and lysosomes (Lee et al., 2021). ORF 8 gene in SARS-CoV-2 is poorly conserved among coronaviruses over 366 nucleotides in length, demonstrating structural plasticity and high variety, both of which have been shown to be significant in pathogenesis (Pereira et al., 2020). ORF9 is an ancillary protein synthesized from an alternative reading frame in the N gene; it suppresses the activation of interferon (IFN) regulatory factors and nuclear factor kappa light chain enhancer of activated B cells (NF-Kβ) (Shi et al., 2014). The function of ORF10, which is uniquely expressed by SARS-CoV-2, remains unclear. Some studies demonstrated that overexpression of ORF10 inhibited the expression of type I interferon (IFN-I) genes and IFN-stimulated genes in a significant manner (Figure 2.2) (Li et al., 2022).

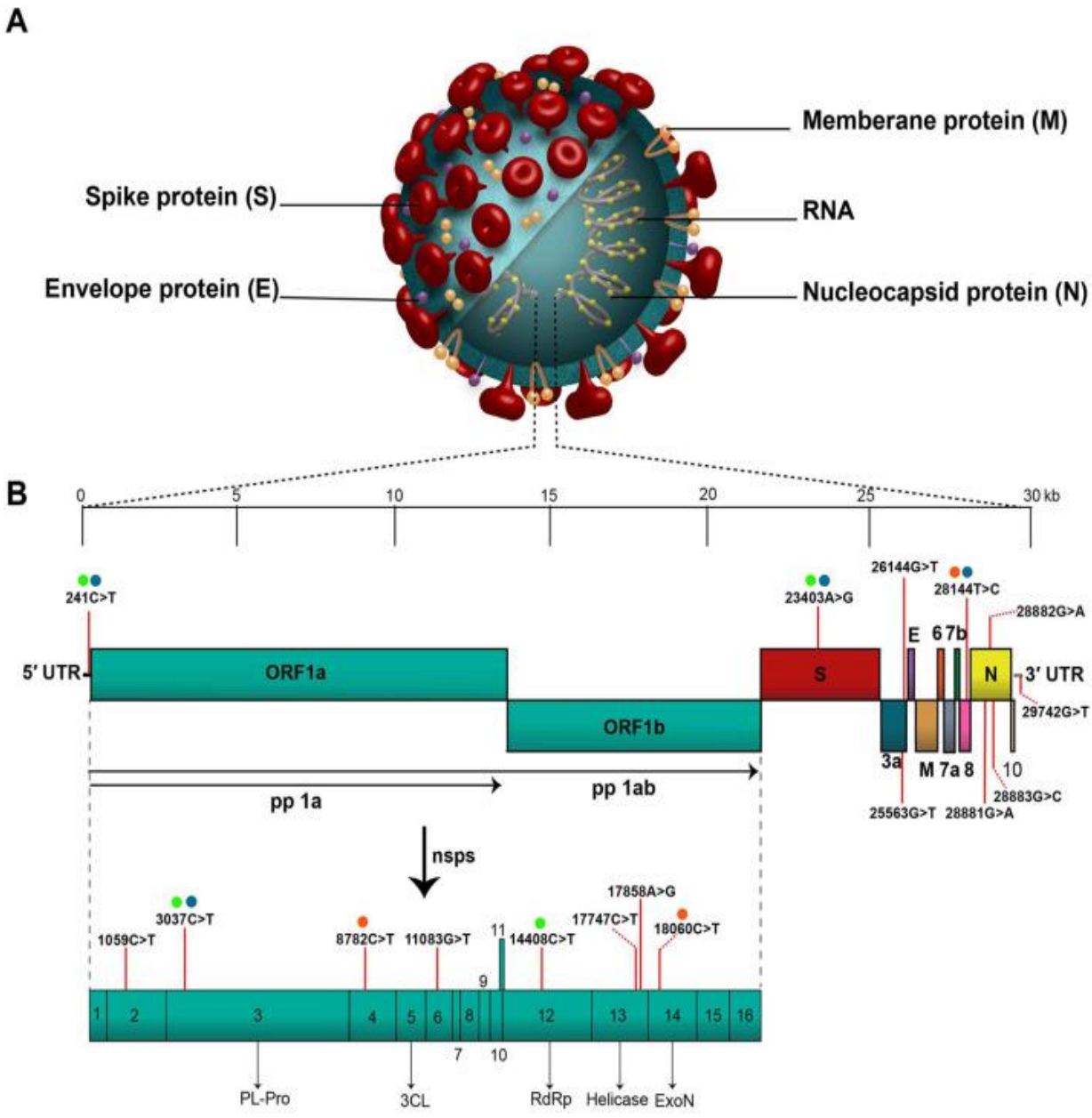

Figure 2. 2: SARS-CoV-2's structure and genomic organization are presented schematically (Rahimi et al., 2021).

2. 4. Replication Cycle

SARS-CoV-2 enters the host cells by the S protein. Viral cell entrance is mediated by a large number of glycosylated S proteins that coat the virus and bind to the host cell receptor ACE2 (Letko et al., 2020). Proteins from the host cell, such as the transmembrane serine protease2 TMPRSS2, facilitate the SARS-CoV-2 invasion processes (Majumder and Minko, 2021).

The transmembrane domain (TM), which is anchored in the viral envelope, is thought to interact with the furin protein (FP), which is partitioned in the host membrane to aid in the creation of the fusion pore (Reuven et al., 2012).

TMPRRS2, ACE2, and FPs are mostly defined as key proteins in the life cycle and severity of the virus. When the virus enters the host cell, TMPRRS2 cleaves the S glycoprotein at S1/S2 subunit, then the S1 directly binds to the ACE2 and/or FPs on the surface most of the host cell specifically type II pneumocytes in the lung, then the S2 undergoes a conformational change and mediates viral fusion (Yan et al., 2020). Immediately after releasing of the viral nucleocapsid, +ssRNA works as functional mRNA with respect to ORF1a and ORF1b encoding polyprotein pp1a 440–500 kDa and pp1ab 740–810 kDa, respectively. After that, two viral proteases, papain-like protease (PLpro) and main-protease (Mpro) break down the polyproteins into sixteen nonstructural proteins Nsps (Nsp1-Nsp16) (Zumla et al., 2016). Full-length RNA copies of the genome are created and employed as a template for full-length RNA genomes, with the resultant complex driving RNA creation via replication and transcription (Luk et al., 2019).

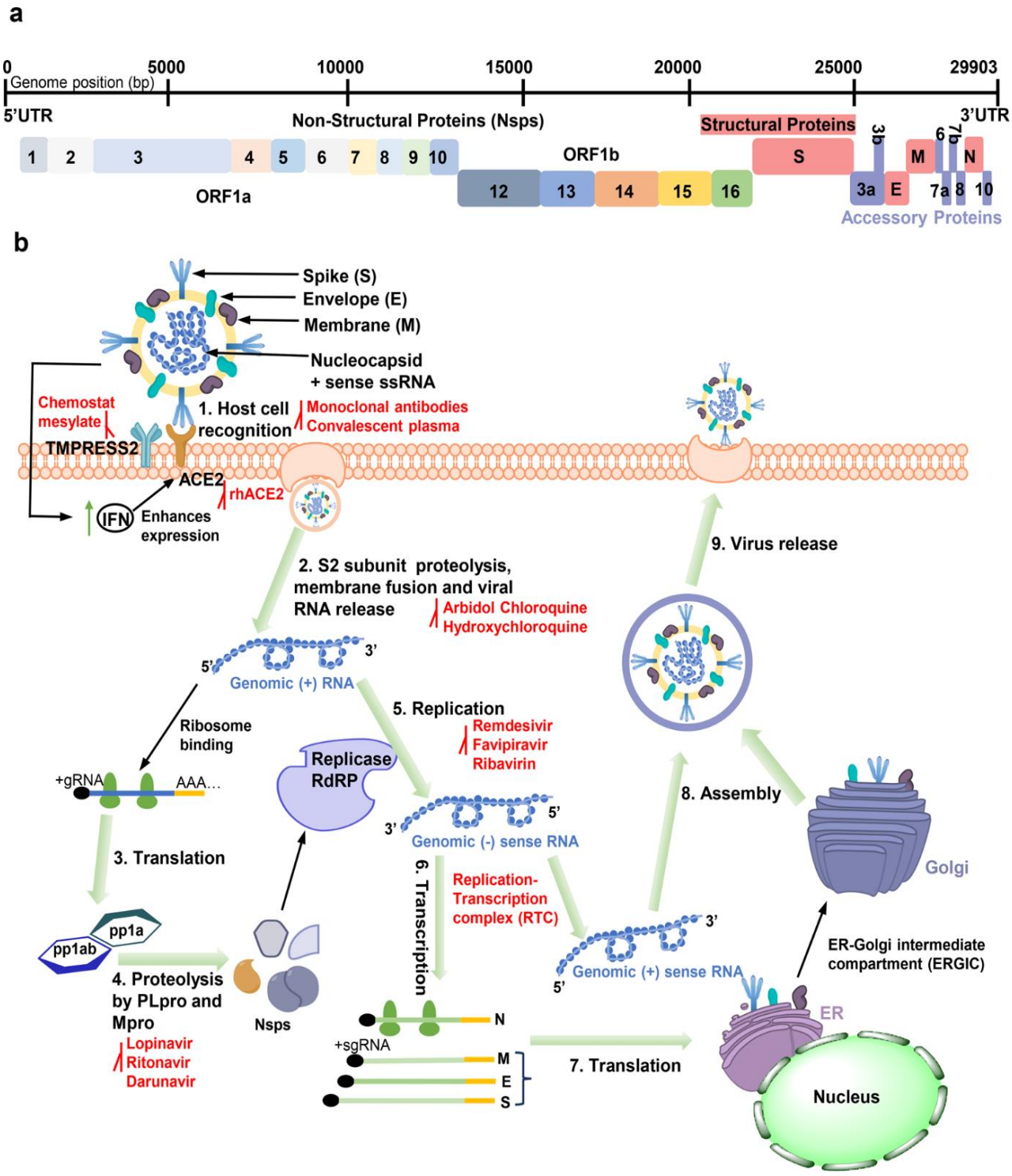

Figure 2. 3: The replication cycle of SARS-CoV-2 and potential targets (Yan et al., 2020).

Four structural proteins, including the spike (S), membrane (M), envelope (E), and nucleocapsid (N), are synthesized by sub genomic RNAs using the host's transcription and translation machinery. Several accessory proteins, including ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10, are also synthesized (Wu et al., 2020b; Michel et al., 2020). After molecular assembly, progeny virions are transported in vesicles with a smooth inner wall, and by the use of a secretory route are transported to the plasma membrane, where they are then expelled through exocytosis and transported to other areas of the body. (Astuti and Ysrafil, 2020; Naqvi et al., 2020). (Figure 2.3) explain the replication cycle of SARS-CoV-2.

2. 5. Symptoms and Clinical Types

The period of the disease following virus acquisition 1 to 14 days. This poses a major challenge, People infected with SARS-CoV-2 may exhibit light to moderate flu-like symptoms, with fever, diarrhea, coughing, and shortness of breath being the most typical ones. Severe cases that are reported in individual with high-risk include systemic inflammation and respiratory failure. This may result in multi-organ damage (Durmaz et al., 2020). Less common symptoms include sputum production, headache, hemoptysis, diarrhea, anorexia, sore throat, chest pain, chills and nausea, and vomiting in studies of patients in China (Huang et al., 2020a).

Dyspnea and pneumonia often manifested themselves within a median of 8 days following the beginning of sickness, after an incubation period of 1-14 days (typically around 5 days) (Wu et al., 2020b). By the end of the first week, severe instances of the illness may manifest as dyspnea and/or hypoxia. Acute

respiratory distress syndrome, coagulation problems, septic shock, and multi-organ failure are all potentially fatal complications of this illness (Guan et al., 2020). Middle-aged and older patients with chronic conditions such cirrhosis, tumor surgery, high blood pressure, diabetes, coronary heart disease, and Parkinson's disease have accounted for the majority of fatal cases (Adhikari et al., 2020).

Clinically, COVID-19 patients are classified in to 4 main categories; mild, moderate, sever and critical. However, in some references it is classified in to mild, moderate and sever, as critical cases are basically severe cases but need critical care and admitted in to ICU (Lin et al., 2021).

1- Mild disease: pneumonia-like symptoms, high body temperature, muscle pain, headache, and absence of radiograph features.
2- Moderate disease: defined by a dry cough, shortness of breath and tachypnea, respiratory rate of less than 30 breaths per minute, a resting mean oxygen saturation of less than 93%. Hospitalization depends up on the patient needs but no need to ICU admission.
3- Severe disease: defined by the presence of acute respiratory distress syndrome (ARDS) lead to respiratory failure (a resting mean oxygen saturation of less than 93%), septic shock, and/or multiple organ failure necessitating intensive care unit admission.

2. 6. Pathogenicity

SARS-CoV-2 is spread mostly by the inhalation of infected droplets or aerosols from someone else's coughing or sneezing (Li et al., 2020a). Direct transmission via respiratory droplets is fotified by productive SARS-CoV-2

replication in both the upper and lower respiratory tract, and the increasing number of cases indicating human-to-human transmission among close contacts who are coughing actively, as well as the evidence of productive SARS-CoV-2 replication in both the upper and lower respiratory tract, lend credence to the theory of direct dissemination by respiratory droplet (Wang et al., 2020a). More than half of all h CoV-19 attacks are caused by the silent transmission of illness in the pre-symptomatic and asymptomatic phases (Moghadas et al., 2020). The first cells in human body targeted by SARS-CoV-2 during usual infection are likely to be multiciliated cells in the trachea or nasopharynx, or sustentacular cells in the mucosa of nasal olfactory (Ahn et al., 2021).

An efficient immune response against invading pathogens requires the early activation of innate immunity that non-specifically pose antiviral effect through releasing proinflammatory molecules or chemicals that in turn activates the specific adaptive immune response against invading pathogens, both of which contributing infection clearance and preventing reinfection by the same pathogen (Ricci et al., 2021).

Virus particles can go down from the nose and throat and spread gradually via the tracheobronchial branches to the lower respiratory system if the virus is not stopped by innate or adaptive human responses. However, the lower respiratory tract is a potential entry point for the virus. Infection of the alveoli can result in inflammation and a reduction in gas exchange. It has been demonstrated, both in vivo and in vitro, that hCoV-2 infects alveolar type 2 (AT2) cells first (Huang et al., 2020b, Lamers et al., 2021). AT2 cells generate pulmonary mucus necessary for lubricating the lung, which reduces surface tension in the alveoli during breathing. AT1 cells covers most of the alveolar

surface and aid gas exchange. Moreover, AT2 cells are the progenitors of AT1 cells in the adult human lung (Barkauskas et al., 2013).

It is the recognition of pathogen-associated molecular patterns (PAMPs) by host receptors that initiates the innate immune response. (Carty et al., 2021). The Toll-like receptor, C-type lectin receptor, NOD-like receptor, and RIG-I-like receptor are all examples of pattern recognition receptors. These receptors, when activated, set off a chain reaction that ultimately results in the release of type I and type III IFNs, as well as the formation and activation of inflammasome complexes such as the NOD-like receptor P3 inflammasome, which in turn stimulates the release of IL-1 β and IL-18 (Rodrigues et al.,2021). Dendritic cells (DC) are able to detect the presence of viruses even in the absence of active viral replication and release type I IFNs alongside all nucleated cells during viral infection (Severa et al., 2021).

Patients with both severe and moderate SARS-CoV-2 infections had considerably higher monocyte counts and lower natural killer cell (NK) counts. Moreover, the proportion of NK cells was low in patients with fatal COVID-19, but rose in those who made a full recovery (Carsetti et al., 2020).Patients with moderate and severe illness showed comparable expression profile of inflammatory cytokines up to 10 days after the beginning of disease, suggesting that this is a significant aspect of COVID-19 immunopathogenesis, TNF-α, IL-6, and IL-10 levels invariably declined in patients with moderate disease and instead remained elevated in those with severe COVID-19 (Lucas et al., 2020).

Although CD4+ cells react to virtually all SARS-CoV-2 proteins, this response is particularly prominent against the S, N, and M proteins. A CD4+

T-cell response can be observed 2–4 days after the beginning of symptoms, and a CD8+ T-cell response can be seen in 1 day (Sette and Crotty, 2021). Meanwhile, activation of B lymphocytes occurs resulting in the development of neutralizing antibodies (Robbiani et al., 2020; Rogers et al., 2020). Antibodies of the IgG and IgM subtypes are generated, especially against the S and N proteins as is the case with other acute viral infections (Zhang et al., 2020). Therefore, neutralizing epitopes on the SARS-CoV-2 RBD domain appear to be highly immunogenic and easily identified by antibodies, especially those matching to the ACE2 receptor binding footprint. However, in a significant percentage of eradicated COVID-19 cases, circulating SARS-CoV-2 neutralizing antibody titers are low (Dan et al., 2021; Wajnberg et al., 2020).

When SARS-CoV-2 enters the body, it triggers a Cytokine storm which is described as a condition that happens during severe cases of SARS-CoV-2 infection as a result of the proliferation and hyperactivity of innate and adaptive immune cells (Feng et al., 2020). Acute respiratory distress syndrome (ARDS), multiple organ failure, and death result from the cytokine storm activation in severe cases of SARS-CoV-2 infection, much as they do in SARS-CoV-1 and MERS-CoV infections (Li et al., 2020b). Leukocyte counts is greater, aberrant respiratory detection is seen, and raised plasma pro-inflammatory cytokine levels are detected in SARS-CoV-2-infected patients (Huang et al., 2020a). Acute COVID-19 causes mortality by a cascade of events known as a cytokine storm, which damages the lungs and other organs like the kidney, liver, and heart (Mehta et al., 2020).

2. 7. Risk Factors

2. 7. 1. Age

Increasing evidence suggested that age is a significant risk factor for SARS-CoV-2 disease severity (Zhou et al., 2020). It was revealed that 56%–62% of COVID-19 patients' fatalities were found in people older than 65 years old. (Perrotta et al., 2020; Lim et al., 2021).

In older aged people, immunosenescence and comorbid disorders are more likely to promote viral induced cytokine storm resulting in life-threatening respiratory failure and multisystemic involvement. Furthermore, the evidence of a gradual decrease in the number of cilia and ciliated cells in the airway with aging and upper airway size decreases with increasing age in both men and women and relatively linear increase in nasal cavity volume with increasing age coupled with an age-dependent decrease of nasal resistance might represent determinants for a higher prevalence of COVID in the elderly population (Perrotta et al., 2020).

2. 7. 2. Gender

Similar findings may be made about the SARS-CoV-2 infection, for which men have a higher death risk than women (Jin et al., 2020; Mehra et al., 2020). These findings point to an underlying sensitivity that is sex-dependent (Zhou et al., 2020). Furthermore, during an illness, sex hormones like estrogen can boost person immune system's innate and adaptive defenses while testosterone can dampen them (Bartz et al., 2020).

2. 7. 3. Obesity

People who are overweight (BMI 25 kg/m^2-29.9 kg/m^2) or obese (BMI 30 kg/m^2 or more) pose a significant threat to public health, especially during COVID-19 epidemic (Goyal et al., 2020). More patients admitted to intensive care units were overweight or obese, suggesting a correlation between them (Simonnet et al., 2020).

The main characteristic observed in severe COVID-19 is the crushing triggering of the body's inflammatory responses with dysregulation of innate and adaptive immunity (Samprathi and Jayashree, 2021). The risk of cardiometabolic disorders, illness severity, and hospitalization is raised in obese individuals due to the role of inflammatory, immunological, and coagulopathic processes. In recent years it has been shown that in healthy obese subjects there are increasing levels of pro-inflammatory cytokines such as IL- 6 and TNF alpha and as a result, high levels of acute-phase reactants such as CRP and haptoglobin. (Cohen et al., 2021).

2. 7. 4. Other risk factors

Studies have shown that the probability of a severe course of COVID-19 and fatal consequences were most strongly influenced by the presence of comorbidities. Generally, infection with SARS-CoV-2 resulted in ACE2 down-regulation because the virus binds to ACE2 receptors, which promotes the production of inflammatory cytokines and worsens clinical conditions. (Hirano and Murakami, 2020). Studies showed that in Diabetes and hypertensive renal illness, ACE2 enzyme level is altered, the enzyme that

protects the circulatory system from the impact of angiotensin II (Mizuiri et al., 2008; Reich et al., 2008; Wang et al., 2011), making those two patient groups more susceptible to the SARS-CoV-2 infection consequences (Li et al., 2020c).

Diseases of the cardiovascular system (such as heart disease and high blood pressure), the lungs (such as chronic obstructive pulmonary disease), the liver, the brain, diabetes mellitus, cancer and weakened immune system (whether from an underlying immune deficiency or from taking medications that suppress the immune system, like cortisol, on a regular basis) are at extremely high risk for fatal infections and ICU admission (Ejaz et al., 2020, Wang et al., 2020b, Singh et al., 2021).

2. 8. SARS-CoV-2 variants' classifications and definitions

As they evolve, all viruses accumulate mutations, the vast majority of which are either detrimental to or have no effect on viral fitness. In addition, the viruses gain a selection advantage through amino acid swaps, insertions, and deletions, which in turn makes them more infectious, transmittable, and, ultimately, the dominant stray (Rochman et al., 2021). SARS-CoV-2 was rapidly adapted to its new human. For continued reactions to SARS-CoV-2 variants, the WHO released variants of interest (VOIs) and variants of concern (VOCs) in late 2020 (WHO, 2020).

2. 8. 1. Variants of concern (VOCs)

Variants of Concerns (VOCs) are virus variants with altered pathogen properties that have been shown to negatively affect the epidemiology (especially increased transmissibility), the clinical presentation (especially increased virulence), or the effectiveness of countermeasures, diagnostic detection methods, vaccines or therapeutics (Parums et al., 2021, WHO, 2021). All of the five variants of concern (VOCs), namely Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) were shown to have mutations in both the NTD and the RBD (Aleem et al., 2022).

2. 8. 1. 1. VOC Alpha (B.1 .1. 7)

The S protein mutation D614G was the first to attract international notice when compared to the Wuhan-Hu-1 prototype strain. This mutation was observed to enhance the expression of the viral S protein within cells and increase viral titer through changes to RBD conformational enhancement and ACE2-binding ability during intracellular replication. (Ou et al., 2021). Since March 2020, D614G mutation has been detected in epidemic viral strains worldwide (Khare et al., 2021). Besides the D614G mutation, lineages B.1 and B.1.1 also possess two other mutations, V367F and D364Y, in the S protein region, which could potentially contribute to greater viral transmission. Compared to the Wuhan-Hu-1 strain, the V367F mutation markedly improved the binding capacity to hACE2. B.1.1.7, originating from B.1 and B.1.1, was first identified in the UK and was given the alternate name VOC Alpha or N501Y.V1 after it resurfaced in August 2020. Moreover, besides D614G, there are further variants have been noticed in the S region,

including N501Y, H69 deletion, V70 deletion, Y144 deletion, A570D, P681H, T716I, S982A, and D1118H(Hemachudha et al., 2022). In the second half of 2020. The B.1.1.7 strain fastly became the most common virus strain in Europe (Zhao et al., 2021).

2. 8. 1. 2. VOC Beta (B. 1. 351)

In August 2020, a lineage known as B.1.351 was discovered in South Africa and was later named VOC Beta or N501Y.V2. This lineage is distinguished by various mutations in the S protein, with the primary mutations being K417N, E484K, and N501Y. In South Africa, these mutations are present in over 80% of VOC Beta strains, and they significantly increase immune evasion, particularly through the E484K site. Changes in amino acids at positions D80A, D215G, LLA241-243del, D614G, and A701V are also present in this lineage (Niu et al., 2021). The E484K mutation causes a change in the charge of the amino acid from negative to positive. This mutation does not impact ACE2 binding, but it reduces the neutralization capacity of humoral immunity by more than 10 times for the Wuhan-Hu-1 strain. On the other hand, the K417N mutation increases the ability of the S protein to bind with ACE2. Both K417 and E484 are still important antigenic escape sites for virus-specific antibodies(Wang et al., 2021a, Zhao et al., 2021).

2. 8. 1. 3. VOC Gamma (P. 1)

In January of 2021, researchers in Brazil discovered a new strain of VOC known as P.1 or N501Y.V3 (Faria et al., 2021). The primary sites of mutation for this include L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I, and V1176F in the S protein. Mutations such as L18F, T20N, and D138Y result in a decrease in the effectiveness of

neutralizing antibodies that are produced by the NTD of the S protein (Wang et al., 2021a). The mutations K417T, E484K, and N501Y located in the RBD region have been found to increase the binding ability with ACE2. These same mutations are referred to as sites that are resistant against neutralizing antibodies. One such mutation, E484K, is linked to a greater ability to avoid being neutralized by the host's immune system.(da Silva et al., 2022). In February 2021, the occurrence of VOC Gamma in sequenced cases in South America reached 40%, but the share of Gamma strain globally drastically fell with the emergence of the Delta strain in the summer of 2021 (Boehm et al., 2021).

2. 8. 1. 4. VOC Delta (B. 1. 617. 2)

The initial Delta sequence was posted in October 2020. During the second wave of infection in India, the Delta variant (B.1.617.2) arose. possessing a unique group of mutations (T478K, P681R, and L452R) that enhance its infectiousness and make it impervious to neutralizing antibodies in people who have been infected or vaccinated. On 4th April 2021, the WHO termed it as VOI Delta, which was then redefined as VOC Delta on 11th May 2021 (WHO, 2021). The Delta strain included primary mutation sites comprising T19R, EFR156-158G, L452R, T478K, D614G, P681R, and D950N, located in the S protein(Shen et al., 2021) (Figure 2.4).

The Delta variant was somewhat resistant to neutralizing antibodies due to three specific mutations: L452R, EFR156-158G, and T478K. Additionally, these mutations contributed to increased expression of the S protein on cell membranes and improved binding capability with the ACE2 receptor (Starr et al., 2020).

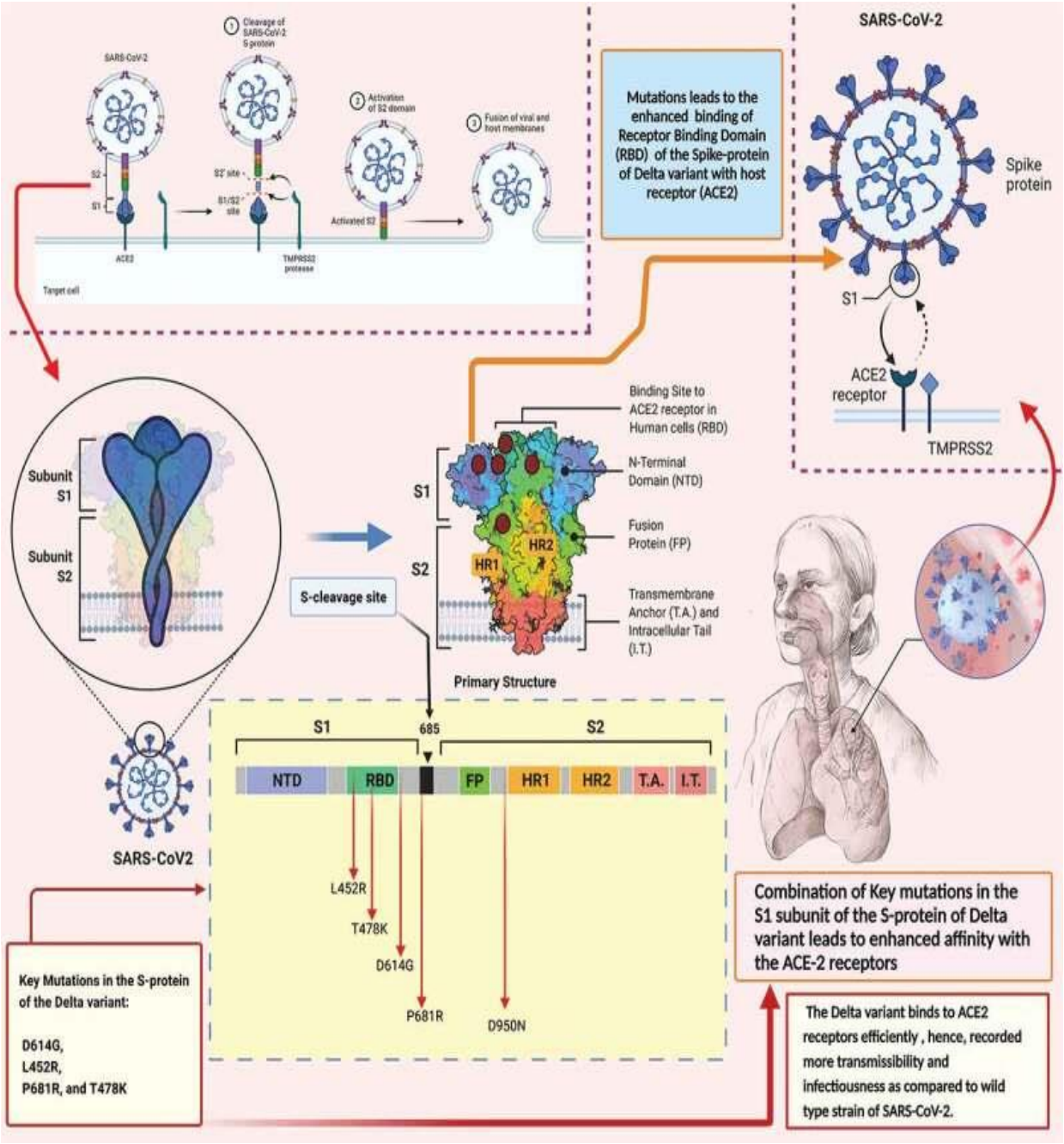

Figure 2. 4: **A diagram illustrating mutations present in the S protein of the Delta variant and their pathogenic effect (Dhawan *et al*.,2022).**

The Delta variant has effectively evolved and now has subgroups or sub lineages like AY.1, AY.2, AY.3, AY.33, AY.34. In England, the AY.1 sub-lineage of the Delta variant has been detected and it had an additional K417N mutation in its spike protein. This variant, also known as the Delta Plus variant, was believed to be the most dangerous due to its potential to escape antibodies. This is because of the K417N mutation which was previously identified in the Beta variant (Arora et al., 2021). The Delta plus strain has also been discovered to have a higher risk of transmission and a strong affinity for lung epithelial cells compared to other VOCs (Chavda and Apostolopoulos, 2022).

2. 8. 1. 5. VOC Omicron (B. 1. 1. 529)

On November 24, 2021, Omicron (B.1.1.529) was categorized as a "variant under monitoring," but the WHO quickly upgraded it to a VOC within two days. This variant had unusual large number of S protein mutation sites than previous VOCs, indicating that it has evolved to adapt to human immunity pressure. Basically, Omicron showed three unique cluster mutation sites; at the RBD (amino acid sites G339D, S371L, S373P, and S375F), receptor-binding motif (amino acid sites Q493R, G496S, Q498R, and Y505H) and (amino acid sites N764K, N856K, Q954H, N969K, and L981F) (Martin et al., 2021). All of the mutation locations on the S protein include the HV69-70del mutation, which is also present in VOC Alpha. At the S1-S2 furin cleavage site, a cluster of mutations (H655Y, N679K, and P681H) are connected to the virus's transmissibility (Gong et al., 2021) (Figure 2.5).

In comparison to the Delta variant, the S protein of the Omicron strain has been discovered to have lower cleavage efficiency and relies heavily on

endocytosis for cell entry. (Meng et al., 2022). Other VOCs have been reported to contain the Omicron A67V and T95I mutations. Omicron was characterized by its excellent ACE2-binding capacity (Cui et al., 2022).

2. 8. 2. Variants of Interest (VOIs)

Variants of Interests (VOIs), also known as circulating variants, refer to virus strains that have minimal impact on transmission, morbidity, and mortality rates. The list of VOI strains was updated frequently, and some strains have been eliminated, including Lota (B.1.526), Eta (B.1.617.1), Epsilon (B.1.427/B.1.429), and Kappa (B.1.617.1), The WHO only kept track of VOI Mu (B.1.621) and VOI Lambda (C.37) as of December 2021 (WHO,2021).

There are multiple reasons why the list of strains that are often updated to VOI may change frequently. One such reason is because the viral genome is constantly evolving in human beings, and these strains are added to the list when they contain certain mutations that are deemed functional. However, it's possible that these strains may only cause a temporary epidemic due to effective non-pharmaceutical interventions, vaccination, and poor adaptability to the environment, or they may be rapidly replaced by more dangerous VOC strains (Dang et al., 2022).

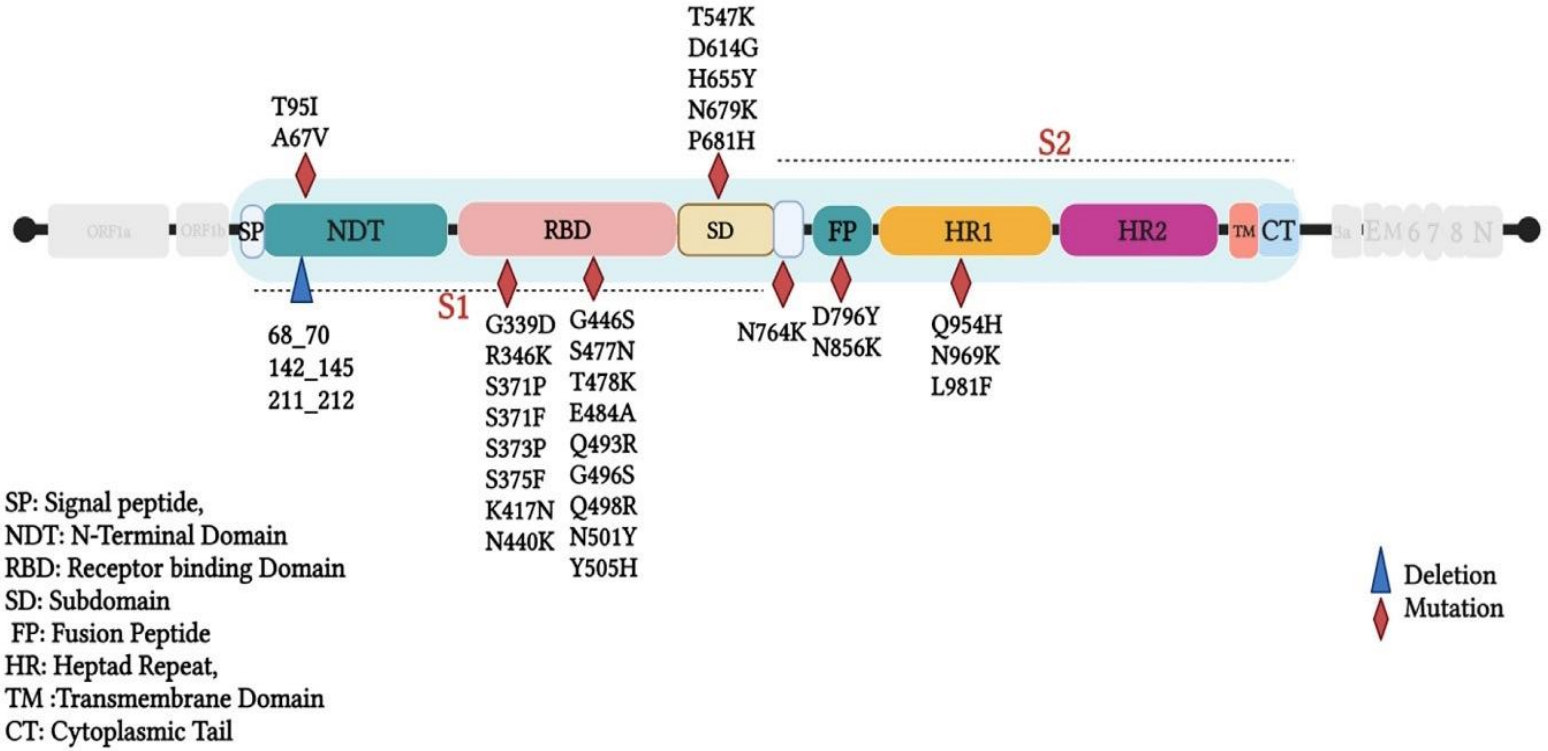

Figure 2.5: An illustration of the Omicron variant's mutations in the S protein (Ahmed et al., 2022).

2. 9. Laboratory Diagnosis

Real-time reverse transcription polymerase chain reaction (rRT-PCR), some hematological and immunological tests, and the immunological approach for detecting viral antigens or antibodies are the three primary methods now utilized to diagnose viral infections (Benzigar et al., 2021).

2. 9. 1. Molecular identification

The molecular technique (RT-qPCR) is employed extensively to identify SARS-CoV-2 RNA in clinical samples of patients suspected to have COVID-19 signs and symptoms (dyspnea, chills, tiredness, myalgia, lymphopenia, dry cough, sneezing, fever). It had high sensitivity and specificity for the quantitative detection of the virus nucleic acid in throat and nasopharyngeal specimens (Corman et al., 2020).

For a whole gene or genome sequencing of SARS-CoV-1, Next Generation Sequencing (NGS) technology is the best standard in the identification of the new variants. NGS gave the chance to sequence all SARS-CoV-2 genes, which included those coding for non-structural proteins and for the intra genes regions (WHO, 2021). In addition, the sequencing of the whole genome, which obtained by NGS technique, was also used to perform phylogeny studies (Berno et al., 2022). In NGS technique, DNA polymerase adds fluorescent nucleotide one by one on to a growing DNA template strand, each incorporated nucleotide is identified by its fluorescent tag. The difference between NGS and sanger sequencing is sequencing volume, NGS sequence millions of fragments simultaneously per run, while the sanger method

sequences only a single fragment of DNA at a time, also NGS offers grater discovery power to detect noval or rare variants with deep sequencing (Berno et al., 2022).

2. 9. 2. Some Laboratory Parameters related to COVID-19 clinical identification.

1. C-reactive protein

At locations of infection or inflammation, C-reactive protein (CRP) levels can increase by a factor of 1,000. In addition to hepatocytes in the liver, smooth muscle cells, macrophages, endothelial cells, lymphocytes, and adipocytes also contribute to the body's CRP production (Sproston and Ashworth, 2018).

Inflammation levels are linked with CRP levels (Rainer et al., 2009). Increases in C-reactive protein levels have been shown to stimulate phagocytosis and the complement system (Gershov et al., 2000). CRP binds to microorganisms and stimulates their phagocytosis clearance (Povoa et al., 2009). Severe patients with COVID-19 who had elevated CRP levels relative to non-sever ones may suggest that CRP might be a biomarker of disease severity and progression (Sadeghi-Haddad-Zavareh et al., 2021).

2. D-dimer

The D-dimer is a blood test that measures a byproduct of the blood coagulation and breakdown process. When a clot in the bloodstream begins to

disintegrate, D-dimer is released (Payus et al., 2019). In the past, D-dimer was employed as a measure of hypercoagulability. Patients with severe COVID-19 illness, especially those in the ICU had considerably higher D-dimer levels (Mishra et al., 2020). Diabetes patients had more serious illnesses and higher D-dimer levels. Chronic hyperglycemia can cause inflammation and endothelial dysfunction, which can result in thrombus development. (Domingueti et al., 2016).

3. Platelets

Blood platelets have vital functions in the circulatory system. Platelets, which originate in the bone marrow as megakaryocytes, circulate throughout the body for around 7 days before being reabsorbed into the bone and are principally responsible for controlling thrombosis and hemostasis (Holinstat, 2017).

Although there are many potential causes for fluctuating platelet counts, in the setting of infectious disorders, a low count may result from impaired platelet generation in the lungs, as has been documented for COVID-19 (Ren et al., 2020; Xu et al., 2020a). Instead, immune system stimulation may cause an antibody-mediated phagocytic response, leading to enhanced platelet clearance (Semple et al., 2007).

4. Lymphocytes

White blood cells called lymphocytes have a uniform appearance but different functions. They comprise T and B lymphocytes as well as natural killer cells. These cells regulate the immune response, eliminate tumor and infected cells directly, and produce antibodies (LaRosa and Orange, 2008).

Some study proved that in hospitalized patients with COVID-19, low lymphocyte counts were independently determined as predictors of mortality. Most of the patients with severe COVID-19 had lymphopenia (Yang et al., 2020a). According to another study, lymphopenia was common in COVID-19 patients in the ICU and the persistence of lymphopenia is a sign of poor prognosis in terms of survival (Wang et al., 2020a). Accumulating data have shown that lymphocytopenia and leukopenia are common in COVID-19 patients (Terpos et al., 2020; Yang et al., 2020b).

Four potential mechanisms leading to lymphocyte deficiency. (1) The virus might directly infect lymphocytes. (2) The virus might directly destroy lymphatic organs. (3) Inflammatory cytokines continued to be disordered, perhaps leading to lymphocyte apoptosis. Basic researches confirmed that tumour necrosis factor (TNF)α, interleukin (IL)-6, and other pro-inflammatory cytokines could induce lymphocyte deficiency. (4) Inhibition of lymphocytes by metabolic molecules produced by metabolic disorders, such as hyperlactic acidemia. The severe type of COVID-19 patients had elevated blood lactic acid levels, which might suppress the proliferation of lymphocytes. Multiple mechanisms mentioned above or beyond might work together to cause lymphopenia, and further research is needed (Tan et al., 2020).

CHAPTER THREE
MATERIALS AND METHODS

MATERIALS AND METHODs

3. 1. Materials

3. 1. 1. Commercial kits

The table below contains a list of every commercial kit utilized in this investigation:

Table 3. 1: **Commercial kits used in this study**

N	Item	Company	Origin
1	Agarose powder	Thermo Fisher Scientific	U. S. A.
2	Blood grouping kit	Spinract	Spain
3	CBC kit	Orphee	Switzerland
4	CRP titer kit	Roche	Germany
5	D-dimer kit	Roche	Germany
6	COVID-19 IgG/IgM Rapid test kit	Inzek B.V.	Netherlands
7	SARS-CoV-2 nucleic acid detection (PCR) kit	Zybio	China
8	IPSOGEN RT kit	QIAGEN	Germany
9	Nextera XT sample preparation kit	Illumina inc.	U. S. A.
10	Nucleic acid extraction kit	Zybio	China
11	NucleoFast 96 PCR clean-up kit	MACHEREY-NAGEL GmbH	Germany

3. 1. 2. Apparatus and Equipments

The table below details the study's apparatus and equipments:

Table 3. 2: Equipments used in this study

N	Item	Company	Origen
1	Antiseptics and cotton		
2	Biochemical analyzer	Hitachi-Cobas e 411	Japan
3	Biochemical analyzer	Hitachi-Cobas c 111	Japan
4	Centrifuge	Hettich	Germany
5	Cool box	Iqloo, Playmate	U.S.A.
6	N95 facemasks, goggles, protective clothes, latex gloves, and waterproof boots are all essential pieces of PPE for combating the COVID-19 virus		
7	Deep freezer (-20 °C)	Thermo Fisher Scientific	U.S.A.
8	Deep freezer (-70˚C).	Gesellschaft Fur Labortechnik (GFL)	Germany
9	Disposable syringes	Suztola Dukangning	China
10	Disposable virus sampling tube	Zybio	China
11	Dry ice	Dry ice company	Erbil, Iraq
12	Eppendorf tube (0.5,1.5and 2 ml)	Eppendorf (Germany)	Germany
13	Gel&clot activator tube	BioZek	Netherlands
14	Hematology analyzer	Mythic 18	Switzerland
15	Ice bags		
16	Ice maker	Scotsman	Italy

17	K3 EDTA tubes	BioZek	Netherlands
18	Laboratory racks, wooden sticks and slides		
19	Laminar flow cabinet	Fisher Scientific	Malaysia
20	Laptop	Lenovo	China
21	Microcentrifuge	Eppendorf	Germany
22	Micropipettes (10-1000, 50-300 and 1-10 μL) with disposable tips.	Mybiosource	U.S.A.
23	MiSeq system	Illumina	U.S.A.
24	Nanodrop 1000 micro volume spectrophotometer	Thermo Fisher Scientific	U.S.A.
25	Nanophotometer P 360	Implen	Germany
26	Nucleic acid isolation system (EXM 3000)	Zybio	China
27	Printer	Espon	Japan
28	Refrigerator	Toshiba. Japan	Japan
29	Roll mixer	Cland &JT	China
30	Rotor-Gene Q	QIAGEN	Germany
31	Sodiun citrate 3.2% tube	BioZek	Netherlands
32	Swabs	Zybio	China

3. 1. 3. Subjects

The study included 104 Covid-19 patients (40 mild, 32 moderate and 32 severe infection) and 34 healthy controls. Necessary information about each participant was recorded (appendix I). Samples were collected only from those who volunteered for the research.

1. Patients

The study included 104 COVID-19 patients visited the Central laboratory or admitted to the three COVID-19 specific hospitals; Al Emirati Hospital, Rozhawa Hospital and Lalav Hospital in Erbil city/Iraq, from 1st of November 2021 to 28th of February 2022.

Inclusion criteria:

- Disease positivity 10-14 days.
- Age groups ≥ 18 years.
- Positive SARS-CoV-2 PCR test.
- Both male and female.

Exclusion criteria:

- Negative SARS-CoV-2 PCR test
- Those with surgical operation, tuberculosis (TB) or other lung diseases.
- Have taken any medication for COVID-19 treatment.

According to the criteria outlined in Section 2.5 of the literature review, patients were divided into three categories: mild, moderate, and severe.

2. Control

Thirty-four healthy individuals have been selected to represent the control group.

Inclusion criteria:

- Age and gender matched to the patient's group.
- Nearly normal BMI.

- Tested negative for anti-COVID-19 IgM and IgG antibodies.

Exclusion criteria:

- History of chronic diseases.

3. 2. Methods

3. 2. 1. Study design, Place and Period

A case-control study that have been done in Erbil city/ Iraq during the 1st of November 2021 to the 1st of July 2022.

3. 2. 2. Body mass index (BMI)

Participants' height and weight were measured using a conventional mechanical scale. Weight was divided by the square of height to get the body mass index (kg/m2). Categorization of BMI was done according to WHO (Schetz et al., 2019) as following:

BMI	**Nutritional status**
< 18.5	Underweight
18.5–24.9	Normal weight
25.0–29.9	Overweight
≥ 30.0	Obese

3. 2. 3. Sample collection and processing

1. Blood

Seven milliliters of blood was taken by vein puncture using a disposable syringe from each subject enrolled in this study (both study and control groups), in which 2 ml were added to tubes containing sodium citrate as anticoagulant to obtain plasma for the determination of d-dimer, another 2 ml were added to EDTA tubes for assessing hematological parameters (CBC) and 3 ml were added to gel tubes and left to stand at room temperature (20-25°C) to allow clotting and centrifuged at 3000 rpm for 15 minute to obtain serum. The obtained serum was isolated and stored at -20°C and used later for the determination of CRP and anti-SARS-CoV-2 IgM and IgG antibodies.

2. Nasal and throat swabs

Description

COVID-19 personal protective equipment (PPE) was used during sample collection (N95 masks, goggles, protective clothing, latex gloves and waterproof boots). Sample collection was done using disposable virus sampling tubes which are used for collection and storage of SARS-CoV-2. The collected samples were sealed in plastic bags (usually one plastic bag per sample) and transported to the laboratory.

Procedure

A-Nasal swab

By gently inserting the swab in to the nasal palate through nasal passage, staying for a while and then slowly rotating 3-5 times, immersing the swab head in the preservation solution and squeezing it several times, forcefully breaking off the tail of the swab and discarding it, then tightening the tube cap.

B-Throat swab

Wiping the swab on both sides of the pharyngeal tonsils and posterior wall of the pharynx, immersing the swab head in the preservation solution and squeezing it several times, forcefully breaking off the tail of the swab and discarding it, then tightening the tube cup. Then the collected samples were immediately transported to the laboratory (within 4-6 hours) for diagnosis. For a long-term storage, the obtained samples were placed at -70˚C. Repeated freezing and thawing was avoided during the long-term storage.

3. 2. 4. Laboratory investigations

3. 2. 4. 1. Hematological parametres

1. **ABO and Rh typing**

Principle

The surface of human blood cells contain specific antigens, the ABO group of a person depends on whether the red blood cells contain one, both,

or neither of the two blood group antigens A and B. Therefore, there are 4 mains ABO groups A, B, AB and O. Rh antigens also are surface antigenes expressed on red blood cells, red cells expressing the Rh antigens are positive Rh, while the blood cells which do not express this surface antigens are Rh negative. The ABO blood group and Rh factor determined by adding anti body against those antigen, then an antigen-antibody complex formation (agglutination) use to detect the blood group and Rh factor positivity.

Procedure

The slide method was used to detect the ABO blood group and Rh factor, which involves mixing a drop of blood taken from EDTA tubes with a drop of antisera on a slide (or a white porcelain support), the slide was devided in to three parts as for each part a drop of blood (from both patients and control group) was mixed with anti-A, anti-B, anti-D seperately. The agglutination or blood clumping pattern which observed was used to determine the ABO and Rh factor posativity.

2. Complete Blood Count (CBC)

Principle

The Complete Blood Count test is the counting of the cells that are present in the blood including red blood cells, white blood cells, platelet count, hemoglobin estimation, as well as hematocrit levels.

Procedure

Platelet count, total WBC count, lymphocyte count and percentage were determined using a fully automated hematology analyzer (Mythic 18/Switzerland) performing hematological analysis on EDTA anticoagulated blood after putting the tubes on a roll mixer for about 5 minutes, the Mythic 18 uses the impedance technique, results were provided within 1 min on the liquid crystal display, printed out on a printer.

3. D-dimer

Principle (catalog number 70640501)

Enhanced immunoturbidimetric test with particles, Uniform-sized latex particles are covered with Fab2 fragments, which are monoclonal antibodies to the D-Dimer epitopes. When D-Dimer-containing samples are added to the test reactant, antigen/antibody complexes are formed, resulting in a rise in turbidity. The rate of absorbance is proportional to the number of D-dimer epitopes present in the sample. The precipitate is determined turbidimetrically.

Kit components (Reagents)

Reagent	Main constituents
R1	TRIS/HCI buffer: 250 mmol/l, pH 8.2, preservatives (liquid).
R2	Latex particles coated with monoclonal anti-human D-Dimer antibodies (mouse): 0.12 %, preservative (liquid).

Procedure

D-dimer is determined by taking 0.5 ml of plasma and putting it in a fully biochemical analyzer (Cobas c 111/Japan) with its special reagents (according to the manufacturer's instructions), then a ready calculated result was obtained.

3. 2. 4. 2. Immunological Parameters

1. C- reactive protein (CRP)

Principle (catalog number 5401615190)

An immunoturbidimetric experiment with particle enhancement was performed. Monoclonal anti-CRP antibodies coated on latex particles agglutinated with human CRP. Aggregate concentrations were measured using turbidimetry.

Kit component (catalog no. 5401615190)

Reagent	Main constituents
R1	TRIS[a]) buffer with bovine serum albumin, preservatives.
SR	Latex particles coated with anti-CRP (mouse) in glycine buffer, immunoglobulins (mouse), preservative.
TRIS=Tris(hydroxymethyl)-aminomethane.	

Procedure

C-reactive protein is determined by taking 0.5 ml of serum and adding it in a fully biochemical analyzer (Cobas c 111/Japan) with its special kit reagents (according to the manufacturer's instructions). a ready calculated result was obtained.

2. SARS-CoV-2 IgG and IgM detection

Principle (catalog number 600092)

The COVID-19 IgG/IgM Rapid Test kit (Whole Blood/Serum/Plasma) is a lateral flow immunochromatographic assay. The test uses anti-human IgM antibody (test line IgM), anti-human IgG (test line IgG) and goat antimouse IgG (control line C) immobilized on a nitrocellulose strip. The burgundy-colored conjugate pad contains colloidal gold conjugated to recombinant COVID-19 antigens conjugated with colloid gold (COVID-19 conjugates) and mouse IgG-gold conjugates. When a specimen followed by assay buffer is added to the sample well, IgM &/or IgG antibodies if present, will bind to COVID-19 conjugates making antigen antibodies complex. This complex migrates through nitrocellulose membrane by capillary action. When the complex meets the line of the corresponding immobilized antibody (anti-human IgM &/or anit-human IgG) the complex is trapped forming a burgundy-colored band which confirm a reactive test result (positive result). Absence of a colored band in the test region indicates a non-reactive test result (negative result). The test contains an internal control (C band) which should exhibit a burgundy-colored band of the immunocomplex goat anti mouse

IgG/mouse IgG-gold conjugate regardless of the color development on any of the test bands.

Procedure

Test cassette, specimen, and/or controls allowed at room temperature (15-30°C) prior to testing to equilibrate the temperature.

For serum and plasma sample took up to the fill line (approximately 10 µl) using the dropper and was transferred to the sample well, two drops of buffer added to the sample well (approximately 80 µl); the timer had started.

For Venipuncture and Finger Prick Whole Blood took in whole blood using the dropper to approximately 1 cm above the fill line then 1 full drop was expelled (approximately 20 µl) into the sample well. Two drops of buffer added to the sample well (approximately 80 µl). Hence, the timer had started.

We waited for colored lines to appear. The results were given in 10 minutes. Positive result was visible as soon as 2 minutes. Any result after 15 minutes was not interpreted.

3. 2. 5. Molecular study

3. 2. 5. 1 Nucleic Acid Extraction (RNA Extraction)

Description:

Nucleic acid extraction from nasopharyngeal and throat swab samples was done in the Central laboratory in Erbil using a specific kit (Zybio/China).

Principle

The magnetic beads in the kit have specific polymeric groups of adsorbed nucleic acid on the surface. The isolation system uses the magnet on the experiment cabin to move the magnetic beads adsorbed with nucleic acid to different reagent wells, then quickly and repeatedly stirring the liquid using the magnetic sleeve to make the magnetic beads and liquid evenly mixed. After lysis, nucleic acid absorption, magnetic beads transfer, wash and elution, high-purity nucleic acid product was finally obtained. The extracted and purified nucleic acid was used for virus detection and Next-generation sequencing.

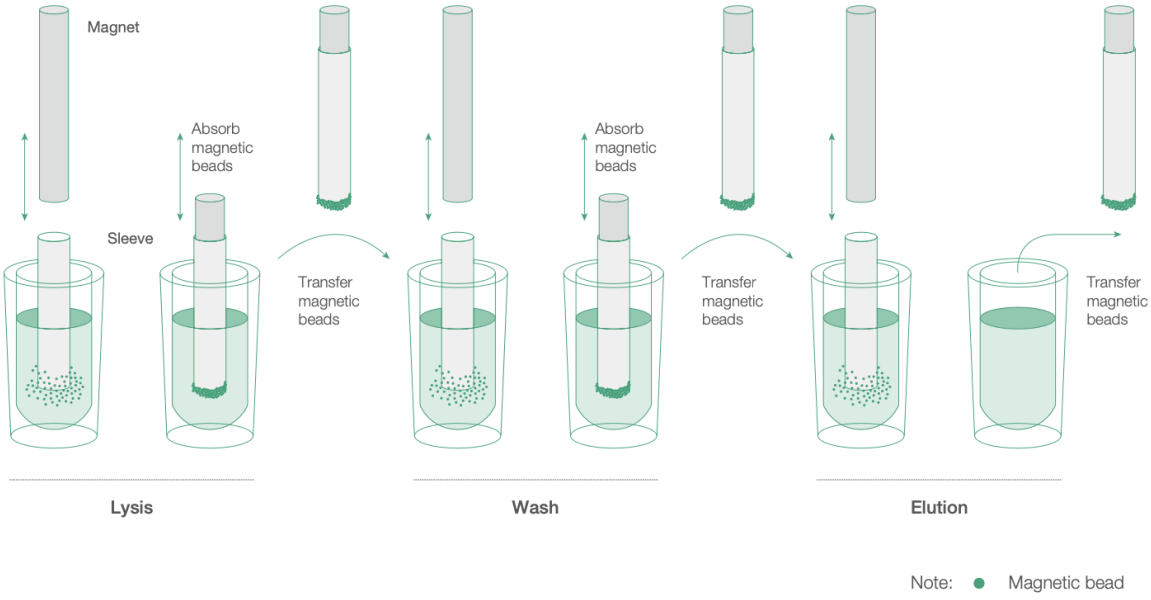

Figure 3. 1: The principle of RNA extraction.

Kit Components

Component	Main constituents
Proteinase K	<5% Proteinase K
Nucleic acid extraction reagent prepackaged in 96-well plates	Extraction reagent I, Extraction reagent II, Elution buffer, Magnetic Beads Solution

Procedure:

1. All of the equipment was warmed up to room temperature. After 3-5 minutes, the 96-well plates were inverted, stirred thoroughly to release the liquid stuck to the aluminum film and well wall, and set aside.

2. The aluminum film then was carefully opened. Fifteen microliters of proteinase K were added to the position A1-H1 and A7-H7 in order, then 200 μL of sample were added in order. The 96-well plates were put in their corresponding position in the instrument and the magmatic rod sleeve was inserted.

3. The system was turned on and the process took about 9 minutes.

4. After centrifuging out a few magnetic beads, the nucleic acid solution from wells A6-H6 and A12-H12 in the inventory was pipetted into a 1.5 mL centrifuge tube and sent on to the next step in the process of NGS.

Figure 3. 2: EXM3000 isolation system.

3. 2. 5. 2 Checking up RNA quality and concentration

After RNA extraction, a Nanophotometer P360 (Implen/Germany) was employed to assess the RNA concentration and purity. Firstly, the instrument was turned on and the Nano volume was chosen. Then, after choosing Nucleic acids option, the RNA caption with lid factor 10 (reference must be given before measuring any sample by using blank, which is a nucleic acid free water) was taken. The blank was used to reset the instrument. Optical density was measured at 260 and 280nm. Generally, a ratio of A260/A280 of approximately 1.8-2 indicates pure RNA. Concentration of RNA was given as ng/µl. According to the above procedure, 40 ng/µl of RNA concentration was acceptable for next molecular processes. After resetting and fixing all factors, two microliters of each RNA sample was applied in the nanodrop system, and the results of our sample concentrations were at least 90 ng/µl.

3. 2. 5. 3 Real-Time Polymerase Chain Reaction (RT-PCR)

Description:

The test was done using SARS-CoV-2 Nucleic acid detection kit (Zybio/China). It can specifically detect the target genes (ORF 1ab and N genes) of the virus. This kit was readily used for SARS-CoV-2 detection in clinical laboratories.

Principle:

The process mainly consisted of two steps; reverse transcription and PCR amplification.

Kit Components:

Components	Main constituents
SARS-CoV-2 PCR reaction solution	Tris-HCL buffer, dNTPs, Mg^{2+}, primers and probes
SARS-CoV-2 enzyme Solution	Reverse transcriptase, Taq DNA polymerase, Uracil N-glycosylase (UNG)
SARS-CoV-2 negative Control.	0.9 % (w/v) NaCl
SARS-CoV-2 positive Control.	Armored virus contained SARS-CoV-2 target fragment sequence.
SARS-CoV-2 internal Control.	Armored virus contained internal control fragment sequence

Procedures

1. Reagent preparation: this step was conducted in reagent preparation zone. SARS-CoV-2 PCR buffer was brought to room temperature, mixed and centrifuged for several seconds. Later, the number (N) of reagents required (N=Number of samples +Number of Negative Control + Number of Positive Control) was calculated as described below. The prepared reaction solution was mixed and centrifuged for several seconds.

Component	Volume (µL) / test
SARS-CoV-2 PCR reaction solution	8
SARS-CoV-2 enzyme solution	2
Total	10

2. Briefly, 10 µL of RNA extract, negative control and positive control were added to each PCR reaction tube with filter tips, and covered, then transferred to the amplification detection zone after transient centrifugation to avoid producing bubbles in the tubes.
3. Amplification: this step was conducted in amplification zone. PCR reaction tube putted into the Rotor-Gene Q (Qiagen/Germany) instrument then the number and sequence of the sample recorded and PCR amplification parameters set according to the following program recommended by the company.

	Reactions	Temperature	Time	Cycle
1	UNG reaction	37 ˚C	1 min	1
2	Revers transcription	50 ˚C	5 min	1
3	Initial denaturation	95 ˚C	2 min	1
4	Denaturation	95 ˚C	5 sec	45
5	Amplification and fluorescence detection	60 ˚C	30 sec	

4. Result Analysis: the results were automatically saved, while target curves and corresponding internal standard curves were analyzed separately. Start value, end value, and threshold value of baseline were adjusted according to the analyzed image (e.g., Start value range 3-15, End value range 5-20). Later, the "Analyze" button was activated to obtain results and make parameters meet the requirement in quality control procedure (as described below), then results in "Plate" window was recorded.

No	Controls	Control Standards
1	Positive control	Target (FAM, ROX) and internal control (VIC): The amplification curve both in typical S shape and Ct<40
2	Negative control	Target (FAM, ROX): Ct=45 or not detected. Internal control (VIC): Ct <40

Result was claimed positive when the Ct of tow targets (FAM, ROX) <40. For accurate results we took Ct value between 15-25.

Results	Criteria
Positive	S-shaped and Ct 40 amplification curves in the FAM and ROX fluorescence channels indicate SARS-CoV-2 positivity. FAM indicate N gene, ROX indicated ORF1 ab test result.
Negative	FAM and ROX fluorescence channels were not detected or Ct=45, and VIC channel Ct<40, suggesting SARS-CoV-2 is negative.
Gray Zone	FAM or ROX fluorescence channel 40 ≤ Ct values <45, and VIC channel Ct values <40 indicated that the result is in gray zone and needed re-testing. If the results were the same and showed typical S-shaped curve, it was judged as positive, otherwise it was negative.
Invalid	Ct value =45 or no value in FAM and ROX fluorescence channels, and Ct values ≥40 or no value in VIC channel, indicated that the result is invalid, and re-testing is needed.

3. 2. 5. 4: Next Generation Sequencing (NGS) of the spike gene.

This part of the study was totally done by Intergen genetic diagnosis and research center in Ankara/Turkey. The following details were sent by the center.

1. **Complementary DNA (cDNA) preparation**: RNA extracts were transformed to cDNA using standard protocols of IPSOGEN RT kit (Qiagen/Germany). The prepared cDNA samples were stored in -20˚C until PCR processing.
2. **Primer design and PCR:** PRIMER - Primer Designer V.2.0 (Scientific & Educational Software) was used to create primers targeting a specific location.
3. **Amplification**: PCR was carried out using specific primers and master mix solution applying the following components:

Component	**For each reaction (µl)**
dH20	15
5x Tampon (Thermo Inc.)	5
dNTP mix, each 10mM	0,5
Forward Primer (5 µM)	1.5
Reverse Primer (5 µM)	1.5
PhireII HS DNA Polymerase (Thermo Inc.)	0.5
cDNA sample	1
Total	25

As for cycling conditions, the initial denaturation was performed at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 45 seconds, gradient annealing at 60°C for 45 seconds, and an extension at 72°C for 45 second, with a final extension at 72°C for 10 minutes.

4. The product obtained following PCR was checked using a 2% agarose gel electrophoresis. Then, products were mixed into one tube for each sample according to their quality obtained from the gel image.
5. Samples were purified using NucleoFast® 96 PCR clean-up kit (MACHEREY-NAGEL GmbH/Germany). Purified PCR pool was quantified by using Nanodrop 1000 micro volume spectrophotometer (Thermo Inc./U.S.A.) and diluted according to Illumina's recommendations prior to sequencing.
6. **NGS and analysis:** Nextera XT sample preparation kit (Illumina Inc./U.S.A.) was used to get the samples ready for NGS analysis. NGS of the samples was carried out using MiSeq (Illumina Inc/ U. S. A.) according to protocols and procedure of the kit. The data which was obtained from MiSeq was in FASTA format. The sequences were aligned to the Wuhan type SARS-CoV-2 genome. The alignment was done on NC_045512.2 with the Burrow -Wheeler aligner.

Following receiving the spike gene sequence of the analyzed samples with the ready aligned results, all the alignments have been re-checked using BLAST in NCBI and the amino acid record using UNIPROT.

3. 2. 6 Statistical analysis

Computerized statistical evaluation was done using Statistical Package for Social Sciences (SPSS) 25 and Graph pad prism 8.

1. Mean, standard deviation, interquartile range, percentage, and other descriptive statistics were utilized to show the data.

2. Appropriate inferential statistics were used to examine the results, and they included:

- Pearson Chi-square test ($\chi 2$) was used to find out the correlation between the demographic criteria and COVID-19 and simple linear regression was used to test the correlation between different lab parameters and hospitalization.
- Paired t-test and One-way ANOVA followed by Tukey's test, as parametric tests, whereas *Mann–Whitney* test and Kruskal-Wallis followed by Dunne's test as non-parametric tests were used to find out the difference between the means of different variables.

The P value < 0.05 was considered statistically significant, less than 0.01 was considered highly significant and greater than 0.05 was considered statistically non-significant.

CHAPTER FOUR
RESULTS

RESULTS

4. 1. Demographics and clinical characteristics among different groups of COVID-19 patients

A total of 104 people were included in the trial (40 with mild illness, 32 with moderate infection, and 32 with severe infection), and 34 healthy people acted as a control group.

The demographics and medical features among different groups of COVID-19 patients are presented in table 4. 1. This study showed that most of COVID-19 patients were located between the age interval of 42-53 year (42%). Young age groups; 18-29 and 30-41 years, mostly experienced mild infections (88.89% and 71.4%, respectively), whereas severe infection was mostly seen among old age groups; 54-65 and ≥ 66 years (54.5% and 50%, respectively). The relation between COVID-19 and age was statistically significant (*P* value= 0.000). The study also showed a non significant relation between COVID-19 and gender (*P* value= 0.96) although males had a higher rate of infection than females (57.7% and 42.3%, respectively), but no specific characterization has been seen regarding the severity of infection.

A statistically significant relation has not been observed between COVID-19 and ABO blood groups and Rh positivity (*P* value= 0.41 and 0.47, respectively). Blood groups A and O had the same distribution among the patients (35.6%) followed by group B (26%) and the least recorded group was AB (2.9%). No specific characterization regarding the severity of infection has been observed among ABO groups. Ninety-three patients were Rh+ and 11 were Rh-. Most of the Rh- patients showed mild infection (54.55%).

Moreover, no significant relation has been observed between COVID-19 and number of family members affected (*P* value= 0.277).

The history of patients with COVID-19 showed that 71 (68.3%) patients had single or multiple comorbidities. Mild patients showed least comorbidities (25%) most of which were single factors such as obesity, type 2 diabetes mellitus (DM), smoking and pregnancy. Complicated, serious and multiple comorbidities were common among hospitalized patients (90.6% of moderate and 100% of severe patients). Patients in the severe group were mostly admitted to the intensive care unit (ICU). Statistical analysis showed a significant relation between COVID-19 infection and disease comorbidities. Details on the type and rate of occurrence of each comorbidity are presented in table 4.1.

A number of signs and symptoms has been related to COVID-19 and expressed and/or recorded relatively differently among different patient groups. Cough, headache, body ache and fever were the most common symptoms recorded in all the three patient groups. Shortness of breath (dyspnoea) was specifically observed in hospitalized patients (47.54% and 52.46% for moderate and severe infections, respectively) and was significantly related to COVID-19 (*P* value 0.000). On the other hand, the loss of sense of smell (anosmia) and sense of taste (ageusia) were tow symptoms specifically described by patients with mild infection both of which were significantly related to COVID-19 (P value= 0.002).

Death rate showed a significant relation with COVID-19 (*P* value 0.000). No death has been recorded among patients with mild infection, but death rate was high among sever patients (17(53.1%)) as a consequence of multiple

comorbidities. The lowest mean±SD of PO_2 was recorded among patients with severe COVID-19 (67.94±13.35) that required the urgent need for artificial oxygen supply. Patients with moderate infection required oxygen supply intermittently as the drop in SpO_2 was not high and continuous (mean±SD: 85.91±6.35).

Table 4. 1: Demographic and clinical characteristics among different groups of COVID-19 patients.

Character	Mild n(%)	Moderate n(%)	Severe n(%)	Total n(%)	Chi² *P* value
Age groups (years)					
18-29	16(88.89)	2(11.11)	0(0)	18 (100)	0.000
30-41	10(71.4)	4(28.6)	0(0)	14(100)	
42-53	10(45.4)	6(27.3)	6(27.3)	22(100)	
54-65	3(13.6)	7(31.9)	12(54.5)	22(100)	
≥66	1(3.6)	13(46.4)	14(50)	28(100)	
Total	40(38.46)	32(30.77)	32(30.77)	104 (100)	
Gender					
Female	17(38.7)	13(29.5)	14(31.8)	44(100)	0.96
Male	23(38.3)	19(31.7)	18(30)	60(100)	
ABO &Rh					
A	14(37.84)	13(35.14)	10(27.02)	37(100)	0.41
AB	2(66.67)	1(33.33)	0(0)	3(100)	
B	9(33.33)	11(40.74)	7(25.93)	27(100)	
O	15(40.54)	7(18.92)	15(40.54)	37(100)	
Rh-	6(54.55)	2(18.2)	3(27.3)	11(100)	0.47
Rh+	34(36.6)	30(32.3)	29(31.2)	93(100)	
Number of Family member affected					
No	13(28.9)	18(40)	14(31.1)	45(100)	0.277
Yes	26(47.3)	13(23.6)	16(29.1)	55(100)	
All	1(25)	1(25)	2(50)	4(100)	

Comorbidities	10 (25)	29 (90.6)	32 (100)	71 (68.3)	0.000
Obesity					
Underweight	4 (66.7)	1(16.7)	1(16.7)	6(100)	
Normal	24(46.2)	14(26.9)	14(26.9)	52(100)	
Overweight	10(32.3)	11(35.5)	10(32.3)	31(100)	0.239
Obese	2(13.11)	6(40)	7(46.7)	15(100)	
Smoking	2(11.8)	6(35.3)	9(52.9)	17(100)	0.028
Diabetes mellitus (DM)	3(12)	8(32)	14(56)	25(100)	0.002
Chronic Kidney Diseases	0(0)	2(13.33)	13(86.67)	15(100)	0.000
Respiratory Problems	0(0)	2(16.67)	10(83.33)	12(100)	0.000
Cerebrovascular diseases	0(0)	1(16.67)	5(83.33)	6(100)	0.014
Cardiovascular disease	1(5.6)	3(16.7)	14(77.8)	18(10)	0.000
Pregnancy	1(33.33)	2(66.67)	0(0)	3(100)	0.322
Cancer	0(0)	2(66.67)	1(33.33)	3(100)	0.288
Thyroid Diseases	0(0)	1(50)	1(50)	2(100)	0.529
Autoimmune diseases	0(0)	1(100)	0(0)	1(100)	0.321
Mental illness	0(0)	1(50)	1(50)	2(100)	0.53
Rheumatoid arthritis	0 (0)	0(0)	1(100)	1(100)	0.32
Liver diseases	0 (0)	0(0)	1(100)	1(100)	0.32
Signs and symptoms					
Cough	27(34.62)	26(33.33)	25(32.05)	78(100)	0.36
Headache	36(52.17)	19(27.5)	14(20.3)	69(100)	0.000
Body ache	24(39.3)	15(24.6)	22(36.1)	61(100)	0.201
Fever	20(35.71)	18(32.14)	18(32.14)	56(100)	0.82
Sneezing	13(61.90)	5(23.81)	3(14.3)	21(100)	0.039
Abdominal pain	1(100)	0(0)	0(0)	1(100)	0.446
Diarrhea	6(37.5)	4(25)	6(37.5)	16(100)	0.78

Vomiting	1(11.11)	4(44.44)	4(44.44)	9(100)	0.21
Dyspnea	0(0)	29(47.54)	32(52.46)	61(100)	0.000
Anosmia	7(100)	0(0)	0(0)	7(100)	0.002
Ageusia	7(100)	0(0)	0(0)	7(100)	0.002
Shivering	4(80)	1(20)	0(0)	5(100)	0.124
Mortality rate	0(0)	2(6.3)	17(53.1)	19(18.3)	0.000
SpO_2 (mean±SD)	97.35±1.14	85.91±6.35	67.94±13.35		0.013

4. 2. Laboratory parameters among COVID-19 patients

The finding of selected laboratory parameters among different patient groups and healthy controls are shown in table 4. 2. There was a significant increase in CRP in moderate and sever patients compared to both control group and patients with mild infection. D-dimer value was elevated among all patient groups compared to the control group. The increase in hospitalized patients was obvious and the statistical analysis showed a significant difference among all four groups. Regarding Platelet count, there was no significant distinction among all the groups despite the low platelet count recorded among severe patients compared to other groups.

Both lymphocyte count and lymphocyte % has been decreased among hospitalized COVID-19 patients compared to control group, in which the difference in lymphocyte count was not statistically significant among all the groups, but the difference in lymphocyte % was significant among hospitalized patients compared to both patients with mild infection and healthy controls.

Table 4. 2: Laboratory parameters among COVID-19 patients

Parameter	Control (34)	Mild (40)	Moderate (32)	Severe (32)
CRP (mg/L)	0.35 [a] (0.27-0.422)	0.168 [a] (0.05-0.37)	4.262 [b] (0.60-10.98)	7.114 [b] (3.83-9.92)
D-dimer (ng/ml)	28.5 [a] (6-37.2)	120 [b] (60-205)	1105 [c] (600-2428)	3193 [c] (1310-5000)
Platelet count (10^9/L)	228.5 [a] (147-260)	256 [a] (215-315)	249.5 [a] (193-324)	172 [a] (128.5-227)
Lymphocyte count (10^9/L)	2.42±0.53 [a]	2.26±.069 [a]	1.17±1.0 [a]	0.91±0.81 [a]
Lymphocyte %	31.41±1.00 [a]	35.5±9.33 [a]	13.65±11.35 [b]	10.52±12.94 [b]

The same letters mean no significant difference.

The different letters mean significant difference at $p<0.05$.

The data are expressed as mean ± SEM for parametric analysis using one way ANOVA followed by Tukey's test, while for non-parametric analysis the data are expressed as median (interquartile range) and analyzed with Kruskal-Wallis followed by Dunne's test.

4. 3. Laboratory parameters and COVID-19 outcome

Table 4. 3 illustrates the measurements of selected laboratory parameters among patients with COVID-19 who survived following infection compared to those who died. Statistical analysis showed a highly to very highly significant difference between the mean measurements of all the laboratory tests among survivors compared to non-survivors (*P* value= 0.001, 0.001,

0.002, 0.009 and 0.000 for CRP, D-dimer, platelet count, lymphocyte count and lymphocyte %, respectively)

Table 4. 3: Laboratory parameters and COVID-19 outcome

Parameter	Outcomes of COVID-19 infection	N	Median (IQR) Mean± SEM	*P value*
CRP (mg/dl)	Survivors	85	0.62 (0.17-5.84)	0.001
	Non-survivors	19	8.10 (4.89-10.28)	
D-dimer (ng/ml)	Survivors	85	483 (123-1272)	0.001
	Non-survivors	19	5000 (1310-5000)	
Platelets count (10^9/L)	Survivors	85	264 (192-306)	0.002
	Non-survivors	19	182 (127-227)	
Lymphocyte count (10^9/L)	Survivors	85	2.15±0.52	0.009
	Non-survivors	19	0.74±0.10	
Lymphocyte %	Survivors	85	24.3±1.73	0.000
	Non-survivors	19	6.19±0.74	

IQR: interquartile range, SEM: standard error of mean.

The data are expressed as mean ± SEM for parametric analysis using un-paired t-test, while for non-parametric analysis the data are expressed as median (interquartile range) and analyzed using *Mann–Whitney* test.
Significant difference at $p<0.05$, Highly significant difference at $p<0.01$, Very highly significant difference at $p<0.001$.

4. 4. Correlation between D-dimer with each of CRP, platelet count, lymphocyte count, and lymphocytes % among hospitalized COVID-19 patients

Table 4. 4 and figures 4. 1, 4. 2, 4.3 and 4. 4 illustrate the correlation between D-dimer with the other four lab parameters. It was found that there is a significant positive correlation between D-dimer and CRP (r= 0.69, *P* value= 0.000). On the other hand, a significant negative correlation was observed between D-dimer and each of platelet count (r= -0.28, *P* value= 0.004), lymphocyte count (r= -0.73, *P* value= 0.000) and lymphocyte % (r= -0.76, *P* value= 0.000).

Table 4. 4: Correlation between D-dimer with each of CRP, Platelet count, Lymphocyte count, and Lymphocytes % among hospitalized COVID-19 Patients

Parameter	D-dimer	
	r	***P value***
CRP (mg/dl)	0.69	0.000
Platelets count (10^9/L)	-0.28	0.004
Lymphocyte count	-0.73	0.000
Lymphocyte %	-0.76	0.000

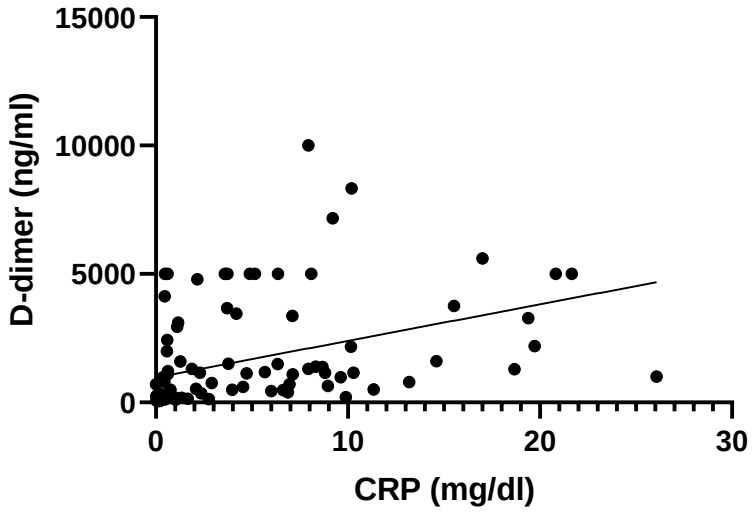

Figure 4. 1: Correlation between D-dimer and CRP.

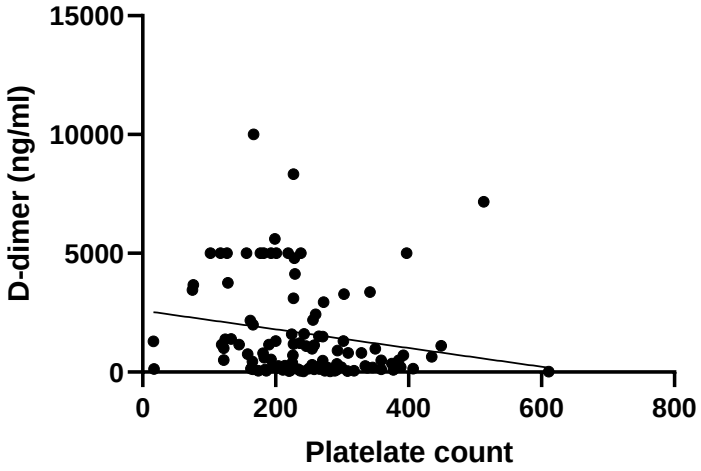

Figure 4. 2: Correlation between D-dimer and platelet count.

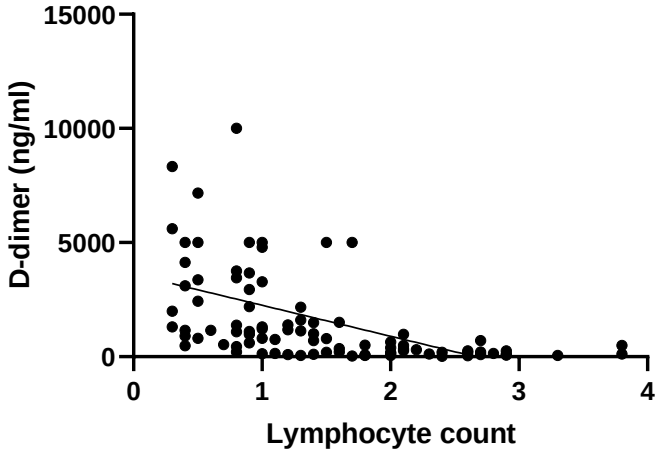

Figure 4. 3: Correlation between D-dimer and lymphocyte count.

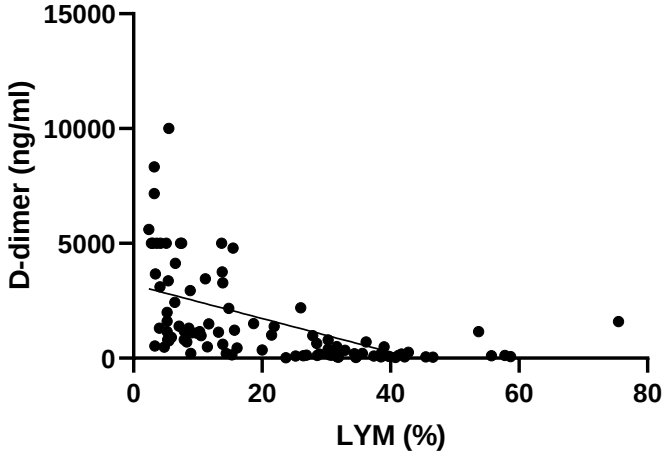

Figure 4. 4: Correlation between D-dimer and lymphocyte %.

4. 5. Molecular study

The molecular technique (RT-qPCR) is employed extensively to identify SARS-CoV-2 RNA in clinical samples of patients. It had high sensitivity and specificity for the quantitative detection of the virus nucleic acid in throat and nasopharyngeal specimens. The Details about the 15 nasopharyngeal and throat swab specimens from mild and sever COVID-19 patients selected from different time intervals subjected to RNA extraction, NGS and analysis of S gene are shown in table 4. 5.

Table 4. 5: VOCs identified following NGS and analysis of SARS-CoV-2 S gene from mild and sever COVID-19 patients.

Sample no.	VOCs	COVID-19 Patient Group	Mortality
1	Delta (B.1.617.2)	Mild	Survived
2	Delta (B.1.617.2)	Mild	Survived
3	Delta (B.1.617.2)	Mild	Survived
4	Delta (B.1.617.2)	Mild	Survived
5	Delta (B.1.617.2)	Mild	Survived
6	Delta (B.1.617.2)	Mild	Survived
7	Delta (B.1.617.2)	Mild	Survived
8	Delta (B.1.617.2)	Mild	Survived
9	Delta (B.1.617.2)	Sever	Survived
10	Delta (B.1.617.2)	Sever	Died
11	Delta (B.1.617.2)	Sever	Died
12	Delta (B.1.617.2)	Sever	Died
13	Delta (B.1.617.2)	Sever	Died
14	Omicron (B.1.1.529)	Sever	Died
15	Omicron (B.1.1.529)	Sever	Died

As the samples were collected from the 1st of November to the 28th of February, it seemed that it was the period of transition from SARS-CoV-2 Delta (B.1.617.2) variant to the Omicron (B.1.1.529) variant. Delta (B.1.617.2) variants were identified from 13 samples and 2 were Omicron (B.1.1.529). Variants were identified according to the WHO classification and definition of each VOC depending on unique mutations specified for each variant.

Following the S gene sequence alignment of the analyzed samples with the Wuhan-Hu-1 strain (wild-type) SARS-CoV-2 using BLAST in NCBI, various mutations were identified, some of which were repeated among the same variant, some were single and specific to one strain and others were conserved in all VOCs (table 4.6). The total nucleotide number of the S gene was found to be 3816 and 3813 for each of Delta and Omicron variants. The total number of mutations detected in Delta variants varied from 10 to 14 mutations (figure 4. 5) The analyzed Delta variant isolated were not similar in the number and type of mutations, except two isolates (no. 10 and 11) which were identical in the number (10 mutations) and type of mutations isolated from two members of the same family both of which have been passed away following admission to ICU. The previous ten mutations identified from the previous two Delta isolates were collectively found in some other Delta isolates. On the other hand, both Omicron variants were totally the same in the number (35 mutations) and type of mutations.

On the whole, Different mutation classes has been observed including nonsynonymous single nucleotide variations (SNV) that constituted the most abundant type of mutation, multiple synonymous SNVs, 4 non-frameshift

deletions one in Delta variants (156_158del) and 3 in Omicron (68-70del, 142-145del and 211-212del), and a single non-frameshift insertion (ins214 EPE) that has been detected in Omicron.

Samples were selected from two clinically different patient groups, mild and sever, in an attempt to find out mutations related to severity and/or mortality, but such relation has not been fixed.

Table 4. 6: Details about Mutations Identified in S gene Following Sequence Alignment of the Analyzed Samples with the Wuhan-Hu-1 Strain (wild- type) SARS-CoV-2.

Sample no.	Total N. no.	Variant position in whole genome	N. change (Ref/Alt)	Variant type	A.A. position	A.A. change
1	3816	21618	C/G	nonsynonymous	19	T/R
		21721	C/T	synonymous	53	D/D
		21800	G/T	nonsynonymous	80	D/Y
		21846	C/T	nonsynonymous	95	T/I
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22247	T/C	synonymous	229	L/L
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23191	C/T	synonymous	543	F/F
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R
		24410	G/A	nonsynonymous	950	D/N
2	3816	21618	C/G	nonsynonymous	19	T/R
		21721	C/T	synonymous	53	D/D

		21846	C/T	nonsynonymous	95	T/I
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R
		24410	G/A	nonsynonymous	950	D/N
		25352	G/T	nonsynonymous	1264	V/L
3	3816	21618	C/G	nonsynonymous	19	T/R
		21721	C/T	synonymous	53	D/D
		21846	C/T	nonsynonymous	95	T/I
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R
		24410	G/A	nonsynonymous	950	D/N
		25469	C/T	nonsynonymous	77	V/I
4	3816	21618	C/G	nonsynonymous	19	T/R
		21846	C/T	nonsynonymous	95	T/I
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R
		24410	G/A	nonsynonymous	950	D/N
		24863	C/T	nonsynonymous	1101	H/Y
5	3816	21618	C/G	nonsynonymous	19	T/R
		21647	A/G	nonsynonymous	29	T/A

		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22311	C/T	nonsynonymous	250	T/I
		22444	C/T	synonymous	294	D/D
		22597	C/T	synonymous	345	T/T
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R
		23625	C/T	nonsynonymous	688	A/V
		24410	G/A	nonsynonymous	950	D/N
		25146	A/C	nonsynonymous	1195	E/A
6	3816	21618	C/G	nonsynonymous	19	T/R
		21647	A/G	nonsynonymous	29	T/A
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22104	G/T	nonsynonymous	181	G/V
		22311	C/T	nonsynonymous	250	T/I
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R
		24410	G/A	nonsynonymous	950	D/N
7	3816	21618	C/G	nonsynonymous	19	
		21846	C/T	nonsynonymous	95	T/I
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R
		24410	G/A	nonsynonymous	950	D/N

		24872	G/T	nonsynonymous	1104	V/L
8	3816	21618	C/G	nonsynonymous	19	T/R
		21647	A/G	nonsynonymous	29	T/A
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22311	C/T	nonsynonymous	250	T/I
		22468	G/T	synonymous	302	T/T
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R
		24410	G/A	nonsynonymous	950	D/N
9	3816	21618	C/G	nonsynonymous	19	T/R
		21647	A/G	nonsynonymous	29	T/A
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22311	C/T	nonsynonymous	250	T/I
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R
		23909	G/A	nonsynonymous	783	A/T
		24410	G/A	nonsynonymous	950	D/N
10	3816	21618	C/G	nonsynonymous	19	T/R
		21647	A/G	nonsynonymous	29	T/A
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22311	C/T	nonsynonymous	250	T/I
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R

		24410	G/A	nonsynonymous	950	D/N
11	3816	21618	C/G	nonsynonymous	19	T/R
		21647	A/G	nonsynonymous	29	T/A
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22311	C/T	nonsynonymous	250	T/I
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23604	C/G	nonsynonymous	681	P/R
		24410	G/A	nonsynonymous	950	D/N
12	3816	21618	C/G	nonsynonymous	19	T/R
		21721	C/T	synonymous	53	D/D
		21846	C/T	nonsynonymous	95	T/I
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23403	A/G	nonsynonymous	614	D/G
		23533	C/T	synonymous	657	N/N
		23604	C/G	nonsynonymous	681	P/R
		24410	G/A	nonsynonymous	950	D/N
13	3816	21618	C/G	nonsynonymous	19	T/R
		21647	A/G	nonsynonymous	29	T/A
		21987	G/A	nonsynonymous	142	G/D
		22029-22034	AGTTCA/-	non frameshift deletion	156_158 del	-
		22088	C/T	nonsynonymous	176	L/F
		22311	C/T	nonsynonymous	250	T/I
		22917	T/G	nonsynonymous	452	L/R
		22995	C/A	nonsynonymous	478	T/K
		23401	G/T	nonsynonymous	613	Q/H
		23403	A/G	nonsynonymous	614	D/G

		23534	A/C	nonsynonymous	658	N/H
		23604	C/G	nonsynonymous	681	P/R
		23873	G/T	nonsynonymous	771	A/S
		24410	G/A	nonsynonymous	950	D/N
14	3813	21762	C/T	nonsynonymous	67	A/V
		21765-21770	TACATG/-	non frameshift deletion	68_70del	_
		21846	C/T	nonsynonymous SNV	95	T/I
		21987-21995	GTGTTTATT/-	non frameshift deletion	142_145del	_
		22194-22196	ATT/-	non frameshift deletion	211_212del	_
		22204	_/GAGCCAGAA	non frameshift insertion	_	R214delinsR
		22578	G/A	nonsynonymous	339	G/D
		22599	G/A	nonsynonymous	346	R/K
		22673	T/C	nonsynonymous	371	S/P
		22674	C/T	nonsynonymous	371	S/F
		22679	T/C	nonsynonymous	373	S/P
		22686	C/T	nonsynonymous	375	S/F
		22813	G/T	nonsynonymous	417	K/N
		22882	T/G	nonsynonymous	440	N/K
		22898	G/A	nonsynonymous	446	G/S
		22992	G/A	nonsynonymous	477	S/N
		22995	C/A	nonsynonymous	478	T/K
		23013	A/C	nonsynonymous	484	E/A
		23040	A/G	nonsynonymous	493	Q/R
		23048	G/A	nonsynonymous	496	G/S
		23055	A/G	nonsynonymous	498	Q/R
		23063	A/T	nonsynonymous	501	N/Y
		23075	T/C	nonsynonymous	505	Y/H
		23202	C/A	nonsynonymous	547	T/K
		23403	A/G	nonsynonymous	614	D/G
		23525	C/T	nonsynonymous	655	H/Y

		23599	T/G	nonsynonymous	679	N/K
		23604	C/A	nonsynonymous	681	P/H
		23854	C/A	nonsynonymous	764	N/K
		23948	G/T	nonsynonymous	796	D/Y
		24130	C/A	nonsynonymous	856	N/K
		24424	A/T	nonsynonymous	954	Q/H
		24469	T/A	nonsynonymous	969	N/K
		24503	C/T	nonsynonymous	981	L/F
		25000	C/T	synonymous	1146	D/D
15	3813	21762	C/T	nonsynonymous	67	A/V
		21765-21770	TACATG/-	non frameshift deletion	68_70del	_
		21846	C/T	nonsynonymous SNV	95	T/I
		21987-21995	GTGTTTATT/-	non frameshift deletion	142_145del	_
		22194-22196	ATT/-	non frameshift deletion	211_212del	_
		22204	_/GAGCCAGAA	non frameshift insertion	_	R214delinsR
		22578	G/A	nonsynonymous	339	G/D
		22599	G/A	nonsynonymous	346	R/K
		22673	T/C	nonsynonymous	371	S/P
		22674	C/T	nonsynonymous	371	S/F
		22679	T/C	nonsynonymous	373	S/P
		22686	C/T	nonsynonymous	375	S/F
		22813	G/T	nonsynonymous	417	K/N
		22882	T/G	nonsynonymous	440	N/K
		22898	G/A	nonsynonymous	446	G/S
		22992	G/A	nonsynonymous	477	S/N
		22995	C/A	nonsynonymous	478	T/K
		23013	A/C	nonsynonymous	484	E/A
		23040	A/G	nonsynonymous	493	Q/R
		23048	G/A	nonsynonymous	496	G/S
		23055	A/G	nonsynonymous	498	Q/R

		23063	A/T	nonsynonymous	501	N/Y
		23075	T/C	nonsynonymous	505	Y/H
		23202	C/A	nonsynonymous	547	T/K
		23403	A/G	nonsynonymous	614	D/G
		23525	C/T	nonsynonymous	655	H/Y
		23599	T/G	nonsynonymous	679	N/K
		23604	C/A	nonsynonymous	681	P/H
		23854	C/A	nonsynonymous	764	N/K
		23948	G/T	nonsynonymous	796	D/Y
		24130	C/A	nonsynonymous	856	N/K
		24424	A/T	nonsynonymous	954	Q/H
		24469	T/A	nonsynonymous	969	N/K
		24503	C/T	nonsynonymous	981	L/F
		25000	C/T	synonymous	1146	D/D

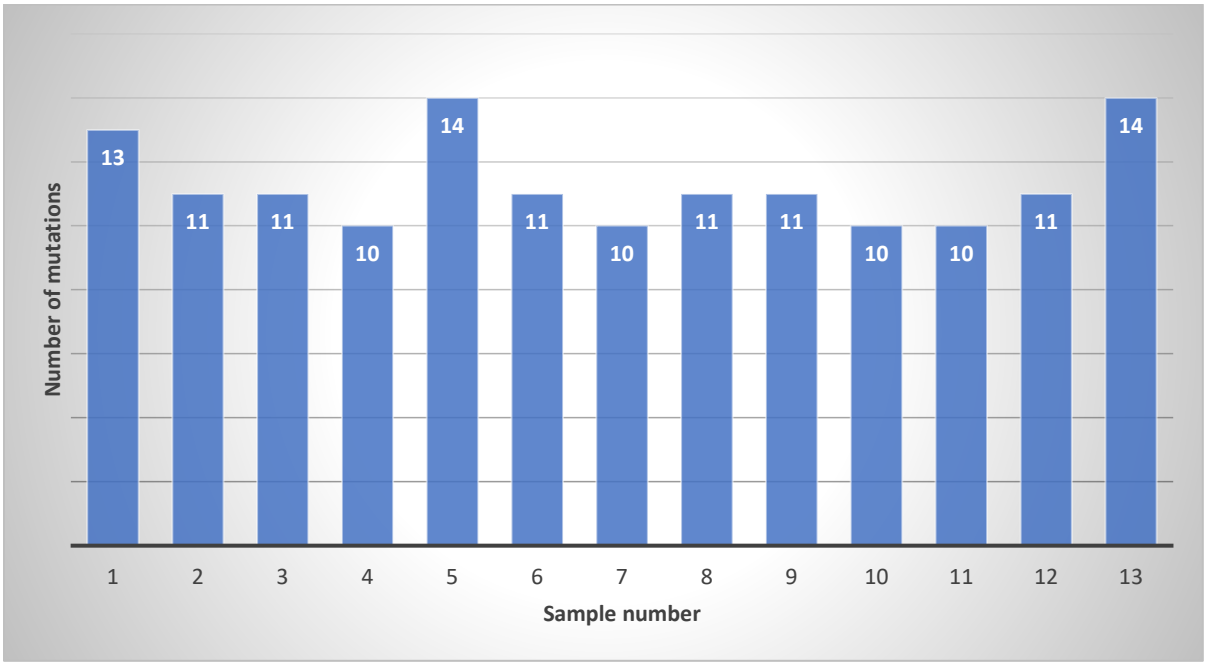

Figure 4. 5: Total number of mutations identified following NGS and analysis of S gene of Delta variants.

The spike amino acid variations were found to be variable among the isolates of the same or different variants. Table 4. 7. presents the frequency and specificity of amino acid variations in Delta and Omicron variants. It was found that D614G and T478K were the most frequent variations and were present in all the 15 SARS-CoV-2 isolates. D614G was the first mutation that appeared after Wuhan-Hu-1 strain and conserved in all previously identified variants from Alpha variant (B.1.1.7) to Omicron, but T478K was first identified in Delta variant and conserved in Omicron variant as well.

Variations T19R, G142E, L452R, P681R, D950N and 156-158del were detected only in Delta isolates. L452R, T478K and P681R were the basis of identification of Delta variants as they were identified by WHO as signature mutations of Delta variant. It is of worth to highlight a number of variations observed among Delta variants that were isolate-specific; never repeated in two or more isolates, naming D80Y, L176F, G181V, L229L, D294D, T302T, T345T, F543F, Q613H, N657N, N658H, A688V, A771S, A783T, H1101Y, V1104L, E1195A and V1264L. Details about amino acid variation types and frequencies in the spike of Delta variants are presented in figure 4.6.

On the other hand, both identified Omicron variants had unusual number of amino acid changes in spike protein including A67V, G339D, R346K, S371P, S371F, S373P, S375F, K417N, N440K, G446S, S477N, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F, D1146D, 214inseEPR, 68-7-del, 142-145del and 211-212del.

Table 4. 7: Frequency and Specificity of Amino Acid Variations in Delta (B.1.617.2) and Omicron (B.1.1.529) Variants.

No.	Spike amino acid variation	Variation frequency	Delta (B.1.617.2)	Omicron (B.1.1.529)
1.	T19R	13	+	-
2.	T29A	7	±	-
3.	D53D	4	±	-
4.	A67V	2	-	+
5.	D80Y	1	±	-
6.	T95I	8	±	+
7.	G142D	13	+	-
8.	L176F	1	±	-
9.	G181V	1	±	-
10.	214EPE	2	-	+
11.	L229L	1	±	-
12.	T250I	7	±	-
13.	D294D	1	±	-
14.	T302T	1	±	-
15.	G339D	2	-	+
16.	T345T	1	±	-
17.	R346K	2	-	+
18.	S371P	2	-	+
19.	S371F	2	-	+
20.	S373P	2	-	+
21.	S375F	2	-	+
22.	K417N	2	-	+
23.	N440K	2	-	+
24.	G446S	2	-	+
25.	L452R	13	+	-
26.	S477N	2	-	+
27.	T478K	15	+	+
28.	E484A	2	-	+
29.	Q493R	2	-	+
30.	G496S	2	-	+
31.	Q498R	2	-	+
32.	N501Y	2	-	+

33.	Y505H	2	-	+
34.	F543F	1	±	-
35.	T547K	2	-	+
36.	Q613H	1	±	-
37.	D614G	15	+	+
38.	H655Y	2	-	+
39.	N657N	1	±	-
40.	N658H	1	±	-
41.	N679K	2	-	+
42.	P681R	13	+	-
43.	P681H	2	-	+
44.	A688V	1	±	-
45.	N764K	2	-	+
46.	A771S	1	±	-
47.	A783T	1	±	-
48.	D796Y	2	-	+
49.	N856K	2	-	+
50.	D950N	13	+	-
51.	Q954H	2	-	+
52.	N969K	2	-	+
53.	L981F	2	-	+
54.	H1101Y	1	±	-
55.	V1104L	1	±	-
56.	D1146D	2	-	+
57.	E1195A	1	±	-
58.	V1264L	1	±	-
59.	68-70del	2	-	+
60.	142-145del	2	-	+
61.	156-158del	13	+	-
62.	211-212del	2	-	+

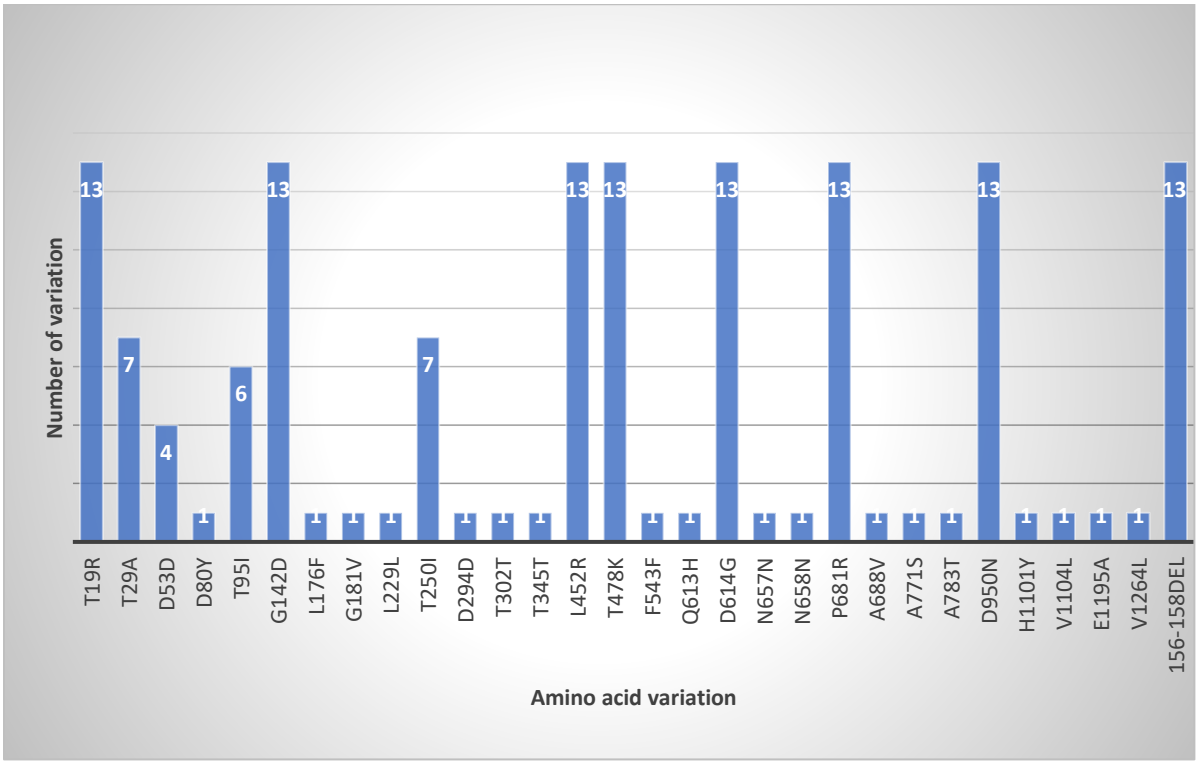

Figure 4. 6: A summary of amino acid variation types and frequencies in the spike of Delta variants identified in this Study.

CHAPTER FIVE
DISCUSSION

DISCUSSION

The COVID-19 pandemic that began in Wuhan, China, in late 2019 triggered a global health crisis (Guan et al., 2020). The infection was found to be mostly mild in healthy people, but it did quickly progress to acute respiratory distress syndrome (ARDS) and serious multi-organ issues because of the rapid viral replication and high rate of systemic inflammation due to the cytokine storm. The latter was greatly associated with people with certain underlying factors such as old age, DM, respiratory and circulatory disorders which resulted in increased rates of hospitalization and death (Wang and Ma, 2008, Ciceri et al., 2020).

Severe COVID-19 patients in this investigation were older than those who were infected mildly. This agrees with other studies where age and severity are correlated with worse clinical outcomes (Ballaz et al., 2021). Elderly persons tend to have a higher risk of contracting infectious diseases because they are a vulnerable population group with weakened immune systems. (Flook et al., 2021). Older persons may have more tissue damage from infections due to weakened immunological defenses against infectious diseases and aging-related chronic pro-inflammatory immune system conditions with persistently low innate immune activation. (Shaw et al., 2010).

The disease progression and mortality rate in those COVID-19 individuals are thought to be influenced by age as well. In earlier research, the percentage of infections that ultimately result in severe and life-threatening illness, or death, increased speedily with age, notably among people over 50 (Francone et al., 2020). This study also shed the light on comorbidities that

were associated with the severity of COVID-19. According to the present finding, diabetes, chronic kidney diseases and cardiovascular diseases were the mostly recorded comorbidities that imply the highest risk for a severe clinical presentation in patients with COVID-19. The association of comorbidities with the severity of the clinical presentation has also been reported by other studies that have concluded that people with severe COVID-19 frequently have concomitant conditions, such as cardiovascular illnesses. (Cai et al. 2020) , diabetes, cerebrovascular diseases and chronic kidney diseases (Wang et al., 2020). Moreover, the same correlation have been also fixed for other human respiratory diseases caused by influenza (Mertz et al., 2013), SARS-CoV-1 (Peiris et al., 2003), and MERS-CoV (Alraddadi et al., 2016).

Regarding the association of ABO blood groups, results have showed that there is no significant association with the disease, however, blood groups A and O had the same and highest distribution among the patients. This finding is not in a concordance with Wu et al. (2020c) who found that patients in group A may be more susceptible to become infected with COVID-19 than those in group O. On the other hand, a meta-analysis has been published on the greater sensitivity of the ABO blood group to infection which does not necessarily coincide with greater mortality or severity (Golinelli et al., 2020). Regarding the Rh factor, this study uncovered that a statistical relationship was not found between severity and the Rh factor (table 4. 1). This result is similar to the finding of Yaylacı et al. (2020) who documented that Rh factor has no relation with disease severity.

Although males were more likely to become infected, this study found no correlation between gender and illness severity. Ishaq et al. (2021) found something very similar and showed that there were no significant differences in the IgG levels in both genders. Also, an Iranian team showed that gender may not have a roll in the severity and mortality (Javanian, 2020). Clinical studies have also described that the prognosis was worse among patients older than 60 and mainly males (Stokes et al., 2020). The X chromosome and sexual hormone defenses (estrogens act as promoter while testosterone have suppressive effect) both are important in the innate and adaptive immune systems activation, and Jaillon and his colleagues showed that women are less vulnerable to viral infections as a result (Jaillon et al., 2019).

In the present study, adequate prognostic performance of the immunological markers (CRP) and the hematological markers (lymphocyte, platelet and D-dimer) has been demonstrated. A significant increase in the level of CRP was recorded among hospitalized patients and was found to be associated with the severity and mortality of COVID-19. The same finding was reported by each of Liu et al. (2020) and Zeng et al. (2020) as they found that measuring CRP is important for predicting disease severity and mortality. Increased plasma CRP levels is due to excessive immune response in COVID-19, called a cytokine storm, which arises from the overproduction of proinflammatory early-response cytokines such as TNF, IL-6, and IL-1β which stimulate hepatocytes to produce excessive CRP (Deb et al., 2022).

D-dimer was associated with severity and mortality in the current study. D-dimer is produced as a result of the breakdown of fibrin, it was used as a marker of thrombosis in COVID-19 (Gorog et al., 2022). The level of D-dimer

also elevated in other pathological conditions (Jiang et al., 2021). The study by Lehmann et al. (2021) confirmed that the D-dimer level may be an important predictor of thromboembolic events in patients with COVID-19. It was evidenced in the study that in non-surviving patients the D-dimer level increased, while the lymphocytes decrease (Wang et al., 2020a). According to the work performed by Görlinger et al. (2020) there was a few patients presented D-dimer values of ≥2.0 ng/ml. Therefore, for patients with markedly elevated D-dimers (cut-off: 2.0 ng/mL), hospital admission should be considered even in the absence of other serious symptoms. Similarly, it was found in a study by Mareev et al. (2020) at the time of admission, values higher than 2.0 µg/mL were reported, where the authors emphasized that D-dimer could effectively predict in-hospital mortality in patients with COVID-19.

In the current study, lymphopenia was associated with mortality. Similarly, Fajgenbaum and June (2020) proved that in hospitalized patients with COVID-19, low lymphocyte counts were independently determined as predictors of mortality. Most of the patients with severe COVID-19 had lymphopenia (Yang et al., 2020a). According to a study by Wang et al. (2020a), lymphopenia was common in COVID-19 patients in the ICU and the persistence of lymphopenia is a sign of poor prognosis in terms of survival.

Our results showed that thrombocytopenia was associated with mortality. The precipitation of platelets in the endothelial and thrombotic alterations of SARS-CoV-2 has been widely demonstrated, mainly their interaction with neutrophils that form extracellular neutrophil traps (NETs) in a deregulated way to trigger thrombosis and microcirculation disturbances (Gong et al.,

2020, Ji et al., 2020). In COVID-19, it was reported that platelets are activated and aggregated chaotically, and their mean volume increased, and their absolute count decreased (Grommes et al., 2012, Wool and Miller, 2021).

The SARS-CoV-2 virus compromises human cell by the interaction of the spike protein with the angiotensin-converting-2 (ACE2) protein receptor on the human cell. Thus, for the purpose of developing treatment strategies, scientists now specifically target the spike protein., preventive measures, and better understanding the disease pathogenesis (Mansbach et al., 2021, Zhang et al., 2021). Although RNA viruses like SARS-CoV-2 have a rather high rate of mutation, the question that remains unanswered is whether or not the modified strains change the viral characteristics. (Chakraborty et al., 2021). According to reports, this gene's mutation potential is 4-5 times higher than that of other genomic locations. (Hillus et al., 2021).

Infection with SARS-CoV-2 Delta variant (B.1.617.2) was first identified in India in late 2020 and caused an increase in COVID-19 morbidity and mortality worldwide (Saville et al., 2022). On April 4, 2021, the WHO described it as VOI Delta, and on May 11, 2021, it redefined it as VOC Delta. It remained the dominant epidemic strain until early December 2021 accounting for 81% of all epidemic strains worldwide (Saha et al., 2021). On the other side, on December 16, 2021, the Omicron variety (B.1.1.529) was identified for the first time in Botswana and South Africa and was swiftly disseminating throughout South Africa and the rest of the world. The United States, United Kingdom, France, Germany, Australia, Japan, and China are just some of the 77 countries that have reported seeing it (Kupferschmidt, 2021). Since then, Omicron has dominated and remained the VOC causing

SARS-CoV-2 infection worldwide (Wang et al., 2021b). the study results were agreeing with this timing.

The most concerning variant was the Delta variant as disease severity, hospitalization and mortality rates were obviously increased with Delta variant compared to the other variants (Twohig et al., 2022). Surprisingly, this variant has been associated with a 120% more risk of hospitalization, a 287% increased risk of ICU admission, and a 137% increased mortality risk (Fisman and Tuite, 2021) compared to infections caused by previous VOCs. Delta variant has been identified with unique set of mutations; L452R, T478K, and P681R, that made the virus highly contagious and evade neutralizing antibodies in vaccinated people or previously infected individuals (Planas et al., 2021). In addition, many other substitutions such as T19R, G142D, L452R, T478K, D614G, P681R, D950N and a deletion in the nucleotide position 156 and 157 in the S gene have been also reported (He et al., 2021). All the above-mentioned mutations were found in all the 13 Delta variants sequenced in this study.

L452R mutation located in the RBD and switches an arginine for a leucine. This mutation was shown to restructure the genome that led to increasing interaction with the ACE-2 receptor (Lan et al., 2020, Pascarella et al., 2021). It has also shown to aid viral replication and viral transmissibility (Motozono et al., 2021). According to a previous study, it was reported that this mutation aids the virus to escape from immune cells such as CD4, allowing virus survival and activity in the body (Saberiyan et al., 2022). The Delta variant also has a mutation that increases its infectiousness; proline is replaced by arginine at position 681 (P681R), which has been shown to promote furin-

mediated cleavage of the S-protein and, in turn, better fusion of the virus particle with the host cell (Bian et al., 2021, Shiehzadegan et al., 2021, Cascella et al., 2022). The P681R mutation in the Delta form has been linked to a partial decrease in neutralizing antibodies (NAbs) (Wami et al., 2021). All of these three important mutations were detected in the 13 delta samples in this study.

The Omicron variant had an unusual number of changes in the amino acid sequence in spike protein. There have been discovered a total of 30 non-synonymous mutations namely, A67V, T95I, G339D, R346K, S371P, S371F, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F, and only D1146D as a synonymous mutation along with 68_70, 142_145, and 211_212 deletions and 214 EPE insertion, all those changes were detected in the 2 omicron samples in this study. How the Omicron VOC came to have such a large number of mutations, notably on the Spike protein, is still a mystery. Less selection pressure was placed on the virus after this variation was found for the first time in an immunocompromised patient in South Africa, less selective pressure was on the virus. Still, the prolonged duration of the infection could be the reason for such a huge evolution. In general, the process of viral evolution was sped up by an intensifying and ongoing selective pressure on the virus at both the population and individual levels (Ahmed et al., 2021).

In the present study there were 15 mutations in the RBD of Omicron spike protein, including S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, N501Y, and Y505H. This result is

similar to the finding of Mohapatra et al. (2022). In addition to further changes that were brought on by earlier VOCs (Ahmed et al., 2021) that can potentially influence ACE2 receptor binding and antibody response (Harvey et al., 2021, Li et al., 2021). Previously discovered mutations in other VOCs, such as K417N, N440K, G446S, T478K, and E484A, have been linked to immunological escapes and evasion of neutralizing antibodies. (Wang and Cheng, 2022). Besides, 68/70 del mutation in the S gene was used as a target region for PCR detection of Omicron variant (Metzger et al., 2021) .

The Omicron variation was distinguished by three distinct sets of mutations at the RBD (amino acid sites G339D, S371L, S373P and S375F), receptor-binding motif (amino acid sites Q493R, G496S,Q498R and Y505H) and (amino acid sites N764K, N856K, Q954H, N969K, AND L981F) (Martin et al., 2021). The ins214EPE insertion mutation has not been seen in any other SARS-CoV-2 strains. Distal to the antibody-binding location in the N-terminal domain (NTD) is where the EPE insertion was found in Omicron (McCallum et al., 2021). However, the loop where the insertion was present mapped to a known human T-cell epitope on SARS-CoV-2. Further studies will be necessary to understand whether this insertion may help SARS-CoV-2 escape T-cell immunity (Garushyants et al., 2021).

Table 4. 7. and figure 4. 6. showed the frequency of spike amino acid mutations that were found to be variable among the isolates of the same or different variants. It was found that D614G and T478K were the most frequent variations and were present in all the 15 SARS-CoV-2 isolates. The asparagine-to-glycine substitution at amino acid position 614 (D614G) was one of the most critical mutations that has become so dominant in all VOCs

and VOIs globally, including both Delta and Omicron variants (Chakraborty et al., 2022). Furthermore, Studies have revealed that the D614G mutation dramatically boosts the virus particles' ability to replicate in the respiratory system, particularly in the upper section, which can be associated with the enhanced transmission potential. However, this sort of mutation might not have an impact on how severe the infection caused by the version with the D164G mutation is. (Omotuyi et al., 2020, Korber et al., 2020).

The T478K substitution has been reported by other literatures to be found in both Delta and Omicron variants (Di Giacomo et al., 2021) which agree with this study finding. Mutations T19R, G142D, L452R, T478K, D614G, P681R, D950N and a deletion in the nucleotide position 156 and 157 were only detected in the 13 Delta isolates. Other mutations such as T29A, D53D, T95I and T250I were detected only in some delta strains. These results were in accordance with other studies that reported that above 8 mutations were found in almost all delta variants (Amoutzias et al., 2022).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS AND RECOMMENDATIONS

6. 1. CONCLUSIONS

1. The NGS and analysis of the S gene identified two SARS-CoV-2 VOCs in Erbil City during study period; Delta (B.1.617.2) and Omicron (B.1.1.529).
2. On the whole, different mutation classes have been observed in which non-synonymous SNV exhibited the most abundant type.
3. The total nucleotide number of the S gene was found to be 3816 and 3813 for each of Delta and Omicron variants.
4. Delta variants were not identical in the S gene mutations, except for two isolates recovered from the same family members.
5. L452R, T478K and P681R, the three Delta variant signature mutations identified by WHO, were found in all Delta variants in this study.
6. D80Y, L176F, G181V, L229L, D294D, T302T, T345T, F543F, Q613H, N657N, N658H, A688V, A771S, A783T, H1101Y, V1104L, E1195A and V1264L were isolate-specific amino acid variations in Delta variants and never repeated in two or more isolates.
7. The two Omicron variants were identical in the type and number of mutations in the S gene.
8. Omicron variants exhibited 35 mutations of different classes; some of which were conserved mutations but most were totally new.
9. D614G and T478K were the most frequent SNVs and were present in all the 15 SARS-CoV-2.
10. No specific mutations were found to be associated with severity and mortality of COVID-19.
11. Gender, ABO and Rh were not associated with COVID-19.

12. Mild COVID-19 was recorded mostly in young adults, but sever infection was associated with extreme ages.
13. Nearly all hospitalized patients exhibited multiple comorbidities, most of which were significantly related to the disease and diseases severity.
14. A significant relation was found between each of CRP, D-dimer and lymphocyte % with COVID-19 severity.
15. Generally, CRP, D-dimer, lymphocyte and platelet count were significant predictors of mortality in patients with COVID-19.

6. 2. RECOMMENDATIONS:

1. Care strongly recommended with elderly people with SARS-CoV-2 infection, especially those with multiple comorbidities.
2. Virus screening on the molecular level necessary not be ignored as this virus continuously undergo genetic changes and highly virulent strains may appear at any time.
3. Phylogenetic analysis of the sequenced strains is necessary to know the genetic relatedness with the internationally identified and recorded SARS-CoV-2 isolates.

REFERENCES

REFERENCES

- Adhikari, S.P., Meng, S., Wu, Y.J., Mao, Y.P., Ye, R.X., Wang, Q.Z., Sun, C., Sylvia, S., Rozelle, S., Raat, H. and Zhou, H., 2020. Epidemiology, causes, clinical manifestation and diagnosis, prevention and control of coronavirus disease (COVID-19) during the early outbreak period: a scoping review. Infectious diseases of poverty, 9(1), 1-12.
- Ahmad, L., 2021. Implication of SARS-CoV-2 immune escape spike variants on secondary and vaccine breakthrough infections. Frontiers in immunology, 4563.
- Ahmed, J.Q., Maulud, S.Q., Al-Qadi, R., Mohamed, T.A., Tayib, G.A., Hassan, A.M., Taha, L.S., Qasim, K.M. and Tawfeeq, M.A., 2022. Sequencing and mutations analysis of the first recorded SARS-CoV-2 Omicron variant during the fourth wave of pandemic in Iraq. Brazilian Journal of Infectious Diseases, 26.
- Ahn, J. H. et al. 2021. Nasal ciliated cells are primary targets for SARS-CoV-2 replication in the early stage of COVID-19. J. Clin. Invest. 131, 148517.
- Akira, S., Uematsu, S. and Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell, 124(4), 783-801.
- Aleem, A., AB, A.S. and Slenker, A.K., 2022. Emerging variants of SARS-CoV-2 and novel therapeutics against coronavirus (COVID-19).
- Alizon, S., Haim-Boukobza, S., Foulongne, V., Verdurme, L., Trombert-Paolantoni, S., Lecorche, E., Roquebert, B. and Sofonea, M.T., 2021. Rapid spread of the SARS-CoV-2 Delta variant in some French regions, June 2021. Eurosurveillance, 26(28), 2100573.
- Alraddadi, B.M., Watson, J.T., Almarashi, A., Abedi, G.R., Turkistani, A., Sadran, M., Housa, A., Almazroa, M.A., Alraihan, N., Banjar, A. and

Albalawi, E., 2016. Risk factors for primary Middle East respiratory syndrome coronavirus illness in humans, Saudi Arabia, 2014. Emerging infectious diseases, 22(1), 49.

- Amoutzias, G.D., Nikolaidis, M., Tryfonopoulou, E., Chlichlia, K., Markoulatos, P. and Oliver, S.G., 2022. The remarkable evolutionary plasticity of coronaviruses by mutation and recombination: insights for the COVID-19 pandemic and the future evolutionary paths of SARS-CoV-2. Viruses, 14(1), 78.
- Arora, P., Kempf, A., Nehlmeier, I., Graichen, L., Sidarovich, A., Winkler, M. S., Schulz, S., Jack, H.-M., Stankov, M. V. & Behrens, G. M. 2021. Delta variant (B. 1.617. 2) sublineages do not show increased neutralization resistance. Cellular & molecular immunology, 18**,** 2557-2559.
- Astuti, I., 2020. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2): An overview of viral structure and host response. Diabetes & Metabolic Syndrome: Clinical Research & Reviews, 14(4), 407-412.
- Ballaz, S.J., Pulgar-Sánchez, M., Chamorro, K., Fernández-Moreira, E., Ramírez, H., Mora, F.X. and Fors, M., 2021. Common laboratory tests as indicators of COVID-19 severity on admission at high altitude: a single-center retrospective study in Quito (ECUADOR). Clinical Chemistry and Laboratory Medicine (CCLM), 59(8), 326-329.
- Barkauskas, C.E., Cronce, M.J., Rackley, C.R., Bowie, E.J., Keene, D.R., Stripp, B.R., Randell, S.H., Noble, P.W. and Hogan, B.L., 2013. Type 2 alveolar cells are stem cells in adult lung. The Journal of clinical investigation, 123(7), 3025-3036.
- Bartz, D., Chitnis, T., Kaiser, U.B., Rich-Edwards, J.W., Rexrode, K.M., Pennell, P.B., Goldstein, J.M., O'Neal, M.A., LeBoff, M., Behn, M. and

Seely, E.W., 2020. Clinical advances in sex-and gender-informed medicine to improve the health of all: a review. JAMA internal medicine, 180(4), 574-583.

- Benton, D.J., Wrobel, A.G., Xu, P., Roustan, C., Martin, S.R., Rosenthal, P.B., Skehel, J.J. and Gamblin, S.J., 2020. Receptor binding and priming of the spike protein of SARS-CoV-2 for membrane fusion. Nature, 588(7837), 327-330.
- Benzigar, M.R., Bhattacharjee, R., Baharfar, M. and Liu, G., 2021. Current methods for diagnosis of human coronaviruses: pros and cons. Analytical and bioanalytical chemistry, 413, 2311-2330.
- Bermejo-Martin, J.F., González-Rivera, M., Almansa, R., Micheloud, D., Tedim, A.P., Domínguez-Gil, M., Resino, S., Martín-Fernández, M., Ryan Murua, P., Pérez-García, F. and Tamayo, L., 2020. Viral RNA load in plasma is associated with critical illness and a dysregulated host response in COVID-19. Critical Care, 24(1), 1-13.
- Berno, G., Fabeni, L., Matusali, G., Gruber, C. E. M., Rueca, M., GiombiniI, E. & Garbuglia, A. R. 2022. SARS-CoV-2 Variants Identification: Overview of Molecular Existing Methods. Pathogens, 11**,** 1058.
- Bian, L., Gao, F., Zhang, J., He, Q., Mao, Q., Xu, M. and Liang, Z., 2021. Effects of SARS-CoV-2 variants on vaccine efficacy and response strategies. Expert review of vaccines, 20(4), 365-373.
- Boehm, E., Kronig, I., Neher, R.A., Eckerle, I., Vetter, P. and Kaiser, L., 2021. Novel SARS-CoV-2 variants: the pandemics within the pandemic. Clinical Microbiology and Infection, 27(8), 1109-1117.
- Brian, D.A. and Baric, R.S., 2005. Coronavirus genome structure and replication. Coronavirus replication and reverse genetics, 1-30.
- Brown, C.M., Vostok, J., Johnson, H., Burns, M., Gharpure, R., Sami, S.,

Sabo, R.T., Hall, N., Foreman, A., Schubert, P.L. and Gallagher, G.R., 2021. Outbreak of SARS-CoV-2 infections, including COVID-19 vaccine breakthrough infections, associated with large public gatherings—Barnstable County, Massachusetts, July

- Cai, Q., Huang, D., Ou, P., Yu, H., Zhu, Z., Xia, Z., Su, Y., Ma, Z., Zhang, Y., Li, Z. and He, Q., 2020. COVID-19 in a designated infectious diseases hospital outside Hubei Province, China. Allergy, 75(7), 1742-1752.
- Carsetti, R., Zaffina, S., Piano Mortari, E., Terreri, S., Corrente, F., Capponi, C., Palomba, P., Mirabella, M., Cascioli, S., Palange, P. and Cuccaro, I., 2020. Different innate and adaptive immune responses to SARS-CoV-2 infection of asymptomatic, mild, and severe cases. Frontiers in immunology, 11, 610300.
- Carty, M., Guy, C. and Bowie, A.G., 2021. Detection of viral infections by innate immunity. Biochemical pharmacology, 183, 114316.
- Cascella, M., Rajnik, M., Aleem, A., Dulebohn, S.C. and Di Napoli, R., 2022. Features, evaluation, and treatment of coronavirus (COVID-19). Statpearls [internet].
- Chakraborty, C., Bhattacharya, M. and Sharma, A.R., 2022. Present variants of concern and variants of interest of severe acute respiratory syndrome coronavirus 2: their significant mutations in S-glycoprotein, infectivity, re-infectivity, immune escape and vaccines activity. Reviews in Medical Virology, 32(2), 2270.
- Chakraborty, C., Saha, A., Sharma, A.R., Bhattacharya, M., Lee, S.S. and Agoramoorthy, G., 2021. D614G mutation eventuates in all VOI and VOC in SARS-CoV-2: Is it part of the positive selection pioneered by Darwin?. Molecular Therapy-Nucleic Acids, 26, 237-241.
- Chan, J.F.W., Kok, K.H., Zhu, Z., Chu, H., To, K.K.W., Yuan, S. and

Yuen, K.Y., 2020. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. Emerging microbes & infections, 9(1), 221-236.

- Chan-Yeung, M. and Xu, R.H., 2003. SARS: epidemiology. Respirology, 8, 9-14.
- Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., Qiu, Y., Wang, J., Liu, Y. & Wei, Y. 2020. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. The lancet, 395, 507-513.
- Chavda, V. P. & Apostolopoulos, V. 2022. Global impact of delta plus variant and vaccination. Expert Review of Vaccines, 21, 597-600.
- Ciceri, F., Beretta, L., Scandroglio, A. M., Colombo, S., Landoni, G., Ruggeri, A., Peccatori, J., D'angelo, A., DE Cobelli, F. & Rovere-Querin, P. 2020. Microvascular COVID-19 lung vessels obstructive thromboinflammatory syndrome (MicroCLOTS): an atypical acute respiratory distress syndrome working hypothesis. Critical care and resuscitation, 22**,** 95.
- Cohen, E., Margalit, I., Shochat, T., Goldberg, E. and Krause, I., 2021. Markers of chronic inflammation in overweight and obese individuals and the role of gender: a cross-sectional study of a large cohort. Journal of Inflammation Research, 14, 567.
- Collins, F., Lande, E., Rogers, J., Waterston, R. & Conso, I. 2004. Finishing the euchromatic sequence of the human genome. Nature, 431**,** 931-945.
- Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020;25(3):2000045.
- Coronaviridae Study Group (CSG) of the International Committee on

Taxonomy of Viruses. (2020) the species severe acute respiratory syndrome-related coronavirus: Classifying 2019-nCoV and naming it SARS-CoV-2, Nat. Microbiol., 5(4): 536-544.

- Cui, Z., Liu, P., Wang, N., Wang, L., Fan, K., Zhu, Q., Wang, K., Chen, R., Feng, R., Jia, Z. and Yang, M., 2022. Structural and functional characterizations of infectivity and immune evasion of SARS-CoV-2 Omicron. Cell, 185(5), 860-871.
- da Silva, J.F., Esteves, R.J., Siza, C., Soares, E.P., Ramos, T.C., Campelo, E.C., da Costa, C.F., de Alencar, L.C., Cavalcante, R.P., Florêncio, C.R. and Mattos, T.P., 2022. Cluster of SARS-CoV-2 Gamma Variant Infections, Parintins, Brazil, March 2021. Emerging infectious diseases, 28(1), 262.
- Dan, J.M., Mateus, J., Kato, Y., Hastie, K.M., Yu, E.D., Faliti, C.E., Grifoni, A., Ramirez, S.I., Haupt, S., Frazier, A. and Nakao, C., 2021. Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. Science, 371(6529), 4063.
- Dang, S., Ren, L. and Wang, J., 2022. Functional mutations of SARS-CoV-2: implications to viral transmission, pathogenicity and immune escape. Chinese Medical Journal, 135(10),1213-1222.
- Deb, S., Mondal, R., Lahiri, D., Ganguly, U. and Shome, G., 2022. Diverse immunopathological manifestations and immunogenomic predispositions in COVID-19: summarizing the evidence. International Journal of Research in Medical Sciences, 10(6), 1390.
- Dhawan, M., Sharma, A., Priyanka, N., Thakur, N., Rajkhowa, T.K. and Choudhary, O.P., 2022. Delta variant (B. 1.617. 2) of SARS-CoV-2: Mutations, impact, challenges and possible solutions. Human Vaccines & Immunotherapeutics, 18(5), 2068883.
- Di Giacomo, S., Mercatelli, D., Rakhimov, A. and Giorgi, F.M., 2021.

Preliminary report on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Spike mutation T478K. Journal of medical virology, 93(9), 5638-5643.

- Domingo, E. and Perales, C., 2019. Viral quasispecies. PLoS genetics, 15(10), 1008271.
- Domingueti, C.P., Dusse, L.M.S.A., das Graças Carvalho, M., de Sousa, L.P., Gomes, K.B. and Fernandes, A.P., 2016. Diabetes mellitus: the linkage between oxidative stress, inflammation, hypercoagulability and vascular complications. Journal of Diabetes and its Complications, 30(4), 738-745.
- Durmaz, B., Abdulmajed, O. and Durmaz, R., 2020. Mutations observed in the SARS-CoV-2 spike glycoprotein and their effects in the interaction of virus with ACE-2 receptor. Medeniyet medical journal, 35(3), 253.
- Ejaz, H., Alsrhani, A., Zafar, A., Javed, H., Junaid, K., Abdalla, A.E., Abosalif, K.O., Ahmed, Z. and Younas, S., 2020. COVID-19 and comorbidities: Deleterious impact on infected patients. Journal of infection and public health, 13(12), 1833-1839.
- Fajgenbaum, D.C. and June, C.H., 2020. Cytokine storm. New England Journal of Medicine, 383(23).2255-2273.
- Faria, N.R., Mellan, T.A., Whittaker, C., Claro, I.M., Candido, D.D.S., Mishra, S., Crispim, M.A., Sales, F.C., Hawryluk, I., McCrone, J.T. and Hulswit, R.J., 2021. Genomics and epidemiology of the P. 1 SARS-CoV-2 lineage in Manaus, Brazil. Science, 372(6544), 815-821.
- Feng, Z., Diao, B., Wang, R., Wang, G., Wang, C., Tan, Y., Liu, L., Wang, C., Liu, Y., Liu, Y. and Yuan, Z., 2020. The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) directly decimates human spleens and lymph nodes. MedRxiv.
- Fisman, D.N. and Tuite, A.R., 2021. Progressive increase in virulence of

novel SARS-CoV-2 variants in Ontario, Canada. MedRxiv, 2021-07.

- Flook, M., Jackson, C., Vasileiou, E., Simpson, C.R., Muckian, M.D., Agrawal, U., McCowan, C., Jia, Y., Murray, J.L., Ritchie, L.D. and Robertson, C., 2021. Informing the public health response to COVID-19: a systematic review of risk factors for disease, severity, and mortality. BMC infectious diseases, 21, 1-23.
- Francone, M., Iafrate, F., Masci, G.M., Coco, S., Cilia, F., Manganaro, L., Panebianco, V., Andreoli, C., Colaiacomo, M.C., Zingaropoli, M.A. and Ciardi, M.R., 2020. Chest CT score in COVID-19 patients: correlation with disease severity and short-term prognosis. European radiology, 30, 6808-6817.
- Garushyants, S.K., Rogozin, I.B. and Koonin, E.V., 2021. Template switching and duplications in SARS-CoV-2 genomes give rise to insertion variants that merit monitoring. Communications biology, 4(1), 1343.
- Gérard, C., Maggipinto, G. and Minon, J.M., 2020. COVID-19 and ABO blood group: another viewpoint. British journal of haematology, 190(2), 93.
- Gershov, D., Kim, S., Brot, N. and Elkon, K.B., 2000. C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. The Journal of experimental medicine, 192(9), 1353-1364.
- Golinelli, D., Boetto, E., Maietti, E. and Fantini, M.P., 2020. The association between ABO blood group and SARS-CoV-2 infection: A meta-analysis. PLoS One, 15(9), 0239508.
- Gong, J., Ou, J., Qiu, X., Jie, Y., Chen, Y., Yuan, L., Cao, J., Tan, M., Xu, W., Zheng, F. and Shi, Y., 2020. Multicenter development and validation of a novel risk nomogram for early prediction of severe 2019-novel coronavirus pneumonia.

- Gong, S.Y., Chatterjee, D., Richard, J., Prévost, J., Tauzin, A., Gasser, R., Bo, Y., Vézina, D., Goyette, G., Gendron-Lepage, G. and Medjahed, H., 2021. Contribution of single mutations to selected SARS-CoV-2 emerging variants spike antigenicity. Virology, 563, 134-145.
- Görlinger, K., Dirkmann, D., Gandhi, A. and Simioni, P., 2020. COVID-19 associated coagulopathy and inflammatory response: what do we know already and what are the knowledge gaps? Anesthesia and analgesia.
- Gorog, D.A., Storey, R.F., Gurbel, P.A., Tantry, U.S., Berger, J.S., Chan, M.Y., Duerschmied, D., Smyth, S.S., Parker, W.A., Ajjan, R.A. and Vilahur, G., 2022. Current and novel biomarkers of thrombotic risk in COVID-19: a Consensus Statement from the International COVID-19 Thrombosis Biomarkers Colloquium. Nature Reviews Cardiology, 19(7), 475-495.
- Goyal, P., Ringel, J.B., Rajan, M., Choi, J.J., Pinheiro, L.C., Li, H.A., Wehmeyer, G.T., Alshak, M.N., Jabri, A., Schenck, E.J. and Chen, R., 2020. Obesity and COVID-19 in New York City: a retrospective cohort study. Annals of internal medicine, 173(10), 855-858.
- Grommes, J., Alard, J.E., Drechsler, M., Wantha, S., Mörgelin, M., Kuebler, W.M., Jacobs, M., von Hundelshausen, P., Markart, P., Wygrecka, M. and Preissner, K.T., 2012. Disruption of platelet-derived chemokine heteromers prevents neutrophil extravasation in acute lung injury. American journal of respiratory and critical care medicine, 185(6), 628-636
- Guan, W.J., Ni, Z.Y., Hu, Y., Liang, W.H., Ou, C.Q., He, J.X., Liu, L., Shan, H., Lei, C.L., Hui, D.S. and Du, B., 2020. Clinical characteristics of coronavirus disease 2019 in China. New England journal of medicine, 382(18), 1708-1720.

- Harvey, W.T., Carabelli, A.M., Jackson, B., Gupta, R.K., Thomson, E.C., Harrison, E.M., Ludden, C., Reeve, R., Rambaut, A., COVID-19 Genomics UK (COG-UK) Consortium and Peacock, S.J., 2021. SARS-CoV-2 variants, spike mutations and immune escape. Nature Reviews Microbiology, 19(7), 409-424.
- He, X., He, C., Hong, W., Zhang, K. and Wei, X., 2021. The challenges of COVID-19 Delta variant: Prevention and vaccine development. MedComm, 2(4), 846-854.
- Hemachudha, P., Petcharat, S., Ampoot, W., Ponpinit, T., Paitoonpong, L. and Hemachudha, T., 2022. Genetic variations from successive whole genome sequencing during COVID-19 treatment in five individuals. New Microbes and New Infections, 45, 100950.
- Hillus, D., Schwarz, T., Tober-Lau, P., Vanshylla, K., Hastor, H., Thibeault, C., Jentzsch, S., Helbig, E.T., Lippert, L.J., Tscheak, P. and Schmidt, M.L., 2021. Safety, reactogenicity, and immunogenicity of homologous and heterologous prime-boost immunisation with ChAdOx1 nCoV-19 and BNT162b2: a prospective cohort study. The Lancet Respiratory Medicine, 9(11), 1255-1265.
- Hirano, T. and Murakami, M., 2020. COVID-19: a new virus, but a familiar receptor and cytokine release syndrome. Immunity, 52(5), 731-733.
- Holinstat, M., 2017. Normal platelet function. Cancer and Metastasis Reviews, 36(2), 195-198.

  Hozhabri, H., Piceci Sparascio, F., Sohrabi, H., Mousavifar, L., Roy, R., Scribano, D., De Luca, A., Ambrosi, C. and Sarshar, M., 2020. The global emergency of novel coronavirus (SARS-CoV-2): An update of the current status and forecasting. International journal of environmental research and public health, 17(16), 5648.

- Hu, B., Huang, S. and Yin, L., 2021. The cytokine storm and COVID-19. Journal of medical virology, 93(1), 250-256.
- Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X. and Cheng, Z., 2020a. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. The lancet, 395(10223), 497-506.
- Huang, J., Hume, A.J., Abo, K.M., Werder, R.B., Villacorta-Martin, C., Alysandratos, K.D., Beermann, M.L., Simone-Roach, C., Lindstrom-Vautrin, J., Olejnik, J. and Suder, E.L., 2020b. SARS-CoV-2 infection of pluripotent stem cell-derived human lung alveolar type 2 cells elicits a rapid epithelial-intrinsic inflammatory response. Cell Stem Cell, 27(6), 962-973.
- Hurst, K.R., Koetzner, C.A. and Masters, P.S., 2009. Identification of in vivo-interacting domains of the murine coronavirus nucleocapsid protein. Journal of virology, 83(14), 7221-7234.
- Ishaq, S.E., Abdulqadir, S.Z., Omar, S.A., Qadir, M.K., khdir Awla, H., Rasul, M.F., Bapir, A.A., Zanichelli, A., Mansoor, M.K., Kaleem, M. and Rizwan, M.A., 2021. Comparative study of SARS-CoV-2 antibody titers between male and female COVID-19 patients living in Kurdistan region of Iraq. Gene Reports, 25, 101409.
- Jaillon, S., Berthenet, K. and Garlanda, C., 2019. Sexual dimorphism in innate immunity. Clinical reviews in allergy & immunology, 56, 308-321.
- Javanian M, Bayani M, Shokri M, et al. Clinical and laboratory findings from patients with COVID-19 pneumonia in Babol North of Iran: a retrospective cohort study. Rom J Intern Med. 2020.
- Ji, D., Zhang, D., Xu, J., Chen, Z., Yang, T., Zhao, P., Chen, G., Cheng, G., Wang, Y., Bi, J. and Tan, L., 2020. Prediction for progression risk in patients with COVID-19 pneumonia: the CALL score. Clinical Infectious Diseases, 71(6), 1393-1399.

- Jiang, R.M., Pourzanjani, A.A., Cohen, M.J. and Petzold, L., 2021. Associations of longitudinal D-Dimer and Factor II on early trauma survival risk. BMC bioinformatics, 22, 1-13.
- Jin, J.M., Bai, P., He, W., Wu, F., Liu, X.F., Han, D.M., Liu, S. and Yang, J.K., 2020. Gender differences in patients with COVID-19: focus on severity and mortality. Frontiers in public health, 152.
- Khare, S., Gurry, C., Freitas, L., Schultz, M.B., Bach, G., Diallo, A., Akite, N., Ho, J., Lee, R.T., Yeo, W. and Team, G.C.C., 2021. GISAID's role in pandemic response. China CDC weekly, 3(49), 1049.
- Korber, B., Fischer, W.M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., Hengartner, N., Giorgi, E.E., Bhattacharya, T., Foley, B. and Hastie, K.M., 2020. Tracking changes in SARS-CoV-2 spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell, 182(4), 812-827.
- Kupferschmidt, K., 2021. Where did 'weird'Omicron come from?.
- Lamers, M.M., van der Vaart, J., Knoops, K., Riesebosch, S., Breugem, T.I., Mykytyn, A.Z., Beumer, J., Schipper, D., Bezstarosti, K., Koopman, C.D. and Groen, N., 2021. An organoid-derived bronchioalveolar model for SARS-CoV-2 infection of human alveolar type II-like cells. The EMBO Journal, 40(5), 105912.
- Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., Zhang, Q., Shi, X., Wang, Q., Zhang, L. and Wang, X., 2020. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. nature, 581(7807), 215-220.
- LaRosa, D.F. and Orange, J.S., 2008. 1. Lymphocytes. Journal of Allergy and Clinical Immunology, 121(2), 364-S369.

- Lee, J.G., Huang, W., Lee, H., van de Leemput, J., Kane, M.A. and Han, Z., 2021. Characterization of SARS-CoV-2 proteins reveals Orf6 pathogenicity, subcellular localization, host interactions and attenuation by Selinexor. Cell & bioscience, 11(1), 1-12.
- Lee, J.Y., Kim, Y.J., Chung, E.H., Kim, D.W., Jeong, I., Kim, Y., Yun, M.R., Kim, S.S., Kim, G. and Joh, J.S., 2017. The clinical and virological features of the first imported case causing MERS-CoV outbreak in South Korea, 2015. BMC infectious diseases, 17, 1-10.
- Lehmann, A., Prosch, H., Zehetmayer, S., Gysan, M.R., Bernitzky, D., Vonbank, K., Idzko, M. and Gompelmann, D., 2021. Impact of persistent D-dimer elevation following recovery from COVID-19. PLoS One, 16(10), 0258351.
- Letko, M., Marzi, A. and Munster, V., 2020. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. Nature microbiology, 5(4), 562-569.
- Leung, K., Shum, M.H., Leung, G.M., Lam, T.T. and Wu, J.T., 2021. Early transmissibility assessment of the N501Y mutant strains of SARS-CoV-2 in the United Kingdom, October to November 2020. Eurosurveillance, 26(1), 2002106.
- Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., Ren, R., Leung, K.S., Lau, E.H., Wong, J.Y. and Xing, X., 2020a. Early transmission dynamics in Wuhan, China, of novel coronavirus–infected pneumonia. New England journal of medicine.
- Li, X., Geng, M., Peng, Y., Meng, L. and Lu, S., 2020b. Molecular immune pathogenesis and diagnosis of COVID-19. Journal of pharmaceutical analysis, 10(2), 102-108.

- Li, B., Yang, J., Zhao, F., Zhi, L., Wang, X., Liu, L., Bi, Z. and Zhao, Y., 2020c. Prevalence and impact of cardiovascular metabolic diseases on COVID-19 in China. Clinical research in cardiology, 109(5), 531-538.
- Li, X., Hou, P., Ma, W., Wang, X., Wang, H., Yu, Z., Chang, H., Wang, T., Jin, S., Wang, X. and Wang, W., 2022. SARS-CoV-2 ORF10 suppresses the antiviral innate immune response by degrading MAVS through mitophagy. Cellular & molecular immunology, 19(1), 67-78.
- Li, Y., Ma, M.L., Lei, Q., Wang, F., Hong, W., Lai, D.Y., Hou, H., Xu, Z.W., Zhang, B., Chen, H. and Yu, C., 2021. Linear epitope landscape of the SARS-CoV-2 Spike protein constructed from 1,051 COVID-19 patients. Cell reports, 34(13), 108915.
- Lim, Z.J., Subramaniam, A., Ponnapa Reddy, M., Blecher, G., Kadam, U., Afroz, A., Billah, B., Ashwin, S., Kubicki, M., Bilotta, F. and Curtis, J.R., 2021. Case fatality rates for patients with COVID-19 requiring invasive mechanical ventilation. A meta-analysis. American journal of respiratory and critical care medicine, 203(1), 54-66.
- Lin, S., Mao, W., Zou, Q., Lu, S. and Zheng, S., 2021. Associations between hematological parameters and disease severity in patients with SARS-CoV-2 infection. Journal of clinical laboratory analysis, 35(1), 23604.
- Liu, Y., Ning, Z., Chen, Y., Guo, M., Liu, Y., Gali, N.K., Sun, L., Duan, Y., Cai, J., Westerdahl, D. and Liu, X., 2020. Aerodynamic analysis of SARS-CoV-2 in two Wuhan hospitals. Nature, 582(7813), 557-560.
- Lu, H., Stratton, C.W. and Tang, Y.W., 2020. Outbreak of pneumonia of unknown etiology in Wuhan, China: The mystery and the miracle. Journal of medical virology, 92(4), 401.
- Lucas, C., Wong, P., Klein, J., Castro, T.B., Silva, J., Sundaram, M., Ellingson, M.K., Mao, T., Oh, J.E., Israelow, B. and Takahashi, T., 2020.

Longitudinal analyses reveal immunological misfiring in severe COVID-19. Nature, 584(7821), 463-469.

- Luk, H.K., Li, X., Fung, J., Lau, S.K. and Woo, P.C., 2019. Molecular epidemiology, evolution and phylogeny of SARS coronavirus. Infection, Genetics and Evolution, 71, 21-30.
- Majumder, J. and Minko, T., 2021. Recent developments on therapeutic and diagnostic approaches for COVID-19. The AAPS journal, 23, 1-22.
- Mansbach, R.A., Chakraborty, S., Nguyen, K., Montefiori, D.C., Korber, B. and Gnanakaran, G., 2021. The SARS-CoV-2 Spike variant D614G favors an open conformational state. Biophysical Journal, 120(3), 298a.
- Mareev, V.Y., Orlova, Y.A., Pavlikova, E.P., Matskeplishvili, S.T., Krasnova, T.N., Malahov, P.S., Samokhodskaya, L.M., Mershina, E.A., Sinitsyn, V.E., Mareev, Y.V. and Kalinkin, A.L., 2020. Steroid pulse-therapy in patients With coronAvirus Pneumonia (COVID-19), sYstemic inFlammation And Risk of vEnous thRombosis and thromboembolism (WAYFARER Study). Kardiologiia, 60(6), 15-29.
- Martin, D., Lytras, S., Lucaci, A., Maier, W., Gruning, B. & Shank, S. 2021. Selection analysis identifies significant mutational changes in Omicron that are likely to influence both antibody neutralization and Spike function (Part 1 of 2). Virological. org [Internet], 5.
- McCallum, M., De Marco, A., Lempp, F.A., Tortorici, M.A., Pinto, D., Walls, A.C., Beltramello, M., Chen, A., Liu, Z., Zatta, F. and Zepeda, S., 2021. N-terminal domain antigenic mapping reveals a site of vulnerability for SARS-CoV-2. Cell, 184(9), 2332-2347.
- Mehra, M.R., Desai, S.S., Kuy, S., Henry, T.D. and Patel, A.N., 2020. Retraction: cardiovascular disease, drug therapy, and mortality in Covid-19. N Engl J Med. DOI: 10.1056/NEJMoa2007621.

- Mehta, P., McAuley, D.F., Brown, M., Sanchez, E., Tattersall, R.S. and Manson, J.J., 2020. COVID-19: consider cytokine storm syndromes and immunosuppression. The lancet, 395(10229), 1033-1034.
- Meng, B., Abdullahi, A., Ferreira, I.A., Goonawardane, N., Saito, A., Kimura, I., Yamasoba, D., Gerber, P.P., Fatihi, S., Rathore, S. and Zepeda, S.K., 2022. Altered TMPRSS2 usage by SARS-CoV-2 Omicron impacts infectivity and fusogenicity. Nature, 603(7902), 706-714.
- Mertz, D., Kim, T.H., Johnstone, J., Lam, P.P., Kuster, S.P., Fadel, S.A., Tran, D., Fernandez, E., Bhatnagar, N. and Loeb, M., 2013. Populations at risk for severe or complicated influenza illness: systematic review and meta-analysis. Bmj, 347.
- Metzger, C.M., Lienhard, R., Seth-Smith, H.M., Roloff, T., Wegner, F., Sieber, J., Bel, M., Greub, G. and Egli, A., 2021. PCR performance in the SARS-CoV-2 Omicron variant of concern?. Swiss medical weekly, 151(4950), 30120-30120.
- Michel, C.J., Mayer, C., Poch, O. and Thompson, J.D., 2020. Characterization of accessory genes in coronavirus genomes. Virology journal, 17(1), 1-13.
- Mishra, Y., Pathak, B.K., Mohakuda, S.S., Tilak, T.V.S.V.G.K., Sen, S., Harikrishnan, P., Singh, R. and Singh, A.R., 2020. Relation of D-dimer levels of COVID-19 patients with diabetes mellitus. Diabetes & Metabolic Syndrome: Clinical Research & Reviews, 14(6), 1927-1930.
- Mizuiri, S., Hemmi, H., Arita, M., Ohashi, Y., Tanaka, Y., Miyagi, M., Sakai, K., Ishikawa, Y., Shibuya, K., Hase, H. and Aikawa, A., 2008. Expression of ACE and ACE2 in individuals with diabetic kidney disease and healthy controls. American Journal of Kidney Diseases, 51(4), 613-623.
- Moghadas, S.M., Fitzpatrick, M.C., Sah, P., Pandey, A., Shoukat, A., Singer, B.H. and Galvani, A.P., 2020. The implications of silent

transmission for the control of COVID-19 outbreaks. Proceedings of the National Academy of Sciences, 117(30), 17513-17515.

- Mohapatra, R.K., Tiwari, R., Sarangi, A.K., Islam, M.R., Chakraborty, C. and Dhama, K., 2022. Omicron (B. 1.1. 529) variant of SARS-CoV-2: concerns, challenges, and recent updates. Journal of medical virology, 94(6), 2336.
- Monchatre-Leroy, E., Boué, F., Boucher, J.M., Renault, C., Moutou, F., Ar Gouilh, M. and Umhang, G., 2017. Identification of alpha and beta coronavirus in wildlife species in France: bats, rodents, rabbits, and hedgehogs. Viruses, 9(12), 364.
- Motozono, C., Toyoda, M., Zahradnik, J., Ikeda, T., Saito, A., Tan, T.S., Ngare, I., Nasser, H., Kimura, I., Uriu, K. and Kosugi, Y., 2021. An emerging SARS-CoV-2 mutant evading cellular immunity and increasing viral infectivity. BioRxiv, 2021-04.
- Naqvi, A.A.T., Fatima, K., Mohammad, T., Fatima, U., Singh, I.K., Singh, A., Atif, S.M., Hariprasad, G., Hasan, G.M. and Hassan, M.I., 2020. Insights into SARS-CoV-2 genome, structure, evolution, pathogenesis and therapies: Structural genomics approach. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1866(10), 165878.
- Nasab, M.G., Saghazadeh, A. and Rezaei, N., 2020. SARS-CoV-2−a tough opponent for the immune system. Archives of medical research, 51(6), 589-592.
- Neuman, B.W., Kiss, G., Kunding, A.H., Bhella, D., Baksh, M.F., Connelly, S., Droese, B., Klaus, J.P., Makino, S., Sawicki, S.G. and Siddell, S.G., 2011. A structural analysis of M protein in coronavirus assembly and morphology. Journal of structural biology, 174(1), 11-22.
- Nieto-Torres, J.L., DeDiego, M.L., Verdiá-Báguena, C., Jimenez-Guardeño, J.M., Regla-Nava, J.A., Fernandez-Delgado, R., Castaño-

Rodriguez, C., Alcaraz, A., Torres, J., Aguilella, V.M. and Enjuanes, L., 2014. Severe acute respiratory syndrome coronavirus envelope protein ion channel activity promotes virus fitness and pathogenesis. PLoS pathogens, 10(5), 1004077.

- Niu, Z., Zhang, Z., Gao, X., Du, P., Lu, J., Yan, B., Wang, C., Zheng, Y., Huang, H. and Sun, Q., 2021. N501Y mutation imparts cross-species transmission of SARS-CoV-2 to mice by enhancing receptor binding. Signal Transduction and Targeted Therapy, 6(1), 284.
- Omotuyi, I.O., Nash, O., Ajiboye, O.B., Iwegbulam, C.G., Oyinloye, E.B., Oyedeji, O.A., Kashim, Z.A. and Okaiyeto, K., 2020. Atomistic simulation reveals structural mechanisms underlying D614G spike glycoprotein-enhanced fitness in SARS-COV-2. Journal of computational chemistry, 41(24), 2158-2161.
- Ou, J., Zhou, Z., Dai, R., Zhang, J., Zhao, S., Wu, X., Lan, W., Ren, Y., Cui, L., Lan, Q. and Lu, L., 2021. V367F mutation in SARS-CoV-2 spike RBD emerging during the early transmission phase enhances viral infectivity through increased human ACE2 receptor binding affinity. Journal of Virology, 95(16), 00617-21.
- Palladino, M., 2021. Complete blood count alterations in COVID-19 patients: A narrative review. Biochemia medica, 31(3).
- Park, S.E., 2020. Epidemiology, virology, and clinical features of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; coronavirus disease-19). Pediatric Infection and Vaccine, 27(1), 1-10.
- Parums, D.V., 2021. Revised World Health Organization (WHO) terminology for variants of concern and variants of interest of SARS-CoV-2. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research, 27, 933622-1.
- Pascarella, S., Ciccozzi, M., Zella, D., Bianchi, M., Benedetti, F., Benvenuto, D., Broccolo, F., Cauda, R., Caruso, A., Angeletti, S. and

Giovanetti, M., 2021. SARS-CoV-2 B. 1.617 Indian variants: Are electrostatic potential changes responsible for a higher transmission rate? Journal of Medical Virology, 93(12), 6551-6556.

- Payus AO, Rajah R, Febriany DC, Mustafa N. Pulmonary Embolism Masquerading as Severe Pneumonia: A Case Report. Open Access Maced J Med Sci. 2019 Feb 15;7(3):396-399.
- Peiris, J.S., Guan, Y. and Yuen, K., 2004. Severe acute respiratory syndrome. Nature medicine, 10(Suppl 12), 88-97.
- Peiris, J.S.M., Chu, C.M., Cheng, V.C.C., Chan, K.S., Hung, I.F.N., Poon, L.L., Law, K.I., Tang, B.S.F., Hon, T.Y.W., Chan, C.S. and Chan, K.H., 2003. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. The lancet, 361(9371), 1767-1772.
- Pereira, F., 2020. Evolutionary dynamics of the SARS-CoV-2 ORF8 accessory gene. Infection, Genetics and Evolution, 85, 104525.
- Perez-Gomez, R., 2021. The development of SARS-CoV-2 variants: the gene makes the disease. Journal of developmental biology, 9(4), 58.
- Perrotta, F., Corbi, G., Mazzeo, G., Boccia, M., Aronne, L., D'Agnano, V., Komici, K., Mazzarella, G., Parrella, R. and Bianco, A., 2020. COVID-19 and the elderly: insights into pathogenesis and clinical decision-making. Aging clinical and experimental research, 32(8), 1599-1608.
- Planas, D., Veyer, D., Baidaliuk, A., Staropoli, I., Guivel-Benhassine, F., Rajah, M.M., Planchais, C., Porrot, F., Robillard, N., Puech, J. and Prot, M., 2021. Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. Nature, 596(7871), 276-280.
- Povoa, P., Pereira, J. and Coelho, L., 2009. C-reactive protein: structure, synthesis and function. C-Reactive Protein: New Research, 307-324.

- Rahimi, A., Mirzazadeh, A. and Tavakolpour, S., 2021. Genetics and genomics of SARS-CoV-2: A review of the literature with the special focus on genetic diversity and SARS-CoV-2 genome detection. Genomics, 113(1), 1221-1232.
- Rainer, T.H., Chan, C.P., Leung, M.F., Leung, W., Ip, M., Lee, N., Cautherley, G.W., Graham, C.A., Fuchs, D. and Renneberg, R., 2009. Diagnostic utility of CRP to neopterin ratio in patients with acute respiratory tract infections. Journal of Infection, 58(2), 123-130.
- Reich, H.N., Oudit, G.Y., Penninger, J.M., Scholey, J.W. and Herzenberg, A.M., 2008. Decreased glomerular and tubular expression of ACE2 in patients with type 2 diabetes and kidney disease. Kidney international, 74(12), 1610-1616.
- Ren, L.L., Wang, Y.M., Wu, Z.Q., Xiang, Z.C., Guo, L., Xu, T., Jiang, Y.Z., Xiong, Y., Li, Y.J., Li, X.W. and Li, H., 2020. Identification of a novel coronavirus causing severe pneumonia in human: a descriptive study. Chinese medical journal, 133(09), 1015-1024.
- Reuven, E.M., Dadon, Y., Viard, M., Manukovsky, N., Blumenthal, R. and Shai, Y., 2012. HIV-1 gp41 transmembrane domain interacts with the fusion peptide: implication in lipid mixing and inhibition of virus–cell fusion. Biochemistry, 51(13), 2867-2878.
- Ricci, D., Etna, M.P., Rizzo, F., Sandini, S., Severa, M. and Coccia, E.M., 2021. Innate immune response to SARS-CoV-2 infection: from cells to soluble mediators. International Journal of Molecular Sciences, 22(13), 7017.
- Robbiani, D.F., Gaebler, C., Muecksch, F., Lorenzi, J.C., Wang, Z., Cho, A., Agudelo, M., Barnes, C.O., Gazumyan, A., Finkin, S. and Hägglöf, T., 2020. Convergent antibody responses to SARS-CoV-2 in convalescent individuals. Nature, 584(7821), 437-442.

- Robson, B., 2020. Computers and viral diseases. Preliminary bioinformatics studies on the design of a synthetic vaccine and a preventative peptidomimetic antagonist against the SARS-CoV-2 (2019-nCoV, COVID-19) coronavirus. Computers in biology and medicine, 119, 103670.
- Rochman, N.D., Wolf, Y.I., Faure, G., Mutz, P., Zhang, F. and Koonin, E.V., 2021. Ongoing global and regional adaptive evolution of SARS-CoV-2. Proceedings of the National Academy of Sciences, 118(29), 2104241118.
- Rodrigues, T.S., de Sá, K.S., Ishimoto, A.Y., Becerra, A., Oliveira, S., Almeida, L., Gonçalves, A.V., Perucello, D.B., Andrade, W.A., Castro, R. and Veras, F.P., 2021. Inflammasomes are activated in response to SARS-CoV-2 infection and are associated with COVID-19 severity in patients. Journal of Experimental Medicine, 218(3).
- Rogers, T.F., Zhao, F., Huang, D., Beutler, N., Burns, A., He, W.T., Limbo, O., Smith, C., Song, G., Woehl, J. and Yang, L., 2020. Isolation of potent SARS-CoV-2 neutralizing antibodies and protection from disease in a small animal model. Science, 369(6506), 956-963.
- Ruan, Q., Yang, K., Wang, W., Jiang, L. and Song, J., 2020. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. Intensive care medicine, 46(5), 846-848.
- Saberiyan, M., Karimi, E., Khademi, Z., Movahhed, P., Safi, A. and Mehri-Ghahfarrokhi, A., 2022. SARS-CoV-2: phenotype, genotype, and characterization of different variants. Cellular & Molecular Biology Letters, 27(1), 1-19.
- Sadeghi-Haddad-Zavareh, M., Bayani, M., Shokri, M., Ebrahimpour, S., Babazadeh, A., Mehraeen, R., Moudi, E., Rostami, A., Barary, M., Hosseini, A. and Bijani, A., 2021. C-reactive protein as a prognostic

indicator in COVID-19 patients. Interdisciplinary Perspectives on Infectious Diseases, 2021.

- Saha, S., Tanmoy, A.M., Hooda, Y., Tanni, A.A., Goswami, S., Al Sium, S.M., Sajib, M.S.I., Malaker, R., Islam, S., Rahman, H. and Anik, A.M., 2021. COVID-19 rise in Bangladesh correlates with increasing detection of B. 1.351 variant. BMJ global health, 6(5), 6012.
- Samprathi, M. and Jayashree, M., 2021. Biomarkers in COVID-19: an up-to-date review. Frontiers in pediatrics, 8, 607647.
- Starr, T. N., Greaney, A. J., Hilton, S. K., Ellis, D., Crawford, K. H., Dingens, A. S., Navarro, M. J., Bowen, J. E., Tortorici, M. A. & Walls, A. C. 2020. Deep mutational scanning of SARS-CoV-2 receptor binding domain reveals constraints on folding and ACE2 binding. cell, 182**,** 1295-1310.
- Saville, J.W., Mannar, D., Zhu, X., Srivastava, S.S., Berezuk, A.M., Demers, J.P., Zhou, S., Tuttle, K.S., Sekirov, I., Kim, A. and Li, W., 2022. Structural and biochemical rationale for enhanced spike protein fitness in delta and kappa SARS-CoV-2 variants. Nature communications, 13(1), 742.
- Schetz, M., De Jonj, A., Deane, A. M., Druml, W., Hemelaar, P., Pelosi, P., Pickkers, P., Reintam-Blaser, A., Roberts, J. & Sakr, Y. 2019. Obesity in the critically ill: a narrative review. Intensive care medicine, 45**,** 757-769.
- Semple, J.W., Aslam, R., Kim, M., Speck, E.R. and Freedman, J., 2007. Platelet-bound lipopolysaccharide enhances Fc receptor–mediated phagocytosis of IgG-opsonized platelets. Blood, The Journal of the American Society of Hematology, 109(11), 4803-4805.
- Sette, A. and Crotty, S., 2021. Adaptive immunity to SARS-CoV-2 and COVID-19. Cell, 184(4), 861-880.

- Severa, M., Diotti, R.A., Etna, M.P., Rizzo, F., Fiore, S., Ricci, D., Iannetta, M., Sinigaglia, A., Lodi, A., Mancini, N. and Criscuolo, E., 2021. Differential plasmacytoid dendritic cell phenotype and type I Interferon response in asymptomatic and severe COVID-19 infection. PLoS pathogens, 17(9), 1009878.
- Shaw, A.C., Joshi, S., Greenwood, H., Panda, A. and Lord, J.M., 2010. Aging of the innate immune system. Current opinion in immunology, 22(4), 507-513.
- Shen, L., Bard, J.D., Triche, T.J., Judkins, A.R., Biegel, J.A. and Gai, X., 2021. Emerging variants of concern in SARS-CoV-2 membrane protein: a highly conserved target with potential pathological and therapeutic implications. Emerging microbes & infections, 10(1), 885-893.
- Shi, C.S., Qi, H.Y., Boularan, C., Huang, N.N., Abu-Asab, M., Shelhamer, J.H. and Kehrl, J.H., 2014. SARS-coronavirus open reading frame-9b suppresses innate immunity by targeting mitochondria and the MAVS/TRAF3/TRAF6 signalosome. The Journal of Immunology, 193(6), 3080-3089.
- Shiehzadegan, S., Alaghemand, N., Fox, M. and Venketaraman, V., 2021. Analysis of the delta variant B. 1.617. 2 COVID-19. Clinics and practice, 11(4), 778-784.
- Simonnet, A., Chetboun, M., Poissy, J., Raverdy, V., Noulette, J., Duhamel, A., Labreuche, J., Mathieu, D., Pattou, F., Jourdain, M. and

  LICORN and the Lille COVID-19 and Obesity Study Group, 2020. High prevalence of obesity in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) requiring invasive mechanical ventilation. Obesity, 28(7), 1195-1199.
- Singh MK, Mobeen A, Chandra A, Joshi S, Ramachandran S (2021) A meta-analysis of comorbidities in COVID-19: which diseases increase the susceptibility of SARS-CoV-2 infection? Comput Biol Med 130:104219.

- Sproston, N.R. and Ashworth, J.J., 2018. Role of C-reactive protein at sites of inflammation and infection. Frontiers in immunology, 9, 754.
- Stokes, E.K., Zambrano, L.D., Anderson, K.N., Marder, E.P., Raz, K.M., Felix, S.E.B., Tie, Y. and Fullerton, K.E., 2020. Coronavirus disease 2019 case surveillance—United States, January 22−may 30, 2020. Morbidity and Mortality Weekly Report, 69(24), 759.
- Tan, L., Wang, Q., Zhang, D., Ding, J., Huang, Q., Tang, Y.Q., Wang, Q. and Miao, H., 2020. Lymphopenia predicts disease severity of COVID-19: a descriptive and predictive study. Signal transduction and targeted therapy, 5(1), 1-3.
- Tang, G., Liu, Z. and Chen, D., 2022. Human coronaviruses: Origin, host and receptor. Journal of Clinical Virology, 105246.
- Terpos, E., Ntanasis-Stathopoulos, I., Elalamy, I., Kastritis, E., Sergentanis, T.N., Politou, M., Psaltopoulou, T., Gerotziafas, G. and Dimopoulos, M.A., 2020. Hematological findings and complications of COVID-19. American journal of hematology, 95(7), 834-847.
- Thomson, E.C., Rosen, L.E., Shepherd, J.G., Spreafico, R., da Silva Filipe, A., Wojcechowskyj, J.A., Davis, C., Piccoli, L., Pascall, D.J., Dillen, J. and Lytras, S., 2020. The circulating SARS-CoV-2 spike variant N439K maintains fitness while evading antibody-mediated immunity. BioRxiv, 2020-11.
- Twohig, K.A., Nyberg, T., Zaidi, A., Thelwall, S., Sinnathamby, M.A., Aliabadi, S., Seaman, S.R., Harris, R.J., Hope, R., Lopez-Bernal, J. and Gallagher, E., 2022. Hospital admission and emergency care attendance risk for SARS-CoV-2 delta (B. 1.617. 2) compared with alpha (B. 1.1. 7) variants of concern: a cohort study. The Lancet Infectious Diseases, 22(1), 35-42.
- Wajnberg, A., Amanat, F., Firpo, A., Altman, D.R., Bailey, M.J., Mansour, M., McMahon, M., Meade, P., Mendu, D.R., Muellers, K. and

Stadlbauer, D., 2020. Robust neutralizing antibodies to SARS-CoV-2 infection persist for months. Science, 370(6521), 1227-1230.

- Walls, A.C., Park, Y.J., Tortorici, M.A., Wall, A., McGuire, A.T. and Veesler, D., 2020. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. Cell, 181(2), 281-292.
- Wami, H., Wallenstein, A., Sauer, D., Stoll, M., von Bünau, R., Oswald, E., Müller, R. and Dobrindt, U., 2021. Diversity and prevalence of colibactin- and yersiniabactin encoding mobile genetic elements in enterobacterial populations: insights into evolution and co-existence of two bacterial secondary metabolite determinants. bioRxiv, 2021-01.
- Wang, D., Hu, B., Hu, C., Zhu, F., Liu, X., Zhang, J., Wang, B., Xiang, H., Cheng, Z., Xiong, Y. and Zhao, Y., 2020a. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus–infected pneumonia in Wuhan, China. Jama, 323(11), 1061-1069.
- Wang, J., Li, Z., Cheng, X., Hu, H., Liao, C., Li, P., Lu, J. and Chen, Z., 2020b. Epidemiologic characteristics, transmission chain, and risk factors of severe infection of COVID-19 in Tianjin, a Representative Municipality City of China. Frontiers in public health, 8, 198.
- Wang, Q., Zhang, Y., Wu, L., Niu, S., Song, C., Zhang, Z., Lu, G., Qiao, C., Hu, Y., Yuen, K.Y. and Wang, Q., 2020c. Structural and functional basis of SARS-CoV-2 entry by using human ACE2. Cell, 181(4), 894-904.
- Wang, G., Lai, F.M.M., Kwan, B.C., Lai, K.B., Chow, K.M., Li, P.K. and Szeto, C.C., 2011. Expression of ACE and ACE2 in patients with hypertensive nephrosclerosis. Kidney and Blood Pressure Research, 34(3), 141-149.
- Wang, H. and Ma, S., 2008. The cytokine storm and factors determining the sequence and severity of organ dysfunction in multiple organ

dysfunction syndrome. The American journal of emergency medicine, 26(6), 711-715.

- Wang, L. and Cheng, G., 2022. Sequence analysis of the emerging SARS-CoV-2 variant Omicron in South Africa. Journal of medical virology, 94(4), 1728-1733.
- Wang, P., Casner, R.G., Nair, M.S., Wang, M., Yu, J., Cerutti, G., Liu, L., Kwong, P.D., Huang, Y., Shapiro, L. and Ho, D.D., 2021a. Increased resistance of SARS-CoV-2 variant P. 1 to antibody neutralization. Cell host & microbe, 29(5), 747-751.
- Wang, Y., Chen, R., Hu, F., Lan, Y., Yang, Z., Zhan, C., Shi, J., Deng, X., Jiang, M., Zhong, S. and Liao, B., 2021b. Transmission, viral kinetics and clinical characteristics of the emergent SARS-CoV-2 Delta VOC in Guangzhou, China. EClinicalMedicine, 40, 101129.
- World Health Organization, 2020. Naming the coronavirus disease (COVID-19) and the virus that causes it. Brazilian Journal of Implantology and Health Sciences, 2(3).
- World Health Organization; 2020.WHO Coronavirus, N., 2019. situation report-1. Geneva
- World Health Organization; Coronavirus, W.H.O., 2021. Dashboard| WHO coronavirus (COVID-19) dashboard with vaccination data.
- Wool, G.D. and Miller, J.L., 2021. The impact of COVID-19 disease on platelets and coagulation. Pathobiology, 88(1), 15-27.
- Wrapp, D., Wang, N., Corbett, K.S., Goldsmith, J.A., Hsieh, C.L., Abiona, O., Graham, B.S. and McLellan, J.S., 2020. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science, 367(6483), 1260-1263.
- Wu, A., Peng, Y., Huang, B., Ding, X., Wang, X., Niu, P., Meng, J., Zhu, Z., Zhang, Z., Wang, J. and Sheng, J., 2020a. Genome composition and

divergence of the novel coronavirus (2019-nCoV) originating in China. Cell host & microbe, 27(3), 325-328.

- Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Hu, Y., Song, Z.G., Tao, Z.W., Tian, J.H., Pei, Y.Y. and Yuan, M.L., 2020b. Complete genome characterisation of a novel coronavirus associated with severe human respiratory disease in Wuhan, China. BioRxiv, 2020-01.
- Wu, B.B., Gu, D.Z., Yu, J.N., Yang, J. and Shen, W.Q., 2020c. Association between ABO blood groups and COVID-19 infection, severity and demise: A systematic review and meta-analysis. Infection, Genetics and Evolution, 84, 104485.
- Wu, H., Xing, N., Meng, K., Fu, B., Xue, W., Dong, P., Tang, W., Xiao, Y., Liu, G., Luo, H. and Zhu, W., 2021. Nucleocapsid mutations R203K/G204R increase the infectivity, fitness, and virulence of SARS-CoV-2. Cell host & microbe, 29(12), 1788-1801.
- Wu, Z. and McGOOGAN, J.M., 2020. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese Center for Disease Control and Prevention. jama, 323.1239-1242.
- Xia, S., Zhu, Y., Liu, M., Lan, Q., Xu, W., Wu, Y., Ying, T., Liu, S., Shi, Z., Jiang, S. and Lu, L., 2020. Fusion mechanism of 2019-nCoV and fusion inhibitors targeting HR1 domain in spike protein. Cellular & molecular immunology, 17(7). 765-767.
- Xu, P., Zhou, Q. and Xu, J., 2020a. Mechanism of thrombocytopenia in COVID-19 patients. Annals of hematology, 99(6). 1205-1208.
- Xu, H., Zhong, L., Deng, J., Peng, J., Dan, H., Zeng, X., Li, T. and Chen, Q., 2020b. High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa. International journal of oral science, 12(1), 1-5.

- Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y. and Zhou, Q., 2020. Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. Science, 367(6485), 1444-1448.
- Yang, X., Yu, Y., Xu, J., Shu, H., Liu, H., Wu, Y., Zhang, L., Yu, Z., Fang, M., Yu, T. and Wang, Y., 2020a. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. The lancet respiratory medicine, 8(5), 475-481.
- Yang, A.P., Liu, J.P., Tao, W.Q. and Li, H.M., 2020b. The diagnostic and predictive role of NLR, d-NLR and PLR in COVID-19 patients. International immunopharmacology, 84, 106504.
- Yaylacı, S., Dheir, H., İşsever, K., Genc, A.B., Şenocak, D., Kocayigit, H., Guclu, E., Suner, K., Ekerbicer, H. and Koroglu, M., 2020. The effect of abo and rh blood group antigens on admission to intensive care unit and mortality in patients with COVID-19 infection. Revista da Associação Médica Brasileira, 66, 86-90.
- Young, B.E., Ong, S.W.X., Kalimuddin, S., Low, J.G., Tan, S.Y., Loh, J., Ng, O.T., Marimuthu, K., Ang, L.W., Mak, T.M. and Lau, S.K., 2020. Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. Jama, 323(15), 1488-1494.
- Zaidi, F.Z., Zaidi, A.R.Z., Abdullah, S.M. and Zaidi, S.Z.A., 2020. COVID-19 and the ABO blood group connection. Transfusion and Apheresis Science, 59(5), 102838.
- Zaki, A.M., Van Boheemen, S., Bestebroer, T.M., Osterhaus, A.D. and Fouchier, R.A., 2012. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. New England Journal of Medicine, 367(19), 1814-1820.
- Zeng, F., Huang, Y., Guo, Y., Yin, M., Chen, X., Xiao, L. and Deng, G., 2020. Association of inflammatory markers with the severity of COVID-

19: A meta-analysis. International Journal of Infectious Diseases, 96, 467-474.

- Zhang, J., Cai, Y., Xiao, T., Lu, J., Peng, H., Sterling, S.M., Walsh Jr, R.M., Rits-Volloch, S., Zhu, H., Woosley, A.N. and Yang, W., 2021. Structural impact on SARS-CoV-2 spike protein by D614G substitution. Science, 372(6541), 525-530.
- Zhang, X., Lu, S., Li, H., Wang, Y., Lu, Z., Liu, Z., Lai, Q., Ji, Y., Huang, X., Li, Y. and Sun, J., 2020. Viral and antibody kinetics of COVID-19 patients with different disease severities in acute and convalescent phases: a 6-month follow-up study. Virologica Sinica, 35(6), 820-829.
- Zhao, S., Lou, J., Cao, L., Zheng, H., Chong, M.K., Chen, Z., Chan, R.W., Zee, B.C., Chan, P.K. and Wang, M.H., 2021. Quantifying the transmission advantage associated with N501Y substitution of SARS-CoV-2 in the UK: an early data-driven analysis. Journal of travel medicine, 28(2), 11.
- Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B., Gu, X. and Guan, L., 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.
- Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R. and Niu, P., 2020. A novel coronavirus from patients with pneumonia in China, 2019. New England journal of medicine.
- Zumla, A., Chan, J.F., Azhar, E.I., Hui, D.S. and Yuen, K.Y., 2016. Coronaviruses—drug discovery and therapeutic options. Nature reviews Drug discovery, 15(5), 327-347.

APPENDIX

APPENDEX I

Questionnaire Form

Number: Date:

General History:

Name:

Age(years): Height(cm): Weight(kg):

BMI (kg/m²):

Gender: Male Female

Social status: Single Married

City:

Employment:

Address: Phone Number:

Hospital/ Lab.:

Medical History of COVID -19:

Times Infection: Time.

Vaccination: Yes No

Type of vaccine:

Date of vaccination:

Number of Doses: First Second

Infection date:

Symptoms: Cough: Headache: body ache: Fever:

Diarrhea: Vomiting: Sneezing:

Breath difficulties: Others:

Family history:

Treatments:

Day of admission to hospital:

Severity of the disease:

Mild Moderate Sever

Lab code number:

Length of stay in hospital:

1week 2week 3week

4week 5week 6week

>6weeks

General Medical History:

Heart disease: Yes No

Auto immune disease: Yes No

Diabetic: Yes No

Respiratory Problems: Yes No

Asthma: Yes No

Renal Abnormalities: Yes No

Liver Abnormalities: Yes No

Thyroid Abnormalities: Yes No

Smoking: Yes No

Drug received before COVID-19:

NOTES:

Appendix II

حكومة اقليم كوردستان/العراق
وزارة التعليم العالي و البحث العلمي
رئاسة جامعة اربيل التقنية
Polytechnic Journal

Kurdistan Regional Government
Ministry of Higher Education & Scientific Research
Erbil Polytechnic University Presidency

حکومەتی هەرێمی کوردستان/ عیراق
وەزارەتی خوێندنی باڵا و توێژینەوەی زانستی
سەرۆکایەتی زانکۆی پۆلیتەکنیکی هەولێر
هۆبەی گۆڤار

No.: 940
Date: 23-1-2023

ژمارە: / /٢٠٢٣ی زایینی
ڕێکەوت: / /٢٧٢٢ی کوردی

Acceptance Letter

To:

Poly Technic Journal

Asma Ameen Ghareeb Department of Medical Laboratory Technology, Erbil Technical Health and Medical Collage, Erbil Polytechnic University.

Sazan Moffaq Abdulaziz Department of Medical Laboratory Technology, Erbil Technical Health and Medical Collage, Erbil Polytechnic University

We are pleased to inform you that your manuscript under the title **"PHOTON Clinical & Laboratory markers as predictors for severity and mortality in COVID-19"** had been accepted for publishing in our journal in our journal volume (13) number (1) after peer review.

Gailan

Asst. Prof. Dr. Gailan Ismail Hassan

Chief of Editorial Board

24. 1. 2023

C/C:

- The journal.
- Special File.
- Outgoing

تسلسل الجيل التالي للجين الشوكي لسارس- كوف-2 المأخوذة من المرضى المصابين بكوفيد-19 مع خصائص بعض التحاليل المختبرية

رسالة

مقدمة الى مجلس الكلية التقنية الصحية والطبية_جامعة اربيل التقنية_كجزء من متطلبات نيل درجة الماجستير فى تقنيات التحليلات المرضية

من قبل

أسماء أمين غريب

بكالوريوس التقنيات الطبية فى التحليلات المرضية

باشراف

الاستاذ المساعد الدكتورة

سازان موفق عبد العزيز

اربيل_كوردستان

نيسان ٢٠٢٣ _ شوال ١٤٤٤

الخلاصة

تسبب مرض فيروس كورونا المستجد 2019 (COVID-19) في مشكلة صحية عالمية في أواخر عام 2019. نتج عن فيروس المتلازمة التنفسية الحادة الوخيمة كورونا 2 (SARS-CoV-2)، وهو فيروس RNA مغلف. تم العثور على أن العرض السريري للمرض متنوع ويتراوح بين خفيف ومتوسط وشديد. تم تسجيل عدد من الأمراض المصاحبة مثل السمنة ومرض السكري واضطرابات الأعضاء والعمر مرتبطة بمعدلات الامراضيه والوفيات.

كان الهدف من هذا البحث هو النظر في العلاقة بين بعض المتغيرات المختبريه مع الإصابة بفيروس SARS-CoV-2 في مدينة أربيل / العراق ، ودراسة انواع متغيرات الفيروس المنتشرة بين السكان المصابين من خلال تسلسل الجيل التالي (NGS)وتحليل الجين الشوكي (S).

اشتمل البحث بشكل أساسي على جمع العينات ، والتصنيف السريري للمرضى ، ودراسة بعض العلامات المختبرية والتعرف الجزيئي للفيروس متبوعًا بالتسلسل الجيني الكامل والتوصيف. تم جمع عينات من الحنجرة وعينات الدم من الحالات المشتبه بها التي قامت بزيارة المختبر المركزي فى اربيل أو تم إدخالها إلى احد المستشفيات الثلاثة الخاصة بفيروس كورونا COVID-19 (المستشفى الاماراتي، مستشفى غرب اربيل، مستشفى لالاڤ) في مدينة أربيل / العراق. تم تأكيد الإصابة في 104 مريضا بعد استخراج الحمض النووي الريبي وتحديد الفيروس بواسطة تقنية RT-PCR. بعد ذلك ، تم تصنيف المرضى سريريًا إلى خفيف (ع= 40) ، متوسط (ع = 32) وشديد (ع = 32). كما تم جمع عينات الدم من 34 عنصرسليم للعمل عليهم كاصحاء للمقارنة . تم تسجيل جميع المعلومات السريرية والديموغرافية اللازمة. تم قياس المعلمات الدموية مثل عدد الخلايا الليمفاوية ونسبتها المئويه والصفائح الدموية وغيرها من المؤشرات الحيوية (CRP و D-dimer). أخيرًا ، تم إرسال مستخلصات الحمض النووي الريبي من 15 حالة خفيفة وشديدة إلى أنقرة / تركيا من أجل التسلسل الجيني الكامل لجين S باستخدام NGS.

ارتبط العمر بشكل كبير بـ COVID-19 (قيمة P = 0.000) ، حيث كانت العدوى الشديدة شائعة في الأعمار القصوى. لم يتم العثور على علاقة بين ABO و Rh والجنس مع COVID-19 (قيمه= 0.47، 0.41 P و 0.96 على التوالي). كان معدل الوفيات مرتفعًا بين المرضى ذوي الحالات الشديدة

(17) (53.1٪)) نتيجة لأمراض مصاحبة متعددة. انخفض نسبه اشباع الاوكسجين (SPO2) بشكل ملحوظ في المجموعات الشديدة والمتوسطة مقارنة بالمجموعات الخفيفة.

فحوصات (CRP، D-dimer، و lymphocyte٪)في المجموعات المتوسطة والشديدة أظهرت علاقة عالية بشدة المرض حيث P =(0.05>) مقارنة بمجموعة التحكم. من ناحية أخرى ، كانت جميع المؤشرات الحيوية المدروسة أعلى بشكل ملحوظ في غير الناجين منها في الناجين (P <0.001). الارتباط بين D-dimer و CRP كان ايجابيا وعاليا جدا(r= 0.69، P value = 0.000) ، بينما لوحظ وجود ارتباط سلبي معنوي مع المؤشرات الحيوية المختبرية الأخرى.

تم تحديد متغيرين للفيروس بواسطه تسلسل NGS وتحليل الجين S وهما ؛ 13 دلتا (B.1.617.2) و 2 أوميكرون (B.1.1.1.529). تم تحديد المتغيرات وفقًا لمواصفات منظمة الصحة العالمية لكل متغيرات الفيروس . على العموم ، لوحظت فئات طفرة مختلفة بما في ذلك غير المرادف الذي يشكل النوع الأكثر وفرة من الطفرات والمرادفات والحذف بدون إطار وإدخال بدون إطار. تم الكشف عن اختلافات في الأحماض الأمينية L452R و T478K و P681R في البروتين الشوكي في جميع عزلات دلتا. من ناحية أخرى ، تظهر متغيرات Omicron مع عدد غير عادي من الطفرات (35 طفرة). تم العثورعلى طفرةD614G في كلا المتغيرين.

لم يرتبط الجنس ، ABO ، و Rh بـ COVID-19 ، ولكن العمر وعدد من الأمراض المصاحبة كانا مرتبطين بشكل كبير مع شدة المرض. ارتبطت جميع المؤشرات الحيوية المختبرية المدروسة بالوفيات. أظهرت متغيرات دلتا اختلافات في طفرة الجين S ، في حين أن كلا المتغيرين Omicron كانا متشابهين تمامًا. لم يتم العثور على طفرات محددة مرتبطة بشدة المرض والوفيات.

شیکردنەوەی NGS بۆ درکە بوماوەی SARS-CoV-2 لە نەخوشانی کۆڤید-١٩ لەگەل تایبەتمەندی هەندێک لە پارامێتەرەکانی تاقیگەیی

نامەیەکە

پێشکەشی ئەنجومەنی کۆلێژی تەکنیکی تەندروستی و پزیشکی هەولێر کراوە لە زانکۆی پۆلیتەکنیکی هەولێر وەکو بەشێك لە پیداویستیەکانی بەدەست هینانی پلەی ماستەر لە شیکاری نەخوشیەکان

لەلایەن

أسماء أمين غريب

بەکالۆریۆس تەکنیکی پزیشکی لە شیکاری نەخوشیەکان

سەرپەرشتیکراوە لە لایەن:

پرۆفیسۆری یاریدەدەر د.سازان موفق عبدالعزیز

هەولێر، کوردستان

گوڵان ٢٧٢٣ **شەوال ١٤٤٤** **نیسان ٢٠٢٣**

پوختە

پەتای کورونا ١٩ لە کۆتاییەکانی ساڵی ٢٠١٩ کێشەیەکی تەندروستی جیهانی دروستکرد، بەهۆی ڤایرۆسی کورونا بو SARS-CoV-2 کە ڤایرۆسێکی پۆشراوی RNA بوو. نیشانەکانی نەخۆشییەکە جیاوازە و دەردەکەوی لە ناوەندی سووک، مامناوەند و تونددەوە. ژمارەیەک نەخۆشی هاوبەشی وەک قەڵەوی، شەکرە، تێکچوونی ئەندامەکان و تەمەن پەیوەندییان بە توندی نەخۆشیەکە و مردنەوە هەبووە.

ئامانجی ئەم توێژینەوەیە بریتی بوو لە گەران بەدوای پەیوەندی نێوان هەندێک لە پارامێتەرەکانی تاقیگەیی لەگەڵ تووشبوون بە SARS-CoV-2 لە شاری هەولێر/عێراق، هەروەها لێکۆڵینەوە لە VOCs سووڕاوەکان لەنێو دانیشتوانی تووشبوودا لە ڕێگەی شیکردنە وەی NGS و شیکاری بو درکەبوماوەی (S).

لە بنەڕەتدا، تویژینەوەکە بریتی بوو لە کۆکردنەوەی نمونە، پۆلێنکردنی کلینیکی نەخۆشەکان، لێکۆڵینەوە لە هەندێک پارامێتەرەکانی تاقیگەیی و ناسینەوەی گەردیلەیی ڤایرۆسەکە و دواتر ڕێکخستن و تایبەتمەندی تەواوی درکەبوماوەی S. پشکنینی قوڕگ و لووت و نموونەکانی خوێن لەو گومان لێکراوانەی کە سەردانی تاقیگەی ناوەندییان کردووە یان لە سێ نەخۆشخانەی تایبەت بە پەتای کورونا-١٩ (نەخوشخانەی ئیماراتی، روژئاوا و لالاف) لە شاری هەولێر/عێراق وەرگیراون. تووشبوونەکە لە 104 نەخۆشدا پشتڕاستکرایەوە دوای دەرهێنانی RNA و ناسینەوە بە RT-PCR. پاشان، نەخۆشەکان لە ڕووی کلینیکیەوە پۆلێن کران بۆ سووک (n=40)، مامناوەند (n=32) و توند (n=32). هەروەها نمونەی خوێن لە ٣٤ کۆنتڕۆڵی تەندروست وەرگیرا. هەموو زانیارییە کلینیکی و دیمۆگرافییە پێویستەکان تۆمارکران. پارامێتەرەکانی خوێن وەک ژمارەی خروکە سبیە لیمفەکان و ریژەیان (%) و ژمارەی و بایۆمارکەرەکانی تر (CRP و D-dimer) پێوانە کران. لە کۆتاییدا، دەرهێنراوەکانی RNA لە 15 حاڵەتی سووک و توند ڕەوانەی ئەنقەرە/تورکیا کران بۆ شیکردنی تەواوی درکەبوماوەی S بە بەکارهێنانی شیکردنی NGS.

لە ئەنجامەکان دەرکەوت کە تەمەن پەیوەندییەکی بەرچاوی لەگەڵ پەتای کورونا-19 هەبوو (P= 0.000)، کە تێیدا تووشبوونی توند لە بەتەمەنەکاندا باو بوو. هیچ پەیوەندییەک لە نێوان ABO، Rh و ڕەگەز لەگەڵ پەتای کورونا-19 نەدۆزراوەتەوە (P= 0.24، 0.12 و 0.96 یەك بەدوای یەك)

، ڕێژەی مردن لە نێوان نەخۆشە توندەکاندا بەرز بوو (17 کەس)(53.1%) وەک دەرئەنجامی چەندین نەخۆشی هاوبەش لەو تەمەنانە.

ریزژەی CRP، D-dimer، و % خروکە سبییە لیمفوییەکان بەرز بو لە ناوەندی نەخوشە مامناوەند و توندەکان(P<0.05) بە بەراورد لەگەڵ گروپی کۆنترۆڵ. لە لایەکی دیکەوە، هەموو بایۆمارکەرەکانی لێکۆڵینەوەکراو لە کەسانی ڕزگارنەبوودا بە شێوەیەکی بەرچاو بەرزتر بوون لە چاو ئەوانەی ڕزگاریان بوو (P<0.001) زۆر بە شیوەیکی بەرز پەیوەندی هەبوو لە نێوان D-dimer وCRP (r= 0.69، P= 0.000)، لە کاتێکدا پەیوەندییەکی نەرێنی بەرچاو لەگەڵ بایۆمارکەرەکانی تری تاقیگەیی بینرا.

شیکاری NGS بۆ بوماوەی S دوو جۆری SARS-CoV-2 ی دەستنیشان کرد؛ 13 دەلتا (B.1.617.2) و 2 ئۆمیکرۆن (B.1.1.529). جۆرەکان بەپێی تایبەتمەندیەکانی ڕێکخراوی تەندروستی جیهانی بۆ هەر VOCیەک دەستنیشان کران. بەگشتی، پۆلە جیاوازەکانی بازدان بینراون لەوانە ناهاوواتا کە زۆرترین جۆری بازدانەکانیان پێکهێناوە، هاوواتا، سڕینەوەی ناچوارچێوەگۆڕین و زیادبونی ناچوارچێوەگۆڕین. گۆڕانکارییەکانی ترشە ئەمینیەکانی L452R، T478K و P681R لە پڕۆتینی سپایک لە هەموو جیاکراوەکانی دەلتا دۆزرانەوە و تایبەت بوون بە جۆرەکە. لە لایەکی دیکەوە، جۆرەکانی ئۆمیکرۆن بە ژمارەیەکی نائاسایی بازدانەوە دەردەکەون (35بازدان). بازدانی D614G لە هەردوو جۆرەکەدا پارێزراو بوو.

ڕەگەز، ABO، و Rh پەیوەندییان بە COVID-19ەوە نەبووە، بەڵام تەمەن و ژمارەیەک نەخۆشی هاوبەش پەیوەندییەکی بەرچاویان بە تووندی نەخۆشییەکە هەبووە. هەموو بایۆمارکەرەکانی تاقیگەیی لێکۆڵینەوەیان لەسەر کراوە پەیوەندییان بە مردنەوە هەبووە. جۆرەکانی دەلتا جیاوازیان لە گۆڕانی بوماوەی Sدا نیشان دا، لە کاتێکدا هەردوو جۆری ئۆمیکرۆن بە تەواوی وەک یەک بوون. هیچ بازدانیکی (گۆڕانکارییەکی) تایبەت نەدۆزراوەتەوە کە پەیوەندی بە تووندی و مردنی پەتای کورونا-١٩ەوە هەبێت.