ELUCIDATING THE ROLE OF ALPHA-1-ANTITRYPSIN, IL-6, TNF-α, TAS AND MDA IN THE PATHOGENESIS OF COVID-19 POSITIVE CASES

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by

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IN THE NAME OF ALLAH, THE MOST BENEFICIENT AND MERCIFUL

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

α	Alpha
µL	Microlitre
p	P-value
˚C	Degree Celsius
g/L	Gram per Litre
nm	Nanometre
mL	Millilitre
pg/mL	Picogram per Millilitre
mmol/L	Millimole per Litre
mmol Trolox Equiv./L	Millimole Trolox Equivalent per Litre
µmol/L	Micromole per Litre

LIST OF ABBREVIATIONS

A1AT	Alpha-1-Antitrypsin
AATD	Alpha-1-Antitrypsin Deficiency
IL-6	Interleukin-6
IL-8	Interleukin-8
TNF-α	Tumor Necrosis Factor Alpha
MDA	Malondialdehyde
TAS	Total Antioxidant Status
ELISA	Enzyme-linked Immunosorbent
ARDS	Acute Respiratory Distress Syndrome
NE	Neutrophil Elastase
CTLs	Cytotoxic T Lymphocyte
NK	Natural Killer Cell
CD8+ T	Cytotoxic T Cell
NKG2A	Natural Killer Cell Receptor Group 2 Member A
CRP	C-Reactive Protein
PCT	Procalcitonin
ESR	Erythrocyte Sedimentation Rate
NO	Nitric Oxide
ROS	Reactive Oxygen Species
ACE-2	Angiotensin-Converting Enzyme-2
HRPZII	Hospital Raja Perempuan Zainab II
NMRR	National Medical Research Register
JEPeM	Human Research Ethics Committee of USM
ECLIA	Electrochemiluminescence Immunoassay

WHO	World Health Organisation
COVID-19	Coronavirus Disease 2019
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
RaTG13	Bat Coronavirus
RT-PCR	Real-Time Polymerase Chain Reaction
PI	Proteinase Inhibitor
BSL-2	Biosafety Level-2
CRM 470	Certified Reference Material 470
LOQ	Limit of Quantitation
HRP	Horseradish Peroxidase
TMB	Tetramethylbenzidine
OD	Optical Density
4-PL	4-Parameter Logistic
ABTS	2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid
ENZ	Reagent Application Mask
TTF3	Tetrathiafulvalene-3
SD	Standard Deviation
IQR	Interquartile Range
S1	Subunit 1 Protein
S2	Subunit 2 Protein
TMPRSS2	Transmembrane Serine Protease 2
RBD	Receptor Binding Domain
S protein	Spike Protein
D614G	Aspartic Acid-to-Glycine Substitution at Amino Acid Position 614
A2a	Adenosine Receptor
CRS	Cytokine Release Syndrome
NF-κβ	Nuclear Factor Kappa-β

LIST OF APPENDICES

MENJELASKAN PERANAN ALPHA-1-ANTITRYPSIN, IL-6, TNF-α, TAS DAN MDA DALAM PATOGENESIS KES POSITIF COVID-19

ABSTRAK

Kajian ini bertujuan untuk menyiasat perbezaan dalam tindak balas tekanan imun dan oksidatif dalam pesakit COVID-19 dengan menganalisis tahap Alpha1-antitrypsin (A1AT) dan varian fenotipnya, serta interleukin-6 (IL-6) dan faktor nekrosis tumor (TNF-α), malondialdehid (MDA), dan jumlah status antioksidan (TAS). Kajian kawalan-kes ini melibatkan 282 peserta, termasuk kontrol, pesakit COVID-19 tahap ringan sehingga sederhana, dan pesakit COVID-19 tahap teruk sehingga kritikal, yang berumur 18 hingga 80 tahun. Sampel darah pesakit COVID-19 yang telah dimasukkan ke hospital di Kelantan dan Selangor antara Julai 2021 dan Jun 2023 telah dikumpulkan. Dalam tempoh yang sama, kontrol telah direkrut. Kajian ini mendapati perbezaan yang ketara pada tahap A1AT antara kontrol dan pada pesakit COVID-19 pada tahap yang teruk ($p<0.01$), tetapi bukan antara kontrol dan pada pesakit yang pada tahap ringan hingga sederhana ($p=0.47$) atau antara pesakit tahap ringan hingga sederhana dan tahap teruk hingga kritikal ($p= 0.33$). Paras IL-6 dan TNF-α adalah lebih tinggi dengan ketara dalam pesakit COVID-19 berbanding dalam kontrol ($p<0.001$), tanpa perbezaan yang ketara merentas peringkat kategori penyakit COVID-19. Di samping itu, tahap TAS adalah kurang pada pesakit dengan COVID-19 berbanding dengan mereka dalam kumpulan kontrol ($p<0.001$). Sementara itu, tahap MDA meningkat dengan ketara dalam pesakit COVID-19 berbanding kontrol ($p<0.001$). Kedua-dua TAS dan MDA tidak menunjukkan perbezaan yang ketara merentas kategori COVID-19. Analisis menunjukkan bahawa fenotip PiMM muncul sebagai fenotip utama dalam kalangan peserta, tanpa mengira status COVID-19

mereka atau menjadi sebahagian daripada kumpulan kontrol. Selain itu, kajian ini mengenal pasti beberapa fenotip normal yang jarang diperhatikan, seperti PiBM, PiCM, PiEM dan PiMX. Tambahan pula, peserta tidak mempamerkan varian yang berkaitan dengan kekurangan A1AT, terutamanya PiS dan PiZ. Penyelidikan ini merupakan langkah asas ke arah memahami asas genetik dan biokimia COVID-19, yang membuka jalan bagi pendekatan perubatan dalam mengurus dan merawat penyakit ini. Kajian lanjut diperlukan untuk membolehkan penemuan ini, yang berpotensi membawa kepada pembangunan terapi yang bersasar dan strategi pencegahan berdasarkan kecenderungan genetik dan profil penanda biokimia.

ELUCIDATING THE ROLE OF ALPHA-1-ANTITRYPSIN, IL-6, TNF-α, TAS AND MDA IN THE PATHOGENESIS OF COVID-19 POSITIVE CASES

ABSTRACT

The study aimed to investigate the disparities in immune and oxidative stress responses in COVID-19 patients by analysing the levels of Alpha1-antitrypsin (A1AT) and its phenotype variants, as well as interleukin-6 (IL-6) and tumour necrosis factor (TNF-α), malondialdehyde (MDA), and total antioxidant status (TAS). This case-control study involved a total of 282 participants, including healthy controls, mild to moderate COVID-19 patients, and severe to critical COVID-19 patients, aged 18 to 80. COVID-19 blood samples were archived from the patients that hospitalized in Kelantan and Selangor between July 2021 and June 2023. During the same period, healthy control was recruited. The study found a significant difference in A1AT levels between control and severe COVID-19 patients ($p<0.01$), but not between control and mild to moderate patients ($p=0.47$) or between mild to moderate and severe to critical patients ($p=0.33$). IL-6 and TNF-alpha levels were significantly higher in COVID-19 patients than in controls ($p<0.001$), with no significant difference across different stages of COVID-19 disease. Additionally, TAS levels were reduced in patients with COVID-19 compared to those in the control group ($p<0.001$). Meanwhile, MDA levels significantly increased in COVID-19 patients compared to control patients ($p<0.001$). Both TAS and MDA showed no significant difference across the COVID-19 group. The analysis indicated that the PiMM phenotype emerged as the predominant phenotype among participants, regardless of their COVID-19 status or being part of the healthy control group. Additionally, this study identified some infrequently observed normal phenotypes, such as PiBM, PiCM, PiEM, and PiMX. Furthermore,

participants did not exhibit variants associated with A1AT deficiency, notably PiS and PiZ. This research lays a foundational step toward understanding the genetic and biochemical underpinnings of COVID-19, paving the way for personalized medicine approaches in managing and treating this disease. Further studies are necessary to build on these findings, potentially leading to the development of targeted therapies and preventive strategies based on genetic predispositions and biochemical marker profiles.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

At the end of 2019, a novel coronavirus identified as 2019-nCov was detected in Wuhan, China (Munster et al., 2020). Due to the severe nature of the situation, the Chinese government declared a public health emergency and initiated a formal investigation into the matter on December 31, 2019. Following that, the World Health Organization (WHO) labelled the illness as Coronavirus Disease 2019 (COVID-19), and the pathogenic virus was designated as SARS-CoV-2 by the International Committee on Taxonomy of Viruses (Gorbalenya et al., 2020). COVID-19 caused a global pandemic that resulted in a dramatic loss of human life globally, and genomic analysis discovered that COVID-19 is phylogenetically connected to severe acute respiratory syndrome-like (SARS-like) bat viruses, suggesting that bats may be the source (Sheeren et al., 2020). The virus known as RaTG13, isolated from a *Rhinolophus affinis* bat tested in the province of Yunnan in 2013, remains the closest known relative to SARS-CoV-2 (Zhou et al., 2020)

COVID-19 is a contagious illness mainly spread through airborne droplets or indirect contact with contaminated surfaces (Hashim et al., 2021). Coronaviruses can invade brain tissue, including microglia, astrocytes, and macrophages, and cause nerve damage through direct nerve infection (Beghi et al., 2020). Headaches, dizziness, seizures, decreased level of consciousness, sudden haemorrhagic necrotising encephalopathy, agitation, and disorientation are among the neurologic symptoms that

COVID-19 patients will experience (Filatov et al., 2020). Following that, COVID-19 patients will be infected with Panton-Valentine leukocidin-secreting Staphylococcus aureus, which will cause necrotising pneumonia (Duployez et al., 2020). Individuals diagnosed with COVID-19 may present with significant pulmonary embolism and subsequent right-sided heart failure (Ulah et al., 2020). COVID-19 can cause cytokine storms, which are intense inflammatory processes accompanied by increased oxidative stress, resulting in lung injuries and organ damage (Ye et al., 2020). Moreover, variables such as age, gender, and coexisting medical problems are significant predictors to the severity and fatality of COVID-19 (Feng et al., 2019). Although COVID-19 infects persons of all ages and genders, evidence indicates that those who have comorbidities are more vulnerable to COVID-19 infection (Biswas et al., 2021).

Alpha1-antitrypsin (A1AT) is an acute phase reactant, constitutive tissue protector, antiviral, and anti-inflammatory molecule that has been suggested as a COVID-19 infection inhibitor. Individuals with genetic A1AT deficiency (AATD) unquestionably lack control over inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) associated with an increase in oxidative stress such as malondialdehyde (MDA) and decrease in total antioxidant status (TAS), which is thought to be a risk factor for lung injury and the severity of COVID-19 infection. Compelling evidence suggests that oxidative stress and the body's ability to defend against reactive oxygen species (ROS) are critical in developing COVID-19 at various stages of disease progression. The imbalance between mechanisms that promote oxidative stress and those that provide defense and repair may result in molecular and cellular injury, as well as the activation of stress responses in inflammatory pathways (Ebrahimi et al., 2021). Understanding the role of A1AT in COVID-19 infection and

its impact on COVID-19 progression might lead to improved knowledge of the pathogenesis of an illness and novel treatment options. Pathogenesis of COVID-19 is the entry of the virus into the cell through spike (S) protein which facilitates by the transmembrane serine protease 2 (TMPRSS2). The TMPRSS2 mediated COVID-19 spike protein binding is crucial for the virus to enter host cells through the angiotensin-converting enzyme 2 (ACE2) which later, induces intense inflammatory production and increase oxidative stress which can lead to tissue damage, ARDS, and multi-organ failure (Hoffmann et al., 2020). A1AT effectively inhibits TMPRSS2 and thus restrict the entry of COVID-19 into the cell, making it a promising anti-COVID-19 treatment that might block viral entrance (Callahan et al., 2020). However, until now there has been a lack of a well-established study that evaluated A1AT levels and AATD as an ideal candidate for understanding the pathogenesis and progression of COVID-19 (Herth et al., 2021).

The patient was categorised into five stages based on Annex 2e clinical management of the confirmed COVID-19 case in adults by the Ministry of Health, Malaysia. This study compares the serum levels of A1AT, pro-inflammatory markers (IL-6 and TNF-α), and oxidative stress markers (MDA and TAS) at different stages of COVID-19 infection with healthy control. This study also aims to investigate the A1AT phenotype in the serum of COVID-19 patients and control. The understanding of the correlation of A1AT level with pro-inflammatory, oxidative stress markers, and A1AT phenotype with clinical findings could provide the possibility that A1AT may represent a novelty in the management approach towards expecting the viral burden. The mechanism by which A1AT modulates the immune response and viral replication could offer a dual benefit in reducing the severity of lung injury and controlling the spread of the virus

within the body. Further research into the application of A1AT in COVID-19 treatment regimens could pave the way for new strategies for managing viral infections, particularly those that lead to severe respiratory complications.

1.2 Problem statement and study rationale

Healthcare providers have encountered challenges in their efforts to mitigate the impact of COVID-19 across various geographical areas while also delivering vital healthcare services to individuals. Individuals infected with COVID-19, especially in severe cases, display acute respiratory distress syndrome (ARDS), and urgent medical intervention is required as it can result in respiratory failure. This condition arises due to a phenomenon known as a “cytokine storm” which entails a robust inflammatory response accompanied by elevated oxidative stress. COVID-19 disease severity has been associated with inflammatory biomarkers, such as interleukins and TNF-α. Despite all these, it is believed that overproduction and elevation of oxidative stress markers such as MDA, and deprived antioxidant systems play an essential role in the pathophysiology of COVID-19 infection and the severity of respiratory disease.

Biomarkers were essential in directing patients' clinical management with COVID-19 and global vaccine development efforts. Unfortunately, most studies have been retrospective, possibly due to a significant gap in prospective cohort studies. Given the intricate characteristics of the COVID-19 pandemic, there is an imperative need for expeditious investigation and analysis of prospective research findings to enhance comprehension and optimize treatment interventions for individuals afflicted with

COVID-19. Among other biomarkers, A1AT was a promising candidate because it protects the lung tissue from destruction. A1AT is an acute-phase protein that plays a role in inflammation control by inhibiting inflammatory molecules such as IL-8, TNF-α, and neutrophil elastase, as well as having an antioxidative stress response and anti-apoptotic properties. A1AT was thought to be an inhibitor of COVID-19 infection. If the hypothesis is correct, genetic variants on A1AT imply that A1AT could have a role in the differing severity of COVID-19 responses among individuals.

1.3 Objectives of the Study

1.3.1 General Objective

This study aims to elucidate the serum levels of A1AT, pro-inflammatory markers, and oxidative stress markers and determine the A1AT phenotypes in COVID-19 patients and healthy controls.

1.3.2 Specific Objectives

1) To compare serum A1AT levels at different stages of COVID-19 infection with the healthy control.

2) To compare the serum pro-inflammatory markers (IL-6 & TNF-α) levels at different stages of COVID-19 infection with the healthy control.

3) To compare the serum oxidative stress markers (TAS & MDA) levels at different stages of COVID-19 infection with the healthy control.

4) To determine the phonotypic of A1AT variants in the peripheral blood among patients with COVID-19.

CHAPTER 2

LITERATURE REVIEW

2.1 COVID-19's alarming global prevalence

In December 2019, a new coronavirus variant was discovered in Wuhan, China, as the causative culprit for pneumonia cases. This pathogen's rapid and widespread dissemination prompted the World Health Organization (WHO) to formally designate SARS-COV-2 as a pandemic on March 11, 2020. The abrupt emergence, sustained person-person transmission, and expeditious global propagation have contributed to the enduring nature of the pandemic. There were 30 556 004 active cases and 5 323 214 deaths worldwide as of December 12, 2021. According to data from China, 81% of individuals diagnosed with COVID-19 experienced mild or moderate symptoms, which include those with undiagnosed pneumonia and those with less severe pneumonia. 14% of cases were classified as severe, while 5% were considered critical (Wu & McGoogan, 2020). The Ministry of Health Malaysia (2021) recorded 4,626 COVID-19 cases in Malaysia as of December 11, 2021, with 31 deaths and 4,690 recovery cases. As of October 13, The Ministry of Health Malaysia (2022) reported coronavirus disease 2019 (COVID-19) cases topped 4.8 million. Moreover, the infection caused 7082 clusters, just 13 of which were active as of October 2022, and the outbreak pattern in Malaysia in 2022 reveals that beginning in February, there was a spike in the total number of daily reported cases led by the Omicron variant (Abd Gani et al., 2023).

The tremendous scientific effort to develop an effective COVID-19 vaccine has resulted in various safe and effective alternatives (Altman & Boyton, 2022). As of April 8, 2022,

WHO had examined nine vaccines against COVID-19: Pfizer/BioNTech, Moderna, Johnson & Johnson, AstraZeneca/Oxford, Sinovac, Sinopharm, Covaxin, Covovax, and Nuvaxovid, all of which fulfilled the safety and effectiveness criteria (WHO, 2022). During the first week of symptoms, the standard for diagnosis is real-time polymerase chain reaction (RT-PCR), which detects viral genomes in respiratory materials, while antibody-based techniques are introduced as additional tools (Carter et al., 2020). According to Wang et al., (2020), the detection rate of COVID-19 in nasal swabs was higher [63 % (5/8)] than in pharyngeal swabs [32 % (126/398)]. Along with radiological findings, signs and symptoms are used to diagnose suspected COVID-19 infection and identify people with COVID-19 pneumonia (Malik et al., 2021).

The swift emergence of successive variants of concern, characterized by an extensive array of mutations (notably, Omicron variants exhibit over 50 mutations in their spike protein), necessitates the development of novel vaccine strategies targeting these variants (Shrestha et al., 2022). This imperative arises from the diminished efficacy of conventional vaccines against these novel variants and the requirement for periodic booster doses to sustain adequate levels of neutralizing antibodies for protection against infection and potential reinfections (Feikin et al., 2022). Although COVID-19 is no longer making headlines, it remains a significant worldwide health threat. Lack of access to lifesaving tools such as diagnostics, therapeutics, and vaccines is still a problem. Vaccination demand is now low globally. Misinformation and deception hinder the capacity to launch an efficient response. Yet, specific demographics, such as the elderly and immunocompromised individuals, are more vulnerable to severe outcomes. Additionally, long-term COVID-19, which refers to persistent and severe symptoms following a COVID-19 infection, can affect everyone.

2.2 Role of A1AT in COVID-19 disease

SARS-CoV-2, the virus responsible for COVID-19, is the third beta-coronavirus and the second sarbecovirus to appear in humans in the 21st century (Zhu et al., 2020). More coronaviruses may probably penetrate the species barrier, causing subsequent pandemics. Although effective COVID-19 vaccinations are available, future strains of SARS and other coronaviruses are expected to continue causing issues (Voskarides K., 2022). It is postulated that the SARS-CoV-2 spike protein attaches to its receptor, ACE2, on cell membranes and is cleaved in a precise order by the serine proteases furin at the S1/S2 site and TMPRSS2 at the S2' site. Pneumocytes, a particular kind of cell seen in the human lung and other tissues, express high levels of ACE2, which the viral spike (S) protein's receptor binding domain (RBD) interacts with (Battacharyya et al., 2021). Gradual interactions with ACE2 molecules cause the S1 head to separate from the fusogenic S2 stalk, enabling fusion activation through additional proteolysis at the S2 site (Peng et al., 2021). Moreover, the D614G mutation in the A2a SARS-CoV subtype has an elastase cleavage site close to the S1-S2 protein. This suggests that neutrophil elastase is very important in this infection (de Loyola et al., 2021). Evidence suggests that A1AT, a major natural serine protease inhibitor (serpin), can prevent SARS-CoV-2 infection and improve COVID-19 disease processes (Bai et al., 2023). A1AT inhibits elastase-induced lung tissue injury, and it cuts around the S1/S2 site for SARS-CoV-2 with the spike protein mutation D614G that increases activation of this spike protein and virus spread (Battacharyya et al., 2021). Figure 2.1 illustrates the proposed mechanisms of A1AT in COVID-19.

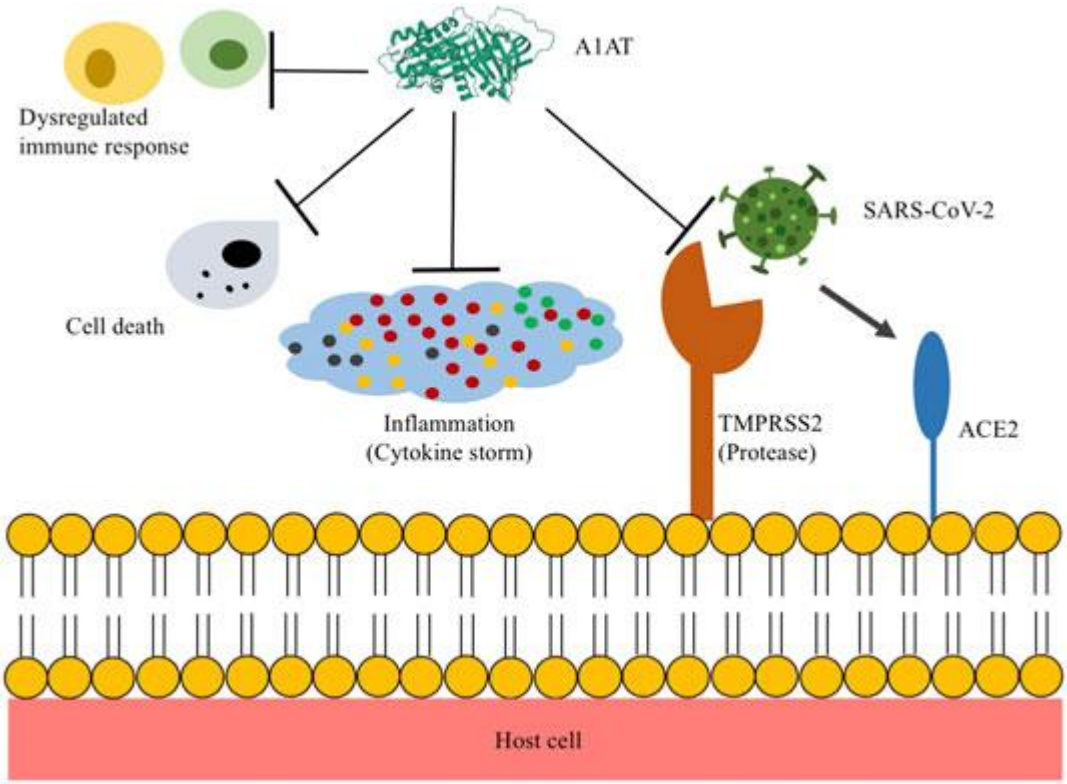

Figure 2.1: Mechanisms of A1AT in COVID-19 (Yang et al., 2020)

Figure 2.1. The ingress of SARS-CoV2 into host cells occurs via the attachment of the viral S-protein to ACE2 present in host cells, facilitated by TMPRSS2. A1AT inhibits TMPRSS2, hence diminishing SARS-CoV-2 infection. A1AT can mitigate acute inflammatory responses, cellular apoptosis, neutrophil elastase trap formation, coagulation activity, and dysregulated immunological responses (Yang et al., 2020).

2.3 Biomarkers in patients with COVID-19

Biomarkers were essential in the early detection, diagnosis, monitoring, and recognition of complications, and the management and disposition of COVID-19 patients. Clinical evaluation is crucial; however, biomarkers can offer additional, new insights that significantly influence various aspects of patient care (Samprathi & Jayashree, 2021). Utilising these biomarkers to analyse COVID-19 may also reduce the likelihood of

virus-induced complications, such as severe hypoxemic respiratory failure and multi-organ dysfunction, including acute cardiac, hepatic failure and renal impairment. In the study of COVID-19, the primary pathological abnormalities were identified, including an impairment of the immune system and severe lung infections (Malik et al., 2021). On the other hand, the temporal variation of biomarkers throughout the illness was crucial in deciding disease progression and therapeutic response (Samprathi & Jayashree, 2021). According to Kong et al., (2020), white blood count, neutrophil count, D-dimer, PCT, CRP, and IL-6 had the highest total amount across increasing clinical stages. In contrast, lymphocyte count had the lowest total amount across increasing clinical stages. Because of the rapid rise in COVID-19 cases worldwide, prospective study results are urgently needed to understand better and treat patients infected with COVID-19 (Malik et al., 2021).

2.4 Inflammatory markers in COVID-19

2.4.1 IL-6

In severe COVID-19, the immune system can overreact to COVID-19, leading to a cytokine storm which is a massive release of pro-inflammatory cytokines (IL-6 & TNF-α) (Ye et al., 2020). IL-6 is an essential cytokine whose production is associated with a variety of inflammatory diseases. It can be generated by stromal cells, practically all immune cells, as well as endothelial cells, fibroblasts, keratinocytes, and tumor cells. The occurrence of cytokine release storm (CRS) in patients with severe COVID-19-induced disease may be attributed to IL-6 amplifier, which enhances the release of pro-inflammatory cytokines like IL-6 through nuclear factor kappa-β (NF-κβ)

hyperactivation (Liu et al., 2020). Furthermore, IL-6 is a target of NF-κβ, and its activation promotes IL-6 synthesis (Hirano & Murakami, 2020). IL-6 is a reliable indicator of the severity and prognosis of COVID-19 induced disease (Biran et al., 2020). This overwhelming inflammatory response can cause extensive lung damage, multi-organ failure, and increased mortality, regardless of A1AT levels (Chen et al., 2021). Immune responses initiate anti-body production after being infected by a virus (Chowdhury et al., 2020). Cytotoxic lymphocytes, such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, are required for viral infection control, and cytotoxic lymphocyte functional exhaustion is associated with disease progression (Zheng et al., 2020). The total number of NK and CD8+ T cells might have been significantly reduced in patients with COVID-19 infection and the function of NK and CD8+ T cells has been depleted in COVID-19 patients due to increased expression of NKG2A (Zheng et al., 2020). In this present study, we evaluate the concentrations of IL-6 in individuals diagnosed with clinical COVID-19, categorizing the cases as mild, moderate, or severe.

2.4.2 TNF-α

TNF-α, produced by macrophages and monocytes, is an early effector that alerts the host's immunity to viral infections by binding to the compatible TNF-α receptor. It can induce cellular apoptosis, modulate innate immune responses, and promote the infiltration of macrophages, dendritic cells, natural killer cells, and neutrophils (Jang et al., 2021). TNF-α, a crucial multifunctional mediator of both acute and chronic systemic inflammatory responses, promotes the synthesis of diverse chemokines and cytokines, and is involved in various physiological processes, such as anti-tumor responses,

inflammation modulation, and immune system homeostasis (Ragab et al., 2020). As one of the most prominent pro-inflammatory cytokines in the innate immune response, dysregulated TNF-α signalling can lead to cytokine release syndrome (CRS). Furthermore, biomarkers associated with inflammation, such as cytokines, CRP, PCT, serum ferritin, and ESR, are correlated with a higher likelihood of severe development of COVID-19 (Qin et al., 2019). Besides, COVID-19 triggers innate and adaptive immune responses, producing TNF-α, and other cytokines, increased vascular permeability that correlate with respiratory failure (Hadjadj et al., 2020). Immune dysregulation is a therapeutic target for COVID-19 patients. The causes for the high levels of inflammatory cytokines are unclear, although they may play a role in organ damage-related cell death (Vatansever & Becer., 2020). In this study, we evaluate the concentrations of TNF-α in individuals diagnosed with clinical COVID-19, categorizing the cases as mild, moderate, or severe. Additionally, we examined the relationship between these cytokine levels and associated risk factors.

2.5 Oxidative Stress and COVID-19

2.5.1 MDA

In oxidative stress effects can be discovered by assessing oxidative stress indicators and antioxidants. Oxidative stress is a key factor in the severity of COVID-19 in some individuals, and it has been associated with pulmonary dysfunction, cytokine storms, and viral sepsis caused by COVID-19 infection (Beltran et al., 2020). MDA, a molecule produced by lipid peroxidation, and nitric oxide (NO), a free radical byproduct that may act as a vasodilator, are important oxidative stress biomarkers (Ahmad et al., 2019). During lipid peroxidation (oxidation of lipids by ROS), excess chemicals are formed,

such as the oxidative marker MDA, and these sorts of compounds can act as harmful agents, changing the structures and functions of numerous cell components (Zarkovic et al., 2021). MDA is an outcome of the cyclo-oxygenase activity in prostaglandin metabolism, and previous investigations have shown that serum MDA levels correlate strongly with COVID-19 severity (Karkhanei et al., 2021). Moreover, the function of ACE-2 is implicated in the development of oxidative stress throughout the progression of COVID-19. This oxidative stress is critical for the host's viral invasion, the virus's multiplication, and the illness's ensuing progression. Oxidative stress marker such as MDA facilitates the oxidation of cysteine residues on viruses and ACE-2 proteins, forming disulfide bonds, which, in turn, strengthens the binding interaction between SARS-CoV-2 and ACE-2, aggravating the pathogenesis of COVID-19 (Suhail et al., 2020). Hence, this study aimed to evaluate the oxidative stress parameters as probable fundamental processes in patients with mild to moderate and severe to critical cases of COVID-19.

2.5.2 TAS

Elevated production of ROS leads to oxidative stress, which can significantly impact respiratory diseases, including COVID-19, especially in the presence of high levels of free radicals due to a deficiency in antioxidant defense such as lower level of TAS (Ntyonga–Pono et al., 2020). Free radicals are a natural by-product of aerobic cell metabolism that the body can handle. Still, when a secondary condition like COVID-19 is present, the abnormally large level of radicals may contribute to the progression and pathogenesis of the COVID-19 disease because antioxidants (TAS) are depleted (Weir et al., 2020). Phagocytes elicit the production of ROS through an oxidative burst to

neutralize pathogens during pathogen exposure (Li et al., 2021). In circumstances where infections demonstrate resilience against this response, the adaptive immunity of the organism is engaged. This adaptive response involves generating and presenting pathogen-derived antigenic peptides, which are synthesized during phagocytosis and digestion, to T cells (Gasteiger & Rudensky, 2014). The activation of T lymphocytes prompts their proliferation and differentiation, culminating in the formation of immunological effector cells. These cells are adept at initiating a robust and antigen-specific immune response (Brownlie & Zamoyska, 2013). Research indicates that patients afflicted with COVID-19 exhibit reduced quantities of T cells, particularly CD8+ T cells, a phenomenon associated with the disease's severity and progression (Liu J. et al., 2020). Gaining knowledge of the underlying processes and pinpointing efficacious remedies for the COVID-19 epidemic is of utmost importance.

2.6 Alpha-1 antitrypsin (A1AT) as an acute phase reactant

Acute phase reactants are specific proteins that serve as markers of inflammation, undergoing significant changes in their concentration in the bloodstream during inflammatory responses. These crucial proteins are synthesized in the liver during acute and chronic inflammatory conditions (Gulhar et al., 2018). A1AT has diverse functions that include anti-inflammatory, immunomodulatory, anti-infective, and tissue-healing properties (Strnad et al., 2020). A1AT is produced by various cell types, including hepatocytes, intestinal and pulmonary alveolar cells, neutrophils, macrophages, and the cornea (Strnad et al., 2020). Notably, the liver synthesises roughly 34 mg of A1AT per kg of body weight daily, leading to a plasma level ranging from 0.9 to 1.75 mg/ml, with

a half-life of 3 to 5 days (Strnad et al., 2020). During the acute-phase reaction, individuals with a normal proteinase inhibitor (PI) genotype (MM) may experience an excessive increase in A1AT levels. Conversely, the magnitude of this rise is significantly diminished in persons with severely deficient alleles (Strnad et al., 2020). Given its anticoagulant properties and its potential to safeguard against inflammation, apoptosis, and the formation of neutrophil extracellular traps, A1AT has emerged as a promising candidate for treating COVID-19 (Yang et al., 2020).

2.7 Common deficiency variants of Alpha-1 antitrypsin

Alpha-1-Antitrypsin Deficiency (AATD) is an inherited genetic condition associated with low serum alpha 1 protease inhibitor levels (A1-PI; also known as [A1AT]) and it suppresses nonspecific degradation by serine protease neutrophil elastase (NE), an enzyme that degrades bronchial and alveolar wall integrity (Chapman et al., 2018). A1AT is codified by the SERPINA1 gene located on human chromosome 14q32 (Gramegna et al., 2018). The consequence of misfolded, aggregated protein is thought to trigger the development of liver illness. Conversely, a deficiency of A1AT in the systemic circulation can lead to an increased susceptibility to lung injury, premature onset of lung emphysema, and the development of chronic obstructive pulmonary disease (Lomas et al., 2016). According to Narayanan & Mistry (2020), the most common mutations known to cause AATD involved in liver disease are the deficient Z (Glu342Lys) and S (Glu264Val) mutations. In contrast to 21 European countries, an analysis of 20 Asian nations revealed a significantly greater incidence across all five phenotypic groups associated with AATD; namely (ZZ, SS, SZ, MS, and MZ) as

reported by (Al-Jameil et al., 2017). The PiM allele is identified as the most common and biologically standard variant. Typically, individuals possess two copies of the M allele, represented as MM, in each cell. It is observed that the S allele leads to a moderate reduction in A1AT production, whereas the Z variant results in a substantial decrease. Heterozygote MS (or homozygote SS variant) produce enough A1AT to protect the lungs. MZ allele carriers have a slightly higher risk of liver and lung dysfunction (Maseeha et al., 2024). Whereas the most common mutations that cause clinical A1AT deficiency are phenotypes PiZ and PiS (Al-Jameil et al., 2017). Diagnostic methods rely heavily on the analysis of PiZ and PiS genotypes in the AATD patient population (Matamala et al., 2020). According to Yang et al., (2021), individuals diagnosed with AATD exhibit heightened susceptibility to COVID-19 due to the upregulated activation of transmembrane protease serine 2 (TMPRSS2) within this patient cohort. The inadequate functional A1AT in AATD sufferers facilitates the ingress of SARS-CoV-2 into cellular structures. Furthermore, A1AT serves as an inhibitor of thrombin and plasmin. Tanash et al., (2016) assert that AATD potentially raises the probability of coagulation complications. The deficiency of sufficient anti-inflammatory, anti-apoptotic, anti-protease, and anti-coagulation attributes in α1-antitrypsin may escalate the risk of extreme acute lung injury. Given the ongoing COVID-19 pandemic, urgent measures are imperative to address the perils confronting AATD patients.

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Study Setting and Study Subject

This case-control study uses the archived sample from Hospital Raja Perempuan Zainab II (HRPZII), Kota Bharu, Kelantan, and Hospital Ampang, Selangor from 1st July 2021 until 30th June 2023. This study has been approved by the National Medical Research Register (NMRR) and the Human Research Ethics Committee of USM (JEPeM) (refer to appendix A).

3.1.2 Study of Population

Cases: Patients who presented to Hospital Raja Perempuan Zainab II (HRPZII), Kota Bharu, Kelantan, and Hospital Ampang, Selangor with positive COVID-19 at recruitment, were enrolled in the study. The diagnosis was established by a trained microbiologist based on a real-time reverse transcription-polymerase chain reaction (RT-PCR) assay from a nasopharyngeal or oropharyngeal swab. The patient was categorised into five stages based on Annex 2e clinical management of the confirmed COVID-19 case in adults by the Ministry of Health, Malaysia (Table 3.1).

Table 3.1: Clinical Staging Associated with COVID-19

Clinical stage		
1	Mild disease	Asymtomatic
2		Symtomatic, No Pneumonia
3	Moderate disease	Symptomatic, Pneumonia
4	Severe disease	Symptomatic, Pneumonia, Requiring supplemental oxygen
5	Critical illness	Critically ill with multi-organ involvement

Based on the different clinical stages, the patients were divided into two categories (Stage 1 to 3: mild to moderate disease) and (Stage 4 to 5: severe to critical illness).

Control: During the same period, healthy control subjects were recruited from healthy volunteers.

3.1.3 Subject criteria

Cases

Inclusion criteria

1. Age between 18-80 years old.
2. Positive COVID-19 based on RT-PCR.

Exclusion criteria

1. Age < 18 years old & > 80 years old.

Control

Inclusion criteria

1. Age between 18-80 years old.
2. Healthy with no history of positive COVID-19 infection.

Exclusion criteria

1. Age < 18 years old & > 80 years old.
2. Acute or chronic infections within the past month.
3. Autoimmune, allergic, neoplastic or endocrine diseases.
4. Immunocompromised or immunosuppressed patients, including patients with diabetes mellitus, malignant diseases, on long-term oral steroids or cytotoxic drugs and AIDS or HIV.

3.1.4 Sample size calculation

For objective 1, the sample size requirement was calculated using G*power software version 3.1 with the calculation based on a One-Way analysis of variance (Test Family: F tests: Statistical test: Anova fixed effects, omnibus, one-way) (Maltais et al., 2018). For a large effect size (f=0.40) to be significant at 5% alpha error probability, 80% power (1-beta error probability) and three number groups, the required sample size was 66 patients. For objective 4, the sample size requirement was calculated using the sample size formula for estimation of a single proportion, $n=(Z_{\alpha}/\Delta)^2 P(1-P)$ (Maltais F et al. 2018) reported that 31.8% of their patients were Alpha-1 antitrypsin deficient based on exon sequencing. For an estimation to a 95% level of confidence ($Z_{\alpha}=1.96$) with 10% margin of error and proportion of 31.8% (P), the required sample size was 84 patients. The largest sample size from calculation was 84 patients (from

the calculation for objective 3). Anticipating 10% dropout due to preanalytical error, the corrected sample size was 94 patients. Since in this study, we plan to do separate analysis according to the stage (Stage 1-3: mild to moderate disease), (stage 4-5: severe to critical illness), the total sample size will be 282, including COVID-19 patients (94 mild to moderate, 94 severe to critical illness) and 94 healthy control. In this study, based on different clinical stages, the patient was divided into two categories: (Stage 1-3: mild to moderate disease) and (Stage 4-5: severe to critical illness). The total sample size was 282 including COVID-19 patients (94 mild to moderate, 94 severe to critical illness) and 94 healthy controls.

3.1.5 Chemicals and reagents

All chemicals and reagents used in this study are listed in Appendix E.

3.1.6 Consumables

All kits and consumables used in this study are listed in Appendix F.

3.1.7 Kits

All kits used in this study are listed in Appendix G.

3.1.8 Laboratory equipments

All laboratory equipments used in this study are listed in Appendix H.

3.2 Methods

3.2.1 Sampling handling for cases

All eligible samples within the study period were selected. The samples were archived from Hospital Raja Perempuan Zainab II (HRPZ II), Kota Bharu, Kelantan, and Hospital Ampang, Selangor. The study used left-over of blood samples from a plain gel separator tube from admitted positive COVID-19 patients for serum A1AT, inflammatory markers, oxidative stress markers, and A1AT phenotype.

All the samples were in triple-layered packaging and transported to the Hospital USM laboratory for safety. Transportation, storage, and processing of the sample followed adequate standard operating procedures (SOPs) following the Laboratory biosafety guidance related to COVID-19 according to WHO guidelines (WHO, 2020).

3.2.1 (a) Triple-layer packaging

Plain tubes were stored vertically and closed with tube cappers and sanitised using 70% alcohol. The tube was then wrapped with biohazard plastic that was sanitised using 70% alcohol – 1st layer. The biohazard plastic sanitised using 70% alcohol and was wrapped with a 2nd layer of biohazard plastic and sanitised using 70% alcohol. The samples were kept in an airtight storage container stored in Biosafety Level-2 (BSL-2) laboratory. BSL-2 laboratories handle pathogens with moderate health risks, such as equine encephalitis, HIV, and Staphylococcus aureus. BSL-2 labs follow Biosafety Level-1 (BSL-1) protocols but require additional precautions. Personnel

must prevent injuries and exposure to infectious materials. The key BSL-2 safety regulations include using personal protective equipment (PPE), like lab coats, gloves, and eye protection, performing aerosol-generating procedures in biological safety cabinets, decontaminating infectious waste, usually with an autoclave, secure, self-latching doors, access to washbasins and eyewash stations and biohazard signage. Access to BSL-2 labs is more restricted than BSL-1, with external staff often prohibited during operations.

3.2.1 (b) Transportation of samples from Hospital Ampang to Hospital USM

The airtight storage container was wrapped in enough absorbent material to absorb all fluid in case of breakage. Then, it was placed in a primary watertight polystyrene box containing dry ice and sealed. Then, the polystyrene box was placed in an outer shipping package, which protects it and its contents from outside influences such as physical damage and water while in transit. Specimen data forms, letters and other types of information that identify or describe the specimen and identify the shipper and receiver were taped to the outside of the outer shipping package. Notification to the investigator and the post office before the delivery of the sample was done a week earlier to facilitate the process of sending and receiving samples.

3.2.1 (c) Storage of samples

All specimens for laboratory investigations should be regarded as potentially infectious (WHO, 2020). The samples were kept at -80 ˚C and stored in the BSL-2

laboratory at the School of Medical Sciences, USM, until analysis. BSL-2 labs are required due to the possible harm that the referred to pathogens pose. COVID-19 samples were handled with appropriate PPE, including disposable gloves, solid-front or wrap-around gowns, scrub suits or coveralls with sleeves that fully cover the forearms, head coverings, shoe covers or dedicated shoes, and eye protection (goggles or face shield). The left-over blood sample was taken only once. The blood sample was discarded after the study had finished.

3.2.2 Sample handling for control

The collection of samples for control is based on convenient sampling. An advertisement or memo regarding this study was placed on social media (Appendix B). The advertisement included the title, objective, and inclusion criteria for the subject. They were given a comfort room where details of the study were explained and given consent. All eligible participants were given an information sheet. In seeking informed consent, each subject was provided with a statement that participation is voluntary, and refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled. If they agreed to participate in this study, the participants were recruited, and consent was obtained (Appendix C). They also need to fill in and answer the questionnaire form provided in (Appendix D). A total of 2 ml of peripheral blood samples were collected in a plain gel separator tube to analyse serum A1AT, inflammatory markers, oxidative stress markers and A1AT phenotype. The control samples were kept in the freezer at -80 ˚C in the laboratory of School the