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GRADUATE EDUCATION INSTITUTE
MEDICAL BIOCHEMISTRY DEPARTMENT
MASTER'S PROGRAM

DETERMINATION OF OXIDATIVE STRESS PARAMETERS AND COPEPTIN LEVELS IN COVID-19 ACCORDING TO THE CLINICAL COURSE

MASTER'S THESIS

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Supervisor: Assist. Prof. Dr. Figen GÜZELGÜL

TOKAT- 2023

ETHICS CONTRACT

According to the Tokat Gaziosmanpaşa University Graduate Education Institute thesis writing guide, I declare that the Master's thesis titled "Determination of Oxidative Stress Parameters and Copeptin Levels in COVID-19 According to the Clinical Course" I have prepared under the supervision of Assist. Prof. Dr. Figen GÜZELGÜL, is an original study in accordance with scientific ethical values and rules, and that I will accept all kinds of legal sanctions if the opposite is determined.

07/08/2023

Thesis Author (Name Surname) Signature

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JURY ACCEPTANCE AND APPROVAL

The thesis study titled "**Determination of Oxidative Stress Parameters and Copeptin Levels in COVID-19 According to the Clinical Course**" prepared by **Marwa Abdelmageed** was held on (07.08.2023) and was voted unanimously / by majority of votes (in which case, choose one) by the Jury given below. It has been accepted as a Master's Thesis in Tokat Gaziosmanpaşa University Graduate Education Institute (Medical Biochemistry Department).

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Approval

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Graduate Institute Director

DEDICATION

I dedicate this dissertation to my beloved grandmother Fathiye who passed away a few days before my dissertation. I wish I could celebrate this moment with you, but I am sure you are feeling me right now and happy about this achievement.

Also, I would like to dedicate this to my whole family, especially my parents, Afaf and Fathy, my husband Amgad, my daughters Rodayna and Maria, my siblings Mustafa, Rania, Hanan, and Eman, who have accompanied me during this journey with love and effort. Thank you for your support and for being with me through the ups and downs.

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Last but not least, I would like to thank the jury members for honoring me by judging my thesis.

ÖZET

KLINIK SEYIRLERINE GÖRE COVID-19'UN OKSIDATIF STRESS VE KOPEPTIN DÜZEYLERININ BELIRLENMESI

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COVID-19 hastalığı, 2019 yılının sonlarında ortaya çıkıp dünyayı tümüyle saran bir pandemiye neden olan ve ve hayatın farklı yönlerini etkileyen bir hastalıktır. Sağlıktan eğitime hatta ekonomiye kadar çok geniş kapsamda etkili olan bu hastalık, sadece enfeksiyon sırasında değil enfeksiyonu geçirdikten sonra bile çok sayıda insanda çeşitli sağlık sorunlarının görülmesine neden olmuştur. Birçok çalışma, hastalığın patofizyolojisinde oksidatif stres ve inflamasyonun kritik rolü olduğunu bildirmiştir. Yeni bir biyobelirteç olan Kopeptin, stres kaynaklı hastalıklarda hastalığın akibetini belirlemede önemli bir rol oynayacağı düşünülmektedir. Çalışmanın amacı, COVID-19'un klinik şiddetinin oksidatif stres, inflamasyon ve kopeptin düzeyleri ile ilişkisini araştırmaktırSARS-CoV-2 için RT-PCR sonuçları pozitif olan hastalar klinik açıdan şiddetli ve hafif seyir olmak üzere gruplandırıldı. Kontrol grubu olarak, COVID-19 enfeksiyonu hiç geçirmemiş veya COVID-19 aşısı olmayan sağlıklı bireyler çalışmaya dahil edildi. Çalışmaya dahil edilen ağır seyir grupları yoğun bakım ünitesinde yatmakta iken, hafif seyir grubu ise hastaneye enfeksiyon kaynaklı hiç yatış yapmamış olgulardan oluşmaktaydı. Oksidatif stres serum Malondialdehit (MDA) ve 4-Hidroksinonenal (4-HNE) ELISA testleri ile değerlendirildi. Antioksidan kapasite için serum Melatonin (MT) ELISA testi yapıldı. Çalışmaya alınan bireylerde serum kopeptin düzeylerini

araştırmak için Copeptin (CPP) ELISA testi kullanıldı. Son olarak inflamasyonu değerlendirmek için serum Interleukin-6 (IL-6) ELISA yapıldı. Sonuçlarımızda, MDA ve 4-HNE düzeyleri hasta gruplarında hafif ve kontrol grubuna göre daha düşük olduğu bulunurken, MT seviyelerinin çalışma grupları arasından istatistiksel açıdan anlamlı bir fark bulunmadığı belirlendi. D vitaminin de çalışma grupları açısından düzeylerinin anlamlı derecede farklı olduğu belirlenmiştir. CPP düzeyinin hasta gruplarında kontrole göre istatistiksel açıdan anlamlı olarak daha düşük olduğu belirlendi. IL-6 düzeyinin, enfeksiyonun ağır klinik tablosunu yansıtmada iyi bir belirteç olabileceği gösterildi. Ayrıca çalışmamızda CRP ve ferritin düzeylerinin de gruplar arasında istatistiksel açıdan anlamlı olarak farklılık gösterdiği ve bu parametlerin de COVID-19'un klinik seyirini ayırt etmede kullanılabileceği belirlendi. Çalışmamız IL-6, CRP ve ferritinin hastalık prognozu için belirleyici olarak kullanılabileceğini gösterdi. Ancak kliniği şiddetli hasta gruplarında MDA ve 4-HNE düzeylerinin düşük olması, MDA ve 4-HNE adüktlerinin oluşmasına ve bu hasta gruplarının sonuçlarında hataya neden olabileceği ön görülmüştür. Ek olarak, tedavi uygulanan grupta MDA ve 4-HNE düzeylerinin tedavi uygulanmayan gruba göre daha düşük olması, kortikosteroid tedavilerinin oksidatif stres parametreleri üzerine etkisi olabileceğini düşündürmekte olup, bu konuda daha kapsamlı çalışmaların yürütülmesi gerektiğini düşündürmektedir.

Anahtar Kelimeler: COVID-19, Oksidatif Stres, İnflamasyon, Kopeptin, Antioksidan.

ABSTRACT

DETERMINATION OF OXIDATIVE STRESS PARAMETERS AND COPEPTIN LEVELS IN COVID-19 ACCORDING TO THE CLINICAL COURSE

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COVID-19 disease, was emerged by the end of 2019 causing a pandemic and affecting different aspects of life. This disease, which has a wide range of effects ranging from health to education and even the economy, has caused various health problems to be observed in many people not only during the infection but even after the infection. Several studies reported the critical role of oxidative stress and inflammation in the pathophysiology of the disease. Copeptin, a novel biomarker, can play an important role in predicting the disease outcome in stress-induced diseases. The aim of this study is to investigate the relationship of clinical severity of COVID-19 with oxidative stress, inflammation and copeptin levels. In addition, the study parameters were statistically evaluated as prognostic biomarkers that can be used in classifying the patients according to the clinical severity of COVID-19. Patients with positive RT-PCR results for SARS-CoV-2 were divided into severe and mild groups. Healthy individuals with neither past COVID-19 infection nor vaccination were included in the study as the control group. While the severe group consisted of patients hospitalized in the intensive care unit, the mild group consisted of those who had never been hospitalized due to infection. Oxidative stress was evaluated by serum Malondialdehyde (MDA) and 4-Hydroxynonenal (4-HNE) ELISA tests. For the antioxidant capacity, serum Melatonin (MT) ELISA test was carried out. Copeptin (CPP) ELISA test was used to investigate serum copeptin levels among

the study individuals. Finally, for evaluating inflammation, serum Interleukin-6 (IL-6) ELISA was performed. Our results showed that MDA and HNE-4 levels were lower in the severe group compared to mild and control groups. Additionally, IL-6 levels were significantly different between the study groups. Also, vitamin D levels showed a statistically significant difference between the study groups, where patient group was characterized by lower vitamin D levels compared to the control group. However, MT levels did not show any significant difference between the study groups. It was determined that the CPP level was significantly lower in the patient group compared to the control group. In addition, our study showed that there was a statistically significant difference between the study groups in terms of CRP and ferritin, which suggests the both parameters may be useful as prognostic markers to classify COVID-19 patients according to the disease severity. However, the fact that the levels of MDA and 4-HNE were observed to be lower among the severe group, suggests that MDA and 4-HNE adducts may have been formed, leading to false estimation of oxidative stress in the severe group. Additionally, the lower levels of MDA and 4-HNE among the treated group compared to the untreated group suggested that more comprehensive studies should be conducted regarding the effect of corticosteroid treatments on oxidative stress parameters.

Keywords: COVID-19, Oxidative Stress, Inflammation, Copeptin, Antioxidant

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LIST OF ABBREVIATIONS

4-HNE: 4-Hydroxynonenal

8-OHdG: 8-Hydroxydeoxyguanosine

ACE2: Angiotensin Converting Enzyme 2

ACTH: Adrenocorticotropic Hormone

ADH: Antidiuretic Hormone

ALT: Alanine aminotransferase

Ang II: Angiotensin II

ANOVA: Analysis of Variance

ApoB: Apolipoprotein B

ARDS: Acute Respiratory Distress Syndrome

AST: Aspartate aminotranferase

AVP: Arginine Vasopressin

BIL: Bilirubin

BMI: Body Mass Index

BUN: Blood Urea Nitrogen

CAT: Catalase

COVID-19: Coronavirus disease 2019

CPP: Copeptin

CRH: Corticotropin Realeasing Hormone

CRP: C-Reactive Protein

DM: Dexamethasone

EDTA: Etylinediaminetetraacetic acid

ELISA: Enzyme Linked Immunosorbent Assay

GABA: Gamma Aminobutyric Acid

GPX: Glutathione Peroxidase

GR: Glutathione Reductase

GSH: Glutathione

HbA1c: Glycated Haemoglobin

HDL: High Denisty level Lipoproteins

HPA-axis: Hypothalamic Pituitary Adrenal axis

ICU: Intensive Care Unit

IL-6: Interleukin-6

kDa: Kilo Dalton

LDH: Lactate Dehydrogenase

LDL: Low Denisty level lipoproteins

MAPK: Mitogen Activated Protein Kinase

MDA: Malondialdehyde

MERS-CoV: Middle East Respiaratory Sydrome Coronavirus

MIS-C: Multisystem Inflammatory Syndrome in Children

MP: Methylprednisoline

MS: Multiple sclerosis

MT: Melatonin

NADPH: Nicontinamide Adenine Dinucleotide Phosphate

NF-κB: Nuclear Factor Kappa Beta

NSPs: Non-Structural Proteins

O.D.: Optical Denisty

ORF: Open Reading Frame

Prxs: Peroxiredoxins

PUFA: Polunsaturated Fatty Acids

PVN: Paraventicular hypothalamic nucleus

RAAS: Renin Angiotensin Aldosterone System

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus type 2

SD: Standard Deviation

SOD: Superoxide Dismutase

SON: Supraoptic Hypothalamic Nucleus

SpO2: Saturation of Peripheral Oxygen

TG: Triglycerides

TMB: Tetramethylbenzidine peroxidase substrate

TMPRSS2: Transmembrane Protease Serine 2

TUKEY-HSD:TUKEY's Honest Significant

UA: Uric Acid

VLDL: Very Low Denisty level Lipoproteins

WHO: World HEalth Organizatio

1. INTRODUCTION

The novel coronavirus disease 2019 (COVID-19), a pathogenic viral infection caused by severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), first emerged in Wuhan City (China) in late 2019. COVID-19 started from a local seafood market in China and spread rapidly across the globe causing high mortality and serious illness (Zhu et al., 2020). In March 2020, the World Health Organization (WHO) declared the disease as a global pandemic (Cucinotta & Vanelli, 2020). Despite applying different treatments, the world faced several pandemic waves due to the emergence of new variants causing continuous increases in the number of infections and deaths. The COVID-19 pandemic impacted all aspects of life, causing unprecedented economic and public health crises together with social, educational, and psychological problems (Haleem et al., 2020).

According to the WHO guidelines, the COVID-19 infection may be symptomatic or asymptomatic. Individuals may remain asymptomatic or turn out to be symptomatic after some time. Those who become symptomatic can be classified into four types according to the disease severity: mild (40%), moderate (40%), severe (15%), and critical (5%) (Organization, 2023).

The most common reported symptoms were cough, fever, fatigue, loss of taste and smell, diarrhea, nausea, and vomiting (Organization, 2023).

SARS-CoV-2 is a novel enveloped positive-sense single-stranded RNA virus from the Beta-coronaviridiae family (Jin et al., 2020). Studies reported that SARS-CoV-2 shares 96.2% and 79.6% sequence identity to bat coronavirus RaTG13 and SARS-CoV (P. Zhou et al., 2020).

Several studies demonstrated that the virus enters the host cells through interactions between the virus spike protein (S) and the angiotensin-converting enzyme 2 (ACE2) receptor in the host cells. Those interactions increase the expression of angiotensin II (Ang II), a component involved in the renin-angiotensin-aldosterone system (RAAS) (Jin et al., 2020), which consecutively activates NADPH oxidase followed by an increase in the reactive oxygen species (ROS) as a host defense system (Gheblawi et al., 2020). Excessive ROS production, together with the low ability of the antioxidant defense system to scavenge those ROS, due to low antioxidants levels, results in redox imbalance ending with oxidative stress. Oxidative stress enhances the production of pro-inflammatory cytokines, like IL-1β, IL-2, IL-6, TNF-α, and IFN-γ, and activates the host immune responses (Kim et al., 2013). Interestingly, the relationship between inflammation and oxidative stress is not straightforward since pro-inflammatory cytokines can also induce oxidative stress and vice versa [9]. Under severe conditions, hyperinflammation occurs due to the uncontrolled production of pro-inflammatory cytokines, a phenomenon known as a cytokine storm. Studies reported that cytokine storm is associated with the disease severity degree. Both oxidative stress and hyperinflammation are known to be associated with endothelial dysfunction, which is observed to be related to the disease severity in COVID-19 cases (Pelle et al., 2022).

High Ang II production not only triggers oxidative stress but also activates the release of antidiuretic hormone (ADH) or AVP (Al-Kuraishy et al., 2021). AVP is a nonpeptide hormone synthesized in the hypothalamus and secreted from the posterior pituitary gland. It plays a role in regulating vascular tones and stress response and maintaining the body's fluid balance. Interestingly, AVP is also released under conditions like dehydration,

psychological stress, and hypertension which are observed among COVID-19 patients (Organization, 2023), (Al-Kuraishy et al., 2021). Due to low stability and prolonged laboratory assays of AVP, Copeptin, the C-terminal of the AVP prohormone co-released with AVP, is a surrogate marker of AVP that reflects its circulating concentration. Several studies reported the association of copeptin with disease severity, its role as a prognostic marker in several diseases, and its ability to predict intensive care unit (ICU) outcomes and mortality (Abdelmageed & Güzelgül, 2023).

2. BACKGROUND

2.1.COVID-19

Coronavirus disease 2019 (COVID-19), a rapidly and highly transmittable disease caused by severe acute syndrome coronavirus 2 (SARS-CoV-2), is considered the most significant worldwide crisis since the 1918 influenza pandemic (Cascella et al., 2022). Globally, several life fields, like economic, healthcare, education, mental health, …etc., were affected by the disease. The disease also had its bad effects on thousands of people whole over the world either for suffering from illness or accompanying or missing some one because of the disease (Haleem et al., 2020).

In December 2019, SARS-CoV-2 was first observed in Wuhan, China (Zhu et al., 2020). It spread rapidly throughout the world, and in March 2020, the COVID-19 outbreak was declared a global pandemic by the World Health Organization (WHO) (Cucinotta & Vanelli, 2020). The novel coronavirus was termed (SARS-CoV-2), as it is genetically related to the coronavirus SARS-COV which was responsible for the acute respiratory distress syndrome (ARDS) and high mortality during the period 2002-2003(Zhu et al., 2020). Recent updates from WHO, state that over 767 million positive cases and about 7 million deaths have been confirmed worldwide (*"Who Coronavirus (COVID-19) Dashboard," World Health Organization, Httpscovid19.Who.Int (Accessed Jun. 4, 2023).*, n.d.). Although COVID-19 is considered a respiratory disease, it stimulates systemic and cell metabolic changes, thus affecting several organs and systems in the host with different severity degrees, leading it to be recognized as a multi-organ or multi-systematic disease (Wu et al., 2020), (Spuntarelli et al., 2020).

2.1.1. SARS-CoV-2

Coronaviruses are RNA viruses; their genomes are considered to be the largest genomes of positive-stranded RNA viruses of about 26-32 kb. They have four genera: Alpha, Beta, Gamma, and Delta coronaviruses (α-, β-, δ-, and γ-CoVs) (Jin et al., 2020). It was found that SARS-CoV-2 belongs to beta coronaviruses family (*sarbecovirus* sub-genus in the genus *beta-coronaviridae*) (Zhu et al., 2020), (Chan, Yuan, et al., 2020), to which the severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) are also related. Alignment of SARS-CoV-2's full genome sequence and available genomes of other beta coronaviruses reported that SARS-CoV-2 shares 96% sequence identity with the bat coronavirus RaTG13, and 79.6% sequence identity with SARS-CoV (P. Zhou et al., 2020).

SARS-CoV-2 virus has a spherical, enveloped structure about 80-120 nm in diameter, with multiple spikes projected outwards (Figure 2.1) (R. Yadav et al., 2021). The SARS-CoV-2 genome (Figure 2.1) is of about 30,000 bp, where the first two-thirds, at the 5' end, consists of two large open reading frames (ORF1a and ORF1b), both encode two overlapping polyproteins which are cleaved by the virus proteases resulting in formation of 16 non-structural proteins (NSPs) (V'kovski et al., 2021). The last third of the genome consists of an ORF which encodes for four structural proteins, together with some accessory proteins (H. Yang & Rao, 2021). NSPs, (nsp 1-16), together with accessory proteins modulate transcription regulation, helicase replication, they also encounter the host antiviral response, besides they are responsible for virus-induced diseases. As for structural proteins, they are responsible for host recognition, membrane fusion, viral entry, and release of viral particles.

The four structural proteins are nucleocapsid protein (N), glycosylated spike protein (S), envelope protein (E), and membrane-bound protein (M) (Chan, Kok, et al., 2020).

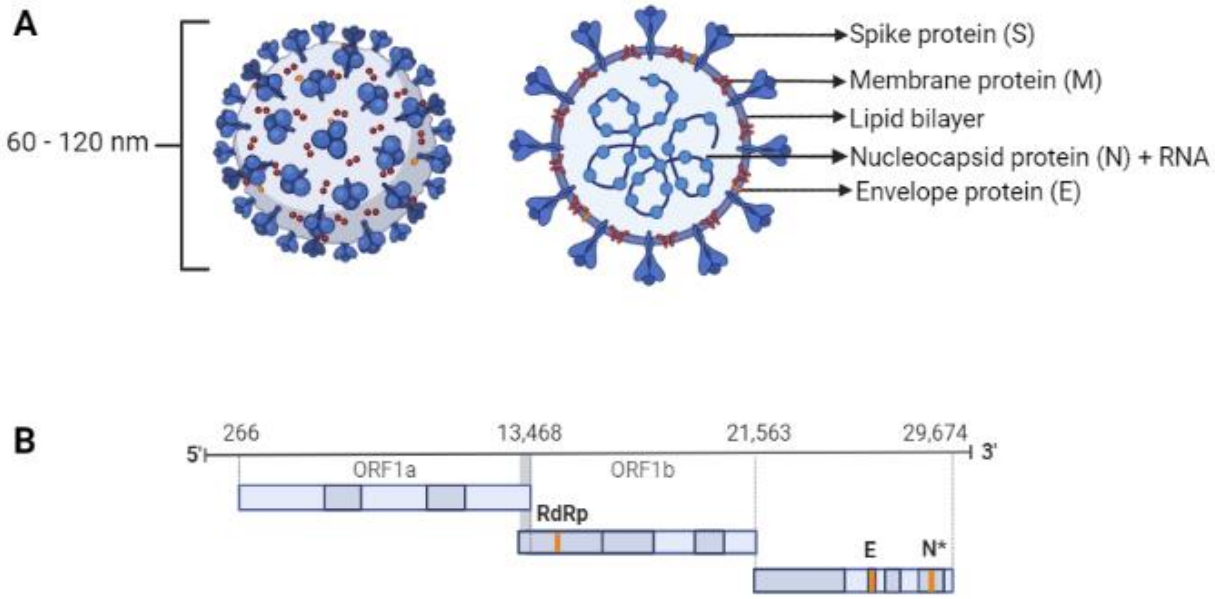


Figure 2.1. SARS-CoV-2 Virus and Genome Structure

A) SARS-CoV-2 Virus Structure B) SARS-CoV-2 Genome

[Created with BioRender com].

2.1.2. SARS-CoV-2 Pathophysiology

Scientists reported that SARS-CoV-2 has 3 entries to the host's body; eyes, nose, and mouth, after staying there for a while it starts to enter the host cell through binding with specific receptors (Arefin, 2022). After body entry, the viral particles infect the epithelial cells found in both nasal and mouth cavity, thus stimulating the human innate response and

interferons (IFN) are released. Interferons induce the expression of Angiotensin-converting enzyme 2 (ACE2), a homolog of ACE which modulates the renin-angiotensin-aldosterone system (RAAS) (Bozkurt et al., 2020), found on the host's cell membrane. ACE2 acts as the main receptor of SARS-CoV-2, leading to the replication and release of more viral particles (Yan et al., 2022). The main mediator of the entry of the viral particles is the viral spike glycoprotein (S protein). As the viral S protein binds to ACE2 receptor, it is cleaved into two subunits (S1 & S2) by a host cell membrane proteinase known as transmembrane protease serine 2 (TMPRSS2) (Figure 2.2.), then the S protein is activated and the viral ribonucleoprotein complex is released into the cell (Coutard et al., 2020), (Lamers & Haagmans, 2022). S1 subunit manages the recognition of host cell receptor besides virus-receptor bindings and interactions. However, S2 subunit is responsible for the host cell membrane and viral particles fusion (Figure 2.2), and the viral entry (Rajpal et al., 2022). This is followed by migration of viral particles to organs with high ACE2 expression through blood circulation (Ni et al., 2020). Probably, the first target cells for the virus are the multiciliated cells found in the upper respiratory tract or the sustentacular cells in the olfactory mucosa. This can be followed by alveolar type 2 cells infection in the lower respiratory tract leading to alveolar damage (Lamers & Haagmans, 2022). As ACE2 is nearly expressed in all body organs with different amounts, this may be the reason of the multi-organ injury observed among the COVID-19 patients (X. Yang et al., 2020). It was observed that ACE2 is highly expressed in kidneys, heart, bladder, esophagus and ileum (Zou et al., 2020), (Hamming et al., 2004), (Zhang et al., 2020). Furthermore, SARS-CoV-2 was detected in different types of specimens and tissues like blood, faeces, urine, liver, heart, etc. (Wang et al., 2020). After being infected, the host immune system is activated and starts to produce

inflammatory cytokines and chemokines in response to the viral attack. In severe and critical COVID-19 cases, excess amounts of inflammatory cytokines and chemokines are released which is commonly known as cytokine storm. This uncontrolled immune system response leads to multi tissue damage which can be considered the main factor affecting mortality and morbidity of COVID-19 (Sodeifian et al., 2022). Additionally, it was reported that the severity of the disease is correlated with the levels of plasma cytokines and inflammatory factors levels (Tufan et al., 2020).

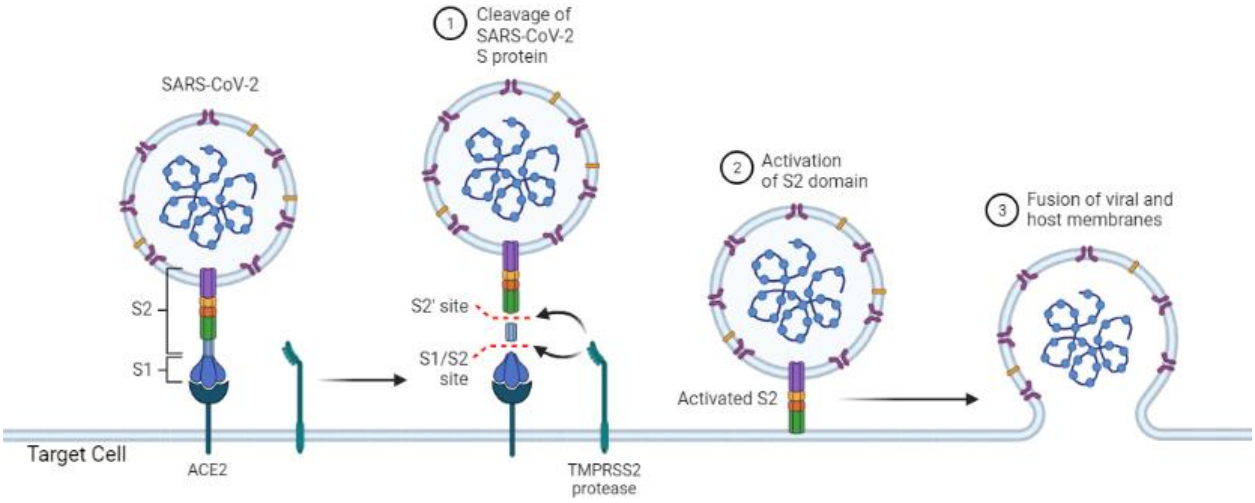


Figure 2.2. SARS-CoV-2 pathophysiology

[Created with BioRender.com]

2.1.3. COVID-19 Symptoms

The most common symptoms are fever (83-99 %), fatigue (44-70%), myalgias (11-35%), anorexia (40-84%), and cough (59-82%) (Organization, 2023), (Lamers & Haagmans,

2022). Hypoxemia, dyspnea (31-40%), dizziness, headache, and sore throat are non-specific and less commonly occurring symptoms (Organization, 2023), (Pijls et al., 2021). Additionally, loss of smell and taste was observed to precede the respiratory symptoms onset (Organization, 2023). However, regarding severe patients, the most common feature is hypoxemia which leads to dyspnea followed by respiratory failure (F. Zhou et al., 2020). It was noted that male sex, advanced age, and obesity are the most common risk factors for developing severe COVID-19 (Williamson et al., 2020), (O'Driscoll et al., 2021). Furthermore, heart failure, hypertension, kidney failure, cardiac arrhythmias, diabetes, malignancy, and chronic pulmonary diseases were the most common comorbidities in COVID-19 patients (Ejaz et al., 2020).

2.1.4. Clinical Findings

SARS-CoV-2 can be transmitted mainly by direct contact through sneezing or coughing, also it can be transmitted through respiratory droplets and aerosols (Lotfi et al., 2020). The average incubation period of the virus may last for 4-5 days before the onset of the symptoms (Lauer et al., 2020), (Li et al., 2020). Clinical features of coronavirus infection may be asymptomatic or symptomatic. Symptomatic cases can be mild (like normal flu), moderate, severe, and critical (such as like acute ARDS and multi-organ malfunctions). According to the WHO guidelines, moderate cases are characterized by signs of pneumonia-like fever, dyspnea, cough, and fast breathing, besides oxygen saturation ≥ 90% (SpO2≥ 90%) (Organization, 2023). However, severe cases are characterized by pneumonia signs accompanied by either SpO2≤90%, respiratory rate > 30/min., or severe respiratory distress.

Complications like ARDS, sepsis, septic shock, acute thrombosis, and multisystem inflammatory syndrome in children (MIS-C), are observed among critical cases which require treatment in intensive care units (ICU) (Organization, 2023). The critical illness usually appears one week after the symptom onset (F. Zhou et al., 2020) and may last four weeks (Excellence, 2021). However, in some cases, the symptoms can last for more than four weeks, which is recently known as 'long COVID' (Excellence, 2021). Additionally, studies showed that severe COVID-19 could develop several extrapulmonary diseases like kidney, heart, gastrointestinal, and liver injury, cardiac arrythmias, coagulopathy, and shock (Berlin et al., 2020).

2.1.5. Laboratory Findings

Systemic hyperinflammation was observed among severe COVID-19 patients. It comprises both excessive release of proinflammatory cytokines like Interleukins (IL-1, IL-6, IL-8) and tumor necrosis factor (TNF) and high plasma levels of inflammatory factors such as ferritin, erythrocyte sedimentation rate, procalcitonin, and C-reactive protein (CRP) (Lamers & Haagmans, 2022). Also, abnormal coagulation parameters like elevated fibrinogen, low platelet count and elevated D-dimer were observed among COVID-19 patients (Levi et al., 2020), (Hu et al., 2020). Some studies showed that elevated TG, ALT and LDH were observed among hospitalized COVID-19 patients. Besides, electrolyte disturbance, like hyponatremia, hypokalemia, and hypoalbuminemia, was observed among severe cases (De Carvalho et al., 2021), (Wagner et al., 2021). In addition, elevated glycated hemoglobin (HbA1c) levels were noticed among hospitalized COVID-19 patients regardless their diabetes mellitus history (Klein et al., 2020).

2.2.Oxidative Stress

Oxidative stress was first defined by Helmut Sies in 1985, as a disturbance between the production of oxidants and antioxidant defenses in favor of oxidants, that may lead to cellular damage (Forman & Zhang, 2021), (Lushchak & Storey, 2021). Most of our bodies' biological processes such as metabolism, breathing, energy production, …etc., produce harmful compounds known as free radicals. Free radicals are unstable highly reactive molecules with an odd number of electrons, that's why they can easily undergo prolonged chain reactions in our bodies (Sharifi-Rad et al., 2020). Sources of free radicals may be endogenous like mitochondria, phagocytic cells, endoplasmic reticulum, etc., or exogenous like pollution, alcohol, smoking, UV rays, certain drugs, etc. (Figure 2.3) (Phaniendra et al., 2015). Reactive oxygen species (ROS) and reactive nitrogen species (RNS), together constitute the free radical and non-radical reactive species (Pham-Huy et al., 2008). ROS are involved in oxidative stress; they are highly reactive oxygen-containing molecules, they may be free radicals with unpaired electrons in the outer shell which make them highly active and unstable for example superoxide (O2•−), hydroxyl (•OH), peroxyl (ROO•), hydroperoxyl (HO2•) and alkoxyl radicals (RO•), or non-radical molecules with even number of electrons so they are more stable but still have high ability to produce free radicals, such as hydrogen peroxide (H_2O_2), organic peroxides (ROOH), and hydroxide ion (OH^-) (Hayyan et al., 2016), (Shields et al., 2021). In low and moderate concentrations ROS are fundamental elements for several physiological processes, such as cell immunity, protection against microorganisms, protein phosphorylation, cell apoptosis, …etc. However, if produced in excessive amounts, ROS can attack macromolecules such as DNA, proteins, carbohydrates, and lipids leading to damage or even apoptosis of cells and tissues (Sharifi-Rad et al., 2020), (Phaniendra et al.,

2015), (Miller et al., 1993). Accordingly, it is stated that oxidative stress is essentially involved in the pathogenesis of several diseases especially chronic ones like diabetes, cancer, cardiac diseases, cataracts, respiratory diseases, rheumatoid arthritis, aging, … etc. (Sharifi-Rad et al., 2020), (Phaniendra et al., 2015).

2.2.1. Role of ROS in Health

Under normal conditions, in endothelial cells, Ang II activates NADPH oxidase leading to the production of ROS especially (O2•−) (Nguyen Dinh Cat et al., 2013). Super oxides activate Raf-1 mitogen activated protein kinase (MAPK) resulting in activation of proteins responsible for cell proliferation control (Harijith et al., 2014), (Son et al., 2011). Additionally, (O2•−) plays an important role in controlling blood pressure, as its production contributes to an increase in vasopressin secretion in central nervous system (Gonzalez et al., 2020). Also, the role of ROS as signaling molecules appears clearly in vasodilation, oxygen sensing, and skeletal muscle physiology (Bassoy et al., 2021). ROS are essentially involved in both innate and acquired responses of the immune system towards inflammation (Bassoy et al., 2021), (Bassoy et al., 2021). Recently, it was observed that ROS can cause apoptosis in cancer cells, which can be used in cancer treatments (Perillo et al., 2020).

2.2.2. Role of ROS in Disease

The major harmful effect of ROS can be observed in oxidation of essential macromolecules of the cell like proteins, lipids, carbohydrates, and DNA which was approved to be main cause of several diseases (Forcados et al., 2021). Oxidation of lipids is known as lipid peroxidation, where ROS, especially hydroxyl (HO•) and hydroperoxyl

($HO•_2$) radicals, attack lipids with carbon-carbon double bonds (C=C) leading to the formation of several oxidation products (Ayala et al., 2014). Lipid hydroperoxides can be considered as main primary products of lipid peroxidation, which leads to generation of some aldehydes like malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Ayala et al., 2014). Those aldehydes affect the permeability of mitochondrial inner membrane, resulting in release of cytochrome *C* which ends by cell death (N. Yadav et al., 2015). Elevated levels of lipid peroxides and aldehydes were found to be significantly involved in several diseases like diabetes, Alzheimer's disease, heart failure, neurodegenerative diseases, respiratory diseases, kidney diseases, rheumatoid arthritis, respiratory diseases, cancer, and some immunological diseases (Forcados et al., 2021), (Pizzino et al., 2017).

ROS also contribute to DNA damage (oxidative damage to DNA) (Dizdaroglu et al., 2002). ROS, especially hydroxyl radicals, peroxyl radicals and super oxides react with DNA bases resulting in formation of DNA adducts and generation of other harmful products like 8-hydroxydeoxyguanosine (8-OHdG) (Cadet & Davies, 2017). This can produce faulty proteins due to improper translation process which increases the possibility of diseases (Ghosh & Shcherbik, 2020). However, generated (8-OHdG) may lead to mutagenesis, which if not repaired can initiate cancer or even lead to metastasis (Pizzino et al., 2017).

Proteins and enzymes also can be oxidized by ROS, especially cysteine and methionine rich proteins, thus modifying their structure giving rise to protein aggregations, malfunctional proteins, and loss of catalytic properties of several enzymes. ROS, especially the hydroxyl radicals attack the proteins peptide bonds, causing its cleavage and formation of carbon centered radicals (C•), which can easily react with oxygen forming alkoxyl radicals (COO•). Alkoxyl radicals can carry out oxidation of other macromolecules. Oxidized

proteins affect the functions of several organs causing a variety of complications like aging which may lead to several diseases like cataract formation, Alzheimer's disease, muscular dystrophy, respiratory distress syndrome, etc. (Forcados et al., 2021), (Juan et al., 2021).

The nuclear factor kappa beta (NF-κB), is a transcription factor functions in regulating the expression of genes responsible for cell differentiation, development, and survival, besides it also controls cytokine production, and inflammation and innate immune responses (Karin & Greten, 2005), (Park & Hong, n.d.). Under oxidative stress, NF-κB is oxidized through its cysteine residue by the ROS attack. NF-κB is then inactivated leading to uncontrolled immune and inflammation responses, and DNA damage which if not repaired may result in cell death (Forcados et al., 2021), (Albensi, 2019).

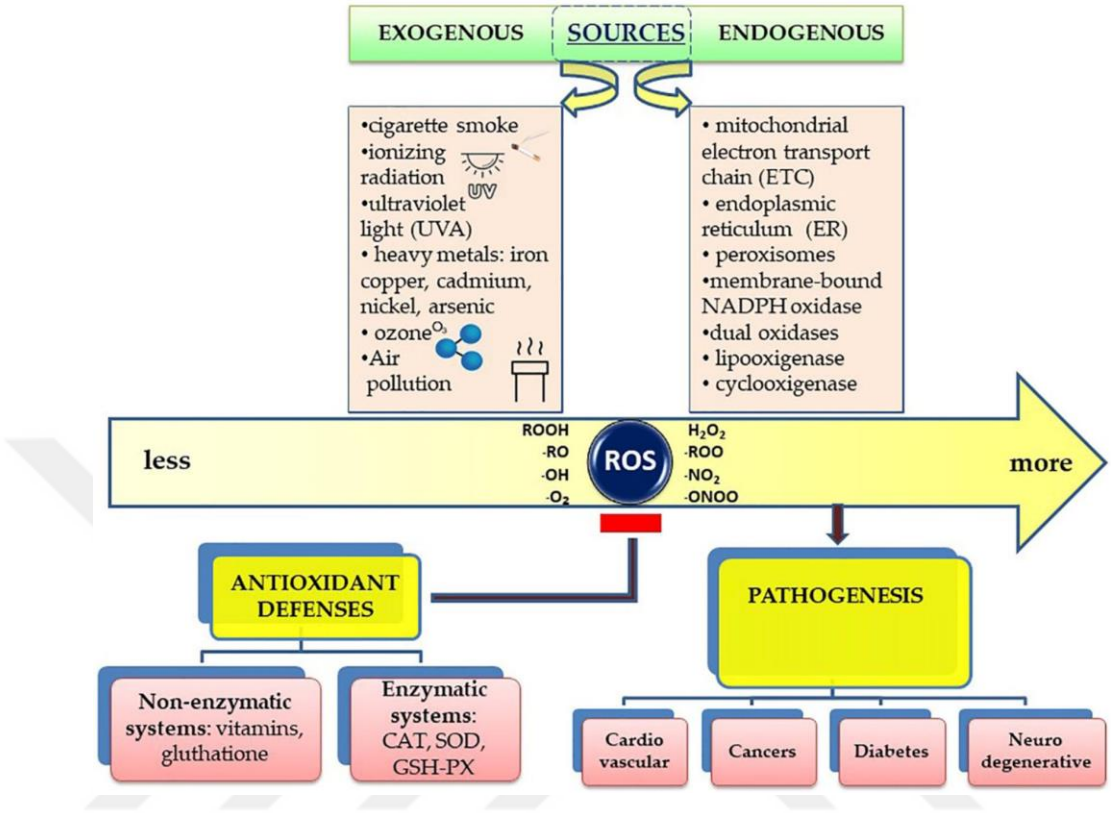


Figure 2.3. Sources of reactive oxygen species and their effect on the human body.

(Sharifi-Rad et al., 2020)

2.3.Antioxidants

Antioxidants are a group of molecules that inactivate the harmful effect of free radicals in the human body by inhibiting or quenching free radical reactions, thus delaying or inhibiting cellular damage (Figure 2.4) (Nimse & Pal, 2015). According to their activity, antioxidants can be classified as enzymatic and non-enzymatic (Nimse & Pal, 2015), (Moussa et al., 2019). Enzymatic antioxidants, such as glutathione peroxidase (GPX), catalase (CAT), glutathione reductase (GR), peroxiredoxins (Prxs), and superoxide dismutase (SOD), scavenge ROS by converting them to H_2O_2 and then to water through a series of reactions using different cofactors like iron, manganese, copper, and zinc. However,

non-enzymatic antioxidants, like metal-binding proteins, glutathione (GSH), uric acid (UA), melatonin (MT), coenzyme Q10, polyamines, and bilirubin (BIL), act by interrupting and inhibiting free radical reactions (Mirończuk-Chodakowska et al., 2018).

Additionally, some vitamins act as non-enzymatic antioxidants, like vitamins C, K, E, A, and D (Sinbad et al., 2019). Vitamin D, which is involved in calcium homeostasis regulation and bone metabolism, was recently reported to have an essential role in the antioxidant defense system (Gil et al., 2018). According to several studies, its role in decreasing the effect of ROS was observed through inhibiting iron-dependent lipid peroxidation, suppressing the gene expression of NADPH oxidase, and stimulating the production of other endogenous antioxidants like GSH (Sinbad et al., 2019), (Mokhtari et al., 2016). Besides its antioxidative function, vitamin D was reported to have an anti-inflammatory action as it down-regulates pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α (Fernandez-Robredo et al., 2020), (Leal et al., 2020). Recently, it was observed that there is a correlation between circulating vitamin D levels and COVID-19 progression, severity, and outcomes (Munshi et al., 2021), (Ali, 2020), (Ilie et al., 2020).

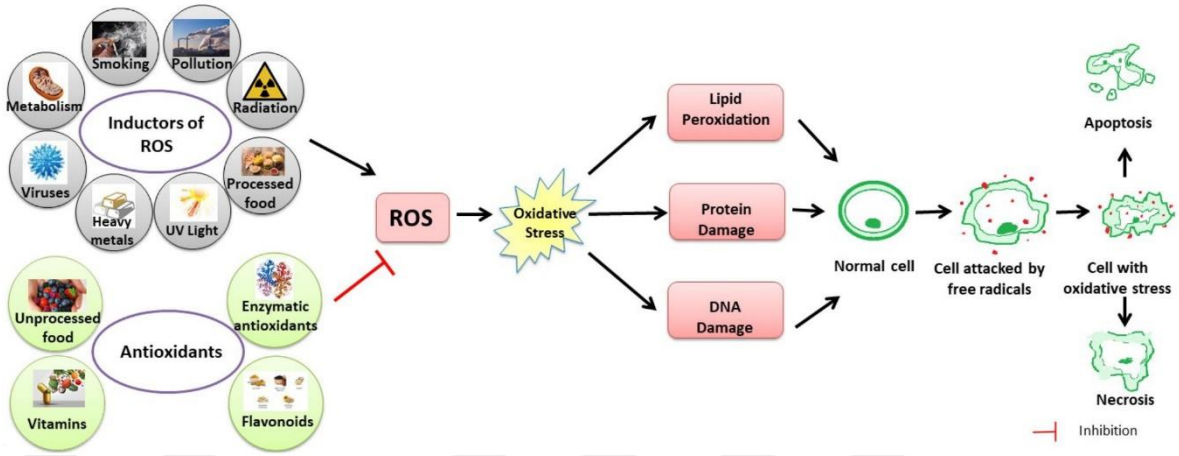


Figure 2.4. Role of antioxidants.

(Sharifi-Rad et al., 2020)

2.4.Inflammation

Inflammation, a process occurs when the immune system recognizes infection or injury and starts to respond by releasing certain chemicals to fight the disease or heal the damaged tissue. It can be acute or chronic (Pahwa et al., 2021). In COVID-19, inflammation is considered to be one of the key features of the disease, especially in severe cases, additionally, it was observed that overactive immune response, abnormalities in inflammatory markers, and cytokine storm are involved in both disease progression and outcomes (Wong, 2021). As mentioned above, in SARS-CoV-2 pathogenesis, interferons and cytokines are released in response to the viral infection. Type-I interferon (IFN-α and IFN-β) is responsible for viral replication inhibition. Due to the differential immune response in COVID-19, type I interferon is secreted immediately after viral RNA recognition in young individuals and cases with no comorbid conditions, followed by inhibition of the viral replication (Mishra et al., 2020). However, in case of old individuals and those who suffer

from comorbid diseases, the release of interferon type I is delayed, thus; viral replication is not inhibited, which may increase the recruitment of more inflammatory cells (monocytes, macrophages, and neutrophils), triggers excess release of proinflammatory cytokines, which is known as cytokine storm (Wong, 2021), (Mishra et al., 2020). Cytokine storm contributes to ARDS and multi-organ damage, which, if not treated, may cause multi-organ failure and death. It was reported that serum levels inflammatory markers such as IL-2R, IL-6, IL-8, and TNF-α, were elevated in severe cases rather than mild cases (Wong, 2021). This in turn stimulates the elevation of serum CRP, ferritin, and LDH levels, which can be also used as inflammation markers (Mishra et al., 2020).

2.5.Oxidative Stress and Inflammation in COVID-19

The relation between inflammation and oxidative stress is rather strong, that if one is present, it promotes the occurrence of the other (Ramos-González et al., 2021), (Biswas, 2016). Excessive amounts of ROS can lead to organ damage, epithelial and lymphocyte dysfunction, and hyperinflammation. On the other hand, hyperinflammation, due to cytokine storm and release of pro-inflammatory markers, leads to oxidative stress. Thus, oxidative stress and inflammation can form a continuous vicious loop together with disease progression (Vollbracht & Kraft, 2022). It was observed that there is a correlation between oxidative stress parameters and proinflammatory cytokines, especially IL-6, among severe COVID-19 patients (Vollbracht & Kraft, 2022). A study investigating monocytes of COVID-19 patients, showed that prolonged oxidative stress and activated inflammasomes produced by infected

monocytes are interrelated, and contribute to disease severity and outcomes (Lage et al., 2022).

2.6.Renin-Angiotensin-Aldosterone System (RAAS)

RAAS is a complex hormonal system that has a crucial role in maintaining physiological homeostasis as it controls the regulation of the cardiac, renal, and vascular physiology. It is responsible for maintaining the blood pressure as well as the fluid and electrolyte homeostasis (Ni et al., 2020). The RAAS system includes two opposite hormonal axes regulated by extrinsic and intrinsic feed-back-feed-forward inputs essential for homeostasis. Angiotensin II (ANG II), is the most effective peptide in the RAAS system (Patel et al., 2017). It has an essential role in both inflammation and oxidative stress through promoting production of proinflammatory cytokines and ROS (Delpino & Quarleri, 2020). RAAS system imbalance may lead to several disease or even sudden death. Additionally, it was reported to have an important role in apoptosis, inflammation and fibrosis (Fountain & Lappin, 2017). A close and well-coordinated interrelation was observed between the RAAS system and the arginine vasopressin system (AVP). Their cooperation was observed to be essential in regulating metabolism, water-electrolyte balance, and vascular system. Additionally, ANG II was found to be a powerful stimulator of AVP secretion (Figure 2.5) (Szczepanska-Sadowska et al., 2018).

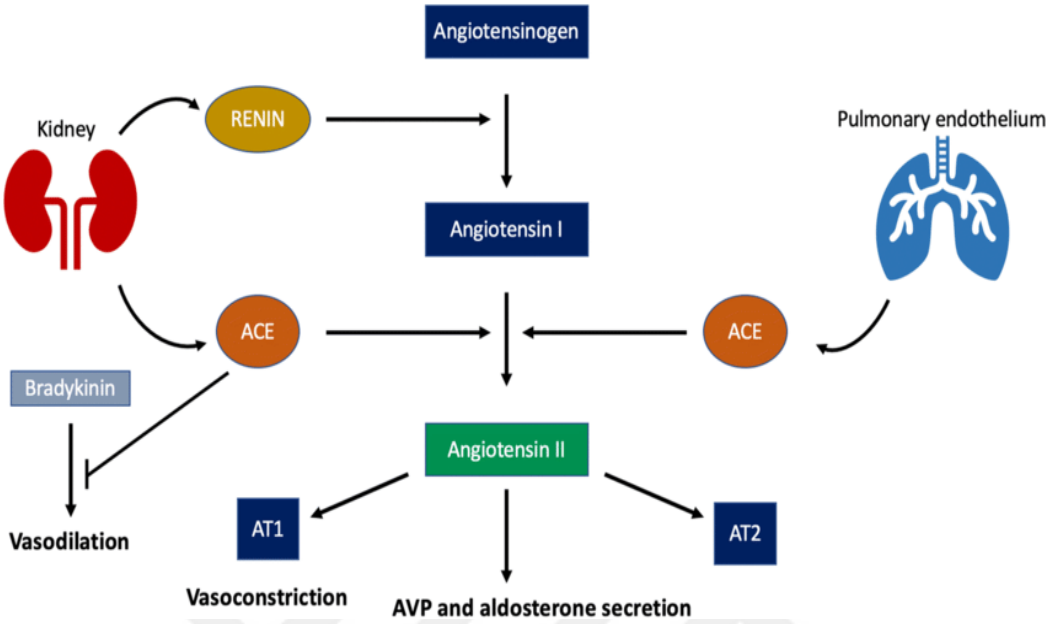


Figure 2.5. Renin-Angiotensin-Aldosterone System.

(Papazisi et al., 2020).

2.7.Parameters

2.7.1. Copeptin

AVP and copeptin are two hormones released from the posterior pituitary gland into the circulation, in equal amounts, in response to osmotic and hemodynamic stimulants in clinical practice. In healthy persons, AVP regulates vascular tone and fluid homeostasis thus maintaining the cardiovascular system equilibrium. Additionally, AVP plays an important role in regulating endocrine, hemostatic, and central nervous systems besides the direct renal and vasoconstrictor effects (Christ-Crain, 2019a).

Fluid balance homeostasis, endocrine stress response, and vascular tone regulation are believed to be the main physiological functions of AVP. In the clinical practice, AVP plays a significant role in controlling osmotic balance, sodium homeostasis, blood pressure,

as well as kidney functions. The role of AVP is mediated by three G-protein coupled receptors: vascular receptor (V1aR), anterior pituitary receptor (V1bR), and antidiuresis-mediating receptor (V2R) (Sparapani et al., 2021).

On the other hand, as mentioned before, a harmonious interaction between the RAAS and AVP systems was observed as they work together to maintain proper regulation of metabolism, the cardiovascular system, as well as the water-electrolyte balance of the body. Thus, both can be co-activated together in response to the same stimulus (Szczepanska-Sadowska et al., 2018).

In clinical practice, AVP measurements are limited because of the short half life time of AVP, low molecular stability of AVP even if stored at -20°C, also more than 99% of circulating AVP is bound to platelets in the circulation, which leads to misestimation of the actual AVP level (Łukaszyk & Małyszko, 2015).

Being released in equimolar amounts with AVP besides its high stability, copeptin was approved to be used to mirror the AVP release.

Nowadays, the main role of copeptin is being a stable surrogate marker of AVP concentration in humans. Furthermore, it was approved to be a biomarker for prognosis of several diseases like pulmonary diseases, cardiovascular diseases, … etc. (Christ-Crain, 2019b), (Abdelmageed & Güzelgül, 2023)

Copeptin was first detected in the pigs' posterior pituitary in 1972 by Holwerda where it (Holwerda, 1972). It is a glycosylated peptide consists of 39- amino acid with a leucine rich core region. Copeptin is found in the C-terminal part of AVP precursor (pro-AVP). The molecular mass of copeptin is about 5 kDa.

Unlike AVP, the physiological function of copeptin in the circulation is still not clear. After being described in 1972, some early researches showed that copeptin may act as a prolactin releasing factor (Nagy et al., 1988), however those results were not approved as they were denied by other experiments (PETTIBONE et al., 1989). Recent studies assumed that copeptin can act as a chaperone protein in the folding and proteolytic AVP maturation (Harris et al., 1998), (Barat et al., 2004). Since copeptin is presumed to be strictly regulated in the circulation, it may have a specific peripheral function. Accordingly, to identify the specific role of copeptin in the circulation much more experiments are needed. At present, copeptin was manifested to show the same response as AVP to stress, osmotic and hemodynamic stimuli. Besides being produced equivalent to AVP, make the copeptin test functional in the prognosis and diagnosis of AVP disorders-related diseases. Although no specific mechanism was approved for copeptin elimination, it was suggested that it could be eliminated through the kidney, since it can be measured in it (Roussel et al., 2014).

Copeptin production is a result of the cleavage of Pro-AVP precursor after being subjected to enzymatic cleavage (Figure 2.6)

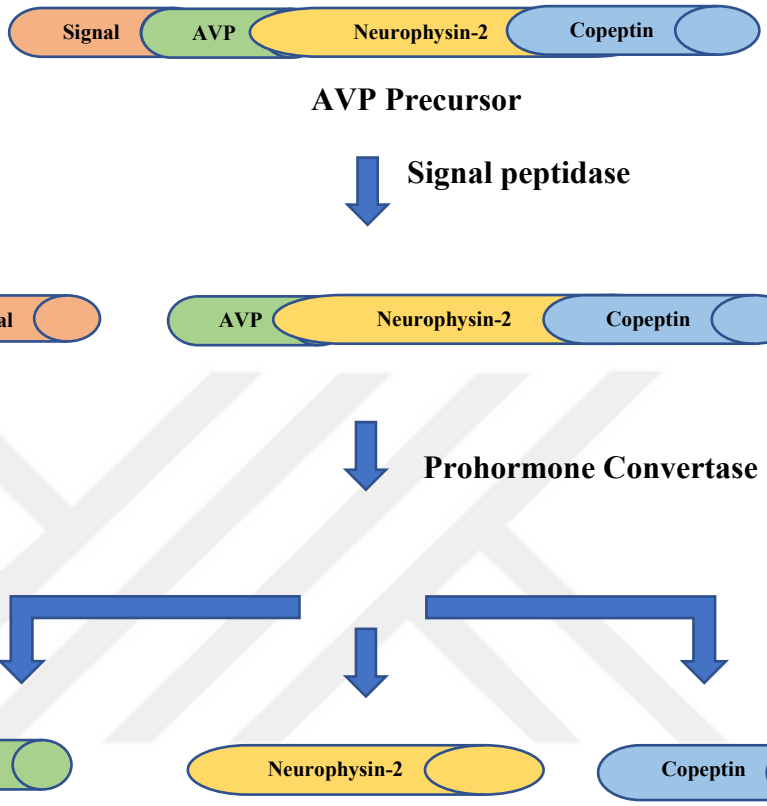


Figure 2.6. AVP precursor is subjected to several enzymatic cleavages resulting in production of AVP, Neurophysin-2 and copeptin.
(Abdelmageed & Güzelgül, 2023)

Pro-AVP precursor is produced in the hypothalamus (Figure 2.7). The release of pro-AVP from the hypothalamus takes place through two different mechanisms. The first mechanism is the posterior pituitary release mechanism, where pro-AVP is produced in the magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) hypothalamic nuclei. The pro-AVP is then processed in the endoplasmic reticulum, where the signal peptide is removed, and a carbohydrate chain is added. Finally, an axonal transport to the posterior pituitary gland occurs, where copeptin, AVP, and Neurophysin-2 are produced due to the enzymatic cleavage of Pro-AVP (Figure 2.6).

The three peptides are stored in the neurohypophysis and are released in response to hemodynamic or osmotic stimuli. Regarding the other mechanism, pro-AVP is synthesized and processed in the parvocellular neurons of hypothalamus, where corticotropin-releasing hormone (CRH) is also produced. AVP is then transported to the pituitary portal system where it acts cooperatively with CRH to stimulate the adrenocorticotropic hormone (ACTH) release from the anterior pituitary gland, which in turn stimulates the release cortisol from the adrenal gland (Sparapani et al., 2021).

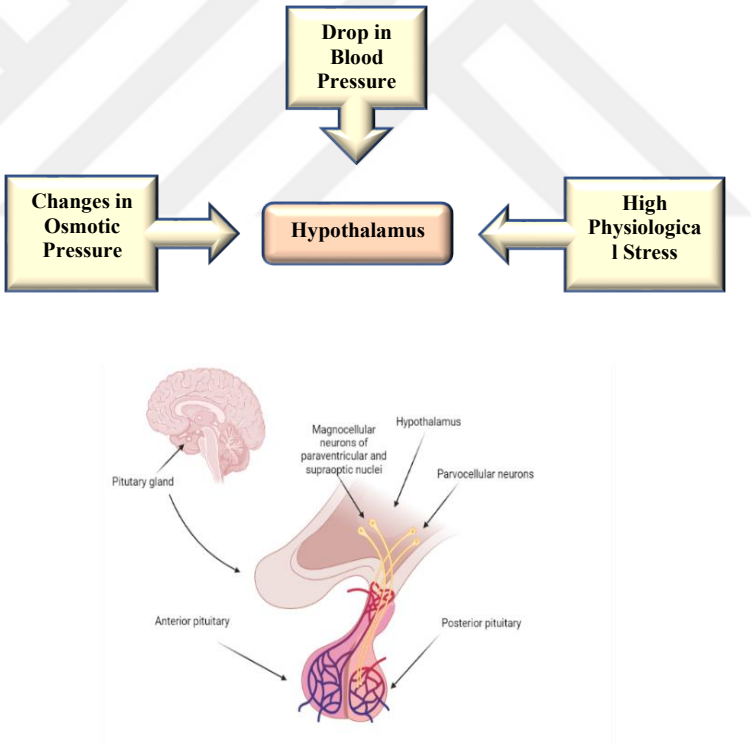


Figure 2.7. Pro-AVP release mechanisms.
(Abdelmageed & Güzelgül, 2023)

Like AVP, copeptin circulating levels respond to blood pressure, stress, and osmolality changes. Copeptin was observed to be more associated with plasma osmolality healthy individuals compared to AVP. Where in healthy controls, copeptin levels are found

to be strongly related to osmolality changes with a rapid rise during thirst and a rapid drop after fluid intake. Also, copeptin may be better than cortisol in determining physiological stress levels. Measuring cortisol as a free hormone is challenging since it has a strong circadian rhythm and it is downstream during stress response (Morgenthaler et al., 2007).

Normal copeptin levels in healthy controls have been found to be 1.70–11.25 pmol/L. In normal situations, for unknown reason, men were observed to have higher copeptin levels than women. It was suggested that higher copeptin levels among men may be due to higher osmolar intake (Dobša & Cullen Edozien, 2012). Similar to copeptin, AVP levels were higher in men than in women. On the other hand, there was no evidence about sex differences in AVP levels. Though AVP levels were reported to show a diurnal rhythm, no regular variability in circadian rhythm was reported in case of copeptin. Additionally, the effect of age differences on copeptin levels was not reported. Furthermore, it was observed that copeptin levels may be elevated by physical exercise. In a large cohort of 6.801 participants a relationship between life-style and diet-related factors such as smoking, alcohol use, and fluid and sodium intake copeptin levels was observed. Besides, copeptin was found to be correlated to high systolic blood pressure, high renal sodium and urea excretion in healthy individuals, and low 24-h urine volume (Mammen et al., 2014).

2.7.2. Malondialdehyde

Malondialdehyde (MDA), or 1,3-propanedial (OHC—CH2—CHO; C3H4O2), is one of the end products of lipid peroxidation, that's why it has been used as a marker of lipid peroxidation induced oxidative stress (Figure 2.8) (Driessen et al., 2013).

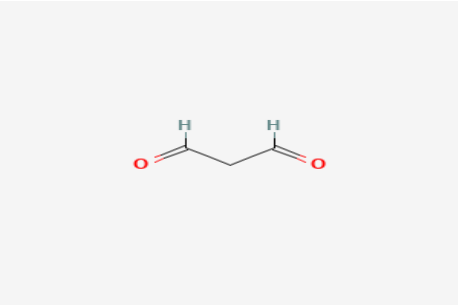


Figure 2.8. Malondialdehyde (MDA).

(*Malonaldehyde @ Pubchem.*Ncbi.*Nlm.Nih.Gov*, n.d.)

Overproduction of free radicals leads to elevated MDA levels. It is also considered to be the most mutagenic product of lipid peroxidase. MDA is chemically reactive due to the presence of the carbonyl group. It can form adducts with macromolecules like DNA and proteins and alter their functions. Under oxidative stress conditions, the level of ROS is elevated. ROS react with polyunsaturated fatty acids (PUFA), present mainly in cell membranes, forming lipid peroxides in a process called lipid peroxidation catalyzed by peroxidase enzymes (Figure 2.3). Lipid peroxides are unstable, so they decompose, forming several compounds like MDA, isoprostanes, and 4-HNE (Singh et al., 2014). Several studies reported the elevation of MDA levels in several diseases like cancers, chronic obstructive pulmonary disease, cardiovascular diseases, asthma, and psychiatry diseases (Singh et al., 2014), (Khoubnasabjafari et al., 2015).

2.7.3. 4-Hydroxynonenal (4-HNE)

4-Hydroxynonenal (4-HNE) (C9H16O2), a secondary product of lipid peroxidation of polyunsaturated fatty acids (Figure 2.9), was discovered by Hermann Esterbauer in the 60's. 4-HNE has three functional groups; a hydroxy group, C=C double bond, and a carbonyl group which contribute to its high reactivity (Figure 2.10).

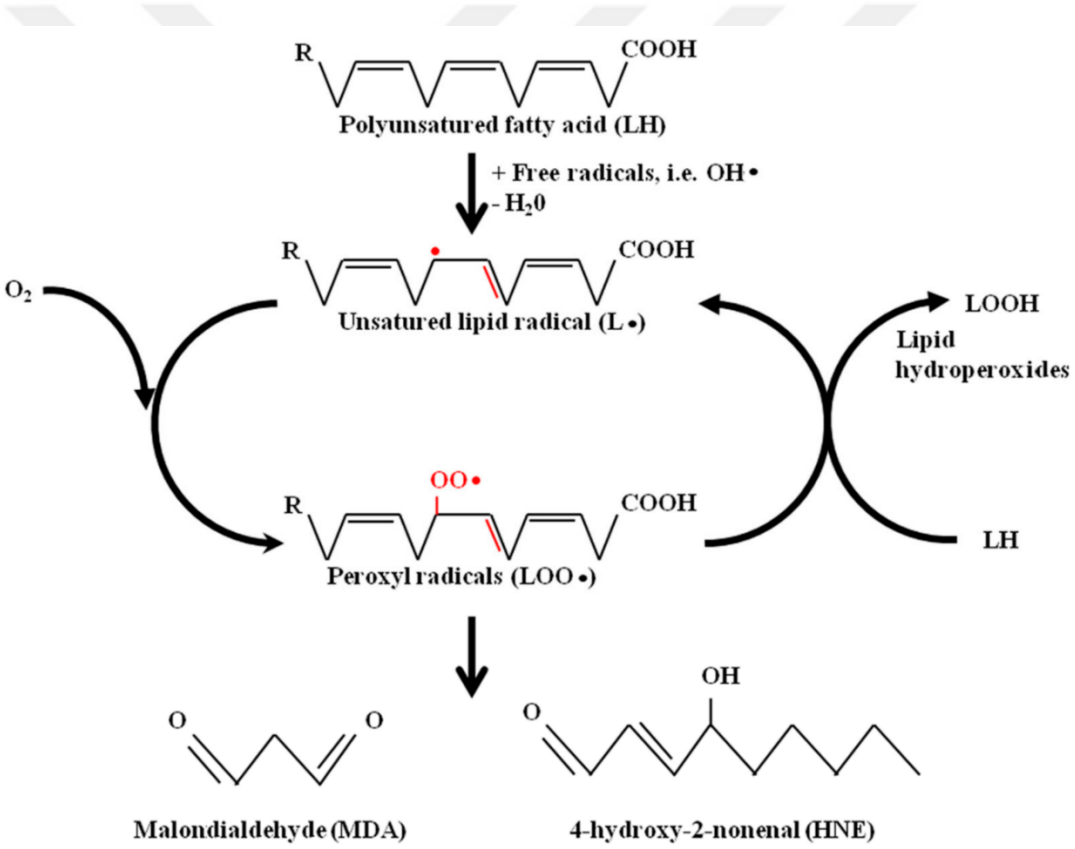


Figure 2.9. Lipid Peroxidation.

(Shoeb et al., 2014)

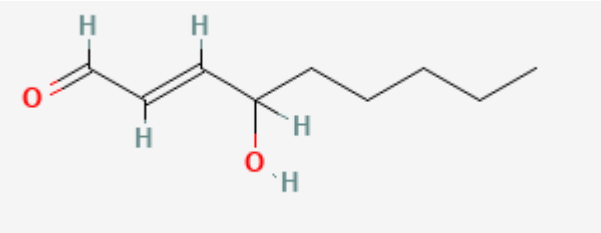


Figure 2.10. 4-Hydroxynonenal structure.

(4-Hydroxynonenal @ Pubchem.Ncbi.Nlm.Nih.Gov, n.d.)

It reacts rapidly with thiols and amino groups. While MDA is the most mutagenic byproduct of lipid peroxidation, 4-HNE is the most toxic. That is related to its ability to form adducts with peptides, proteins, lipids, and nucleic acids, changing their structures and functions and resulting in several pathological conditions. Besides being toxic, it can also act as a signaling and regulatory molecule after adducts formation with macromolecules, especially protein adducts, and that is what contributes to its importance as a biomarker for different oxidative stress-related diseases (Barrera et al., 2018), (Zarkovic et al., 2017). 4-HNE was reported to be involved in the progression and pathogenesis of several diseases like cancer, cataract, osteoporosis, diabetes, atherosclerosis, and inflammatory complications, as well as some neurodegenerative diseases like Parkinson's, and Alzheimer's disease (Jaganjac et al., 2020).

2.7.4. Melatonin (MT)

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine that was first discovered and isolated in 1958(Figure 2.11) (Lerner et al., 1958).

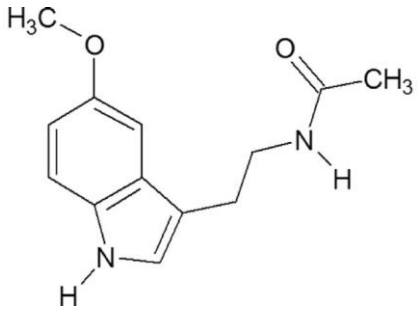


Figure 2.11. Melatonin Structure.

(Allegrone et al., 2019)

It is an endogenous hormone produced mainly by the pineal gland in the brain and released immediately into the blood (Arendt, 1998), (Amaral & Cipolla-Neto, 2018). Additionally, melatonin was observed to be synthesized in extra-pineal tissues like liver, gastrointestinal tract, kidney, retina, ovary, airway epithelium, placenta, platelets, and eosinophilic leukocytes (Acuña-Castroviejo et al., 2014). Melatonin exhibits both circadian and circannual rhythms, thus its production increases in the absence of light (at night) and decreases in the presence of light (at day) (Amaral & Cipolla-Neto, 2018). Accordingly, it is involved in several physiological processes such as body core temperature, sexual maturation, reproductive function, sleep, glucose hemostasis, and energy metabolism (Arendt & Aulinas, 2022). Additionally, the high hydrophilicity and lipophilicity of melatonin facilitates its flow through cell membranes, so it can easily transfer through organs and fluids (Tamura et al., 2012). In 1993, melatonin's function as an antioxidant was discovered (Reiter et al., 2009). Since then, plenty of publications reported the role of melatonin as an antioxidant and anti-inflammatory molecule (Ji et al., 2018), (Kvetnoy et al., 2022). Melatonin is able to reduce oxidative stress either directly or indirectly. It can act as a direct free radical scavenger together with its secondary and tertiary metabolites via

Hydrogen Atom Transfer mechanism (HAT) or Single Electron Transfer mechanism (SET) (Reina & Martínez, 2018). Melatonin can also maintain the redox homeostasis indirectly by inducing the production of endogenous antioxidant enzymes like SOD, GPX, CAT, and GR (Hardeland, 2005). Furthermore, it stimulates glutathione production and inhibits the synthesis of some enzymes which is responsible for free radical production like nitric oxide synthase and lipoxygenase (Reiter et al., 2001). It is worth mentioning that the antioxidant capacity of melatonin is superior to that of other classical antioxidants, including vitamin E, vitamin C, and glutathione. It was reported that one melatonin molecule can scavenge up to 10 ROS. However, in the case of the other antioxidants, one molecule can scavenge one ROS or less (D. Tan et al., 2003), (Gitto et al., 2001). This was related to the cascade reaction of melatonin and its metabolites (D.-X. Tan et al., 2015).

2.7.5. Interleukin 6 (IL-6)

Interleukin 6 (IL-6), is a helical 22-28 kDa phosphorylated and variably glycosylated proinflammatory cytokine. It is characterized by pleiotropic activity and plays a significant role in acute phase reaction, hematopoiesis, cancer progression, inflammation, and bone metabolism. It is responsible for fever and acute phase response in the liver and the induction of acute phase proteins like serum amyloid A, CRP, fibrinogen, and hepcidin in hepatocytes. Additionally, it plays an essential role in the transition from acute inflammation to either chronic inflammatory or acquired immunity diseases. CD8+ T cells, monocytes, neutrophils, eosinophils, fibroblasts, osteoblasts, pancreatic islet beta cells, and many other cells generally express IL-6. Its production is usually correlated with cell activation, and its circulation levels

are controlled by secondary sex hormones, catecholamines, and glucocorticoids. Environmental stress factors like infections and tissue injury induce IL-6 expression. The normal IL-6 range is within 0 - 43.5 pg/mL (Said et al., 2021). IL-6 circulation level may be slightly elevated during the menstruation cycle. Besides, it shows moderate elevations in certain cancers, whereas it is highly elevated after surgeries (Tanaka et al., 2014), (Velazquez-Salinas et al., 2019).

3. METHODOLOGY (METHODS AND MATERIALS)

3.1.Materials

3.1.1. Chemical materials

- 1-Butanol (Merck)
- 2-Thiobarbituric acid (Sigma-Aldrich)
- Ortho-Phosphoric acid (Merck)
- Human CPP(Copeptin) ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China) (Cat. no. EH2880).
- Human MDA (Malonaldehyde) ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China) (Cat. no. EH4147).
- 4-HNE(4-Hydroxynonenal) ELISA Kit 96T (Elabscience Biotechnology Co., Ltd., Wuhan, China) (Cat. no. E-EL-0128).
- Human MT(Melatonin)ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China) (Cat. no. EH3344).
- Human IL-6 (Interleukin 6) ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China) (Cat. no. EH0201).

3.1.2. Tools

- Refrigerated Microcentrifuge (Hettich Micro 22)
- Refrigerated Centrifuge (Hettich Rotana 460 R)
- Deep Freezer (Haier Bio-medical)
- Oven (Memmert)
- Spectrophotometer (Techcomp UV1000)

- Analytical Balance (AND GR200)
- Refrigerator (UĞUR)
- Vortex (VELP)
- Microcentrifuge (Stratagene PicoFuge II)
- Water Bath (Medingen SWB 20)
- Micropipette (Thermo Fisher P10-P100-P1000)
- Microplate Reader (Organon Technika Microwell system)
- Printer (Epson LX-300+)
- Shaker (IKA KS 130 basic)
- Magnetic Stirrer (Yellow line MSH basic)

3.2.Study Design

The study was approved by the ethics committee of Tokat Gaziosmanpasa University under reference no. (22-KAEK-257). Informed consent forms were collected from the patients or their relatives after being instructed about the study.

Individuals of both severe and mild COVID-19 who applied to Tokat Gaziosmanpasa University hospital in the period between March 2021 and September 2021 with positive reverse transcription-polymerase chain reaction (RT-PCR) test for SARS-CoV-2 infection, were included in the study. Information regarding height, weight, age, and comorbidities was collected.

3.3.Participants

An a priori power analysis was conducted using G*Power 3.1.9.2 Software; in the case-control study, to determine the minimum sample size required for the study. Power= 0.95, effect size= 1.086, and alpha= 0.05, the results showed that the minimum sample size for each group should be 25.

A total of 75 participants aged >18, were included in our study. The participants were divided into three groups; severe and mild COVID-19 patients, and non-COVID-19 individuals as a control group. The first group included severe COVID-19 patients n= (25), hospitalized in the ICU of the university hospital. The second group included mild COVID-19 patients n= (25), who were not hospitalized and spent 8-12 days of infection. The third group included non-COVID-19 individuals, as the control group, n= (25), who have never been infected with COVID-19 nor vaccinated. The demographic characteristics of the cases included in the study are summarized in Table 3.1.

Table 3.1. Demographic characteristics of the study cases

	Intensive Care Patients N:25	Mild Course Patients N:25	Control Group N:25
Age	66.4±15.7	34.9±10.2	35±7.6
Gender (Male/Female)	Male: 12 (48%) Female: 13 (52%)	Male: 13 (52%) Female: 12 (48%)	Male: 12 (48%) Female: 13 (52%)
BMI	29.4±6.4	25.1±4.7	24.2±4.2
SpO_2	SpO_2 >95 (5%) SpO_2 90-95 (45%) SpO_2 <90 (50%)	-	-
Death	68 % (17/25)	-	-
Comorbidity	Hypertension (72%) Lung cancer (4%) Multiple sclerosis (4%) Diabetes mellitus (24%) Hepatitis C (4%) Pregnant (8%) Schizophrenia (4%) Chronic obstructive pulmonary disease (12%) Obesity (8%) Benign prostatic hyperplasia (4%) Obstructive Sleep Apnea (4%) Dementia (4%) Coronary artery disease (4%) Congestive heart failure (8%) Asthma (4%)	Asthma (4%) Hepatitis B (4%)	Hyperthyroid (4%) Insulin resistance (4%) Hepatitis B (4%) Asthma (4%) Thalassemia (4%)
Treatment	Corticosteroid (Prednol/Dekort) (64%) Midazolam (52%) Antiviral (48%)	-	-
Variants	British variant (12%) Delta variant (4%)	-	-

3.4.Sample Collection and Processing

For both ELISA test and biochemical analysis, 5mL of venous blood was collected from each participant in vacuum tubes. Blood samples were centrifuged at 14000 rpm for 10 minutes (Hettich Zentrifugen- Rotanta 460R- Germany), and serum was separated and stored at -80 °C until analysis time.

3.5.Biochemical Analysis

Blood glucose, alanine transaminase (ALT), aspartate aminotransferase (AST), triglycerides (TG), High density lipoprotein (HDL), Low density lipoprotein (LDL), Very low-density lipoprotein (VLDL), ferritin, c-reactive protein (CRP), blood urea nitrogen (BUN), and vitamin D, were analyzed in the clinical biochemistry laboratory of Tokat Gaziosmapasa University hospital using the ROCHE Cobas 6000/c501/e601 chemistry analyzer. Copeptin was measured by Human CPP(Copeptin) ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China). Malondialdehyde was measured using Human MDA(Malonaldehyde) ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China). MDA test was repeated using Thiobarbituric acid assay (TBA). 4-Hydroxynonenal was analyzed using 4-HNE(4-Hydroxynonenal) ELISA Kit 96T (Elabscience Biotechnology Co., Ltd., Wuhan, China). Melatonin was measured by Human MT(Melatonin)ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China). Interleukin-6 was measured using Human IL-6 (Interleukin 6) ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China). ORGANON TEKNIKA 12 420

microplate reader at 450 nm was used for reading the optical density (O.D.) absorbance of the five ELISA tests.

3.5.1. Assessment of Copeptin (CPP)

Plasma copeptin was measured with sandwich enzyme-linked immune-sorbent assay technology using Human CPP(Copeptin) ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China) (Cat. no. EH2880). The optical density (O.D.) absorbance was read using ORGANON TEKNIKA 12 420 microplate reader at 450 nm. And copeptin was expressed in picogram per milliliter (pg/mL). Calculations were done using the Microsoft excel 2016 program.

- **Sandwich Enzyme-Linked Immune-Sorbent Assay Principle**

The 96-well plate was pre-coated with capture antibody. The biotin conjugated antibody was used as detection antibodies. Standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, followed by washing process with Wash Buffer. Horseradish peroxidase-Streptavidin (HRP-Streptavidin) was added and the Wash Buffer was used to remove away any unbound conjugates. to visualize HRP enzymatic reaction, Tetramethylbenzidine peroxidase substrates (TMB) were used. TMB was catalyzed by HRP to produce a blue color product. Adding acidic stop solution turns this blue color into yellow. The density of the yellow color is proportional to the target amount of sample captured in plate.

- **Preparation of Working Solutions**

All the reagents and samples were left at room temperature 20 minutes before the assay.

- **Sample Preparation:**

Samples were diluted at 1:3, where 75 µl of sample was diluted with 25 µl with sample dilution buffer and mixed thoroughly.

- **Wash Buffer Preparation:**

30 ml concentrated Wash Buffer (25X) was diluted to 750 ml Wash Buffer using distilled water.

- **Standards Preparation:**

1 ml of Sample Dilution Buffer was added to one Standard tube (labelled as zero tube), then it was left for 10 minutes at room temperature and mixed thoroughly. 7 Eppendorf tubes were labeled 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and blank. 0.3 ml of Sample Dilution Buffer was added to each tube. 0.3 ml was transferred from the zero labelled tube to the 1/2 labelled tube and mixed thoroughly, and then 0.3 ml were transferred from this tube to the next one, and mixed thoroughly, and so on. No solution was transferred to the blank labelled tube (Figure 3.1.).

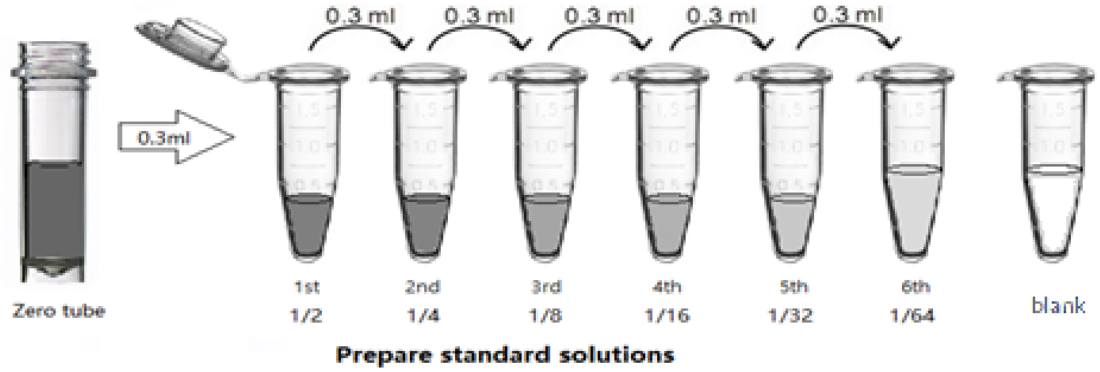


Figure 3.1. Concentrations of Standard Solutions (Copeptin Assay)

- **Biotin-labelled Antibody Working Solution Preparation:**

Biotin-detection antibody was diluted with Antibody Dilution Buffer at 1:100, and then mixed thoroughly 1 hour before the assay.

- **HRP-Streptavin Conjugate (SABC) Working Solution Preparation:**

SABC solution was diluted with SABC dilution Buffer at 1:100 and then mixed thoroughly 30 minutes before the assay.

- **Assay Procedure**

The 96 well microplate was labelled, where 7 wells were for the standards and the rest 89 wells were for the samples. 100 µl of each standard was added to each standard well. 100 µl of each sample (75 µl Sample + 25 µl Sample Dilution Buffer) were added to test sample wells. The plate was sealed with a cover and incubated for 90 minutes at 37 ° C. The plate content was discarded and washed 2 times with Wash Buffer (350 µl washing buffer

for each well). 100 µl of Biotin-labeled Antibody working solution was added to each well, then the plate was covered, taped gently, and incubated for 60 minutes at 37 ° C. The plate content was discarded and the plate was washed 3 times with Wash Buffer. 100 µl of SABC working solution was added to each well and the plate was covered, taped gently and incubated at 37 ° C for 30 minutes. The plate content was discarded and the plate was washed 5 times with Wash Buffer. In a dark place, 90 µl TMB substrate was added into each well, the plate was covered, taped gently, and incubated at 37 ° C in dark for 15 minutes. After the color change was noticed 50 µl of stop solution was added into each well and the color turned yellow immediately (Figure 3.2.). The O.D. absorbance was read using Microplate Reader immediately at 450 nm.

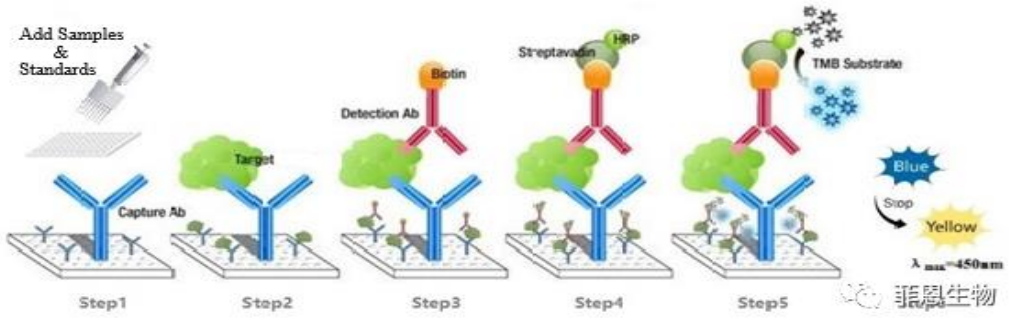


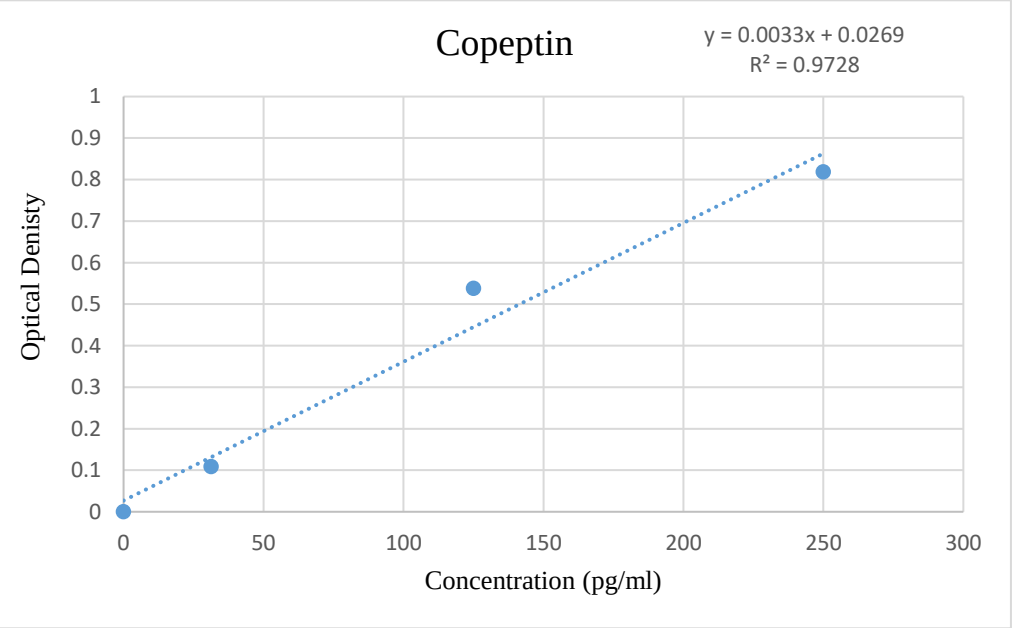
Figure 3.2. Summary of Copeptin Assay Procedure

- **Calculations**

The relative O.D. = O.D. of each well – O.D. of blank well

A standard curve was plotted using the Microsoft excel 2016 program, where the Y-axis represented the O.D. of each standard solution and the X-axis represented the respective concentration of the standard solution. The target concentrations of the samples were

interpolated from the standard curve (Graph 3.1.). The interpolated concentrations of the samples were multiplied by 1.33 as a dilution factor.



Graph 3.1. Standard Curve of Copeptin.

3.5.2. Assessment of Malondialdehyde (MDA)

Plasma Malondialdehyde was measured by Competitive-ELISA detection method, using Human MDA (Malonaldehyde) ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China) (Cat. no. EH4147). The optical density (O.D.) absorbance was read using ORGANON TEKNIKA 12 420 microplate reader at 450 nm. and Malondialdehyde was expressed in nanograms per milliliter (ng/mL). Calculations were done using the Microsoft excel 2016 program.

- **Competitive-ELISA Detection Method Principle**

The microtiter plate provided in this kit has been pre-coated with target. During the reaction, target in the sample or standard competes with a fixed amount of target on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to target. Excess conjugate and unbound sample or standard are washed from the plate, and HRP-Streptavidin (SABC) is added to each microplate well and incubated. Then TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of target in the samples is then determined by comparing the OD of the samples to the standard curve.

- **Preparation of Working Solutions**

All the reagents and samples were left at room temperature 20 minutes before the assay.

- **Sample Preparation:**

Samples were diluted at 1:1, where 25 µl of sample was diluted with 25 µl with sample dilution buffer and mixed thoroughly.

- **Wash Buffer Preparation:**

30 ml concentrated Wash Buffer (25X) was diluted to 750 ml Wash Buffer using distilled water.

- **Standards Preparation:**

1 ml of Sample Dilution Buffer was added to one Standard tube (labelled as zero tube), then it was left for 10 minutes at room temperature and mixed thoroughly. 7 Eppendorf

tubes were labeled 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and blank. 0.3 ml of Sample Dilution Buffer was added to each tube. 0.3 ml was transferred from the zero labelled tube to the 1/2 labelled tube and mixed thoroughly, and then 0.3 ml were transferred from this tube to the next one, and mixed thoroughly, and so on. No solution was transferred to the blank labelled tube (Figure 3.3.).

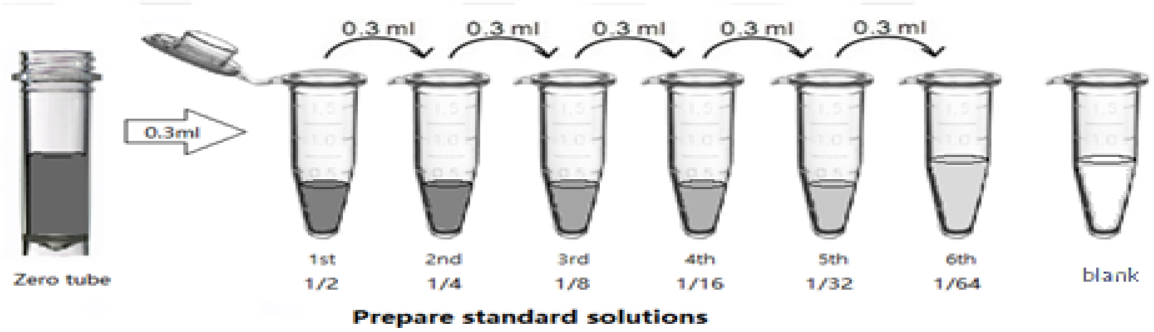


Figure 3.3. Concentration of Standard Solutions (Malondialdehyde Assay)

- **Biotin-labelled Antibody Working Solution Preparation:**

Biotin-detection antibody was diluted with Antibody Dilution Buffer at 1:100, and then mixed thoroughly 1 hour before the assay.

- **HRP-Streptavin Conjugate (SABC) Working Solution Preparation:**

SABC solution was diluted with SABC Dilution Buffer at 1:100 and then mixed thoroughly 30 minutes before the assay.

- **Assay Procedure**

The 96 well microplate was labelled, where 7 wells were for the standards and the rest 89 wells were for the samples. The plate was washed 2 times with Wash Buffer. Then 50 µl of

each standard was added to each standard well. 50 µl of each sample (25 µl Sample + 25 µl Sample Dilution Buffer) was added to test sample wells. 50 µl of Biotin-labeled Antibody working solution was added immediately to each well, then the plate was covered, taped gently, and incubated for 45 minutes at 37 ° C. The plate content was discarded and washed 3 times with Wash Buffer (350 µl washing buffer for each well). 100 µl of SABC working solution was added to each well and the plate was covered, taped gently, and incubated at 37 ° C for 30 minutes. The plate content was discarded and the plate was washed 5 times with Wash Buffer. In a dark place, 90 µl TMB substrate was added into each well, the plate was covered, taped gently, and incubated at 37 ° C in dark for 15 minutes. After the color change was noticed 50 µl of stop solution was added into each well and the color turned yellow immediately (Figure 3.4.). The O.D. absorbance was read using Microplate Reader immediately at 450 nm.

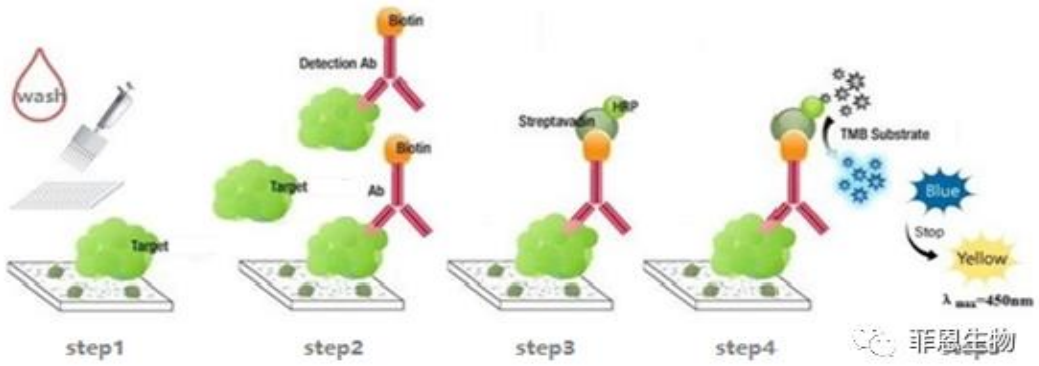
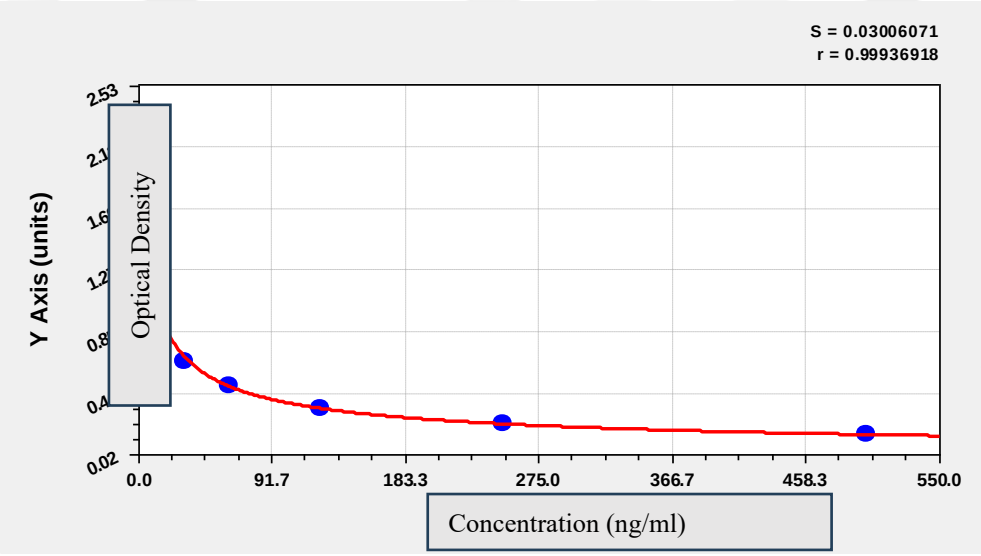


Figure 3.4. Summary of Malondialdehyde Assay Procedure.

- **Calculations**

The relative O.D. = O.D. of each well – O.D. of blank well

A standard curve was plotted using the Curve Expert 1.3 program, where the Y-axis represented the O.D. of each standard solution and the X-axis represented the respective concentration of the standard solution. The target concentrations of the samples were interpolated from the standard curve (Graph 3.2.). The interpolated concentrations of the samples were multiplied by 2 as a dilution factor.



Graph 3.2. Standard Curve of Malondialdehyde (ELISA Assay)

3.5.3. Assessment of 4-Hydroxynonenal (4-HNE)

Plasma 4-Hydroxynonenal (4-HNE) was measured by Competitive-ELISA principle, using 4-HNE(4-Hydroxynonenal) ELISA Kit 96T (Elabscience Biotechnology Co., Ltd., Wuhan, China) (Cat. no. E-EL-0128). The optical density (O.D.) absorbance was read using ORGANON TEKNIKA 12 420 microplate reader at 450 nm. And 4-HNE was expressed in nanograms per milliliter (ng/mL).

The standard curve was plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The target concentration of the samples can be interpolated from the standard curve. Calculations were done using the Microsoft excel 2016 program.

- **Competitive-ELISA Detection Method Principle**

This ELISA kit uses the Competitive-ELISA principle. The micro plate provided in this kit has been pre-coated with 4-HNE. During the reaction, 4-HNE in the sample or standard competes with a fixed amount of 4-HNE on the solid phase supporter for sites on the Biotinylated Detection Ab specific to 4-HNE. Excess conjugate and unbound sample or standard is washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) are added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of 4-HNE in the samples is then determined by comparing the OD of the samples to the standard curve.

- **Preparation of Working Solutions**

All the reagents and samples were left at room temperature 20 minutes before the assay.

- **Sample Preparation:**

No dilutions were performed.

- **Wash Buffer Preparation:**

30 ml concentrated Wash Buffer (25X) was diluted to 750 ml Wash Buffer using distilled water.

- **Standards Preparation:**

The Reference Standard was centrifuged for 1 minute at 10,000×g. 40 ng/mL working solution was prepared by adding 1 ml of Reference standard and Sample Diluent to the Reference Standard and left for 2 minutes, then mixed using vortex meter at low speed. Serial dilutions of 20, 10, 5, 2.5, 1.25, 0.63, and 0 (Blank) were prepared as follows. 7 Eppendorf tubes were labeled 20, 10, 5, 2.5, 1.25, 0.63, and Blank. 0.5 ml of Reference standard and Sample Diluent was transferred to each tube. 0.5 ml of the 40 ng/mL working solution was transferred to the first tube, and mixed thoroughly to produce 20 ng/mL working solution. Then 0.5 ml was transferred to the next tube and mixed thoroughly, and so on. No solution was transferred to the blank labelled tube (Figure 3.5.).

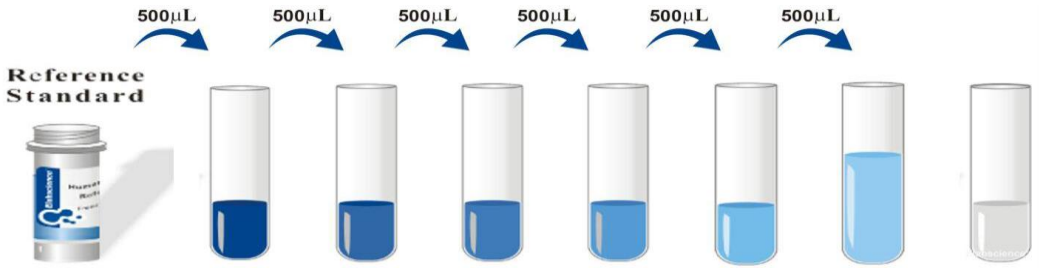


Figure 3.5. Concentrations of Standard Solutions (4-Hydroxynonenal assay)

- **Biotinylated Detection Ab Working Solution Preparation:**

100× concentrated Biotinylated Detection Ab was centrifuged at 800×g for 1 minute, then it was diluted with Biotinylated Detection Ab Diluent at 1:100 to produce 1× working solution.

- **HRP Conjugate Working Solution Preparation:**

100× concentrated HRP Conjugate was centrifuged at 800×g for 1 minute, then it was diluted with HRP Conjugate Diluent at 1:100 to produce 1× working solution.

- **Assay Procedure**

The 96 well microplate was labelled, where 7 wells were for the standards and the rest 89 wells were for the samples. 50 µl of each standard was added to each standard well. 50 µl of each sample was added to test sample wells. Biotinylated Detection Ab working solution was added immediately to each well. The plate was sealed with a cover and incubated for 45 minutes at 37 ° C. The plate content was discarded and washed 3 times with Wash Buffer (350 µl washing buffer for each well). 100 µl of HRP Conjugate working solution was added to each well, then the plate was covered, taped gently, and incubated for 30 minutes at 37 ° C. The plate content was discarded and the plate was washed 5 times with Wash Buffer. In a dark place, 90 µl of Substrate Reagent was added to each well the plate was covered, taped gently and incubated at 37 ° C for 15 minutes away from light. After the color change was noticed 50 µl of stop solution was added into each well and the color turned yellow immediately (Figure 3.6.). The O.D. absorbance was read using Microplate Reader immediately at 450 nm.

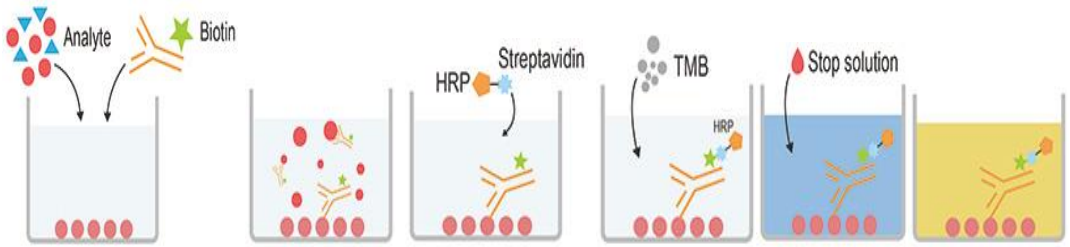
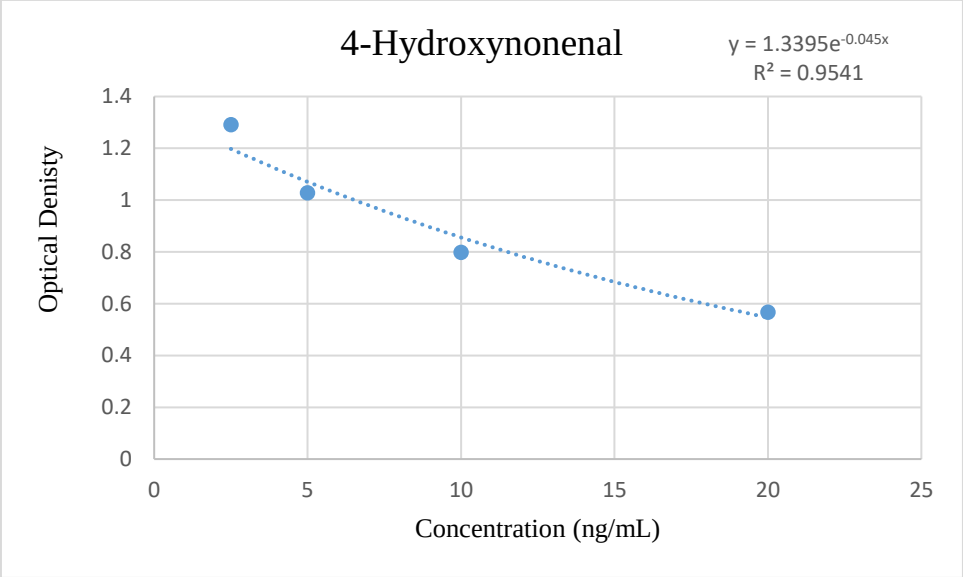


Figure 3.6. Summary of 4-Hydroxyneonal Assay Procedure

- **Calculations**

The relative O.D. = O.D. of each well – O.D. of blank well. A standard curve was plotted using the Microsoft excel 2016 program, where the Y-axis represented the O.D. of each standard solution and the X-axis represented the respective concentration of the standard solution (Graph 3.3.). The target concentrations of the samples were interpolated from the standard curve.



Graph 3.3. Standard Curve of 4-Hydroxynonenal.

3.5.4. Assessment of Melatonin (MT)

Plasma copeptin was measured with Competitive-ELISA detection method, using Human MT(Melatonin)ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China) (Cat. no. EH3344). The optical density (O.D.) absorbance was read using ORGANON TEKNIKA 12 420 microplate reader at 450 nm. And melatonin was expressed in picogram per milliliter (pg/mL). Calculations were done using the Microsoft excel 2016 program.

- **Competitive-ELISA Detection Method Principle**

  This kit was based on Competitive-ELISA detection method. The microtiter plate provided in the kit has been pre-coated with target. During the reaction, target in the sample or standard competes with a fixed amount of target on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to target. Excess conjugate and unbound sample or standard are washed from the plate, and HRP-Streptavidin (SABC) is added to each microplate well and incubated. Then TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of target in the samples is then determined by comparing the OD of the samples to the standard curve.

- **Preparation of Working Solutions**

  All the reagents and samples were left at room temperature 20 minutes before the assay.

- **Sample Preparation:**

  No sample dilution.

- **Wash Buffer Preparation:**

30 ml concentrated Wash Buffer (25X) was diluted to 750 ml Wash Buffer using distilled water.

- **Standards Preparation:**

1 ml of Sample Dilution Buffer was added to one Standard tube (labelled as zero tube), then it was left for 10 minutes at room temperature and mixed thoroughly. 7 Eppendorf tubes were labeled 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and blank. 0.3 ml of Sample Dilution Buffer was added to each tube. 0.3 ml was transferred from the zero labelled tube to the 1/2 labelled tube and mixed thoroughly, and then 0.3 ml were transferred from this tube to the next one, and mixed thoroughly, and so on. No solution was transferred to the blank labelled tube (Figure 3.7.).

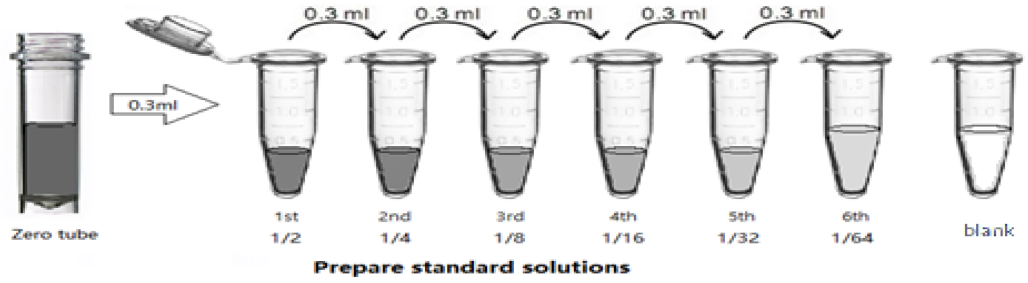


Figure 3.7. Concentrations of Standard Solutions (Melatonin Assay)

- **Biotin-labelled Antibody Working Solution Preparation:**

Biotin-detection antibody was diluted with Antibody Dilution Buffer at 1:100, and then mixed thoroughly 1 hour before the assay.

- **HRP-Streptavin Conjugate (SABC) Working Solution Preparation:**

SABC solution was diluted with SABC dilution Buffer at 1:100 and then mixed thoroughly 30 minutes before the assay.

- **Assay Procedure**

The 96 well microplate was labelled, where 7 wells were used for the standards and the rest (89 wells) were for the samples. The plate was washed 2 times with Wash Buffer (350 µl washing buffer for each well) before loading standards and samples. 50 µl of each standard was added to each standard well. 50 µl of each sample were added to test sample wells. 50 µl of Biotin-labeled Antibody working solution was added to each well immediately, then the plate was covered, taped gently, and incubated for 45 minutes at 37 ° C. The plate content was discarded and washed 3 times with Wash Buffer. 100 µl of SABC working solution was added to each well and the plate was covered, taped gently and incubated at 37 ° C for 30 minutes. The plate content was discarded and the plate was washed 5 times with Wash Buffer. In a dark place, 90 µl TMB substrate was added into each well, the plate was covered, taped gently, and incubated at 37 ° C in dark for 15 minutes. After the color change was noticed 50 µl of stop solution was added into each well and the color turned yellow immediately (Figure 3.8.). The O.D. absorbance was read using Microplate Reader immediately at 450 nm.

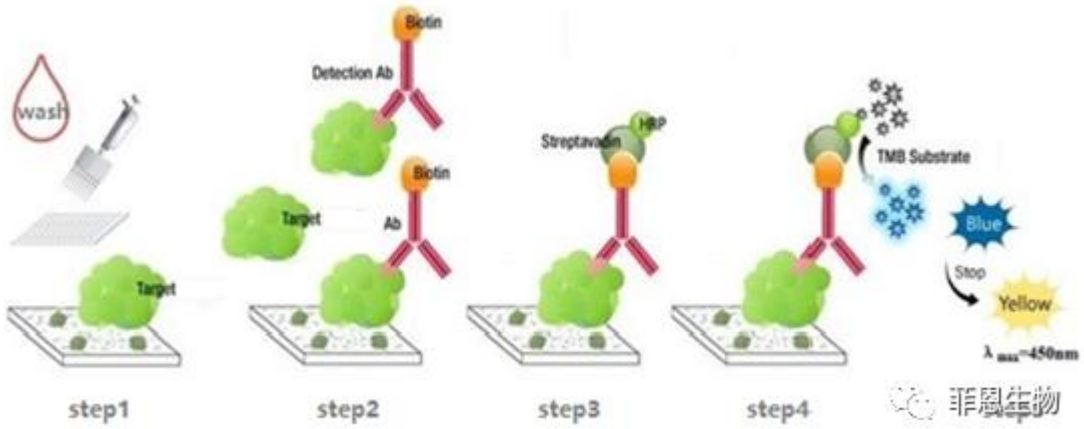
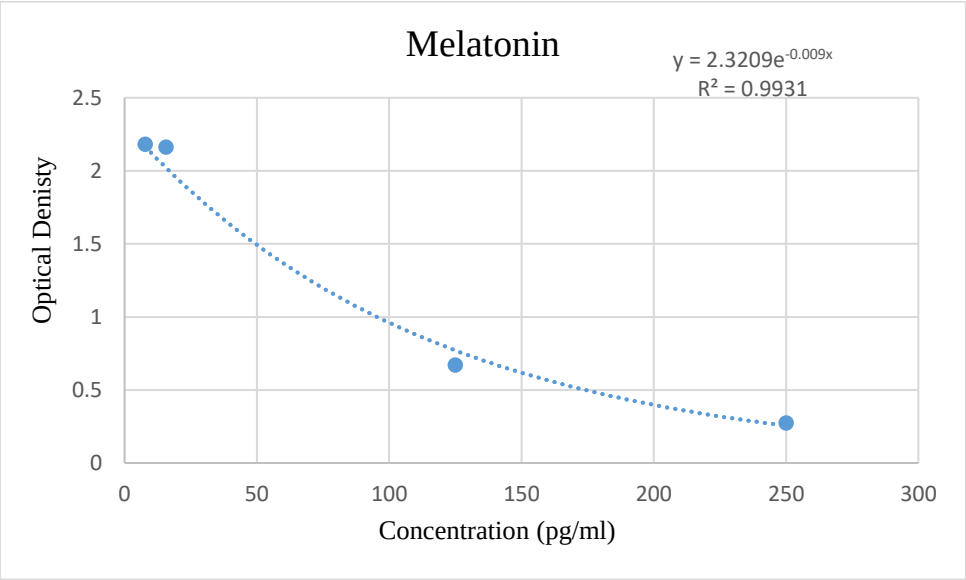


Figure 3.8. Summary of Melatonin Assay Procedure

- **Calculations**

The relative O.D. = O.D. of each well – O.D. of blank well. A standard curve was plotted using the Microsoft excel 2016 program, where the Y-axis represented the O.D. of each standard solution and the X-axis represented the respective concentration of the standard solution. The target concentrations of the samples were interpolated from the standard curve (Graph 3.4.).



Graph 3.4. Standard Curve of Melatonin.

3.5.5. Assessment of Interleukin 6 (IL-6)

Plasma interleukin was measured with sandwich enzyme-linked immune-sorbent assay technology using Human IL-6 (Interleukin 6) ELISA Kit 96T (Wuhan Fine Biotech Co., Ltd., Wuhan, China) (Cat. no. EH0201). The optical density (O.D.) absorbance was read using ORGANON TEKNIKA 12 420 microplate reader at 450 nm. And interleukin was expressed in picogram per milliliter (pg/mL). Calculations were done using the Microsoft excel 2016 program.

- **Sandwich Enzyme-Linked Immune-Sorbent Assay Principle**

The 96-well plate was pre-coated with capture antibody. The biotin conjugated antibody was used as detection antibodies. Standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, followed by washing process with Wash

Buffer. Horseradish peroxidase-Streptavidin (HRP-Streptavidin) was added and the Wash Buffer was used to remove away any unbound conjugates. to visualize HRP enzymatic reaction, Tetramethylbenzidine peroxidase substrates (TMB) were used. TMB was catalyzed by HRP to produce a blue color product. Adding acidic stop solution turns this blue color into yellow. The density of the yellow color is proportional to the target amount of sample captured in plate.

- **Preparation of Working Solutions**

All the reagents and samples were left at room temperature for 20 minutes before the assay.

- **Sample preparation:**

No dilution was performed.

- **Wash Buffer Preparation:**

30 ml concentrated Wash Buffer (25X) was diluted to 750 ml Wash Buffer using distilled water.

- **Standards Preparation:**

1 ml of Sample Dilution Buffer was added to one Standard tube (labeled as zero tube), then it was left for 10 minutes at room temperature and mixed thoroughly. 7 Eppendorf tubes were labeled 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and blank. 0.3 ml of Sample Dilution Buffer was added to each tube. 0.3 ml was transferred from the zero-labeled tube to the 1/2-labeled tube and mixed thoroughly, and then 0.3 ml was transferred from this tube to the next one, and mixed thoroughly, and so on. No solution was transferred to the blank labeled tube (Figure 3.9.).

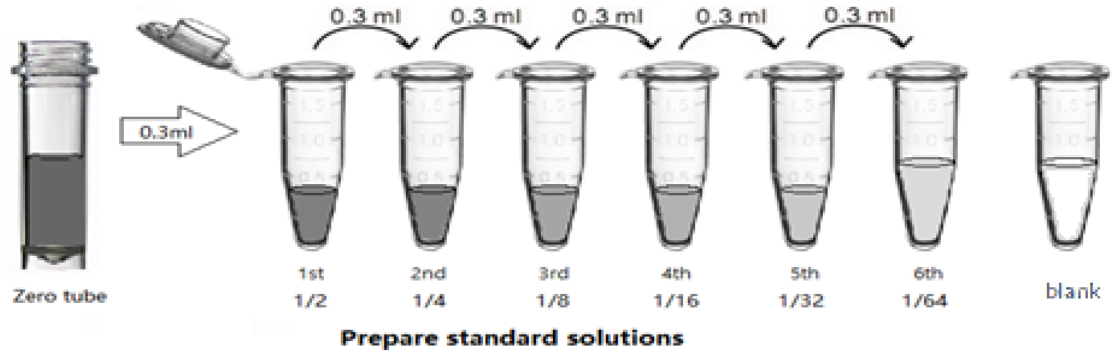


Figure 3.9. Concentrations of Standard Solutions (IL-6 Assay).

- **Biotin-labelled Antibody Working Solution Preparation:**

Biotin-detection antibody was diluted with Antibody Dilution Buffer at 1:100 and mixed thoroughly 1 hour before the assay.

- **HRP-Streptavin Conjugate (SABC) Working Solution Preparation:**

SABC solution was diluted with SABC dilution Buffer at 1:100 and mixed thoroughly 30 minutes before the assay.

- **Assay Procedure**

The 96-well microplate was labeled, where 7 wells were for the standards and the rest 89 wells were for the samples. 100 µl of each standard was added to each standard well. 100 µl of each sample was added to test sample wells. The plate was sealed with a cover and incubated for 90 minutes at 37 ° C. The plate content was discarded and washed 2 times with Wash Buffer (350 µl washing buffer for each well). 100 µl of Biotin-labeled Antibody working solution was added to each well, then the plate was covered, taped gently, and incubated for 60 minutes at 37 ° C. The plate content was discarded and the plate was washed

3 times with Wash Buffer. 100 µl of SABC working solution was added to each well and the plate was covered, taped gently, and incubated at 37 ° C for 30 minutes. The plate content was discarded and the plate was washed 5 times with Wash Buffer. In a dark place, 90 µl TMB substrate was added into each well, the plate was covered, taped gently, and incubated at 37 ° C in the dark for 15 minutes. After the color change was noticed 50 µl of stop solution was added to each well and the color turned yellow immediately (Figure 3.10.). The O.D. absorbance was read using Microplate Reader immediately at 450 nm.

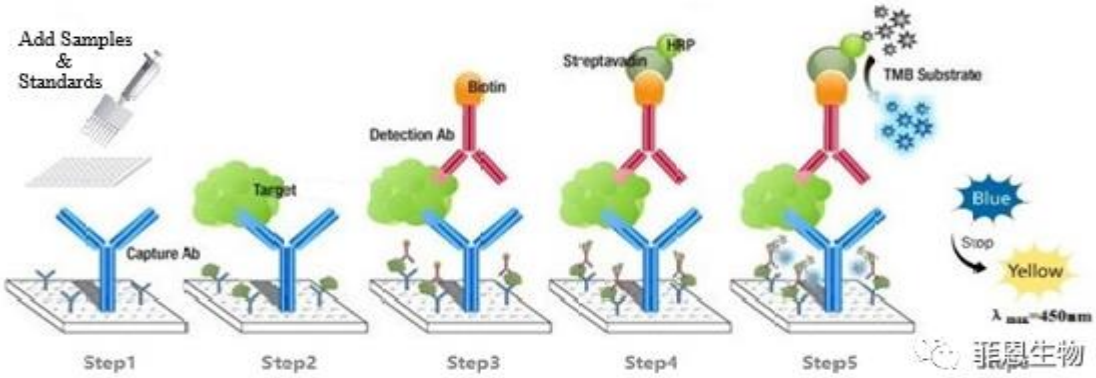
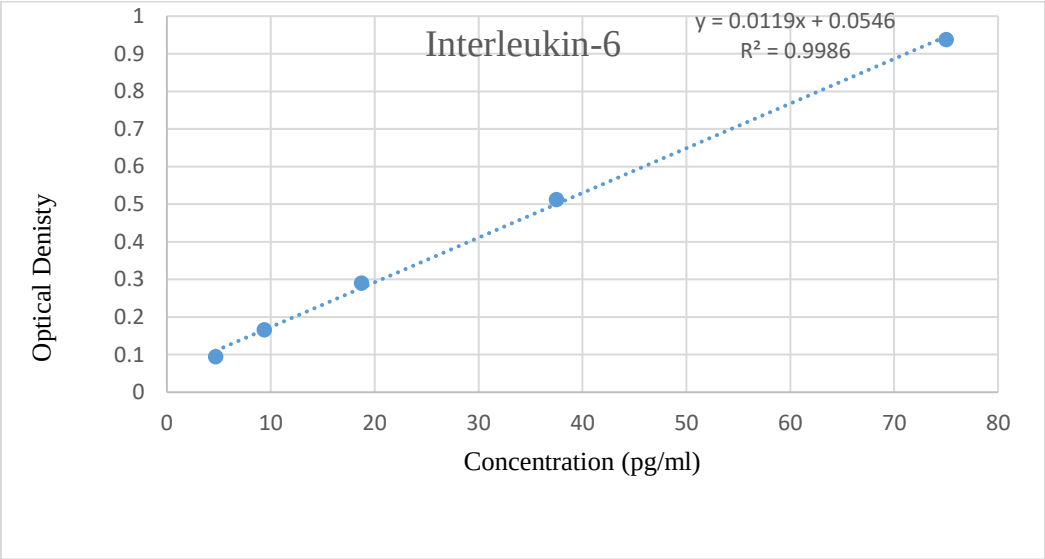


Figure 3.10. Summary of IL-6 assay procedure.

- **Calculations**

The relative O.D. = O.D. of each well – O.D. of blank well. A standard curve was plotted using the Microsoft excel 2016 program, where the Y-axis represented the O.D. of each standard solution and the X-axis represented the respective concentration of the standard solution. The target concentrations of the samples were interpolated from the standard curve (Graph 3.5.).



Graph 3.5. Standard Curve of Interleukin-6.

3.5.6. Thiobarbituric acid (TBA) Assay for MDA

MDA estimation was repeated using the TBA assay. The principle of the assay depends on the reaction between MDA and TBA forming a pinkish red chromogen (TBARS). This chromogen can be measured at 532 – 535 nm.

- **Procedure**:

  1- In a glass test tube 0.25 ml sample or standard were added.

  2- 1.5 ml of 0.1 % phosphoric acid was added.

  3- 0.5 ml of 0.6 % TBA was added.

  4- The tubes were mixed well by vortex.

  5- The tubes were covered well and incubated in water bath at 95 °C for 45 minutes. (a pink color was observed during the incubation).

  6- The tubes were removed from the water bath and cooled under the tap water.

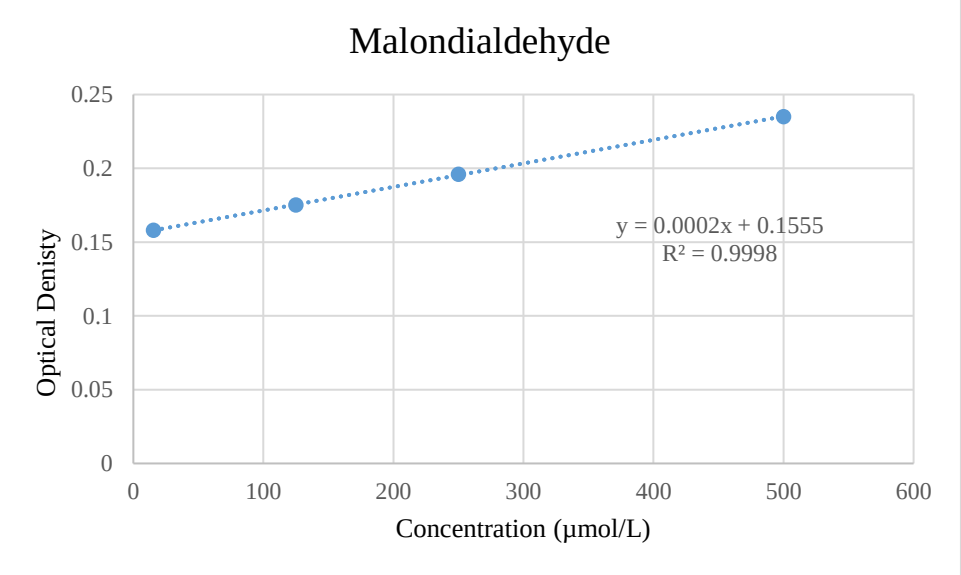
7- 1 ml n-butanol was added to each tube and vortex well.

8- The tubes were centrifuged at 1500 x g for 10 minutes.

9- 1 ml was taken from the supernatant and measured at 537 nm in a quartz cuvette. N-butanol was used as blank.

10- The cuvette was washed well with n-butanol after every reading.

11- Standard curve was drawn and MDA concentrations were calculated from the curve (Graph 3.6.).



Graph 3.6. Standard Curve of Malondialdehyde (N-Butanol Assay)

3.6.Statistical Analysis

Statistical data were conducted using IBM SPSS Statistics 20 software and Microsoft excel 2016. For homogeneity of variances, Levene's test was used, and the One-Sample Kolmogorov-Smirnov test was used for normality. Normally distributed parameters

were analyzed via parametric tests and expressed as mean±SD. Non-normally distributed parameters were expressed as median and interquartile range (IQR) and analyzed using non-parametric tests. To compare the differences between the control and patient groups, Independent Sample T-test was used for normally distributed parameters, Mann-Whitney U Test was used for non-normally distributed parameters, and $p < 0.05$ was considered significant. To determine the significance of mean/median values of the study parameters between the three groups of the study, One Way-ANOVA test followed by a post hoc test (TUKEY HSD) was performed in the case of normally distributed parameters. For non-normally distributed parameters, Kruskal-Wallis test was conducted. The results were considered statistically significant at $p < 0.05$. The relationships between the study parameters were evaluated using Pearson (for homogenously distributed parameters), and Spearman (for non-homogenously distributed parameters) correlation coefficients. Statistical significance was regarded as $p < 0.05$ and $p < 0.01$.

4. RESULTS

Table 4.1. Parameter comparison between the patient and control groups

	Control Group N: 23	Patient Group N: 50	P Value
Age	35 (20-49)	47 (17-89)	0.002**
Gender (M/F)	12/13	25/25	0.0865**
BMI	25.12±4.17	27.44±7.32	0.180*
Glucose (mg/dl)	94 (87.1-108.2)	118 (89.5-175.0)	0.011**
AST (IU/L)	18.0 (11.0-38.0)	20.5 (1.0-1026.0)	0.324**
ALT (IU/L)	16.0 (8.0-84.0)	17.5 (5.0-505.0)	0.483**
TG (mg/dl)	134.0 (72.0-373.0)	166.0 (52.0-1368.0)	0.066**
BUN (mg/dl)	14.0 (8.0-21.0)	15.0 (6.0-94.0)	0.029**
CRP (mg/dl)	2.0 (1.0-9.0)	32.0 (1.0-437.0)	<0.001**
Ferritin (ng/ml)	69.0(6.0-152.0)	231.5 (6.0-4135.0)	<0.001**
IL-6 (pg/ml)	3.0 (1.10-7.8)	5.25 (1.2-160.6)	0.002**
VLDL (mg/dl)	27.0 (14.0-75.0)	33.5 (10.0-274.0)	0.070**
HDL (mg/dl)	49.48±16.10	36.75±11.12	<0.001*
LDL (mg/dl)	106.35±26.62	79.5±37.17	0.003*
Vitamin D (ng/ml)	27.54±13.44	20.34±9.47	0.010*
CPP (pg/ml)	116.66±35.36	111.25±58.02	0.026*
MT (pg/ml)	186.29±39.77	186.73±44.44	0.967*
MDA (ng/ml)	30.33±9.37	28.15±14.17	0.023*
4-HNE (ng/ml)	25.36±16.19	22.23±15.02	0.423*

*Independent T Test was used. $p < 0.05$ was considered significant.
** Mann-Whitney U Test was used. $p < 0.05$ was considered significant.

Patients group and control group were analyzed in terms of the study parameters. Mann-Whitney U Test was used for non-normally distributed parameters, and Independent T Test was used for normally distributed parameters (Table 4.1). According to the results mentioned in Table 4.1, a statistically significant difference was observed between the patient's group and the control group in terms of age, blood glucose, CRP, ferritin, IL-6, HDL, LDL, vitamin D, CPP, and MDA ($p < 0.05$)

Table 4.2. Multiple comparison of study groups

	ICU Group N:25	Mild Group N:25	Control Group N: 25	P Value
Age	69.0 (28.0-89.0)	34.0 (17.0-54.0)	35 (20-49)	<0.001*
Gender (M/F)	12/13	13/12	12/13	0.947**
BMI	29.38 (21.45-53.30)	25.09 (16.53-35.88)	24.15(19.56-34.63)	0.005*
Glucose (mg/dl)	164.0 (69.0-341.0)	93.0 (56.0-191.0)	90.0 (69.0-170.0)	<0.001*
AST (IU/L)	25.0 (4.0-1026.0)	15.0 (1.0-48.0)	18.0 (11.0-38.0)	0.004*
ALT (IU/L)	20.00 (5.0-505.0)	16.0 (6.0-77.0)	16.0 (8.0-84.0)	0.474*
TG (mg/dl)	137.0 (82.0-594.0)	189.0 (52.0-1368.0)	134.0 (72.0-373.0)	0.044*
BUN (mg/dl)	27.8 (10.0-94.0)	13.0 (6.0-17.0)	14.0 (8.0-21.0)	<0.001*
CRP (mg/dl)	105.0 (15.0-437.0)	2.0 (1.0-50.0)	2.0 (1.0-9.0)	<0.001*
Ferritin (ng/ml)	505.0 (64.0-4135.0)	83.0 (6.0-479.0)	69.0(6.0-152.0)	<0.001*
IL-6 (pg/ml)	18.8 (2.7-160.6)	3.0 (1.2-9.10)	3.0 (1.10-7.8)	<0.001*
VLDL (mg/dl)	27.0 (17.0-119.0)	38.0 (10.0-274.0)	27.0 (14.0-75.0)	0.046*
HDL (mg/dl)	34.0 (8.0-64.0)	37.0 (18.0-57.0)	46.0 (27.0-89.0)	0.005*
LDL (mg/dl)	66.0 (6.0-123.0)	82.0 (12.0-189.0)	104.0 (74.0-206.0)	<0.001*
Vitamin D (ng/ml)	19.6±10.83	21.1±8.05	27.54±13.43	0.034**
CPP (pg/ml)	96.0 (15.0-223.0)	115.0 (17.0-232.2)	119.0 (59.3-188.0)	0.330*
MT (pg/ml)	177.07±49.22	196.36±37.61	186.29±39.77	0.282**
MDA (ng/ml)	20.02±10.83[a,b]	36.28±13.13	30.33±9.37	<0.001**
4-HNE (ng/ml)	16.72±14.73	27.74±13.43	25.36±16.19	0.027**

*Kruskal-Wallis test was used. $p< 0.05$ was considered significant.
**ANOVA test was used. $p< 0.05$ was considered significant.

Levene's test was performed to evaluate the homogeneity of the study variances, Followed by ANOVA or Kruskal-Wallis test to compare the study groups in terms of the study parameters. Parameters with Levene's test $p > 0.05$ were evaluated by ANOVA test followed by TUKEY-HSD (post hoc test), and parameters with $p < 0.05$ were tested using the Kruskal Wallis test (Table 4.2). A statistically significant difference was observed between the three groups in terms of age (p <0.001), BMI (p <0.05), glucose (p <0.001), AST (p <0.05), TG (p <0.05), BUN (p <0.001), CRP (p <0.001), ferritin (p <0.001), IL-6 (p <0.001), VLDL(p <0.05), HDL (p <0.05), LDL (p <0.001), vitamin D (p <0.05), MDA (p <0.001), and 4-HNE (p <0.05).

Table 4.3. Comparing study parameters between treated, untreated, and control groups

	Treated N:16	Untreated N:31	Control Group N: 25	P Value
Age	69.5 (38.0-89.0)	40 (17.0-81.0)	35 (20-49)	<0.001*
Gender (M/F)	7/9	17/14	12/13	0.755**
BMI	29.38 (27.34-42.97)	25.23 (16.53-53.30)	24.15 (19.56-34.63)	0.019*
Glucose (mg/dl)	156.0 (69.0-278.0)	94.0 (56.0-341.5)	90.0 (69.0-170.0)	0.004*
AST (IU/L)	25(4-340)	18(1-1026)	18.0 (11.0-38.0)	0.196*
ALT (IU/L)	22(5-267)	16(6-505)	16.0 (8.0-84.0)	0.528*
TG (mg/dl)	157.0 (87.0-311.0)	175.0 (52.0-1368.0)	134.0 (72.0-373.0)	0.128*
BUN (mg/dl)	28.0 (10.0-90.0)	14.0 (6.0-94.0)	14.0 (8.0-21.0)	0.004*
CRP (mg/dl)	118.0 (34.0-437.0)	3.0 (1.0-238.0)	2.0 (1.0-9.0)	<0.001*
Ferritin (ng/ml)	469(64-1628)	192(6-36797)	69.0(6.0-152.0)	<0.001*
IL-6 (pg/ml)	26.45 (4.8-142.10)	3.4 (1.2-160.6)	3.0 (1.10-7.8)	<0.001*
VLDL (mg/dl)	31.5 (17.0-62.0)	35.0 (10.0-274.0)	27.0 (14.0-75.0)	0.134*
HDL (mg/dl)	36.5±12.44	36.70±11.0	49.48±16.10	0.001**
LDL (mg/dl)	61.0 (38.0-123.0)	80.0 (6.0-189.0)	104.0 (74.0-206.0)	<0.001*
Vitamin D (ng/ml)	17.44±7.70	19.99±7.92	27.54±13.43	0.005**
CPP (pg/ml)	105.25 (15.0-222.90)	109.0 (17.0-232.2)	118.9 (59.3-187.9)	0.770*
MT (pg/ml)	150(85-235)	188(90-290)	180(133-275)	0.095*
MDA (ng/ml)	16.7 (10.6-34.6)	30.2 (10.8-64.5)	27.2 (16.9-59.0)	<0.001*
4-HNE (ng/ml)	14.13±10.75	27.75±14.95	25.36±16.19	0.011**

*Kruskal-Wallis test $p<0.05$

**ANOVA test $p<0.05$

The effect of the corticosteroid treatment on the study parameters was investigated among the study groups. Patient group was divided into treated and non-treated. Levent's test for homogeneity was performed. Homogenous parameters (p >0.05), were tested by ANOVA test followed by TUKEY HSD test. For non-homogenous parameters (p <0.05), Kruskal-Wallis Test was used. p <0.05 was considered significant for both tests. Age (p <0.001), BMI (p <0.05), glucose (p <0.05), BUN (p <0.05), CRP (p <0.001), IL-6 (p <0.001), HDL (p <0.05), LDL (p <0.001), vitamin D (p <0.05), MDA (p <0.001), and 4-HNE (p <0.05) showed a statically significant difference between treated, untreated, and control groups.

Table 4.4. Comparing study parameters between treated and untreated ICU patients

	Treated N: 16	Untraeted N: 6	P Value
Age	50(38-72)	61(60-62)	0.052
Gender M/F	7/9	4/2	0.406
BMI	30(27-34)	31(21-40)	0.776
SpO2	84.94±9.46	91.75±3.5	0.941
Glucose (mg/dl)	152(118-195)	302(268-336)	0.894
AST (IU/L)	28.0 (4.0-551.0)	31.0 (11.70-1026.0)	0.883
ALT (IU/L)	17.5 (5.0-267.0)	22.0 (7.0-505.0)	1.000
TG (mg/dl)	186(87-284)	127(125-129)	0.810
BUN (mg/dl)	12.5(10-15)	21(18-24)	0.470
CRP (mg/dl)	133.5(53-213)	52.5(33-72)	0.406
Ferritin (ng/ml)	487.0 (64.0-4135.0)	656.0 (196.3-921.0)	0.804
IL-6 (pg/ml)	26.4(10.9-42.7)	6.6(2.7-10.5)	0.689
VLDL (mg/dl)	37(17-57)	25.5(25-26)	0.728
HDL (mg/dl)	38(28-53)	35(32-38)	0.247
LDL (mg/dl)	64.5(48-93)	84(60-108)	1.000
Vitamin D (ng/ml)	15.4(6-35)	45(35-55)	0.376
CPP (pg/ml)	134(31.5-166)	104(96-112)	0.282
MT (pg/ml)	148(146-152)	186(184-188)	0.168
MDA (ng/ml)	16.3(11-18)	23.25(13-33.2)	0.035
4-HNE (ng/ml)	8.5(6.7-21)	8.6(3.6-13.6)	0.010

* Mann-Whitney U Test $p < 0.05$

The effect of corticosteroid treatment was evaluated among the ICU patients. ICU group is divided into treated and untreated and the study parameters were investigated between the two groups. Glucose, MDA, and 4-HNE showed significantly difference between treated and untreated groups (p <0.05).

Table 4.5. Comparing Copeptin Levels Between Males and Females

	Female N: 36	Male N: 33	P Value
Copeptin	109.41±50.0	117.04±53.32	0.541

Copeptin levels were compared between males and females. No significant difference was observed

Table 4.6. Evaluation of the relationship between the study parameters

	CPP[a]		MDA[a]		4-HNE[a]		IL-6[b]		BUN[b]		Ferritin[b]		CRP[b]	
	r Value	P Value	r Value	P Value	r Value	P Value	r Value	P Value	r Value	P Value	r Value	P Value	r Value	P Value
CPP[a]	-	-	0.360	0.010*	0.197	0.188*	-	-	-	-	-	-	-	-
MDA[a]	-0.084	0.864*	-	-	0.360	0.010*	-	-	-	-	-	-	-	-
4-HNE[a]	0.197	0.188*	0.360	0.010*	-	-	-	-	-	-	-	-	-	-
MT[a]	-0.403	0.006**	0.273	0.056*	-0.046	0.751*	-	-	-	-	-	-	-	-
IL-6[b]	-	-	-	-	-	-	-	-	0.561	<0.001**	0.706	<0.001**	0.726	<0.001**
Age[b]	-	-	-	-	-	-	0.617	<0.001**	0.746	<0.001**	0.685	<0.001**	0.612	<0.001**
FERRİTİN[b]	-	-	-	-	-	-	0.706	<0.001**	0.656	<0.001**	-	-	0.685	<0.001**
Vit. D[a]	0.134	0.376*	-0.155	0.284*	-0.216	0.132*	-	-	-	-	-	-	-	-
LDL[a]	0.300	0.043*	0.025	0.864	0.144	0.320*	-	-	-	-	-	-	-	-
BUN[b]	-	-	-	-	-	-	0.581	<0.001**	-	-	0.656	<0.001**	0.529	<0.001**
Glucose[b]	-	-	-	-	-	-	0.356	0.011*	0.447	0.001**	0.448	0.001**	0.473	0.001**
AST[b]	-	-	-	-	-	-	0.398	0.004**	0.281	0.048*	0.601	<0.001***	0.440	0.002**
ALT[b]	-	-	-	-	-	-	0.134	0.354*	0.122	0.400*	0.359	0.010*	0.162	0.272*

*Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
a Pearson correlation
b Spearman correlation

The relationship between the study parameters was evaluated using Pearson (for homogenously distributed parameters) and Spearman (for non-homogenously distributed parameters) correlation coefficients (Table 4.6).

A strong positive correlation was observed between age and each of BUN ($r=0.746$; $p < 0.001$), IL-6 ($r=0.617$; $p < 0.001$), ferritin ($r=0.685$; $p < 0.001$), and CRP ($r=0.612$; $p < 0.001$). Ferritin was strongly correlated with BUN ($r=0.656$; $p < 0.001$), IL-6 ($r=0.706$; $p < 0.001$), CRP ($r=0.685$; $p < 0.001$), and AST ($r=0.601$; $p < 0.001$). A moderate correlation was observed between ferritin and each of glucose ($r=0.448$; $p < 0.01$) and ALT ($r=0.359$; $p < 0.05$). IL-6 showed a strong positive correlation with BUN ($r= 0.581$; $p<0.001$) and a moderate correlation with both glucose ($r=0.356$; $p<0.05$) and AST ($r=0.398$; $p<0.01$). MDA and 4-HNE were positively correlated ($r=0.356$; $p<0.05$). MT and CPP were inversely correlated ($r=0.403$; $p<0.01$)

5. DISCUSSION

COVID-19 is a complicated disease. The viral interaction with the target cells, the host immune system response, and the systemic response to the previous events are all intertwined. A wide range of respiratory viral infections were shown to be characterized by hyperinflammation, oxidative stress, and cell death. The main target of SARS-CoV-2 is the ACE2 receptor. Binding between the virus particles and the ACE2 receptor leads to the accumulation of Ang II, which stimulates the activation of NADPH Oxidase. The activation of NADPH Oxidase leads to the production of ROS. Overproduction of ROS and low body's antioxidant capacity leads to imbalance in the redox system which results in oxidative stress. Accumulated ROS initiates lipid peroxidation, producing 4-HNE and MDA as secondary products. Besides their role in the pathogenesis of several diseases, 4-HNE and MDA are good markers to monitor oxidative stress (Shoeb et al., 2014). Oxidative stress was found to be involved in the pathophysiology of COVID-19 (Cecchini & Cecchini, 2020), (Ciacci et al., 2023). Additionally, several studies showed that oxidative stress is associated with the severity of COVID-19 disease. Accordingly, the association between lipid peroxidation and disease progress was demonstrated by several studies. In Martín-Fernández et al.'s 2021 study, higher lipid peroxidation levels were independently associated with a higher risk of intubation or death at 28 days among COVID-19 patients (Martín-Fernández et al., 2021). Also, Mehri et al. 2021, stated that MDA levels were significantly higher in ICU group compared to non-ICU group (Mehri et al., 2021). MDA levels were higher in the patient group compared to the control group in Muhammad et al.'s 2021 study (Muhammad et al.,

2021). In our study, there was a significant difference in MDA levels between patient and control groups. Additionally, a significant difference was observed in MDA levels between ICU, mild, and control groups. However, in our study, lower MDA levels were observed in ICU group compared to both mild and control groups. 4-HNE showed no significant difference between patient group and control group. While there was a significant difference in 4-HNE levels between ICU, mild, and control groups. Like MDA, 4-HNE showed lower levels in ICU group compared to mild and control groups. According to our study, lipid peroxidation parameters were lower in ICU group compared to mild and control groups, which agrees with Žarković et al.'s 2022 study. They reported that 4-HNE levels were lower in deceased patients compared to the recovered ones (Žarković et al., 2022). The authors related this to the high reactivity of 4-HNE in addition to its electrophilic structure, which makes it prefer to react with neutrophilic molecules forming adducts. 4-HNE tends to form covalent bonds with molecules containing thiol group (-SH) which is present in GSH, and amino groups ($-NH_2$) present in proteins like albumin, apolipoprotein B (ApoB) a protein present in LDL, and hemoglobin (Nègre-Salvayre et al., 2017). Also, under severe conditions and high concentrations of plasma 4-HNE, 4-HNE tend to form DNA adducts, resulting in cell apoptosis (Muhammad et al., 2021). Žarković et al. mentioned that 4-HNE protein adduct levels were elevated in both diseased and recovered COVID-19 patients which ensures the occurrence of lipid peroxidation and the binding of 4-HNE to the plasma protein. However, the authors suggested that the metabolism of both 4-HNE and its protein adducts may be different in died patients compared to the survived ones. They related this to the systemic stress and the age difference between both groups since 4-HNE protein adducts formation is age-dependent reaching the peak at the age of 65 – 70 (Žarković et al.,

2022). Unfortunately, we could not perform the 4-HNE adduct test. However, there was an age difference between the groups of our study. Like 4-HNE, MDA also tends to react with the functional groups of proteins, lipoproteins, DNA, and RNA, forming adducts (Roede & Fritz, 2015), (Landau et al., 2013). Accumulation of MDA adducts facilitates protein cross-linking and results in mutagenesis (Landau et al., 2013). Previous studies stated the presence of MDA protein adducts in the liver, even in livers free from any disease (Roede & Fritz, 2015). The elevation of liver enzymes among COVID-19 cases was reported by several studies, which indicates that the liver is highly involved in disease pathogenesis (Elhence et al., 2021). This may be related to the reaction between MDA and liver proteins forming adducts and may explain the low plasma MDA levels among our ICU patients compared to those of mild and control groups.

On the other hand, knowing that 16 of ICU patients (72.7 %) were exposed to several treatments, especially corticosteroid treatments, may also explain the low levels of lipid peroxidation biomarkers among the ICU patients compared to mild and control groups. The active ingredients for Prednol and Dekort are Methylprednisolone (MP) and Dexamethasone (DM), respectively. Several studies reported the role of both MP and DM in inhibiting lipid peroxidation. In the study of Keles et al. 2001, elevated MDA levels among multiple sclerosis (MS) patients were decreased after MP treatment for five to seven days, but they did not mention any explanation for the mechanism of MP treatment (Keles et al., 2001). Hall and Braughler mentioned that MP may be responsible for lipid peroxidation inhibition. They also highlighted the role of glucocorticoids in enhancing the (Na^+ + K^+) ATPase activity, which can suppress lipid peroxidation reactions through inhibiting the NADPH oxidase activity (Hall & Braughler, 1982). The study of Gabbia et al. 2018, reported that

DM treatment can reduce inflammation and oxidative stress. They observed that MDA levels were decreased among cholestatic rats who were treated with DM for 2 weeks compared to those who did not receive DM treatment. Down regulated MDA levels were accompanied with hepatic GSH levels elevation, giving an indication that DM acts through activating the antioxidant agent GSH which in turn reduces ROS content. Additionally, inflammatory cytokines were restored to normal levels after DM treatment (Gabbia et al., 2018). In the study of Pinzón e al. 2021, reported that severe patients who received high MP dose (250 – 500 mg for 3 days) followed by prednisone 50 mg for 14 days, showed lower mortality and faster recovery compared to those who received DM (6 mg). Additionally, the study reported a significant decrease in CRP levels after treatment (Pinzón et al., 2021). Zha et al. mentioned that administrating 40 mg MP for 5 days, did not improve the clinical outcomes of severe COVID-19 patients. Also, they suggested that early corticosteroid administration may affect the patients' outcome, however they were unable to apply it (Zha et al., 2020). Our results showed a significant difference in MDA between treated, untreated COVID-19 patients, and controls. Where MDA levels were lower in treated patients compared to the untreated group and the controls. In addition, the same was observed in case of 4-HNE, where 4-HNE for the untreated group was higher in comparison with both treated and control groups. Evaluating the MDA and 4-HNE levels among ICU patients, MDA levels were lower among the treated group compared to the untreated was higher. The same was observed in case of 4-HNE, where the treated group showed low levels compared to the untreated group. Our results suggest that the oxidative stress may have been affected by the treatments applied to the ICU patients especially the corticosteroid treatment. In addition to the above, IL-6 levels were observed to be lower among treated group compared

to the untreated group. This was in agreement with Gabbia et al.'s study, however, this difference was not statistically significant.

According to the above, we recommend that it may be better to perform MDA and 4-HNE protein adducts test together with free MDA and 4-HNE test for better evaluation of oxidative stress state. The same was suggested by Cipierre et al. 2013. Since aldehydes are highly reactive compounds with a short lifetime and lipid peroxidation varies over time, false and low precise results may be obtained. However, adducts are more stable long-lived products which may provide more precise results (Cipierre et al., 2013). Regarding corticosteroid treatment, while lipid peroxidation levels were significantly different between the treated and untreated groups, the inflammatory response was not significantly different between the two groups, though lower levels were observed among the treated group compared to the untreated one. This may be due to the small number of untreated ICU group patients (6 patients) compared to the treated group (16 patient). Additionally, it may be related to low dose and the short period of the administration of corticosteroid treatment, besides the lack of information about whether the time of receiving treatment was in the early or late stage of the infection. We recommend further studies to investigate the effect of corticosteroid therapy on the oxidative stress state among severe patients.

Generally, it was reported that oxidative stress and inflammation are reinforcing each other, and both are involved in the pathogenesis of several diseases. If one of them appears the other is most likely to occur (Gambini & Stromsnes, 2022). Additionally, several studies demonstrated the role of hyperinflammation and oxidative stress in bad outcomes (Sharifi-Rad et al., 2020). In COVID-19, the involvement of hyperinflammation in the pathogenesis of the disease was reported by several studies. Also, the association between

hyperinflammation and bad prognosis in COVID-19 was observed in previous studies (Panda et al., 2021). Hyperinflammation is a result of uncontrolled production of proinflammatory cytokines which is known as cytokine storm. High levels of inflammatory markers were observed more frequently in severe cases (Qin et al., 2020). Tan et al. reported that high levels of IL-6 and CRP were effective indicators of severe COVID-19 (M. Tan et al., 2020). The same results were obtained by Zeng et al., they demonstrated that high CRP, IL-6, and ferritin were associated with worse outcomes and disease severity, IL-6 was an independent parameter to predict severe COVID-19. Additionally, they mentioned that an early decline in IL-6 levels may predict better outcomes (Zeng et al., 2020). In the study of Arshad et al., high serum CRP, IL-6, and ferritin levels at admission were effective in mortality prediction (Arshad et al., 2020). In a study evaluating the effectiveness of oxidative stress and inflammation biomarkers in predicting ICU admission and mortality, Ducastel et al. mentioned that the inflammatory biomarkers were over oxidative stress markers in predicting poor prognosis and mortality (Ducastel et al., 2021).

In the present study, IL-6 levels showed elevated levels among the patient group compared to the control group ($p < 0.05$). Besides, IL-6 levels were significantly higher in the ICU group compared to mild and control groups ($p < 0.001$). In addition, CRP and ferritin levels were significantly different among the study groups. CRP was higher in the ICU group, in comparison with the mild group and the control group ($p < 0.001$). The same was observed for ferritin, the ICU group showed higher ferritin levels compared to the mild and the control group ($p < 0.001$). Comparing IL-6, CRP, and ferritin between treated and non-treated groups of ICU patients did not show any significant difference, however, ferritin and IL-6 were observed to be lower among the treated group compared to the untreated group,

which may be due to low dose and short period of treatment (at the time of blood sampling) together with the big difference in the number of individuals between the treated group (16) and the untreated one (6).

According to the above, our results agree with Tan et al. and Zeng et al. that IL-6 can be an independent marker for COVID-19 severity (M. Tan et al., 2020), (Zeng et al., 2020).

Additionally, our results are in agreement with the study of Ducastel et al., as they suggested that inflammatory parameters were more effective in predicting disease deterioration and mortality (Ducastel et al., 2021).

The redox state is imbalanced during a wide range of chronic disease especially viral infection. To better evaluate the redox state both oxidative stress and antioxidant markers should be examined. Several studies reported the role of melatonin as antioxidant and hoe it can be effective in decreasing the levels of oxidative stress and lipid peroxidation. In the study of Taysi et al 2003, levels of MDA were observed to decrease in rates treated with MT compared to the group with no melatonin administration (Taysi et al., 2003). Suwannakot et al reported that antioxidant levels were restored in rats with oxidative stress after receiving melatonin, they also stated the levels of oxidative stress were decreased in rats with melatonin administration compared to those who did not receive melatonin treatment (Suwannakot et al., 2021). In our study MT and vitamin D were examined to monitor the antioxidant capacity. While there was not any significant difference in MT levels among our study groups, vitamin D levels showed significant difference between our groups.

Vitamin D were higher among controls compared to patients (p <0.05). This was in agreement with a systematic review and meta-analysis that reports the association of lower

vitamin D levels with high risk of COVID-19 infection (N. Liu et al., 2021). Another systematic review demonstrated that individuals with insufficient or low vitamin D levels were more susceptible to hospitalization and ICU admission (Dissanayake et al., 2022). Our results showed that vitamin D levels differed significantly among the ICU, mild, and controls ($p < 0.05$), which agrees to Dissanayake et al.'s study (Dissanayake et al., 2022). On the other hand, vitamin D levels did not show any significant difference between the treated and untreated groups of the ICU patients.

As outlined above, ACE2 is the main body entry of COVID-19. After binding with the viral particles, the ACE2 levels are decreased causing imbalance in the RAAS system (Beyerstedt et al., 2021). Ang II, the main regulator of the RAAS system, is accumulated due to low ACE2. Elevated Ang II levels were observed to boost oxidative stress and inflammation (Yin et al., 2010), (Han et al., 1999). Furthermore, high Ang II levels stimulate aldosterone and vasopressin secretion (Fountain & Lappin, 2017). Elevated Ang II levels were reported to be associated with severe COVID-19 patients (Camargo et al., 2022). Additionally, CPP, a surrogate marker of AVP or vasopressin, was reported to be elevated among severe COVID-19 cases compared to mild cases. Hammad et al. examined the CPP levels among severe COVID-19 cases and mild to moderate cases on admission. According to their results, CPP levels were higher in severe cases compared to mild to moderate cases. They suggested the probability of using CPP as a biomarker on admission to distinguish severe COVID-19 cases from mild cases (Hammad et al., 2022). In a review discussing the effect of respiratory diseases on the release of ACTH hormone, Proczka et al. reported the elevation of AVP and CPP in pulmonary diseases, and COVID-19 disease. In addition, they hypothesized that AVP may have an important role in maintaining the hemostasis of the

respiratory system (Proczka et al., 2021). Gregoriano et al. also reported the association of the elevated copeptin levels with COVID-19 severity, however, they mentioned that higher copeptin levels were also observed among CAP patients thus elevated CPP levels are not specific to COVID-19 cases (Gregoriano et al., 2021). In our study, CPP levels showed significant difference between the patient and control groups (p <0.05). However, CPP levels were lower among patient group compared to the controls. Regarding the disease severity, CPP levels did not show any significant difference among the three study groups. Besides, CPP levels were lower in both severe and mild groups compared to the control group. As mentioned above, 59 % of ICU patients received Midazolam (Dormicum) treatment which is used for anesthesia purposes. Benzodiazepine, the active ingredient of midazolam, was shown to inhibit vasopressin release in rats. Yangi and Onaka mentioned that benzodiazepines modulate γ-aminobutyric acid (GABA) receptor, which is responsible for the release of vasopressin and oxytocin (Yagi & Onaka, 1996), accordingly this may lead to low CPP levels. However, comparing CPP levels between patients who were treated with midazolam and non-treated showed no significant difference between the two groups.

Although glucocorticoid treatment is known to inhibit the AVP release (Erkut et al., 1998), our examinations did not show any significant difference between CPP levels in treated and non-treated group. On the other hand, several studies reported the elevated levels of cortisol among COVID-19 patients and its association with the severity of the disease. In a systematic review and meta-analysis, Dashatan et al. reported the association of elevated cortisol levels with severe COVID-19 patients compared to mild to moderate cases (Amiri-Dashatan et al., 2022). In a cohort of 535 individuals, 403 with COVID-19 and 132 as control, cortisol levels were higher in COVID-19 patients compared to the control group. In

addition, Tan et al. demonstrated the elevated cortisol levels were associated with high mortality (T. Tan et al., 2020). Since high cortisol levels suppress AVP release, in the process of HPA-axis regulation (Chang et al., 2021), we suggest that cortisol levels were extremely elevated in response to stress which resulted in suppression of AVP release and therefore decrease in CPP levels in severe and mild patients compared to controls. Unfortunately, cortisol levels were not examined during our study. According to the above, we suggest that the investigation of the relationship between cortisol and vasopressin during COVID-19 infection or another similar infection. In several studies, CPP levels were observed to be higher in men than in women. Interestingly, in our study mean CPP levels in men were higher (117.04±53.32) compared to women (109.41±50), however, this difference was non-significant.

In harmony with Liu et al. results, our examination showed a positive correlation between IL-6 and each of ferritin (r= 706, p <0.001) and CRP (r = 726, p <0.001) (T. Liu et al., 2020). In addition, positive correlations were observed between age and each of BUN (r = 746, p <0.001), IL-6 (r = 617, p <0.001), ferritin (r = 685, p <0.001), and CRP (r = 612, p <0.001). Furthermore, MDA was positively correlated with 4-HNE (r = 360, p <0.01), however, CPP was negatively correlated with MT (r = -403, p <0.006). In line with Effenberger et al.'s Study, our results showed a significant positive correlation between AST and each of ferritin (r =601, p <0.001), IL-6 (r =398, p <0.004), and CRP (r =440, p <0.002) (Effenberger et al., 2021). Glucose levels were also correlated with each of BUN (r =447, p <0.001), ferritin (r =448, p <0.001), and CRP (r =473, p <0.001). Since ferritin and CRP were reported to be associated with COVID-19 severity (Assal et al., 2022), our results give evidence that high glucose levels may be associated with COVID-19 severity. The same

suggestion was reported by Chen et al. (Chen et al., 2020). Also, positive correlations were observed between BUN and each of ferritin (r =656, p <0.001) and CRP (r =529, p <0.001).

Although our study is the first to investigate all these parameters together in association with the severity of COVID-19, it has some limitations that should be addressed. The first limitation was the absence of follow up and multiple sampling. Another limitation was the inability to perform conformational tests for antioxidant capacity like SOD or GPx. Due to the COVID-19 pandemic, we have faced delays in the kits' shipment and sample collection. Accordingly, some samples were stored for a long time which may have affected our results. The number of ICU patients who received corticosteroid therapy was much higher than those who were not treated, which may have affected our results statistically. Further studies with appropriate number of individuals are suggested for better evaluation of the effect of corticosteroid therapy on COVID-19 cases.

6. CONCLUSION

To our knowledge, our study is the first to investigate the severity of COVID-19 in terms of this number of parameters together.

Our results showed that oxidative stress parameters, MDA and 4-HNE, seemed to be less accurate than inflammatory parameters, IL-6, in predicting the disease outcomes. For oxidative stress evaluation the superiority of MDA and 4-HNE adduct tests over free MDA and 4-HNE tests need to be examined. Melatonin was non-significant in our study; however, it was inversely correlated with Copeptin. Further studies are needed to investigate this negative relationship.

IL-6 may act as an independent prognostic marker in predicting COVID-19 outcomes. High IL-6 levels were directly proportional to the disease severity. The same was observed for CRP and ferritin.

Furthermore, vitamin D deficiency was associated with severe cases, thus maintaining vitamin D at normal levels may decrease the risk of severe COVID-19. Further studies are needed to investigate the effect of COVID-19 infection on the HPA-axis, especially the relationship between cortisol and AVP/copeptin release according to the disease severity. The positive correlation between AST and the severity parameters CRP, IL-6, and ferritin, supports the evidence that liver is highly affected during COVID-19 infection, which foreshadows the probability of the development of liver diseases as a result of COVID-19 infection.

Additionally, the positive relationship between BUN and the severity parameters not only provides evidence that kidneys are affected during the infection, but also may indicate

the risk of kidney disease after COVID-19 infection. The relationship between glucose levels and the severity parameters supports the hypothesis that individuals with comorbid diseases are at high risk for severe COVID-19 infection. Besides, it may also indicate the probability of diabetes development after infection. Further comparative studies are suggested to investigate the effect of corticosteroid on oxidative stress system (oxidant-antioxidant balance). In addition, our findings showed that multiple organs are affected by COVID-19, which may develop chronic diseases after being severely infected.

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