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Presented by

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THEME

Evaluation of vitamin D, CRP and D-dimer levels in COVID-19 patients in the City of Mostaganem

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DEDICATION

It is with genuine gratitude and warm regard that I dedicate this work to my beloved mother and siblings who sacrificed everything for me to be where I am. Words cannot begin to describe my appreciation and gratitude for all you have done for me. Your love and support have helped me to reach my goals and for that, I say, “Thank you very much”. I also thank you for the countless prayers that you have made on my behalf.

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Majoma Ezekiel **ماجوما إيزيكيل**

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Abstract

Vitamin D is a group of fat-soluble prohormones responsible for increasing intestinal absorption of calcium, magnesium, and phosphate which is also responsible for many other biological effects including the regulation of several physiological processes like cell proliferation, differentiation, and immunity modulation. COVID-19 is a highly contagious viral illness caused by SARS-CoV-2, which has had a catastrophic effect on the world's demographics resulting in more than 6.31 million deaths worldwide as of June 2022. Low serum vitamin D levels have been associated with increased vulnerability to respiratory infections and vitamin D treatment has been found to decrease other viral respiratory infections, especially in persons with vitamin D deficiency.

The objective of this work was to investigate hypovitaminosis D in COVID-19 by evaluating the levels of vitamin D, CRP and D-dimer and determining the potential relationship between these parameters and the risk of COVID-19 infection. Our study population mainly included 18 COVID-19-positive patients (11 women and 7 men) whose ages ranged from 17 to 83 years old. The obtained results reveal a prevalence of hypovitaminosis D (< 30ng/ml) as well as elevated CRP levels (> 6ng/l) in the majority of these COVID-19-positive patients. In the other results elevated D-dimer levels (> 0.5mg/l) were observed in the patients with COVID-19.

The results of this study suggest that COVID-19 positivity is significantly associated with hypovitaminosis D, elevated CRP levels, and elevated D-dimer levels. Whether the treatment of this hypovitaminosis D will play some role in the prevention of the viral disease or improve the prognosis of patients with COVID-19 remains to be demonstrated in large randomized controlled trials, which will be certainly necessary to determine whether vitamin D levels could affect COVID-19 risk and precisely define the role of vitamin D treatment in futures waves of SARS-CoV-2 infection.

Keywords: Vitamin D, COVID-19, CRP, D-Dimer, Hypovitaminosis D.

Résumé

La vitamine D est un groupe de prohormones liposolubles responsables de l'augmentation de l'absorption intestinale du calcium, du magnésium et du phosphate, qui est également responsable de nombreux autres effets biologiques, notamment la régulation de plusieurs processus physiologiques tels que la prolifération cellulaire, la différenciation et la modulation immunitaire. Le COVID-19 est une maladie virale hautement contagieuse causée par le SRAS-CoV-2, qui a eu un effet catastrophique sur la démographie mondiale, entraînant plus de 6,31 millions de décès dans le monde en juin 2022. De faibles taux sériques de vitamine D ont été associés à une augmentation il a été constaté que la vulnérabilité aux infections respiratoires et le traitement à la vitamine D diminuent d'autres infections respiratoires virales, en particulier chez les personnes présentant une carence en vitamine D.

L'objectif de ce travail était d'étudier l'hypovitaminose D dans le COVID-19 en évaluant les niveaux de vitamine D, de CRP et de D-dimères et en déterminant la relation potentielle entre ces paramètres et le risque d'infection au COVID-19. Notre population d'étude comprenait principalement 18 patients positifs au COVID-19 (11 femmes et 7 hommes) dont l'âge variait de 17 à 83 ans. Les résultats obtenus révèlent une prévalence d'hypovitaminose D (< 30ng/ml) ainsi que des taux élevés de CRP (> 6ng/l) chez la majorité de ces patients positifs au COVID-19. Dans d'autres résultats, des niveaux élevés de D-dimères (> 0,5 mg/l) ont été observés chez les patients atteints de COVID-19.

Les résultats de cette étude proposent que la positivité COVID-19 soit sensiblement associée à l'hypovitaminose D, aux niveaux élevés de CRP, et aux niveaux élevés de D-dimère. Que le traitement de cette hypovitaminose D jouera un rôle dans la prévention de la maladie virale ou améliorera le pronostic des patients atteints de COVID-19 reste à démontrer dans de grands essais contrôlés randomisés, qui seront certainement nécessaires pour déterminer si les niveaux de vitamine D pourraient affecter le risque de COVID-19 et définir précisément le rôle du traitement à la vitamine D dans les futures vagues d'infection par le SRAS-CoV-2.

Mots-clés : Vitamine D, COVID-19, CRP, D-Dimère.

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LIST OF ABBREVIATIONS

ARDS: Acute respiratory distress syndrome

ALP: Alkaline phosphatase

APC: Antigen presentation cells

AMPs: Antimicrobial peptides

Ca^{2+}: Calcium ion

CAMP: Cathelicidin antimicrobial peptide

CQ: Chloroquine

CD40: Cluster of differentiation 40

COVID-19: Corona virus disease

DCs: Dendritic cells

DBP: Vitamin D Binding Protein

ELFA: Enzyme Linked Fluorescent Assay

ERGIC: Endoplasmic reticulum-Golgi intermediate compartment

FbDP: Fibrin degradation products

hBD-2: Human beta defensin-2

HCQ: Hydroxychloroquine

IL: Interleukin

IFN: Interferon

Mpro: Main protease

MHC: Major Histocompatibility Complex

MERS-CoV: Middle East respiratory syndrome coronavirus

MAP: Mitogen-activated protein

ORFs: Open reading frames

OPG: Osteoprotegerin

PAMPs: Pathogen associated molecular patterns

PPARα and γ: Peroxisome proliferator-activated receptorα and γ

PMCA1b: Plasma membrane Ca^{2+} ATPase 1b

pp1a: Polyproteins 1a

pp1b: Polyproteins 1b

Pdia3: Protein Disulphide Isomerase Family A Member 3

RANK: Receptor activator of nuclear factor-kappa B

RANKL: Receptor activator of nuclear factor-kappa B ligand

RTC: Replication-transcription complex

RXR: Retinoid X receptor

SARS-CoV 2: Severe acute respiratory syndrome coronavirus 2

+ssRNA: Single-stranded positive-sense RNA

SPR: Solid Phase Receptacle

STR: Sealed Reagent Strips

TF: Prothrombotic factor

TM: Thrombomodulin

TRPV5: Transient Receptor Potential Channel vanilloid subtype 5

TRPV6: Transient Receptor Potential Vanilloid subfamily member 6

TNF: Tumor necrosis factor

Th1: Type 1 T helper

UVB: Ultraviolet-B radiation

VDR: Vitamin D receptor

1,24,25(OH)3D: 1,24,25-trihydroxyvitamin D

1, 25(OH)2D: 1,25-dihydroxyvitamin D

24,25(OH)2D: 24,25-dihydroxyvitamin D

25(OH)D: 25-hydroxyvitamin D

7-DHC: 7-dehydrocholesterol

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General Introduction

Vitamin D is a group of fat-soluble prohormones which were identified after the discovery of the anti-rachitic effect of cod liver oil in the early part of the 20th century. It was first identified as a vitamin early in the 20th century but is now recognized as a pro-hormone. The vitamin found in cod liver oil was designated "D" following vitamin A, B and C, which had been discovered earlier.

The main physiological role of vitamin D is to regulate calcium and phosphorus homeostasis and promote bone homeostasis. For many years, the function of vitamin D was considered to be limited to calcium and phosphorus homeostasis. However vitamin D receptors were discovered in various cell types like keratinocytes, lymphocytes and pancreatic cells which led to the discovery of many biological roles of vitamin D in addition to its known actions in classic target tissues. These non-classical functions of vitamin D includes the regulation of several physiological processes like cell proliferation, differentiation and immunity modulation.

COVID-19 is defined as an illness caused by a novel coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; formerly called 2019-nCoV). As of 13 June 2022, SARS-CoV-2 has infected over 535 million people worldwide and has claimed the death of over 6.31 million people.

Though COVID-19 is primarily a respiratory illness, it can affect multiple organ systems including gastrointestinal, hepatic, cardiac, neurological, and renal systems. Thrombotic complications and coagulopathies including disseminated intravascular coagulopathy are common in COVID-19, likely reflecting activation of the coagulation cascade due to viremia or cytokine storm, or possibly due to superinfection and organ dysfunction.

D-dimer is an indirect marker of fibrinolysis and fibrin turnover; this molecule exhibits unique properties as a biological marker of hemostatic abnormalities as well as an indicator of intravascular thrombosis. D-dimer is a soluble fibrin degradation product widely used as a biomarker for thrombotic disorders and it results from the systematic degradation of vascular thrombi through the fibrinolytic mechanism. It is a biomarker of fibrin formation and degradation that can be measured in whole blood or in plasma, hence D-dimer serves as

a valuable marker of activation of coagulation and fibrinolysis in a number of clinical scenarios.

CRP is a pentameric protein synthesized by the liver, whose level rises in response to inflammation. Several studies have indicated that SARS-CoV-2 infection triggers inflammatory processes in those infected with COVID-19. CRP is an acute-phase reactant protein that is primarily induced by the IL-6 action on the gene responsible for the transcription of CRP during the acute phase of an inflammatory/infectious process.

This prospective study aims to evaluate vitamin D, CRP and D-dimer levels in COVID-19 positive patients and evaluate the potential relationship between these parameters and COVID-19 positivity in these patients. This modest work is divided into two parts. The first part is divided into three chapters, the first chapter presents a general information on vitamin D (namely its chemical structure, sources, metabolism, biological roles, regulation, etc.). The second chapter contains the general information on COVID-19, CRP and D-dimer and the third chapter concerns the interrelation of vitamin D and COVID-19. The second part concerns the materials used, methods adopted to carry out our different assays (vitamin D, CRP and D-dimer), the results, and the general discussion ending with the conclusion.

CHAPTER 1

VITAMIN D

CHAPTER 1: VITAMIN D

1.1 Generalities

Vitamin D is a group of fat-soluble secosterols with several existing forms identified after the discovery of the anti-rachitic effect of cod liver oil in the early part of the 20th century. The major compounds in humans are vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol) (**Fig. 1**). Technically, vitamin D is not a vitamin, since it is not an essential dietary factor and is synthesized endogenously in the skin but is now recognized as a pro-hormone. However, vitamin D also meets the criteria of a vitamin, since humans can derive their vitamin D requirement through their diet **(Friedl and Zitt, 2017)**.

Vitamin D3 is a steroid hormone formed when 7-dehydrocholesterol (7-DHC) in the skin is exposed to solar ultraviolet B (UVB, 290-320 nm), and then converted to pre-vitamin D3. In a heat-dependent process, pre-vitamin D3 is immediately converted to vitamin D. Excess UVB rays transform pre-vitamin D3 into biologically inactive metabolites, tachysterol and lumisterol.

Vitamin D2 is a fat-soluble vitamin that is plant-derived and produced exogenously by irradiation of ergosterol, and enters the circulation through diet. Ergocalciferol is the form of vitamin D usually found in vitamin supplements **(Zhang and Naughton, 2010).**

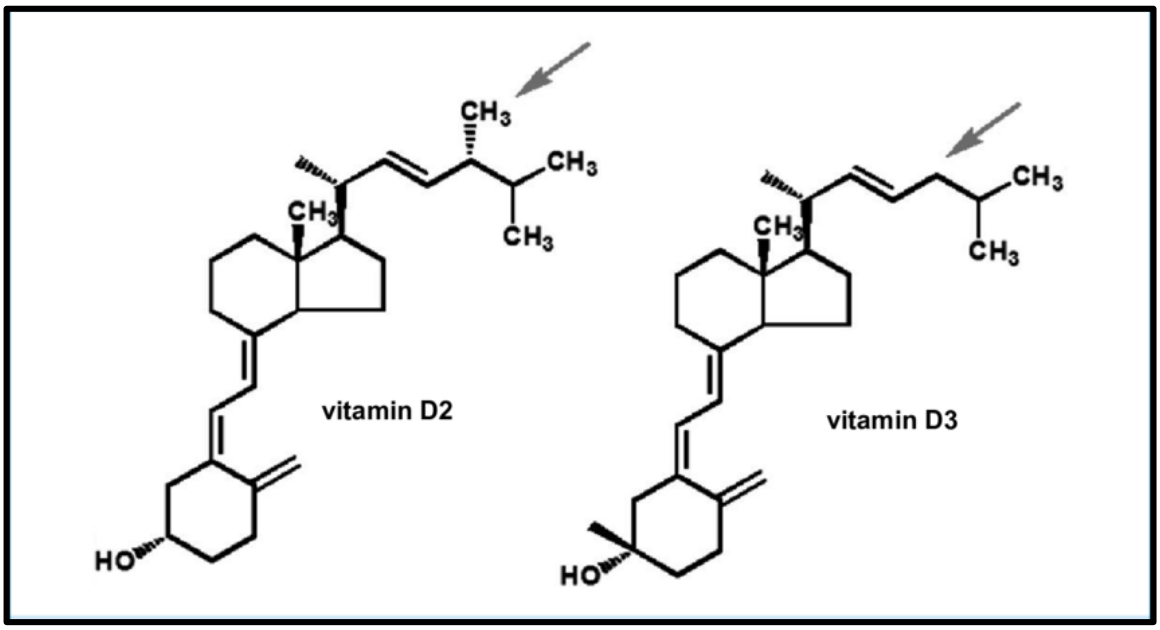

Figure 1: Chemical structure of vitamin D2 and vitamin D3 **(Jorge *et al.*, 2018)**.

1.2 Sources of vitamin D

There are 3 main ways the body can get vitamin D: diet, supplements and sun exposure

1.2.1 Diet

Only a limited number of foods naturally contain vitamin D though some foods are fortified with the vitamin D (**Tab. 1)**. Good sources of vitamin D3 are fish, egg yolk, and offal such as liver. Fortified foods are meant to help boost vitamin D and mineral intake. They're designed to add nutrients that don't naturally occur in the product **(Christel, 2006)**.

Table 1: Summary of the foods containing of vitamin D.

Vitamin D levels in natural foods.	
Foods	Vitamin D
Salmon	988 IU (100 gram)
Herring and sardines	272 IU (100 gram)
Cod liver oil	450 IU/ (1 tbsp)
Tuna	268 IU (100 gram)
Egg yolks	18 IU (1 Egg yolk)
Mushrooms	2300 IU (100 gram)
Vitamin D levels in fortified foods.	
Cow's milk	117 IU (1 cup)
Soy milk	107-117 IU (1 cup)
Orange Juice	100 IU (1 cup)
Cereal and oatmeal	Between 40 and 100IU

1.2.2 Supplements

In recent years, dietary supplements containing vitamin D have become more common and have been more frequently consumed because for most people, the best way to get

enough vitamin D is taking a supplement because it is hard to eat enough through food. The form of vitamin D used in supplement products can be either vitamin D2 or vitamin D3. Before deciding on a vitamin D supplement, it's a good idea to get vitamin D levels tested to know whether your levels are deficient, insufficient, sufficient, or optimal **(Ross *et al.*, 2011)**.

1.2.3 Sun exposure

Sunlight exposure is the primary source of vitamin D for most people. Solar UVB stimulates the production of vitamin D3 from 7-DHC in the epidermis of the skin **(Fig. 2)**. During exposure to sunlight 7-DHC in the skin absorbs UVB radiation and is converted to pre-vitamin D3 and this takes place mainly in the keratinocytes of the stratum basale and stratum spinosum layers of the epidermis. The energy of the UV radiation is used in a non-enzymatic reaction to open the B-ring of the cholesterol precursor 7-DHC, creating the thermodynamically unstable molecule pre-vitamin D3 **(Carlberg, 2019).** Pre-vitamin D3 isomerizes to form vitamin D3 and once formed it immediately enters extracellular space and diffuses from the epidermis into the dermal capillary bed **(Wacker *et al.*, 2013)**.

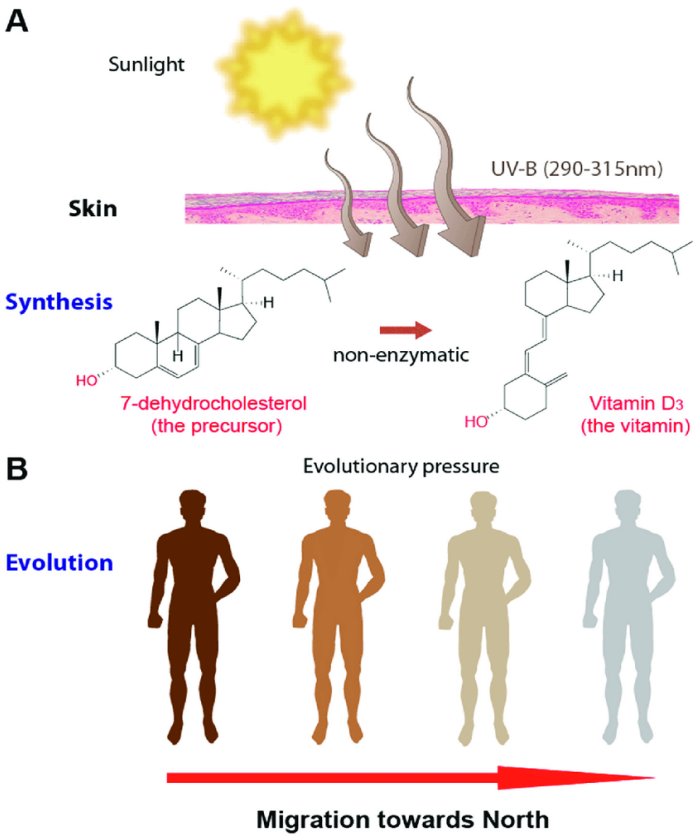

Figure 2: Synthesis of vitamin D by the human skin **(Carlberg, 2019)**

1.3 Definition of vitamin D status

An individual's vitamin D status is best evaluated by measuring the circulating 25-hydroxyvitamin D (25(OH) D3) concentration **(Tab. 2)**. Although controversy surrounds the definition of low vitamin D status, there is increasing agreement that the optimal circulating 25(OH)D3 level should be approximately 30 ng/mL or above **(Binkley *et al*., 2010)**.

Table 2: Currently recognized limit values for the level of 25(OH)D3

Vitamin D status	25(OH) D levels (ng/ml)
Deficiency	< 20
Insufficiency	20 to 29
Sufficiency	**30 à 100**
Potential Toxicity	>100

1.4 Vitamin D requirements

The amount of vitamin D required each day depends on the age of the person **(Tab. 3)**. Average daily recommended amounts are listed below in micrograms (mcg) and International Units (IU):

Table 3: Recommended daily 25(OH)D3 requirements by age **(Amsttuz *et al*., 2011)**

Age	Recommended Amount
0-1 years	10 mcg (400 IU)
1-18 years	15 mcg (600 IU)
19-50 years	15 mcg (600 IU)
50–70 years	15 mcg (600 IU)
>70 years	20 mcg (800 IU)

1.5 Factors affecting vitamin D levels

Latitude - As latitude increases, the amount of vitamin D UV decreases dramatically, which may inhibit vitamin D synthesis in humans. Therefore, a larger dose of UV relative to erythemal UV is required to produce the same amount of vitamin D in a high latitude location. **(Kimlin *et al*., 2007)**

Colour of skin - Skin pigmentation, i.e., melanin, absorbs the UVB that initiates vitamin D synthesis, and hence decreases the vitamin D that is made for a given exposure compared to less pigmented skin. As a result, dark-skinned people tend to require more UVB exposure than light-skinned people to generate the same amount of vitamin D **(Webb *et al*., 2018)**.

Age - Aging reduces vitamin D production in the skin. There is a decrease in the concentration of 7-DHC in the epidermis in old compared with young individuals and a reduced response to UV light, resulting in a 50% decrease in the formation of pre-vitamin D3 **(Gallagher, 2013)**.

Obesity - Vitamin D is fat soluble and distributed into fat, muscle, liver, and serum. All of these compartments are increased in volume in obesity, so the lower vitamin D likely reflects a volumetric dilution effect and whole body stores of vitamin D may be adequate **(Walsh *et al*., 2017)**.

Pollution – Air pollution is one of the chief actors in determining the percentage of the ground level of UVB. Carbon particulates in the air from the burning of fossil fuels, wood, and other materials scatter and absorb UVB rays. The level of air pollution is inversely related to the extent of solar UVB that reaches the earth's surface and thus high levels of atmospheric pollution can reduce ground levels of UVB significantly and this in turn lowers vitamin D cutaneous synthesis **(Hosseinpanah *et al*., 2010)**.

People at risk of vitamin D deficiency are

- Breastfed infants
- Older adults
- People with limited sun exposure
- People with dark skin
- People with conditions that limit fat absorption and people who are obese

1.6 The metabolism of vitamin D

The three main steps in vitamin D metabolism: 25-hydroxylation, 1α-hydroxylation, and 24-hydroxylation are all performed by cytochrome P450 mixed-function oxidases (CYPs). These enzymes are located either in the endoplasmic reticulum (ER) (e.g., CYP2R1) or in the mitochondria (e.g., CYP27A1, CYP27B1, and CYP24A1) **(Bikle, 2014).** Whether synthesized in the skin (vitamin D3) or absorbed through the diet in the small intestines via chylomicrons (vitamin D2 and D3), vitamin D is transported in the blood by a carrier protein called a vitamin D binding protein (DBP) to the liver. In the liver vitamin D is hydroxylated at C-25 by one or more cytochrome P450 vitamin D 25 hydroxylases (including CYP2R1, CYP2D11 and CYP2D25), resulting in the formation of 25(OH)D3 **(Fig. 3)**, a reserve form of vitamin D whose plasma half-life is two to three weeks. It has been suggested that CYP2R1 is the key enzyme required for 25 hydroxylation of vitamin D since a homozygous mutation of the CYP2R1 gene was found in a patient with low circulating levels of 25(OH)D3 and classic symptoms of vitamin D deficiency **(Christakos *et al*., 2010).** This 25(OH)D3 is the major circulating form of vitamin D and provides a clinically useful marker for vitamin D status **(Acar and Özkan, 2021)**.

The 25(OH)D3 is transported by the DBP to the kidney where the final step of active vitamin D formation takes place in the proximal tubules of the kidney, led by the enzyme 1-alpha hydroxylase to produce the active molecule 1,25-dihydroxyvitamin D (1,25(OH)2D3 **(Acar and Özkan, 2021)**. The 25(OH)D3 is hydroxylated at the carbon 1 position of the A ring, resulting in the formation of the hormonally active form of vitamin D, (1,25(OH)2D3) which is responsible for most, if not all of the biological actions of vitamin D **(Christakos *et al*., 2010)**. At least two proteins, cubilin and megalin, facilitate the entry of the DBP-25(OH)D3 complex through the renal tubule cellular receptors. The reduction of these proteins leads to a urinary loss of 25(OH)D3 and, consequently, to its deficiency. Renal tubular cells contain 2 hydroxylases that are part of the cytochrome P450 system: 1-alpha-hydroxylase (CYP27B1) and 24-alpha-hydroxylase (CYP24A1) **(Dominguez *et al*., 2021)**.

The mitochondrial CYP27B1 catalyzes the hydroxylation of the 25(OH)D3 to produce 1,25(OH)2D3 whose plasma half-life is about four hours. The enzyme 24-hydroxylase (CYP24A1) is responsible for the catabolism of 25(OH)D3 and 1,25(OH)2D3. Both the metabolites are 24-hydroxylated at carbon 24 by this enzyme, resulting in 24,25(OH)2D3 and 1,24,25(OH)3D3, respectively. 24-Hydroxylation is the first step in the catabolism of

25(OH)D3 and 1,25(OH)2D3, followed by oxidation and further hydroxylation ending in the production of the inactive metabolite calcitroic acid **(Friedl and Zitt, 2017).** Unlike CYP27A1 and CYP27B1, which are located primarily in the liver and kidney respectively, CYP24A1 is ubiquitous, thereby controlling active vitamin D levels throughout the body **(Tissandie *et al*., 2006).**

Alongside this major renal production, minor sites of 1,25(OH)2D3 production have been identified in the placenta, brain, prostate, keratinocytes, osteoblasts and macrophages that express CYP27B1. However, this extra renal production does not usually contribute to the formation of plasma 1,25(OH)2D3 **(Tissandie *et al*., 2006)**.

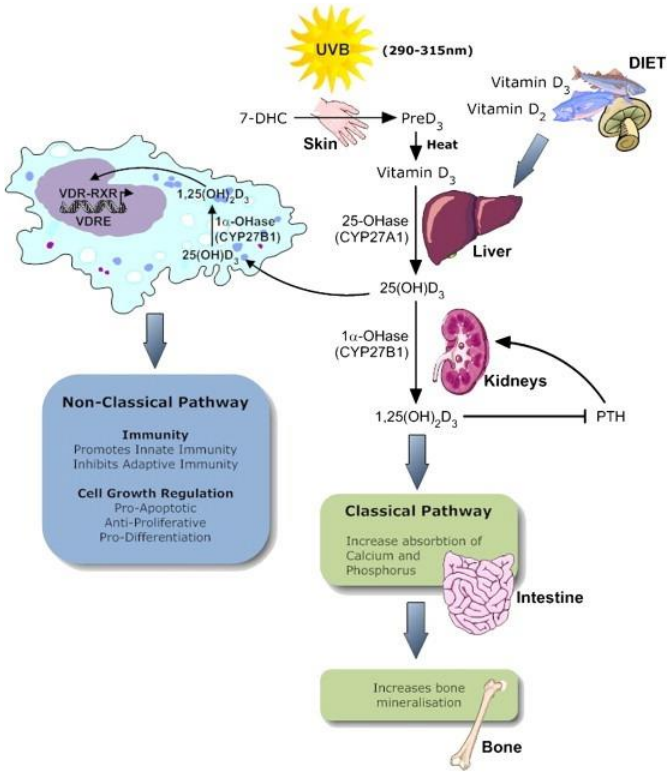

Figure 3: Vitamin D metabolism and biological effects **(Field *et al*., 2011)**

1.7 Mechanism of action of vitamin D

The active metabolite of vitamin D, 1,25(OH)2D3 exhibits both genomic (mediated through the vitamin D receptor (VDR) with transcriptional effects inside the cell nucleus) and non-genomic effects (when the VDR induces rapid signalling, situated on the cell membrane and/or cytoplasm) **(Trochoutsou *et al*., 2015)**.

1.7.1.1 Genomic

All genomic actions of 1,25(OH)2D3 are mediated by the transcription factor VDR which belongs to the superfamily of nuclear receptors **(Carlberg and Campbell, 2013**). This VDR is expressed in most cell types and therefore expressed in all tissues thereby making almost all cells potential targets of calcitriol. The ubiquitous distribution of the VDR explains the large number of genes whose regulation is directly or indirectly dependent on 1,25(OH)2D3. This results in effects of vitamin D on the regulation of genes involved in metabolic pathways as varied as calcium metabolism, proliferation, cell differentiation, inflammation, apoptosis or angiogenesis, to name just a few examples **(Landrier, 2014)**.

In the cell, 1,25(OH)2D3 binds to VDR **(Fig. 4)** and the VDR-1,25(OH)2D3 complex is translocated to the cell nucleus where it associates with the retinoic acid receptor, the retinoid X receptor (RXR) to form a heterodimeric complex. The RXR-VDR heterodimer in the presence of ligand is recruited to the vitamin D response elements in the target genes to activate or to repress their expression through interaction with additional co-regulators **(Umar *et al.*, 2018)**.

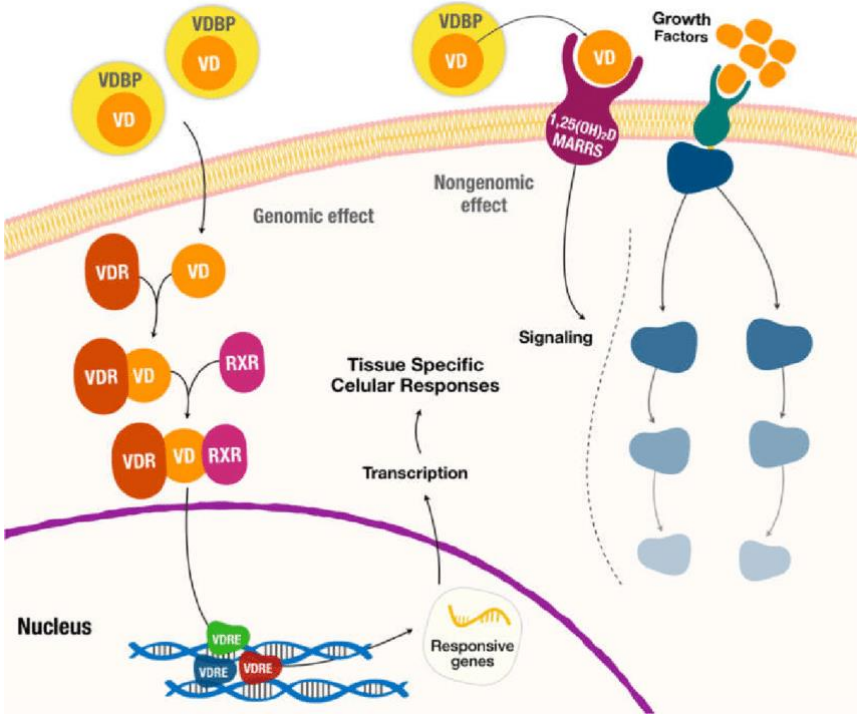

Figure 4: Hormonal actions of vitamin D with genomic and non-genomic effects. **(Botelho *et al.*, 2020)**

1.7.1.2 Non Genomic

1,25(OH)2D3 also exerts effects that are too rapid (seconds to minutes) to involve a genomic action **(Bikle, 2014)**. It is recognised that 1,25(OH)2D3 also exerts non-genomic effects and these effects of 1,25(OH)2D3 are mediated by a membrane receptor, membrane-associated rapid response steroid binding protein (MARRS), also known as protein disulphide isomerase family A Member 3 (Pdia3) **(Fig.4)**. The role of this receptor has been well described in the enterocyte, where it participates in the rapid uptake of calcium **(Landrier, 2014)**. This phenomenon has also been described in other cell types such as osteoblasts, hepatocytes or pancreatic β cells, however the ubiquitous nature of this type of regulation has not yet been established.

It has been postulated that non-genomic mechanisms require the interaction between 1,25(OH)2D3 and intracellular or membrane-bound proteins, thereby providing efficient and rapid response to the stimulus **(Donati *et al*., 2021)**. 1,25(OH)2D3 fixes on the Pdia3 receptor, activates it and after being activated the receptor leads to the activation of signalling molecules, such as phospholipases C (PLC) and A2 (PLA2), and the rapid generation of second messengers such as (calcium ion (Ca^{2+})) and cyclic adenosine monophosphate (cyclic AMP)), which is also accompanied by the activation of protein kinases, such as protein kinase A, mitogen-activated protein (MAP) kinases, protein kinase C (PKC) and Ca^{2+}-calmodulin kinase II **(Hii and Ferrante, 2016)**.

The most noticeable non-genomic rapid effect of 1,25(OH)2D3 is the increase of intracellular Ca^{2+} concentrations at subnanomolar concentrations by modulating its release from intracellular stores and its uptake in intestinal epithelia, referred to as "transcaltachia" **(Donati *et al*., 2021)**

1.7.2 Biological actions of vitamin D

Intestine. Ca^{2+} absorption occurs by an active, carrier-dependent process and a passive, paracellular process. The active process is vitamin-D-dependent, but the passive process is not **(Connie *et al*., 2011)**. Vitamin D is essential to enhance the efficiency of the small intestine to absorb dietary calcium and phosphate. Ca^{2+} uptake requires the epithelial calcium channel TRPV6 (Transient Receptor Potential Vanilloid subfamily member 6) and, to a lesser extent, TRPV5 **(Fig. 5)**. Thereafter, calbindin D transports Ca^{2+} across the cell,

and the plasma membrane Ca^{2+} ATPase 1b (PMCA1b), and the Na /Ca^{2+} exchanger mediate the final delivery of Ca^{2+} to the bloodstream uptake **(Dusso *et al*., 2005).**

Transcellular Ca^{2+} transport is a saturable process comprised of three 1,25(OH)2D3 regulated steps:

a) entry of Ca^{2+} through the apical membrane calcium channel TRPV6
b) binding to the Ca^{2+} binding protein calbindin-D9k,
c) extrusion of Ca^{2+} across the basolateral membrane by PMCA1b **(Christakos *et al*., 2016)**.

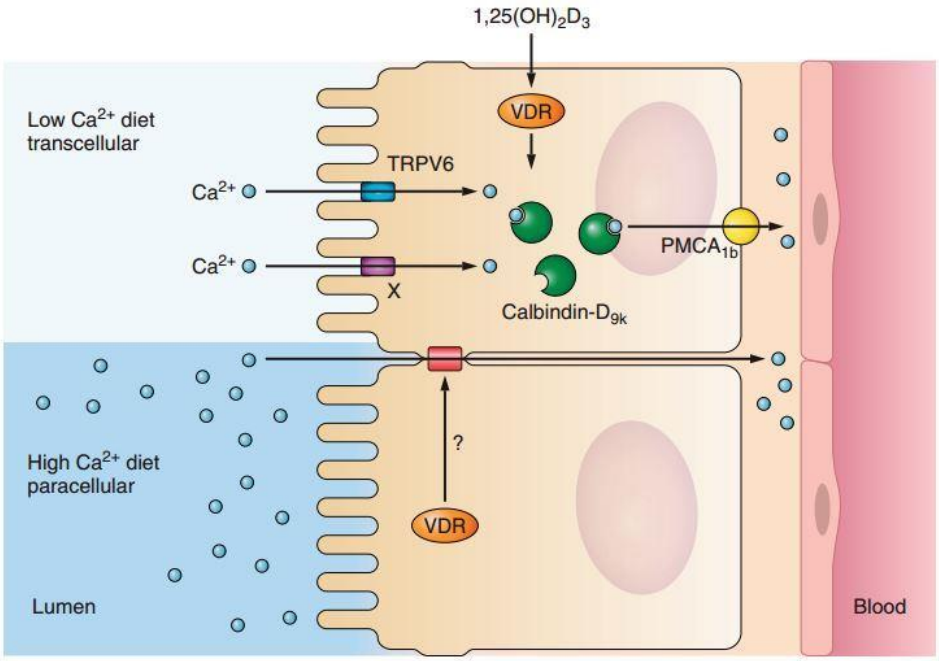

Figure 5: Effects of 1,25(OH)2D3 in the intestine.**(Christakos *et al*., 2016)**.

Kidney. The most important endocrine effect of 1,25(OH)2D3 in the kidney is a tight control of its own homeostasis through simultaneous suppression of CYP27B1 and stimulation of CYP24A1 and very likely through its ability to induce megalin expression in the proximal tubule **(Dusso *et al*., 2005)**. 1,25(OH)2D3 enhances the reabsorption of calcium in distal tubular segments of the nephron **(Kumar *et al*., 2012).** 1,25(OH)2D3 also increases the expression of the renal calcium-transport protein, calbindin-D28k **(Fig. 6)**. Furthermore, 1,25(OH)2D3 promotes the PTH-dependent calcium transport in the distal tubule where active calcium reabsorption occurs **(Anderson *et al*., 2003)**. Epithelial Ca^{2+} channel (ECaC) (or TRPV5) is an important target in 1,25(OH)2D3-mediated calcium reabsorption. Several putative VDR binding sites have been located in the human promoter of the renal ECaC.

Decrease in circulating levels of 1,25(OH)2D3 concentrations result in a marked decline in the expression of the channel at the protein and mRNA levels **Dusso *et al*., 2005)**.

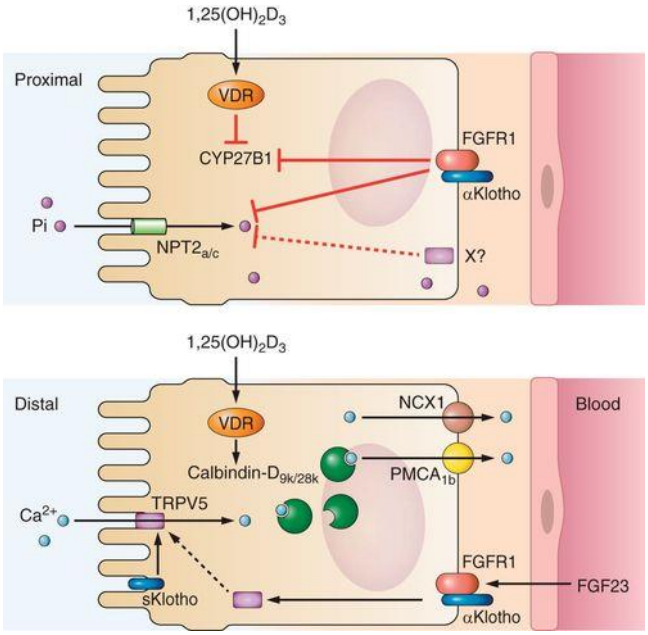

Figure 6: Effects of 1,25(OH)2D3 in the kidney **(Christakos *et al*., 2016)**

Skeletal. 1,25(OH)2D3 regulates osteoclastogenesis by reciprocal regulation of receptor activator of nuclear factor-kappa B (RANK) ligand (RANKL) and osteoprotegerin (OPG). 1,25(OH)2D3-VDR increases the expression of RANKL on the surfaces of the osteoblast **(Fig. 7)**. RANKL interaction with its receptor, RANK, promotes maturation of osteoclast progenitor cells to mature osteoclasts (the bone-resorbing cells). 1,25(OH)2D3-VDR also represses the expression of OPG, a decoy receptor that binds RANKL and prevents RANK-mediated osteoclastogenesis **(Dusso *et al*., 2005).**

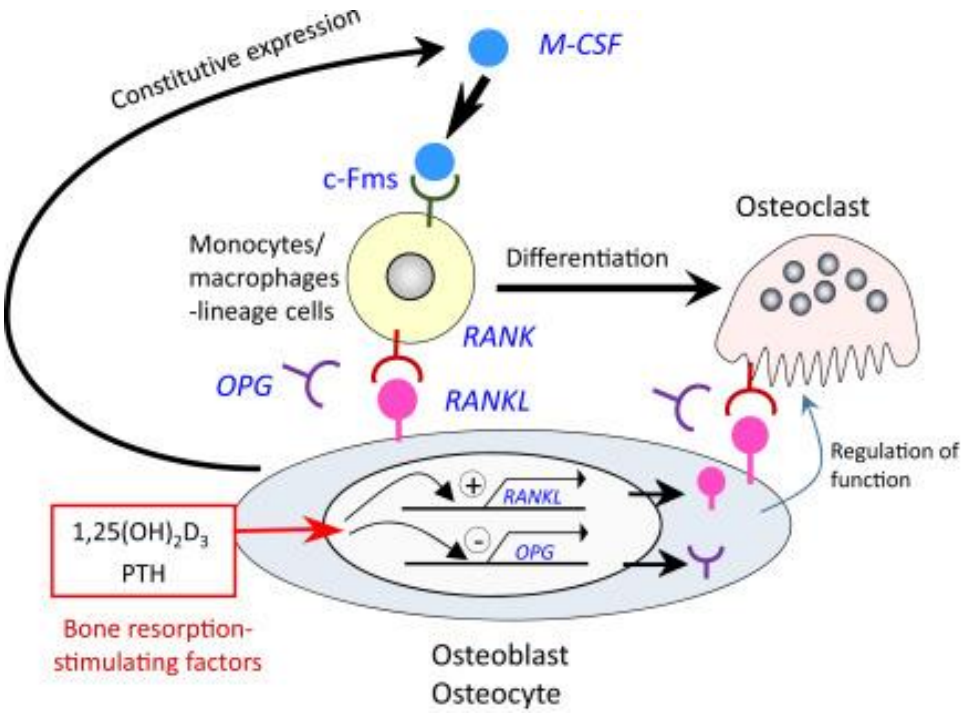

Figure 7: Effects of 1,25(OH)2D3 in osteoclastogenesis **(Yuko *et al*., 2018).**

For many years, the function of vitamin D was considered to be limited to calcium and phosphorus homeostasis. However vitamin D receptors were discovered in other various cell types and this led to the discovery of many other biological roles of vitamin D in addition to its known actions in classic target tissues. These non-classical functions of vitamin D includes the regulation of several physiological processes like cell proliferation, differentiation, and immunity modulation **(Umar *et al*., 2018)**. 1,25(OH)2D3 mediates its function by binding to the VDR. Following interaction with 1,25(OH)2D3-VDR dimerizes with RXR and translocates to the nucleus where it binds to vitamin D response elements in vitamin D responsive genes. Depending on the target gene either co-activators or co-repressors are attracted to the VDR/RXR complexes to induce or repress gene transcription **(Kongsbak *et al*., 2013)**.

1.8 Regulation of vitamin D metabolism

The regulation of vitamin D3 metabolism essentially depends on the enzymes involved in its synthesis (CYP27A1 and B1) or its catabolism (CYP24A1). This regulation involves hormones especially parathormone (PTH) which responds to variations in calcium

homeostasis and molecules of lipid origin with autocrine or paracrine activity via nuclear receptors

1.8.1 Synthesis regulation

Hepatic synthesis of 25(OH)D3 is only loosely regulated, and blood levels of this molecule largely reflect the amount of vitamin D produced in the skin or ingested. In the liver, CYP27A1, involved in its synthesis, is modulated at the transcriptional stage by nuclear receptors. Examples of these nuclear receptors are peroxisome proliferator-activated receptors α and γ (PPARα and γ), whose ligands are polyunsaturated fatty acids, hepatic nuclear factor 4α (HNF4α) activated by phosphorylations and small heterodimer partner (SHP), a nuclear receptor having a transcriptional repression activity **(Fig. 8)**. The expression of CYP27A1 is stimulated by the nuclear receptors HNF4α and PPARγ and inhibited by PPARα and SHP hormone **(Tissandié *et al*., 2006)**.

In the kidney, the activity of CYP27B1 which is responsible for the production of 1,25(OH)D3 is tightly regulated by PTH, calcitonin, and 1,25(OH)2D3 **(Mina et al., 2013)**. Under low serum calcium conditions, or low levels of vitamin D, PTH secreted by the parathyroid glands stimulates the synthesis of the 1α-hydroxylase, resulting in the increase of 1,25(OH)2D3 activation **(Gil *et al*., 2018)**. Conversely, hypercalcemia, hypophosphatemia and/or an increase in the plasma concentration of 1,25(OH)2D3 inhibit the release of PTH. In addition phosphates, calcium and 1,25(OH)2D3 can also act directly on the enzyme and therefore on the circulating level of the active hormone **(Tissandié *et al*., 2006)**. PTH intervenes by increasing the activity of the CYP27B1 promoter via the phosphorylation of the transcription factor cAMP-dependent response element binding protein (CREB).

1,25(OH)2D3 is known to regulate its own production i.e. its high concentration inhibits its own synthesis and also inhibits the activity of CYP27B1. 1,25(OH)2D3 stimulates the expression of CYP27A1 and also the production of 24,25(OH)2D3 an inactive form of vitamin D which reduces its own concentration **(Christakos, 2017)**. Many other factors such as calcitonin (a hormone produced by thyroid C cells), and fibroblastic growth factor 23 (FGF-23) are also involved in the regulation of CYP27B1. Calcitonin stimulates the expression of 1α-hydroxylase but also that of PTH. FGF-23 which is a factor released by growing bone can also adjust vitamin D homeostasis by suppressing renal

expression of 1α-hydroxylase and inducing 24-hydroxylase, thus reducing serum calcitriol levels and subsequently serum Ca^{2+} under hyperphosphatemia conditions **(Gil *et al.*, 2018)**.

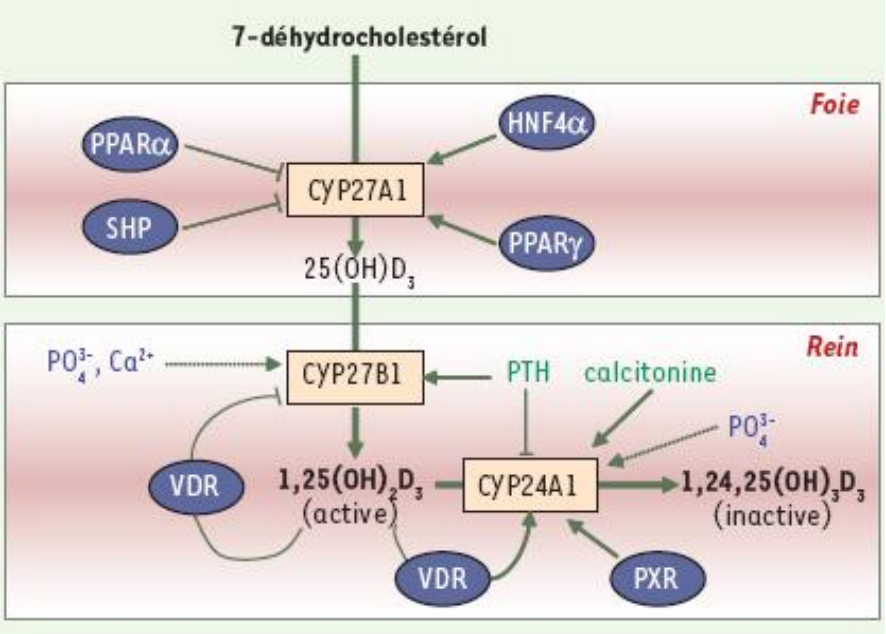

Figure 8: Regulation of vitamin D3 metabolism. **(Tissandié *et al.*, 2006)**

1.8.2 Catabolism regulation

The degradation of vitamin D3 in the kidneys depends on the regulation of CYP24A1. The CYP24A1 gene is regulated in the kidney by the same hormones that control CYP27B1 expression, though it is regulated in a reciprocal fashion. Thus, whereas PTH induces CYP27B1 expression, it strongly suppresses CYP24A1 **(Meyer *et al.*, 2019).** Phosphate intake also inhibits the activity and expression of CYP24A1 as opposed to its effect on CYP27B1 **(Tissandié *et al.*, 2006)**. In contrast, 1,25(OH)2D3 as well as FGF-23 strongly induce the expression of CYP24A1 **(Kägi *et al.*, 2018).** High levels of 1,25(OH)2D3 in contrast, decrease PTH and raise FGF-23 levels, collectively suppressing CYP27B1-mediated production of 1,25(OH)2D3 while increasing CYP24A1-directed degradation **(Meyer *et al.*, 2019)**. The main transcription factor involved in the regulation of the gene encoding CYP24A1 is the VDR. 1,25(OH)2D3 stimulates the transcription of CYP24A1 via its attachment to the heterodimer formed by VDR and RXR **(Tissandié *et al.*, 2006)**.

CHAPTER 2

COVID-19

CHAPTER 2: COVID-19

2.1 Generalities

COVID-19 is defined as an illness caused by a novel coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was first identified amid an outbreak of respiratory illness cases in Wuhan City, Hubei Province, China **(Li *et al.*, 2020)**. It was initially reported to the WHO on December 31, 2019. On January 30, 2020, the WHO declared the COVID-19 outbreak a global health emergency. On March 11, 2020, the WHO declared COVID-19 a global pandemic, its first such designation since declaring H1N1 influenza a pandemic in 2009 **(Cascella *et al.*, 2022)**. As of June 2022, SARS-CoV-2 has infected over 551 million people worldwide and has claimed the death of over 6.31 million people.

COVID is an acronym. 'CO' stands for corona, 'VI' for virus, 'D' for disease, and '19' for 2019 (year first identified). This disease has also been referred to as '2019 novel coronavirus' or '2019-nCoV.

Coronaviruses are enveloped viruses containing a non-segmented positive-sense and single-stranded ribonucleic acid (RNA). Coronaviruses are categorized into four important genera that include Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus **(Pal *et al.*, 2020)**. Gamma and Deltacoronaviruses infect birds and might infect mammals but have never been reported to cause any illnesses in humans. On the other hand, Alpha and Betacoronaviruses are capable of causing respiratory illnesses in humans and gastrointestinal illnesses in animals. In 2002 and 2012, respectively, two highly pathogenic coronaviruses with zoonotic origin, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), emerged in humans and caused fatal respiratory illness, making emerging coronaviruses a new public health concern in the twenty-first century. At the end of 2019, a novel coronavirus designated as SARS-CoV-2 emerged in the city of Wuhan, China, and caused an outbreak of unusual viral pneumonia **(Hu *et al.*, 2021)**. SARS-CoV-2 is therefore the third Betacoronavirus to infect humans and it probably originated from bats and over time accumulated mutations that gave it the capacity for zoonotic transmission.

However COVID-19 has overwhelmingly surpassed SARS and MERS in terms of both the number of infected people and the spatial range of epidemic areas. The ongoing outbreak of COVID-19 has posed an extraordinary threat to global public health.

2.2 SARS CoV2

SARS-CoV-2 virus, the causative agent of the COVID-19 pandemic has a genomic organization consisting of 16 non-structural proteins (nsps), 4 structural proteins, and 9 accessory proteins **(Gorkhali *et al.*, 2021)**. SARS-CoV-2 is a highly transmissible and pathogenic coronavirus that threatens human health and public safety **(Hu *et al.*, 2021)**. SARS-CoV-2 is a β coronavirus which are a group of enveloped viruses that have a non-segmented, single-stranded, positive-sense RNA as their nuclear material and an average diameter of 60–140 nm **(Fig. 9)**. On electron microscopy, these viruses show a characteristic appearance that resembles a crown (corona in Latin means crown) due to the presence of club-shaped surface protein **(Pal *et al.*, 2020)**.

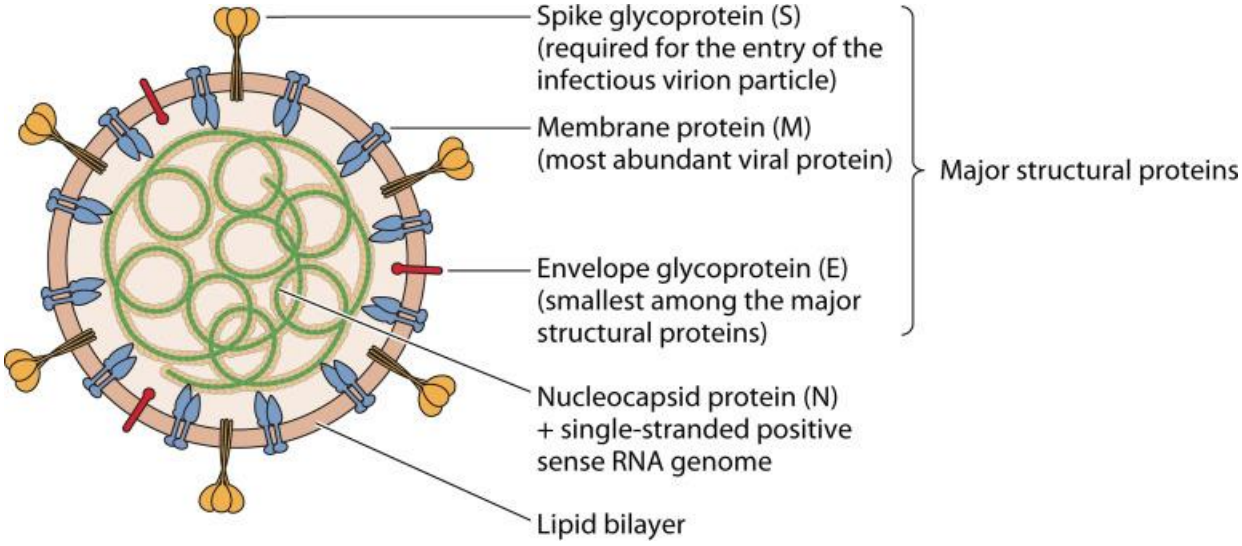

Figure 9: SARS-Cov-2 virus structure **(Dhama *et al.*, 2020)**

2.2.1 SARS-Cov-2 genomic organization

The SARS-Cov-2 is a single-stranded positive-sense RNA (+ssRNA) molecule whose genomic size ranges around 30kbp making it one of the largest known RNA viruses. The genomic structure of SARS-Cov-2 contains at least six open reading frames (ORFs) **(Alanagreh *et al.*, 2020)**. The first ORFs are ORF1a/b, located at the 5' end and it constitutes the first two-thirds of the genome consisting of replicase genes encoding for large polyproteins, pp1a and pp1ab, which are later converted into 16 non-structural proteins by

the process of proteolytic cleavage using multiple proteases: a virally encoded chymotrypsin-like protease and two papain-like proteases **(Gorkhali *et al*., 2021)**.

Other ORFs are located on 3' end encodes at least four structural proteins:

a) Spike glycoprotein that is responsible for recognizing host cell receptors.
b) Membrane proteins that are responsible for shaping the virions.
c) The envelope proteins that are responsible for virions assembly and release.
d) The nucleocapsid proteins that are involved in packaging the RNA genome in the virions and play roles in pathogenicity as an interferon (IFN) inhibitor **(Alanagreh *et al*., 2020)**.

Once the viral genome enters the cytoplasm of the target cell, it translates into two polyproteins pp1a and pp1b. These polyproteins are then processed into 16 non-structural proteins (nsp1-nsp16) to form a replication-transcription complex (RTC) that is involved in genome transcription and replication. For example, nsp3 and nsp5 encode for papain-like protease (PLP) and 3CL-protease, respectively **(Fig. 10)**. Both proteins function in cleaving of polypeptides and block the host innate immune response. Nsp12 encodes for RNA-dependent RNA polymerase (RdRp). Nsp15 encodes for RNA helicase. Consequently, a nested set of subgenomic RNAs (sgRNAs) is synthesized by RTC in the form of discontinuous transcription **(Alanagreh *et al*., 2020)**.

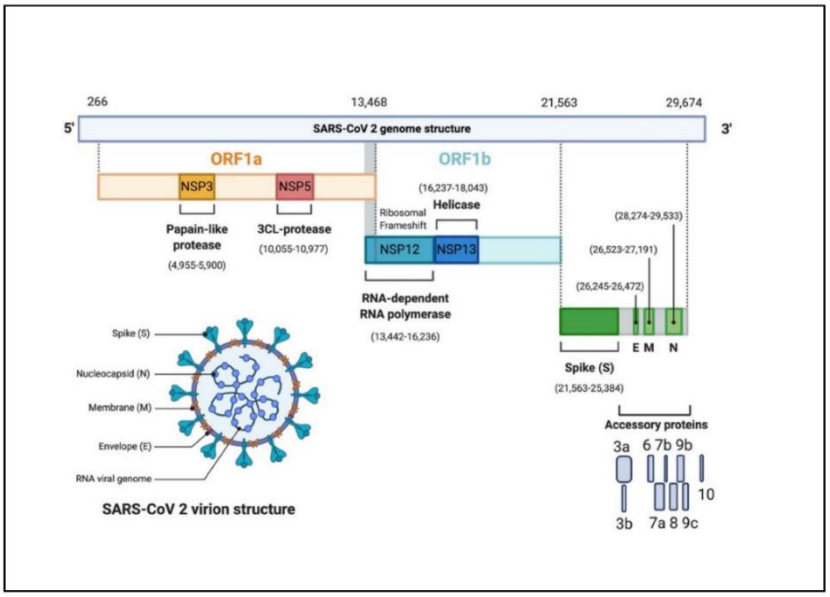

Figure 10: The genomic organization of SARS-CoV-2 **(Alanagreh *et al*., 2020)**

2.2.2 Life cycle of the SARS-CoV2

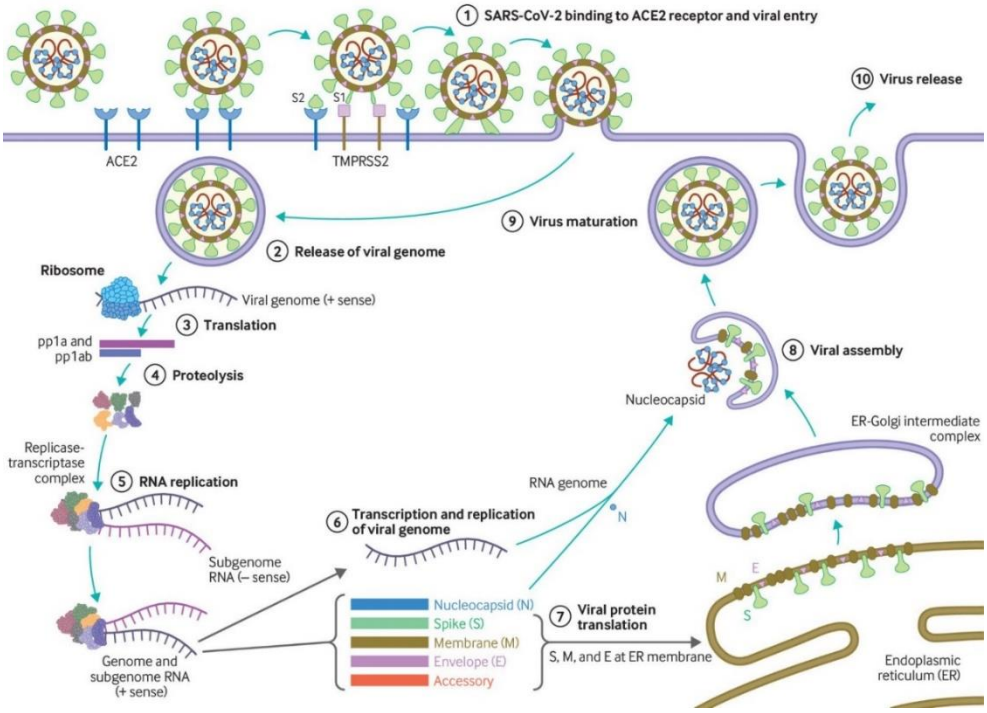

Figure 11: The life cycle of the SARS-Cov-2 **(Cevik *et al*., 2020)**

1) Upon interaction of SARS CoV-2 with the ACE-2 receptor, the S protein undergoes proteolytic activation via a two-step cleavage by host cell enzymes, one for priming at S1/S2 cleavage site and second for activation at a position adjacent to a fusion peptide within the S2 subunit. S protein is cleaved by cellular proteases including transmembrane serine protease 2 (TMPRSS2), cathepsin L and furin which leads to viral entry through clathrin facilitated endocytosis pathway **(Fig. 11)**.
2) Following membrane fusion, the endosomes having a lower pH favours the release of viral genetic material into the cytosol **(Sen *et al*., 2022)**
3) Translation of ORF1a et b of the genomic RNA into pp1a and pp1ab
4) Proteases embedded in viral nsp3 and nsp5 cleave pp1a and pp1ab into 16 non-structural proteins (nsp1 to nsp16) that assemble into RTCs **(Malone *et al*., 2022)**.
5) RNA replication
6) Transcription and replication of the viral genome
7) Viral structural proteins are translated from the RNA and these viral structural proteins are then inserted into the endoplasmic reticulum where they then move to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). Multiple copies of the nucleocapsid protein package genomic RNA into helical structures

(ribonucleoprotein complexes) in the cytoplasm, and interact with hydrophobic membrane proteins (envelope protein) in the ERGIC that serve to direct assembly of the virion.

8) Viral Assembly
9) The maturation of the virus
10) Exocytosis **Cevik *et al*., 2020.**

2.3 Hyper inflammatory pathogenesis of COVID-19

Upon viral infection alveolar epithelial cells, macrophages and monocytes circulating in the blood are activated via toll-like receptors as pattern recognition receptors (PRRs) by the viral pathogen associated molecular patterns (PAMPs) and start to produce a large amount of inflammatory cytokines and chemokines, which attract more immune cells to the site of infection in particular, monocytes and T cells, resulting in widespread lung inflammation **(Hojyo *et al*., 2020)**. COVID-19 patients also have been seen to possess lower levels of regulatory T cells **(Qin *et al*., 2020)**.

Infection with Sars-CoV-2 results in the secretion of large amounts of inflammatory cytokines and chemokines. High levels of IL-2 (interleukin), IL-7, IL-10, G- granulocyte colony-stimulating factor (CSF), tumor necrosis factor (TNF), CXC-chemokine ligand 10 (CXCL10), monocyte chemoattractant protein-1 (MCP1), and macrophage inflammatory protein 1 alpha (MIP1α) in serum were observed in patients with severe COVID-19 **(Niedźwiedzka-Rystwej *et al*., 2022)**. Cytokine release syndrome is a disorder induced by cytokine storms which can cause local tissue damage and systemic non-protective inflammation. Among the elevated levels of inflammatory mediators in COVID-19 patients, the blood levels of IL-6 potentially produced by macrophages or monocytes are noticeably higher in non-survivors compared to survivors and predict the need for mechanical ventilation. The leading cause of death in patients infected with SARS-CoV-2 is severe acute respiratory distress (ARDS). ARDS is a consequence of an exaggerated inflammatory response accompanied by uncontrolled oxidative stress as well as an inflammatory reaction in the lungs **(Hojyo *et al*., 2020)**.

Compared with uninfected individuals, moderate COVID-19 cases show forth an increase in IL-6 and a decrease in total T lymphocyte counts, particularly CD^{4+} T cells and CD^{8+} T cells **(Fig. 12)**. Severe COVID-19 cases have further increased production of IL-6,

IL-2R, IL-10, and TNF-α. There is also a marked decrease of the total T lymphocytes, particularly CD^{4+} T cells and CD^{8+} T cells, and IFN-γ–expressing CD^{4+} T cells. The level of cytokine storm and T cell lymphopenia is associated with pulmonary damage, respiratory distress, and unfavourable outcome **(Savannah *et al.*, 2020)**.

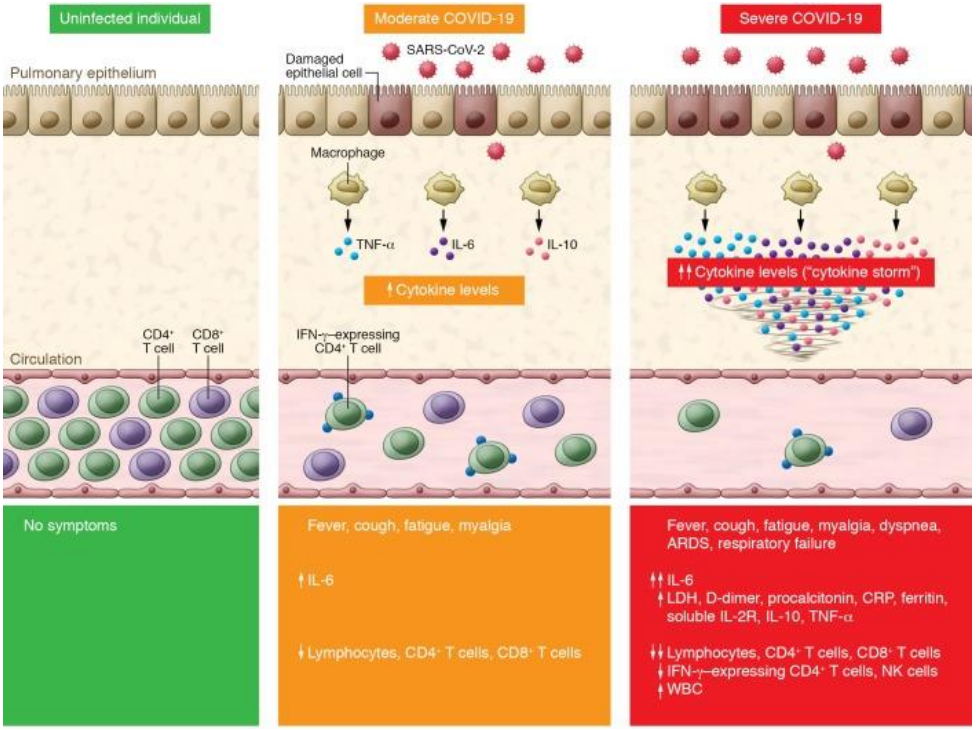

Figure 12: Hyper inflammatory pathogenesis of COVID-19 **(Savannah *et al.*, 2020)**

2.4 C-reactive protein (CRP)

CRP is an acute-phase protein first described by Tillet and Francis, which is produced by hepatocytes and elevated in acute infection or inflammation **(Stringer *et al.*, 2021)**. It is a non-specific marker and is partially elevated via the bioactivity of pro-inflammatory cytokines such as IL-6. Therefore CRP is a sign of both inflammation and a cytokine storm **(Heidari *et al.*, 2022)**. Pro-inflammatory cytokines become a major contributor to the production of CRP during a cytokine storm in COVID-19 infection **(Daneshkhah *et al.*, 2020)**. Secretion begins 4–10 h after an inflammatory insult and peaks at 48 h, with a short half-life of 19 h **(Stringer *et al.*, 2021)**. As an acute-phase protein, the plasma concentration of CRP deviates by at least 25% during inflammatory disorders **(Sproston and Ashworth, 2018).**

CRP is a vital component of the human innate immune system. The human body in response to a microbial pathogens or other inflammatory conditions such as tissue injury, malignancy, necrosis or chronic inflammatory rheumatic diseases, produces cytokines that further trigger the release of CRP from the liver, along with fibrinogen. CRP then binds to the various intrinsic and extrinsic ligands on the surface of the dead cells or pathogens, leading to activation of the classical complement pathway, phagocytosis and opsonisation **(Sen *et al*., 2022)**. Although CRP can initiate the fluid phase pathways of the host defense by activating the complement pathway, it can also initiate cell-mediated pathways by activating complement as well as binding to Fc receptors of IgG. CRP binds to Fc receptors with the resulting interaction leading to the release of pro-inflammatory cytokines **(Sproston and Ashworth, 2018)**.

There are several biomarkers that have been associated with the severity of COVID-19 one of which is CRP and COVID-19 patients who die have been shown to exhibit higher levels of CRP as compared to COVID-19 survivors **(Ali *et al*., 2022).** However, the evidence for CRP as a prognostic marker is yet to be determined **(Stringer *et al*., 2021)**.

Apart from COVID-19, increased CRP levels are used to clinically detect many other inflammatory conditions such as cardiovascular disease and rheumatoid arthritis therefore CRP being a non-specific indicator of solely inflammation cannot be used as the only test to confirm COVID-19. It only indicates the extent of inflammatory response in the body, without confirming the cause. The test in conjunction with signs and symptoms, and other diagnostic tests and physical examinations can confirm the cause of inflammation to be COVID-19 **(Sen *et al*., 2022).**

2.5 D-dimer

D-dimer is a fibrin degradation product, widely used as a biomarker for thrombotic disorders. A D-dimer value of less than 500 ng/mL is usually considered normal, and values increase with increasing age and in pregnancy **(Poudel *et al*., 2021)**. D-dimer levels are commonly elevated in patients infected with SARS-CoV-2 and significantly higher levels are found in those with critical illness and may be used as a prognostic marker for in-hospital mortality **(Yao *et al*., 2020)**. Following the outbreak of the COVID-19 pandemic, D-dimer has been identified as a potential indicator for its prognosis in COVID-19 patients **(Poudel *et al*., 2021)**.

While COVID-19 is primarily a respiratory illness, it can affect multiple organ systems including gastrointestinal, hepatic, cardiac, neurological and renal systems. Thrombotic complications and coagulopathies including disseminated intravascular coagulopathy are common in COVID-19, likely reflecting the activation of the coagulation cascade due to viremia, or a cytokine storm, or possibly due to superinfection and organ dysfunction **(Poudel *et al*., 2021)**. Virus infections usually involve an aggressive pro-inflammatory response and impaired control of the anti-inflammatory response. This could lead to dysfunction of endothelial cells, resulting in excessive thrombin formation. Pulmonary thrombin could even be responsible for oxygen desaturation and acute respiratory distress commonly observed in COVID-19 patients **(Demir *et al*., 2021)**.

COVID-19 patients exhibit multiorgan failure with coagulation abnormalities represented by lower platelets count and increased D-dimer, which are increasingly associated with poor prognosis and explain the microthrombi of the lungs, lower limbs, hands, brain, heart, liver, and kidneys **(Hojyo *et al*., 2020).**

2.6 The symptoms of COVID-19

COVID-19 patients can be classified into mild, moderate, and severe **(Palladino, 2021)**. COVID-19 affects different people in different ways however most infected people will develop mild to moderate illness and recover without hospitalization. Symptoms may appear 2-14 days after exposure to the virus. Symptoms usually begin with non-specific syndromes including fever, dry cough, and fatigue. Multiple systems may be involved, including respiratory (coughing, shortness of breath, sore throat, rhinorrhoea, haemoptysis, and chest pain), gastrointestinal (diarrhoea, nausea, and vomiting), musculoskeletal (muscle ache), and neurologic (headache or confusion).

More common signs and symptoms are fever, coughing and shortness of breath. After the onset of the disease, symptoms are somehow mild and the median time to first hospital admission is 7 days. The disease then progresses to shortness of breath in approximately 8 days, ARDS in 9 days, and to mechanical ventilation in 10.5 days in about 39% of the patients. Patients with severe COVID-19 develop ARDS and worsen in a short period of time and usually die of multiple organ failure **(Wu *et al*., 2020)**.

2.7 Transmission of the Virus

The human-to-human transmission of COVID-19 is the passing of coronavirus disease 2019 from person to person. The principal mode by which people are infected with SARS-CoV-2 is through exposure to respiratory fluids carrying the infectious virus. There are thought to be 3 main transmission routes of the virus in humans **(Bazant and John, 2021)**: droplet transmission, aerosols and direct contact (**Fig. 13**).

2.7.1 Droplet Transmission

SARS-CoV-2 is transmitted between people by respiratory droplets (sputter) emitted during sneezing, coughing or talking by an infected person which reach the mouth, nose or eyes of another person in the immediate vicinity (i.e. within 1 metre) and are inhaled **(Jayaweera *et al.*, 2020)**. In COVID-19 patients these droplets contain the virus and if inhaled or ingested by uninfected people will lead to the development of the disease in these people **(Rahman *et al.*, 2020).** These respiratory droplets are >5-10 μm in diameter and the quantity of droplets emitted varies according to the people and the circumstances (physical effort, singing, etc.). The use of personal protective equipment (PPE) with efficient barriers to the droplets and maintenance of personal and environmental hygiene will limit the rate of infections.

2.7.2 Aerosol Transmission

Smaller droplets (<5μm in diameter) referred to as droplet nuclei or aerosols can stay airborne longer and travel farther away. These aerosols containing the SARS-CoV-2 can be transmitted through airborne transmission. Aerosol transmission is not just from people with symptoms of the disease as even asymptomatic COVID-19-positive people can be the source of infection. In close environments, the virus-containing aerosol may persist in the air for long periods and at high concentrations, further increasing the rate of transmission **(Rahman *et al.*, 2020)**. In the article, "Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1", **(van Doremalen *et al.*, 2020)** reported that SARS-CoV-2 can remain viable and infectious in aerosols for hours.

2.7.3 Direct contact transmission

Direct contact transmission may occur through direct contact with infected people, virus-contaminated objects and surfaces **(Fig. 13)**. Respiratory secretions or droplets

expelled by infected individuals can contaminate surfaces and objects, creating fomites (contaminated surfaces) for example SARS-CoV-2 has been shown to persist on inanimate surfaces for days under controlled laboratory conditions **(Todt *el al*., 2021)**. Viable virus and/or RNA has been detected on surfaces for periods varying from a few hours to a few days, depending on the type of environment (temperature, humidity) and the type of surface (approximately 24 hours on cardboard, and 2 -3 days on plastic or stainless steel). The transmission of COVID-19 can be minimized by frequent use of an alcohol-based hand rub, washing of hands using soap and water, and avoiding touching of eyes, nose, and mouth with contaminated hands **(Rahman *et al*., 2020)**.

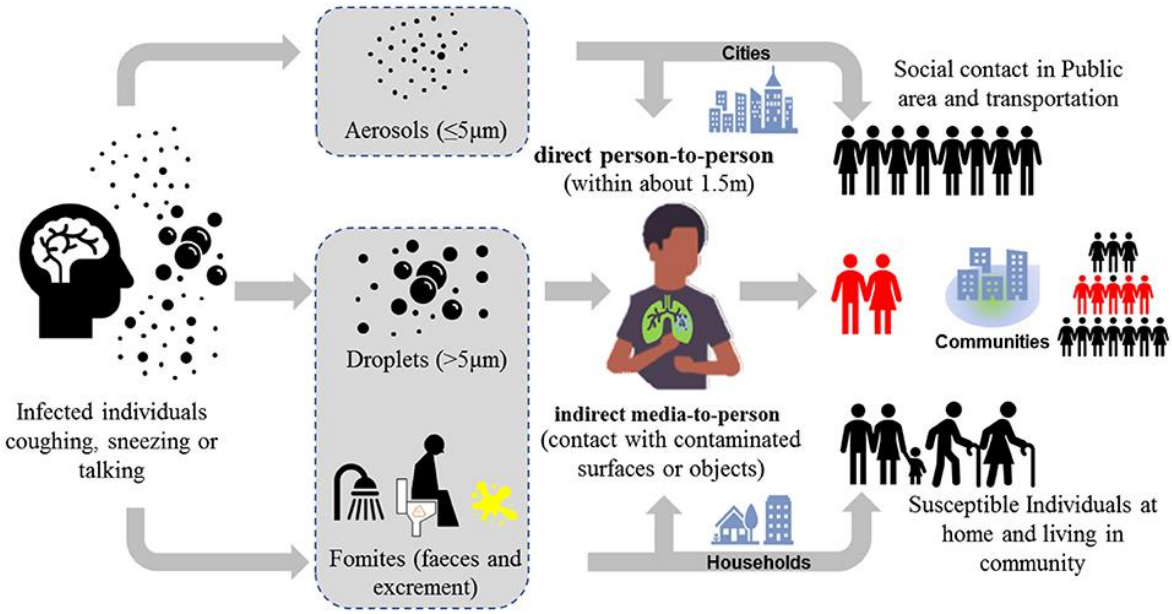

Figure 13: Transmission routes of COVID-19 **(Hu *et al*., 2021).**

2.8 Treatment

2.8.1 Chloroquine and Hydroxychloroquine

Hydroxychloroquine (HCQ) and chloroquine (CQ) are two antimalarial drugs with immunomodulatory effects, commonly used to treat rheumatoid arthritis and systemic lupus erythematosus (SLE) **(Mohammad *et al*., 2021)**. HCQ is an analog of chloroquine that is considered to be less toxic. CQ or HCQ may block SARS-CoV-2 fusion with the host cell and entry into the target cell by elevating the pH in the endolysosomal system **(Li *et al*., 2020)**. Both CQ and HCQ interfere with ACE2 receptor glycosylation thereby preventing SARS-CoV2 binding to target cells **(Singh and Vijayan, 2020)**. In vitro studies have

suggested that both CQ and HCQ may block the transport of SARS-CoV-2 from early endosomes to endolysosomes, possibly preventing the release of the viral genome **(Gasmi *et al.*, 2021)**. However, there have been concerns over the side effects resulting from prolonged usage of these two drugs, which include a higher risk of cardiac arrhythmias and QT interval prolongation.

2.8.2 Remdesivir

Remdesivir is an adenosine nucleoside analog drug showing antiviral activity against Ebola and Marburg viruses, and other RNA viruses **(Hu *et al.*, 2021)**. It binds to the viral RNA-dependent RNA polymerase and inhibits viral replication by terminating RNA transcription prematurely. Remdesivir has demonstrated in vitro activity against SARS-CoV-2 **(Wang *et al.*, 2020)**.

2.8.3 Molnupiravir

Molnupiravir is a small-molecule ribonucleoside prodrug of N-hydroxycytidine (NHC), which has activity against SARS-CoV-2 and other RNA viruses. After oral administration of Molnupiravir, NHC circulates systemically and is phosphorylated intracellularly to NHC triphosphate. NHC triphosphate is incorporated into viral RNA by viral RNA polymerase and subsequently misdirects the viral polymerase to incorporate either guanosine or adenosine during viral replication. This leads to an accumulation of deleterious errors throughout the viral genome that ultimately render the virus non-infectious and unable to replicate **(Bernal *et al.*, 2021)**.

2.8.4 Ritonavir-boosted Nirmatrelvir (Paxlovid)

Nirmatrelvir is an effective and safe antiviral drug that inhibits the main protease (Mpro), 3CL protease, of SARS-CoV-2 **(Hung *et al.*, 2022)**. The SARS-CoV-2 Mpro is a 33.8-kDa protein which is responsible for proteolytic cleavage of viral polyproteins and is essential for viral replication **(Ng *et al.*, 2022)**. It has demonstrated antiviral activity against all coronaviruses that are known to infect humans. Nirmatrelvir is packaged with ritonavir (as Paxlovid), a strong cytochrome P450 (CYP) 3A4 inhibitor and pharmacokinetic boosting agent. Ritonavir is thus combined with nirmatrelvir to enhance its therapeutic concentration **(Hung *et al.*, 2022)**.

CHAPTER 3

VITAMIN D AND COVID-19

CHAPTER 3: VITAMIN D AND COVID-19

3.1 Generalities

For many years, the function of vitamin D was considered to be limited to calcium and phosphorus homeostasis. However, after the discovery of VDRs in other various cell types, many other biological roles of vitamin D have been revealed in addition to its known actions in classic target tissues **(Umar *et al*., 2018)**.

One of these roles is the involvement of vitamin D in the modulating of the innate and adaptive immune responses. This immune-modulatory action of vitamin D originates from two main observations:

a) the presence of VDR in proliferating immune cells (B cells, T cells and antigen-presenting cells) and
b) The ability of immune cells to metabolize vitamin D. The latter function ensures a physiological high concentration of active 1,25(OH)2D3 in a local lymphoid environment, which promotes its specific action and limits any undesirable high concentration-related systemic effects like hypercalcemia and bone resorption. Locally produced vitamin D acts on immune cells either in intracrine, autocrine, and/or paracrine fashion and affects multiple components of innate and adaptive immunity pathways **(Umar *et al*., 2018)**.

3.2 Role of vitamin D in the immune system

3.2.1 Innate immunity

Innate immunity is considered the first defense mechanism against invading microorganisms including bacteria, viruses, fungi and protozoa. This immune system initially recognizes a microorganism or its products via PRR which then trigger a cascade of events that will end with the removal and/or destruction of the invading agents **(Taha *et al*., 2021)**. Vitamin D determines the expression of some antimicrobial peptides (AMPs) such as defensin β2 and cathelicidin, which possess antiviral properties and can act against enveloped viruses, including SARS-CoV-2 **(Laneri *et al*., 2021)**. AMPs are an important component of the innate immunity and are induced upon recognition of pathogen associated molecular patterns. A consensus sequence for the vitamin D response element was identified in the promoter regions of human genes for cathelicidin antimicrobial peptide (CAMP) and β-defensin-2 (DEFB4), and its expression was strongly upregulated by 1,25(OH)2D3. A preliminary study done by **(Zhang *et al*., 2020)** found that oral administration of human

cathelicidin, LL-37, ameliorated systemic symptoms in 11 patients with mild COVID-19. These findings suggest that an improvement in vitamin D status, by providing more substrate (i.e., 25(OH)D3) to immune cells capable of converting it to 1,25(OH)2D3, might be a crucial constituent of the early host defense against SARS-CoV-2 infection through the production of AMPs **(Bae *et al.*, 2022).**

Since SARS-CoV-2 infection begins with the association of its spike 1 (S1) protein with host ACE2, targeting the interaction between S1 and ACE2 is a practical strategy against SARS-CoV-2 infection. (**Wang *et al.*, 2021**) showed encouraging results indicating that human cathelicidin LL37 can simultaneously block viral S1 and cloak ACE2. LL37 binds to the receptor-binding domain (RBD) of S1 with high affinity and decreases subsequent recruitment of ACE2 **(Fig.14)**. Because of blockade of the receptor-binding domain, LL37 inhibits SARS-CoV-2 S pseudovirion infection. Interestingly, LL37 also binds to ACE2 with a very high affinity and cloaks the ligand-binding domain (LBD), thereby decreasing S1 adherence and protecting cells against pseudovirion infection in vitro (**Wang *et al.*, 2021**)

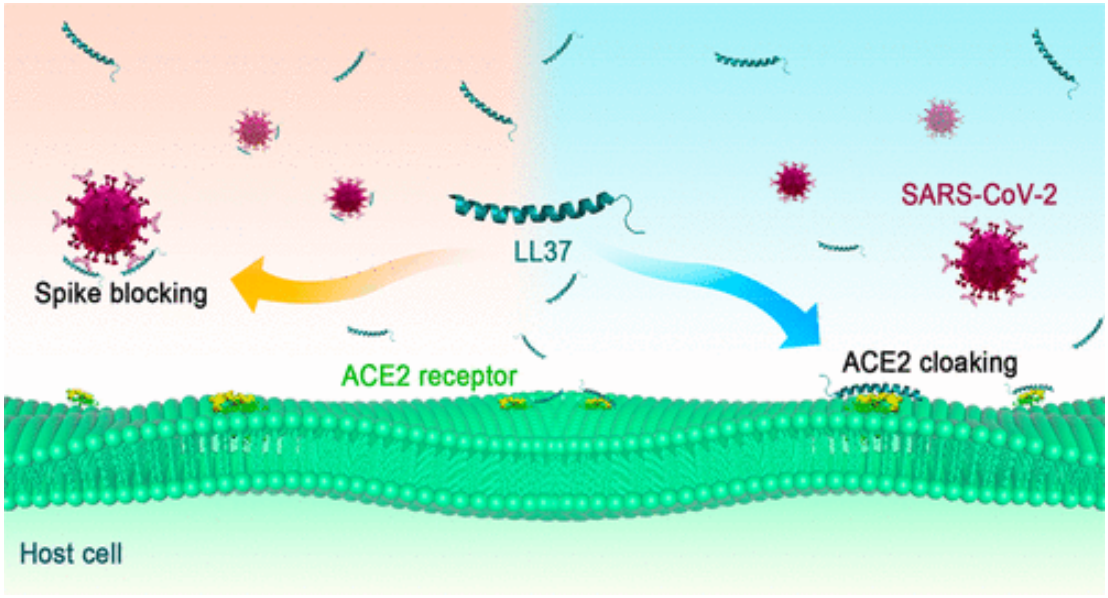

Figure 14: LL37 simultaneously blocking viral S1 and cloaking ACE2 **(Wang *et al.*, 2021)**

LL37 can also inhibit viral infection by targeting the steps that precede the virus entering the cell. It can:

a) create pores within the viral envelope;

b) cause extracellular aggregation of viral particles that block the virus's entry and increase the absorption of the virus by phagocytes;

Human beta defensin 2 (hBD-2) is a naturally occurring epithelial cell-derived host defense peptide that has anti-viral properties. hBD-2 binds the site on the CoV-2-RBD that docks with the ACE2 receptor thereby preventing it from binding to ACE2-expressing cells **(Fig.15)**. They also function as chemokines to augment and alter adaptive immune responses **(Zhang *et al*., 2022).**

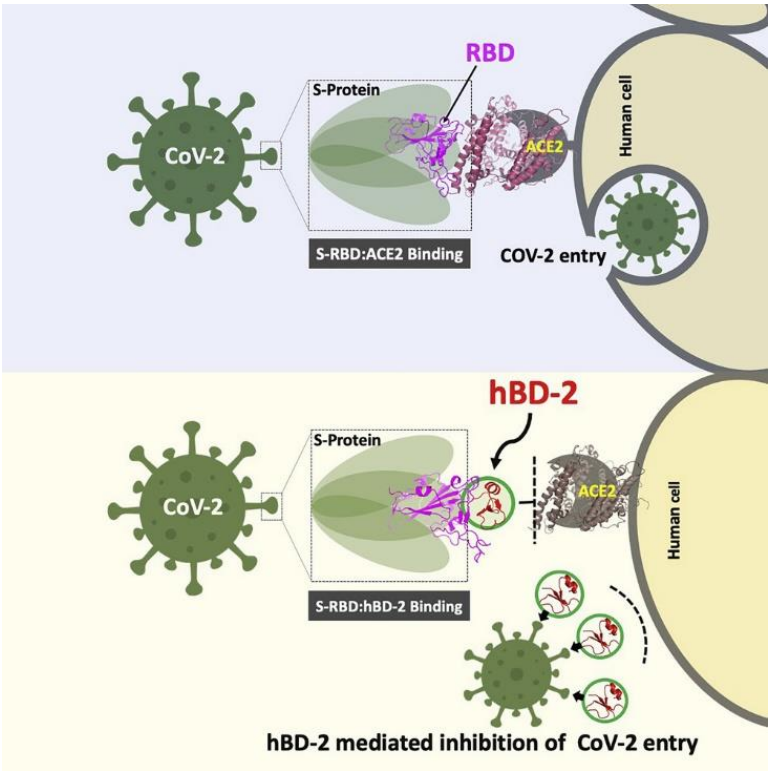

Figure 15: hBD-2mediated inhibition of CoV-2 entry **(Zhang *et al*., 2022)**

Vitamin D also has effects on monocytes and dendritic cells (DCs). It inhibits monocyte production of inflammatory cytokines such as IL-1, IL-6, IL-8, IL-12 and TNFα. It additionally inhibits DC differentiation and maturation with the preservation of an immature phenotype as evidenced by a decreased expression of major histocompatibility complex (MHC) class II molecules, co-stimulatory molecules and IL-12. Inhibition of DC differentiation and maturation is particularly important in the context of autoimmunity and the abrogation of self-tolerance. Antigen presentation to a T cell by a mature DC facilitates an immune response against that antigen while antigen presentation by an immature DC facilitates tolerance. Self-antigens are abundant in the normal state from physiologic cell

death and turnover. However, presentation of these self-antigens is usually by immature DCs so that tolerance to self is maintained **(Aranow, 2011).**

3.2.2 Adaptive Immunity

The adaptive immune system is the second defense mechanism against invading microorganisms which mediates an antigen-specific immune response through APCs, namely DCs, and the antigen recognition cells, T and B lymphocytes. Therefore, activation of these APC causes the production of various cytokines and antibodies and induces cell killing **(Taha *et al*., 2021)**. Vitamin D modulates the adaptive immune response, and since it influences the antigen presentation, it acts as a bridge between innate and adaptive immunity and therefore vitamin D can have either an indirect effect on lymphocytes through paracrine signalling by APC or a direct effect by VDR signalling **(Malaguarnera, 2020)**.

An appropriate immune response to SARS-CoV-2 infection is necessary for viral clearance and mitigates adverse outcomes in patients with COVID-19. Vitamin D and its metabolites are associated with both T and B cell immunity. In general, T cell responses play a pivotal role in combatting viral infections. Dysregulated T cell responses can lead to a pathological response to such infections. Emerging evidence suggests that patients with severe COVID-19 are characterized by the functional exhaustion of T cells and that improvements in vitamin D status can alleviate this process through immunomodulation.

In monocytes and macrophages, 1,25(OH)2D3 downregulates surface expression of MHC II and co-stimulatory molecules (such as cluster of differentiation 40 (CD40), CD80, and CD86), and thus decreases antigen presentation **(Martens *et al*., 2020)**. 1,25(OH)2D3 also impairs the maturation of dendritic cells in a paracrine manner and renders them tolerogenic. Because tolerogenic dendritic cells feature phenotypes resembling immature dendritic cells, 1,25(OH)2D3 reduces the differentiation of naïve T cells into cytotoxic effector T cells**(Bae *et al*., 2022)**. In addition, 1,25(OH)2D3 directly suppresses T cell activation by reducing the T helper cell type 1 (Th1) and Th17 responses **(Fig. 16)**. This is mediated by the binding of 1,25(OH)2D3 to the VDR and subsequent translocation to the nucleus of T cells, which upregulates the expression of the gene for cytotoxic T-lymphocyte antigen 4 (CTLA4), CD38, and IL-10. As CD^{4+} T cells are Th1- skewed in the bronchoalveolar lavage fluid of SARS-CoV2-infected patients, 1,25(OH)2D3 might alleviate uncontrolled excessive immune responses by promoting a transition from pro-inflammatory IFN-γ positive Th1 cells to inhibitory IL-10+ Th1cells. Although T cell

dynamics in COVID-19 need further investigation, the evidence suggests that improving vitamin D status can be beneficial in reducing dysregulated T cell responses **(Bae *et al.*, 2022)**.

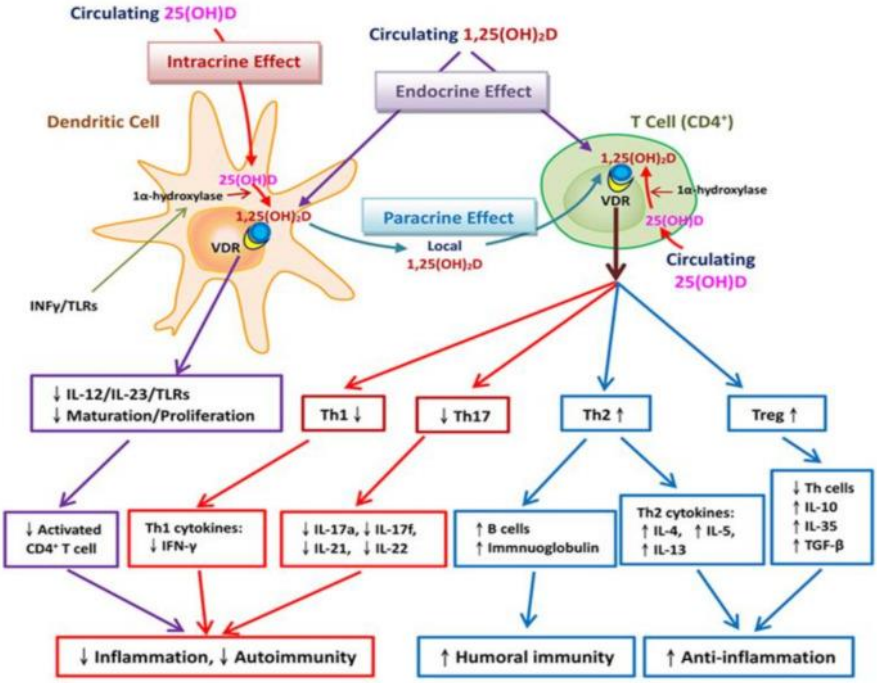

Figure 16: Vitamin D-related adaptive immune responses to COVID-19. **(Peng *et al.*, 2021)**

The presence of VDR in human B-lymphocytes with upregulation of both VDR as well as the 1α-hydroxylation enzyme, suggests a strong influence of vitamin D on B-lymphocytes. It has been shown that 1,25(OH)2D3 induces apoptosis of activated B-lymphocytes, and impedes the generation of plasma cells (by modulation of CD40 and thus NF-κB) and post-switch memory B-lymphocytes, without affecting B-lymphocyte differentiation **(Martens *et al.*, 2020)**. 1,25(OH)2D3 directly reduces the proliferation of B cells and promotes the secretion of IL-10, which in turn suppresses the activation of Th1 and subsequently reduces inflammation. These properties of locally produced 1,25(OH)2D3 might alter B cell responses in patients with COVID-19 **(Bae *et al.*, 2022)**. Of note, B cells are also involved in immunological memory and viral clearance. More studies are needed to elucidate the additional role of improved vitamin D status on B cell function and immunity.

On the other hand, alterations in adaptive immunity and vitamin D status can affect the prognosis of COVID-19 by affecting bone metabolism. Under inflammatory conditions, the release of cytokines, such as TNFα, IL-6, and IL-1, can upregulate osteoclastogenesis

and inhibit osteoblast activities. Among these cytokines, TNFα is a key factor in bone loss and might synergize with the receptor activator of RANKL to induce osteoclastic bone resorption.

3.3 Effects of vitamin D on COVID-19 by its action on CRP levels

Vitamin D could lower CRP levels and prevent inflammation from progressing to pneumonia and ARDS **(Demir *et al.*, 2021)**. The immune-modulatory role of vitamin D clearly shows a decrease in aspects related to the adaptive immunity. Since CRPs are indicators of inflammation, an increase in levels of vitamin D hence leads to the decrease in cytokines which ultimately affects the degree of CRP in the patients **(Fig. 17)**. This entire mechanism acts as an indicator of decreased effects of COVID-19 in patients **(Saxena *et al.*, 2022)**.

Pro-inflammatory cytokines are a major contributor to production of CRP during cytokine storm in COVID-19 infection. CYP27B1 plays an important role in metabolizing vitamin D into calcitriol. Calcitriol then binds and activates VDR in the nucleus, and controls gene expression. Expression of VDR and CYP27B1 can reduce the inflammatory markers. Together, this suggests a possible impact of vitamin D on decreasing pro-inflamatory cytokine production and CRP **(Daneshkhah *et al.*, 2020)**.

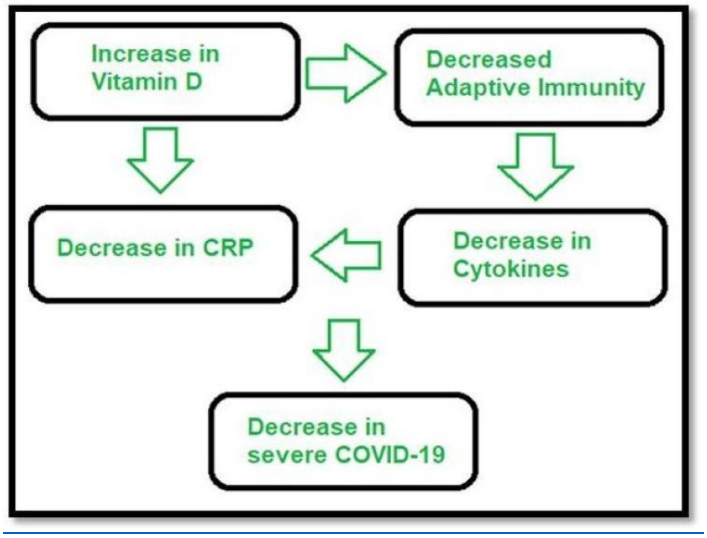

Figure 17: Effects of Vitamin D on COVID-19 by its action on CRP levels **(Saxena *et al.*, 2022).**

3.4 Effects of vitamin D on COVID-19 by its action on D-dimer levels.

D-dimer is a fibrin degradation product, widely used as a biomarker for thrombotic disorders. Elevated D-dimer levels were reported to represent a hypercoagulable state in COVID-19 patients **(Demir *et al*., 2021)**. COVID-19 patients exhibit multiorgan failure with coagulation abnormalities represented by a lower platelet count and increased D-dimer levels, which are increasingly associated with poor prognosis and explain the microthrombi of the lungs, lower limbs, hands, brain, heart, liver, and kidneys.

However vitamin D metabolites have emerged as an effective anticoagulant as is reflected in its ability to regulate different pro- and anti-thrombotic agents of coagulation cascade. It upregulates antigen expression, activity **(Fig. 18)**, and mRNA levels of an anticoagulant, thrombomodulin (TM), and downregulates antigen expression, activity, and mRNA levels of the crucial prothrombotic factor (TF), that initiates the activation of coagulation **(Sengupta *et al*., 2021)**.

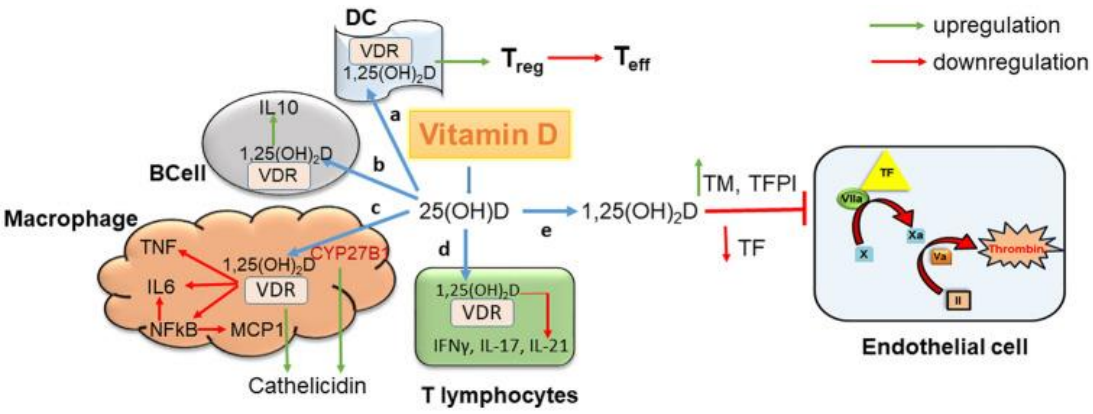

Figure 18: Possible mechanism of the role of vitamin D in the occurrence of thrombosis. **(Sengupta *et al*., 2021).**

CHAPTER 4

MATERIAL AND METHODS

CHAPTER 4: MATERIALS AND METHODS

4.1 Background

Hypovitaminosis D has been associated with increased vulnerability to respiratory infections and vitamin D treatment has been found to decrease other viral respiratory infections, especially in persons with vitamin D deficiency. However not enough importance has been given to the role of vitamin D status in COVID-19 infection. Therefore, this present study aims to investigate hypovitaminosis D in COVID-19 infection by evaluating the levels of vitamin D, CRP and D-dimer in COVID-19 positive patients and determining the potential relationship between these parameters and the risk of COVID-19 infection.

4.2 Objective

The objective of this work was to assess the serum levels of vitamin D, CRP, and D-dimer in COVID-19-positive patients, and possibly determine the potential relationship between these parameters and COVID-19 positivity.

4.3 Population and place of study

Our population of study consisted mainly of 18 COVID-19 positive patients (11 women and 7 men) who did their blood analysis and antigenic test at Laboratoire D'analyse Medicales et D'exploration Biologiques Dr S. Hamrouche Ep Cherfaoui in Mostaganem. The age of the patients ranged from the age of 17 to 83 years old.

4.4 Equipment used

Centrifuges (Heraeus labofuge 400), Vidas, Mö-screen corona antigen test, micropipettes, non-sterile gloves, needle syringes, antiseptic, reagents, heparin tubes

4.5 Methods

4.5.1 Blood Collection

As part of the analyses, 5 ml of blood is taken by venipuncture and collected in heparinized tubes. After collection, the blood is centrifuged at 4000 rotations per minute for a period of less than 10 minutes and the serum obtained is used for the different assays

4.5.2 Dosage of 25(OH) vitamin D

VIDAS 25(OH)D Total is an automated quantitative test for the determination of total 25(OH)D in human serum or plasma using the ELFA (Enzyme-Linked Fluorescent Assay)

technique. It reflects vitamin D produced cutaneously and that obtained from food and supplements for a reliable indication of vitamin D status.

4.5.2.1 Principle of the test

The VIDAS 25(OH)D total assay design is based on a 2-step competitive immunoassay:

1. Serum or plasma 25(OH)D is dissociated from its protein carrier (DBP) then added to alkaline phosphatase (ALP) conjugated vitamin D-specific antibody.
2. Unbound ALP-antibody is then exposed to vitamin D analog coated-solid phase receptor. Solid phase is then washed and substrate reagent is added to initiate the fluorescent reaction. An inverse relationship exists between the amount of 25(OH)D in the sample and the amount of relative fluorescence units detected by the system.

4.5.2.2 Performing a test:

Three operations are enough to perform a test:

1. Pipetting µ of serum sample and dispense into cartridge
2. Place the tip and the cartridge in the system
3. And launch the reaction by clicking on the start button using the computer connected to VIDAS.

4.5.2.3 Reading of results:

After the completion of the test, the results are calculated automatically by the VIDAS machine then compared to a stored calibration curve. The results are then displayed on the computer connected to the VIDAS machine.

4.5.3 D-dimer assay

The VIDAS D-Dimer Exclusion assay is an automated quantitative test for use on the instruments of the VIDAS family for the determination of fibrin degradation products (FbDP) in human plasma (sodium citrate) using the ELFA technique. VIDAS D-dimer exclusion is indicated for use in conjunction with a clinical pre-test probability assessment model to exclude deep vein thrombosis (DVT) and pulmonary embolism (PE) disease in outpatients suspected of DVT or PE.

4.5.3.1 Principle of the test

The assay principle combines a two-step enzyme immunoassay sandwich method with a final fluorescent detection (ELFA). The Solid Phase Receptacle (SPR), a pipette tip-like device, serves as the solid phase as well as the pipetting device for the assay. The assay reagents are ready-to-use and pre-dispensed in the sealed reagent strips (STRs). All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times.

First, the sample is taken by the SPR, diluted and then cycled in and out of the SPR several times. The antigen binds to the anti-FbDP immunoglobulins coated on the SPR. Unbound components are eliminated during a washing step.

In the second step, the conjugate that contains an ALP labelled anti-FbDP monoclonal antibody is cycled in and out of the SPR to form a sandwich. Unbound components are eliminated during the washing steps.

A detection step is then performed. The substrate (4-Methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methyl-umbelliferone), the fluorescence of which is measured at 450 nm. The intensity of fluorescence is proportional to the concentration of antigen present in the sample. At the end of the assay, results are automatically calculated by the instrument in relation to the calibration curve stored in memory. The results are then printed.

4.5.3.2 Reading of results:

After the completion of the test, the results are calculated automatically by the VIDAS machine then compared to a stored calibration curve. The results are then displayed on the computer connected to the VIDAS machine.

4.5.4 Antigenic test for COVID-19

4.5.4.1 Principle of the test

The Coronavirus Ag Rapid Test Cassette (Swab) is an immunochromatographic membrane assay that uses highly sensitive monoclonal antibodies to detect nucleocapsid protein from SARS-CoV-2 in nasopharyngeal (NP) or nasal swab. The test strip is composed of the following parts: namely sample pad, reagent pad, reaction membrane, and absorbing

pad. The reagent pad contains the colloidal-gold conjugated with the monoclonal antibodies against the nucleocapsid protein of SARS-CoV-2; the reaction membrane contains the secondary antibodies for nucleocapsid protein of SARS-CoV-2.

The whole strip is fixed inside a plastic device. When the sample is added into the sample well, conjugates dried in the reagent pad are dissolved and migrate along with the sample. If SARS-CoV-2 nucleocapsid antigen is present in the sample, a complex formed between the anti-SARS-2 conjugate and the virus will be captured by the specific anti-SARS-2 monoclonal antibodies coated on the test line region (T). Absence of the T line suggests a negative result. To serve as a procedural control, a red line will always appear in the control line region (C) indicating that proper volume of sample has been added and membrane wicking has occurred.

4.5.4.2 Test procedure

Allow the test device, test sample and buffer to equilibrate to room temperature (15-30°C) prior to testing.

1) Just prior to testing remove the test device from the sealed pouch and lit it on a flat surface.
2) Push the nozzle which contains the filter onto the extraction tube. Ensure the nozzle has a tight fit.
3) Hold the extraction tube vertically and add 4 drops (approximately 100 μL) of test sample solution tube into the sample well.
4) Start the timer.
5) Read the results at 15 minutes. Do not interpret the result after 20 minutes

4.5.4.3 Interpretation of results

The presence of two lines i.e. control line (C) and test line (T) within the result window indicates a positive result. The presence of only the control line (C) within the result window indicates a negative result. If the control line (C) is not visible within the result window after performing the test, the result is considered invalid. Some causes of invalid results are because of not following the directions correctly or the test may have deteriorated beyond the expiration date. It is recommended that the specimen be re-tested using a new test.

4.5.5 CRP assay

This quantitative test measures C-reactive protein in human serum to assess the body's inflammatory state.

4.5.5.1 Principle

Immunoturbidimetric test: Photometric measurement of the disorder caused by the antigen-antibody reaction in the end point method at 340nm

4.6 Statistical studies

The collected data of this randomized work were subjected to analysis of variance. Duncan's multiple range test was used to distinguish treatment means. The level of $p<0.05$ was taken into account for significance **(SAS, 2008)**. The descriptive results of the quantitative variables were presented in the form of tables and histograms illustrating the means and the standard deviations.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Results

Our population of study included 18 COVID-19-positive patients (11 women and 7 men) divided into 2 age groups (below and above 40 years respectively). In the investigation of hypovitaminosis D, this population diagnosed with COVID-19 was subjected to the testing of certain biological markers essential to the study namely vitamin D, CRP and D-dimer.

5.1.1 Vitamin D level

The vitamin D assay results of our patients distributed according to age and sex are illustrated in **(Fig. 19)**.

Note: Females below 40 years **(FB40)**, Females above 40 years **(FA40)**, Males below 40 years **(MB40)** and Males above 40 years **(MA40)**

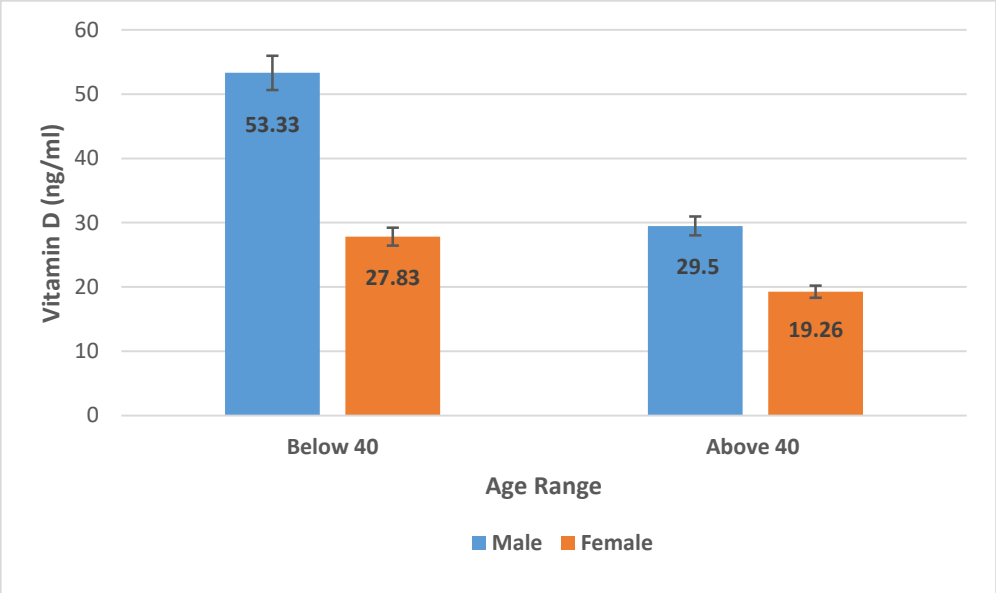

Figure 19: Mean vitamin D levels distributed according to age and sex.

The statistical study ($p<0.05$) of **(Fig. 19)** reveals sufficient vitamin D levels (30-100 ng/ml) in **MB40** who have a mean value of 53.33ng/ml. **FB40** have an insufficiency (20-29 ng/ml) of vitamin D with a mean value of 27.83ng/ml. We also observed an insufficiency (20-29 ng/ml) in vitamin D levels in **MA40** who had a mean value of 29.5ng/ml. Vitamin D deficiency (<20ng/ml) is observed in **FA40** who have a mean value of 19.26ng/ml. The mean vitamin D levels of **MB40** were significantly higher than those of women in the same age

range (53.33ng/ml as compared to 27.83ng/ml respectively). The mean vitamin D levels of **MA40** (29.5ng/ml) are significantly higher than those of the women in the same age range (19.26ng/ml). **MB40** exhibit higher vitamin D levels with a mean value of 53.33ng/ml as compared to **MA40** who have a mean vitamin D level of 29.5ng/ml. Higher vitamin D levels were also observed in **FB40** (mean was 27.83ng/ml) as compared to **FA40** whose mean value is 19.26 ng/ml.

5.1.2 CRP levels

The CRP results of our patients distributed according to age and sex are illustrated in **(Fig. 20)**.

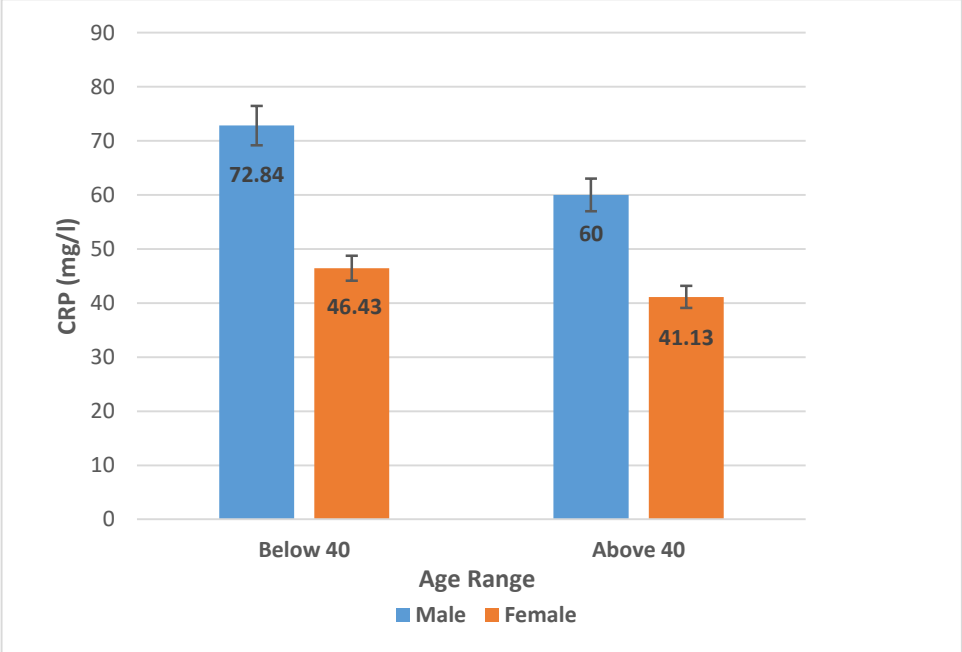

Figure 20: Mean CRP levels distributed according age and sex.

The statistical study ($p<0.05$) of **(Fig. 20)** reveals elevated CRP levels (> 6mg/l) in the majority of these patients infected with COVID-19 (72.84mg/l for **MB40** and 46.43mg/l for **FB40**) and (60mg/l for **MA40** and 41.13mg/l for female patients in the same age category). **MB40** exhibit significantly higher CRP levels (mean value = 72.84mg/l) as compared to **MA40** who have a mean CRP value of 60mg/l. **FB40** had slightly higher CRP levels (mean = 46.43mg/l) than those of the **FA40** (mean = 41.13 mg/l). The mean CRP levels for those

below the age of 40 years old (72.84mg/l in **MB40** and 46.43mg/l in **FB40)** are slightly higher than those for patients above the age of 40 years (mean value = 60mg/l in **MA40** and 41.13mg/l in **FA40)**.

5.1.3 D-dimer levels

The D-dimer results of our patients distributed according to age and sex are illustrated in **(Fig. 21)**.

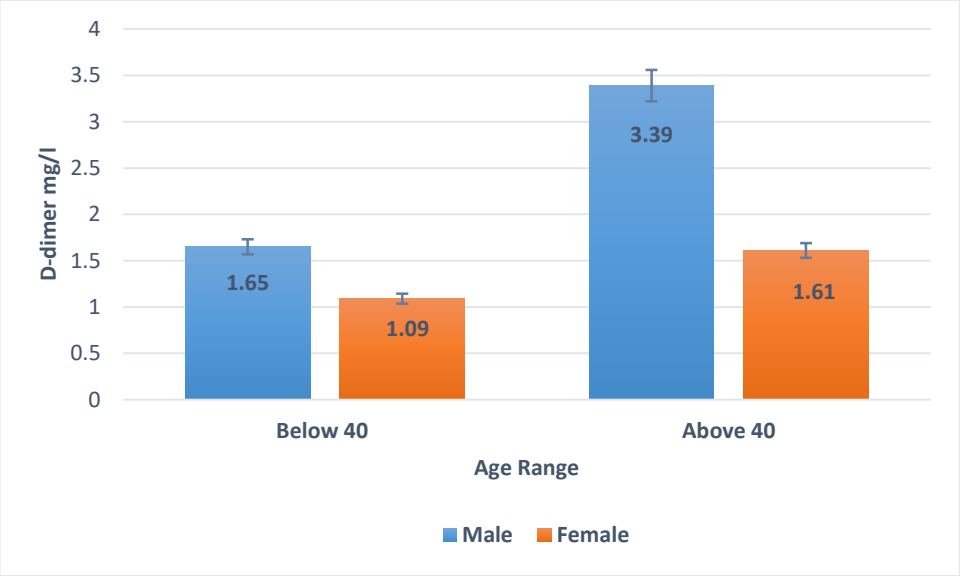

Figure 21: Mean D-dimer levels distributed according to age and sex.

The statistical study ($p<0.05$) of **(Fig. 21)** reveals elevated D-dimer levels (>0.5 mg/l) in all the patients infected with COVID-19. **MB40** have significantly lower D-dimer levels with a mean of 1.65 mg/l as opposed to **MA40** whose mean value is 3.39 mg/l. **FA40** also have significantly higher D-dimer levels (mean = 1.62 mg/l) as compared to those below the age of 40 years old whose mean value is 1.09 mg/l. **FB40** exhibit lower D-dimer levels (1.09 mg/l) as compared to the D-dimer levels in **MB40** (mean value = 1.65 mg/l). The D-dimer levels of **MA40** (mean value = 3.39 mg/l) are significantly higher than those of the women in the same age range (1.62 mg/l).

5.2 General Discussion

Our study focused on a population of 18 patients who tested positive for COVID-19 at Laboratoire D'analyse Medicales et D'exploration Biologiques Dr S. Hamrouche Ep Cherfaoui in Mostaganem. The objective was to evaluate their serum levels of vitamin D, CRP, and D-dimer and possibly determine the potential relationship between these parameters and COVID-19 positivity in these patients.

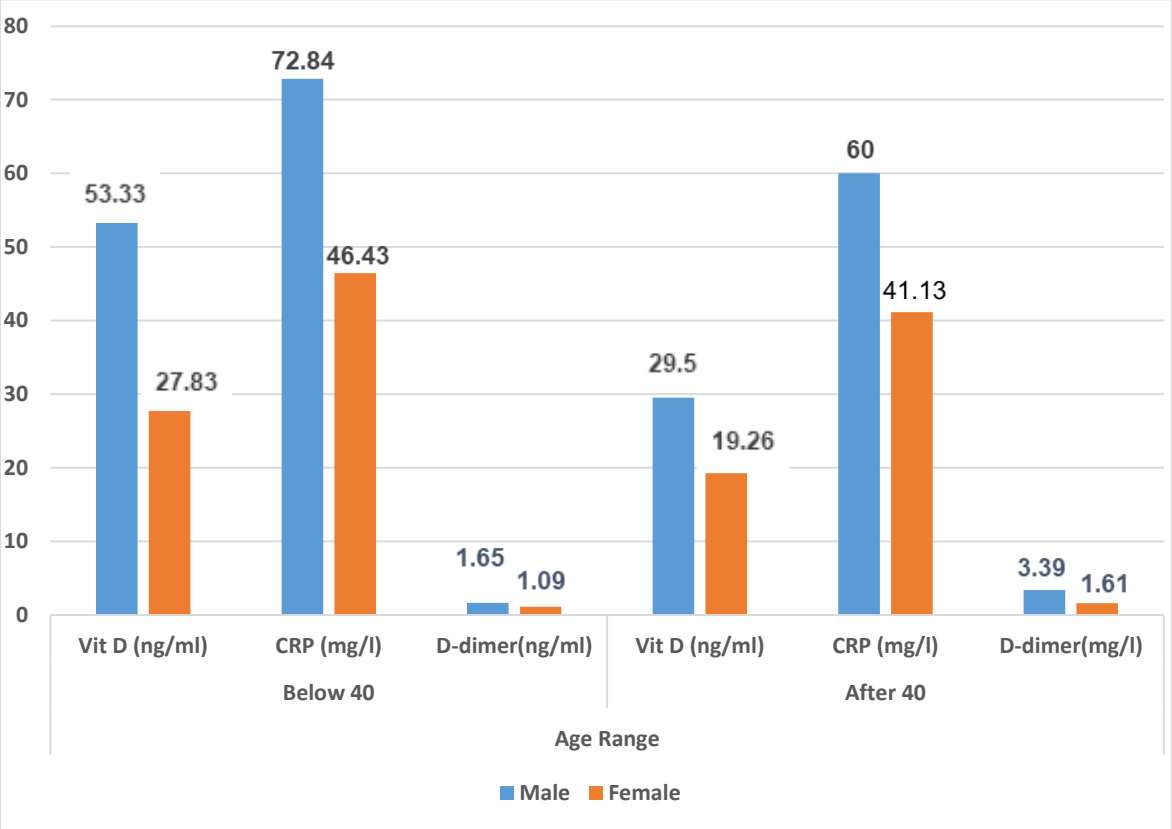

Figure 22: Mean vit D, CRP and D-dimer levels distributed according to age and sex

According to the results of this study hypovitaminosis D (< 30ng/ml) is prevalent in the majority of COVID-19 patients **(Fig. 22)** which was also confirmed by a study done by **(Maghbooli *et al.*, 2020)** who showed that out of their population of 235 patients 67.2% had a 25(OH)D level of less than 30ng/ml. This is also in line with the results of a study done by (**Teama *et al.*, 2021)** which revealed that out of their population of 124 COVID-19-positive patients a high prevalence of hypovitaminosis D was found in 97.6% of the patients. This relationship between vitamin D and COVID-19 infection is anticipated as vitamin D is

involved in the modulation of the innate and acquired immune system and also in the production of antimicrobial peptides **(Kaufman *et al*., 2020)**. Vitamin D also plays a key role in the control of the cytokine storm induced in several inflammatory conditions and also in COVID-19. This activity of vitamin D3 is carried out by inhibiting the production of the pro-inflammatory cytokines such as TNF-α and IFN-γ but, also, by increasing the expression of the anti-inflammatory cytokine IL-10 **(Pagano *et al*., 2020)**.

A retrospective analysis of 348,598 UK biobank participants also showed a higher chance of SARS-CoV-2 in subjects with lower levels of 25(OH)D **(Padhi *et al*., 2020)**. However, after adjusting for potential confounders they found no association between vitamin D concentration and COVID-19 infection **(Hastie *et al*., 2020)**.

Our results also revealed that vitamin D levels in women are lower as compared to those of men in the same age range. This agrees with results found by **(Muscogiuri *et al*., 2019)** whose study findings revealed a lower 25(OH)D concentrations in females as compared to males among the different classes of body mass index (BMI) which they suggested that it was probably due to the higher percentage of fat mass in females as compared to males. However, a study done by **(Hernández *et al*., 2021)** in Santander, northern Spain revealed that serum 25(OH)D values were higher in women than in men. The difference in the results can be attributed to the difference in culture, lifestyle and physical activities of the general Algerian population and the general Spanish population from where the patients came from.

The findings of this study also revealed a higher prevalence of hypovitaminosis D in women above the age of 40 years. These findings agreed with those of a study conducted in a rural area in India by **(Pan *et al*., 2018)** who found that out of their population of 194 participants **70.6**% of the women aged 40 years and above had a concentration of vitamin D lower than 30ng/ml. The results found by **(Pan *et al*., 2018)** revealed that 42.3% of their population were on menopause and vitamin D deficiency is exacerbated in post-menopausal women owing to the loss of oestrogen and age-related changes in the vitamin D receptor and vitamin D synthesis **(Mitra *et al*., 2016)**.

According to the results of this study COVID-19-positive patients had elevated CRP levels (> 6mg/l) which agreed with the findings of **(Guan *et al*., 2020)** who revealed that most of their COVID-19-positive patients had elevated levels of CRP. A study done by

(**Chen *et al*., 2020)** also revealed that out of their population of 73 patients, 86% had higher than normal CRP levels.

The results of this study also show an association between hypovitaminosis D and elevated CRP levels in the majority of the patients as was also confirmed by **(Demir *et al*., 2021)** who concluded that higher CRP levels associated with vitamin D deficiency were mentioned to be linked with increased risks of severe COVID-19 and that COVID-19-positive patients with serum vitamin D levels < 30 ng/ml were shown to have very high levels of CRP. A study done by **(Heidari *et al*., 2022)** also revealed that lower levels of serum vitamin D were associated with higher inflammatory markers, such as CRP and D-dimer. This could be explained by the fact that vitamin D deficiency leads to the generation of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which may result in elevated CRP levels and induce inflammation **(Demir *et al*., 2021)**.

In addition to elevated CRP levels, elevated D-dimer levels were observed in the population of our study which is in line with the findings of **(Alzoughool *et al*., 2021)** who found that high levels of D-dimer are prevalent in COVID-19 patients. These results explain the intravascular coagulation that has been reported in COVID-19 patients as COVID-19 patients have been shown to exhibit multiorgan failure with coagulation abnormalities represented by lower platelets count and increased D-dimer, which are increasingly associated with poor prognosis and explain the microthrombi of the lungs, lower limbs, hands, brain, heart, liver, and kidneys **(Hojyo *et al*., 2020).**

We also found a positive correlation between hypovitaminosis D and elevated D-dimer levels in our population of study which is in agreement with the findings of **(Teama *et al*., 2021)** who found that a decrease in vitamin D levels was associated with more severe COVID-19 cases, with significantly higher blood levels of D-dimer **(Teama *et al*., 2021)**. Lower levels of serum vitamin D were also reported to be associated with elevated D-dimer levels in a study by **(Heidari *et al*., 2022)**. Therefore the results of this present study reveal a prevalence of hypovitaminosis D which is associated with elevated CRP and D-dimer levels in these COVID-19-positive patients.

5.3 Conclusion

Several studies have recently been performed in different populations to decipher the possible role of vitamin D in SARS-CoV-2 infection as vitamin D treatment has been found to decrease other viral respiratory infections, especially in persons with vitamin D deficiency. This relationship is anticipated, given that vitamin D has numerous actions affecting the innate and adaptive immune systems.

In conclusion, the present study has demonstrated that hypovitaminosis D is prevalent in COVID-19-positive patients and that these lower vitamin D levels are associated with elevated CRP and D-dimer levels. Whether the treatment of this hypovitaminosis D will play some role in the prevention or treatment of the viral disease remains to be elucidated in large randomized controlled trials, which will be certainly necessary to determine whether vitamin D levels could affect COVID-19 risk and precisely define the role of vitamin D treatment in futures waves of SARS-CoV-2 infection.

However if controlled trials find this relationship to be causative, the implications are vast and would present a cheap, readily-available method for helping prevent infection, especially for those with vitamin D deficiency.

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