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CD-14 and Toll-4 as Biomarkers for Prognostic Assessment of COVID-19: A Review Article

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***Abstract*— The COVID-19 pandemic has been responsible for almost 700,000 deaths in Brazil since its emergence to the present. As a virus that enters the host through the airways, SARS-CoV-2 is highly transmissible, showing tropism for the ACE-2 enzyme present in greater quantities in the lung alveoli. Since ACE-2 is present in the cells of the connective tissue of veins and arteries, it is widely distributed in the human body, including and mainly in the cells of the oral and nasal mucosa, which constitute the gateway for SARS-CoV-2, which in would then reach the lungs. Furthermore, mucosal epithelial cells have a variety of membrane glycoprotein receptors that bind to viral anti-receptors, among which are the sCD14 biomarkers. This literature review aimed to demonstrate the evasion mechanisms and pathogenicity of SARS-CoV-2 in order to use biomarkers such as sCD14 and Toll-4 to predict the severity of COVID-19 in infected individuals.**

***Index Terms*—CD14, COVID-19, MORBIDITY, SARS-CoV-2, TOLL LIKE RECEPTOR 4.**

I. Introduction

Coronavirus disease 2019 or COVID-19 (coronavirus disease2019) was named by the World Health Organization (WHO) at the end of 2019 (December), the period in which the first cases of pneumonia were reported in the city of Wuhan, Hubei province in the Popular Republic of China. The confirmation of the viral species causing COVID-19 occurred at the beginning of 2020 (January), when the Chinese authorities released the results of the sequencing of the new species of the virus initially named 2019-nCoV and later (in February 2020) SARS-CoV-2 [1].

COVID-19 triggered a pandemic given the high degree of infectivity of SARS-CoV-2. Considering the epidemiological triad where the disease is the result of the interaction between the agent, the susceptible host and the environment, direct transmission (person to person) of this agent occurred mainly through the dispersion of droplets (droplets offluggeor spittles) on the conjunctivae or mucous membranes of the nose or mouth, when sneezing, coughing, spitting, speaking or singing and/or through direct contact such as touching and/or kissing. Indirect transmission occurred through transmission vehicles or fomites. One of the contributing factors to the emergence of communicable diseases are environmental changes such as deforestation/ reforestation, changes in ecosystems and water, floods/droughts, natural disasters, famine and global warming [2].

Mortality attributed to COVID-19 was 6.9 million people according to the WHO from its emergence until March 2023. The Americas region was most affected by the pandemic (43%). The European region and the regions of southeast Africa had rates of 32% and 12% respectively. The western Pacific, eastern Mediterranean and African regions reported lower death rates during the same period, accounting for 14% of the global total. In Brazil, 37,145,514 cases of COVID-19 were reported according to the Ministry of Health, with mortality affecting 699,634 citizens in the country as of March 10, 2023 [3].

This literature review aimed to present the virus that causes COVID-19 as well as its ability to lodge and/or multiply within the human body. The evasion mechanisms of SARS-CoV-2 in the host were elucidated based on the epithelial anatomy and physiology of the naso-oro-pharyngeal mucosa and also the first line of innate response receptors, aiming to construct a simple and low-cost methodology with the purpose to define a biomarker to assess the prognosis of the disease.

II. SARS-CoV-2

Coronavirus 2 (SARS-CoV-2 in its acronym in English: Severe Acute Respiratory Syndrome Coronavirus 2), responsible for Severe Acute Respiratory Syndrome, also called COVID-19, is a virus of the betacoronavirus genus, enveloped RNA, single-stranded positive sense, that is, its genome is messenger ribonucleic acid (mRNA) ready to enter directly into the ribosome cellular and be part of the protein translation process [4, 5].

The structural proteins of the SARS-CoV-2 viruses are: spike proteins (S) present in 33.8%, membrane proteins (M) in 36.9% and nucleocapsid proteins (N) in 60% (Fig. 1). These percentages were obtained using a chromatographic system coupled to a mass spectrometer (LC-MS). In addition, SARS-CoV-2 has 16 non-structural proteins, including: ORF 7a, ORF 7b, ORF 8 and ORF 9 [4].

In broncho-alveolar cells, SARS-CoV-2 can lead to acute lung injury, promoting high amounts of cytokines, up to tissue failure. However, vascular endothelial cells and alveolar macrophages are also important targets of infection and viral activation, contributing to high proportions of pro-inflammatory cytochemical sources and severe symptoms of COVID-19. High concentrations of the Receptor Ligand Domain (DRL or RBD) can induce the pro-inflammatory profile of macrophages and microglia [6].

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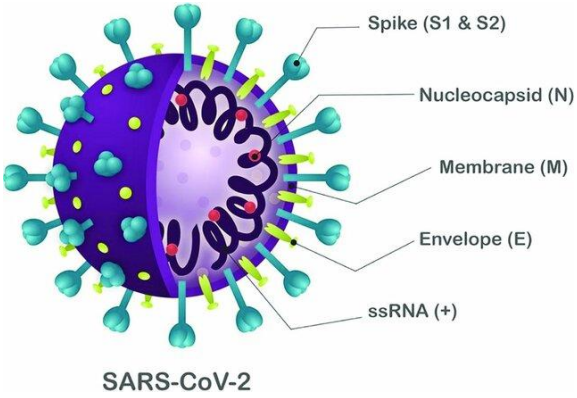

Figure I: Anatomical Characteristics of SARS-CoV-2.
(Figure adapted in doi: *10.3390/coatings12050577*)

III. SPIKE PROTEIN (S)

The S protein is inserted in multiple copies in the membrane of the SARS-CoV-2 virus, giving it crown-like appearance (figure 1). This homotrimeric protein is highly glycosylated, having a sugar shield with at least 22 N glycosylations, 30% of which are mannoseglycans (mannosylations) and several O glycosylations, which can make it difficult to bind to antibodies. The N-terminal domain of the S protein is made up of positively charged amino acids that are linked to the genomic RNA of SARS-CoV-2, appearing comparatively similar to the core of the lipopolysaccharide membrane of Gram-negative bacteria, which also consists of a charged region. of positive ions in addition to phosphorylated sugars and carboxylated sugars [6].

IV. ACESSORY PROTEINS

The non-structural protein ORF 7 of SARS-CoV-2 when cultured with CD14+ peripheral blood monocytes ex-living provided an increase in the production of pro-inflammatory cytokines such as interleukins (ILs): IL-1β, IL-6, IL-8, IL-10, IL-12 and tumor necrosis factor alpha (TNFα), this is because SARS-CoV-2 viral antireceptors integrate into the phospholipid layer of human epithelial cells where they are found with receptor glycoproteins (such as the soluble subunit of CD14) which, even without containing the specific ligand receptor domain, are capable of forming bonds with certain glycoprotein molecules of the cytoplasmic membrane and thus trigger the production of cytokines inflammatory responses to signaling pathways [4, 8, 9, 10].

The accessory protein ORF8, U122 or 6, IL-1β, IL-8 and TNFα) located between the endoplasmic reticulum and the Golgi complex and ORFs 7 through the host cell ubiquitination pathway are capable of interfering with the simultaneous anti-viral immune response in the signaling of cytokines and interferons (IL-6, IL-1β, IL- 8 and TNFα) [6, 9].

V. ECA 2 CELLULAR RECEPTOR

SARS-Cov-2 begins its parasitism by penetrating through the respiratory tract with subsequent spread of the airways and infecting the epithelial cells of the nasal, oral and nasopharyngeal mucosa, owing that ACE2 was found to be expressed in the squamous non-keratinized epithelium basal layer in the oro-nasopharyngeal regions [11].

The angiotensin-converting enzyme 2 (ACE2) is a metallopeptidase that participates in the process of regulating blood pressure (Renin-Angiotensin-Aldosterone) as follows: renin is produced by glandular cells in the juxtamedullary region (macula densa) close to the glomeruli renal, due to low filtration and the need for greater local pressure, this (renin) is carried by the bloodstream to the liver where it meets angiotensinogen and subsequently through the bloodstream it will be cleaved into angiotensin I by the enzyme converter of angiotensin I (ACE1) in the region of the lungs where the aforementioned ACE2 will cleave it to angiotensin II, which will regulate blood pressure in the human circulatory system [11, 12].

VI. CELL FUSION

The furin enzyme present in the adipose tissue of eukaryotic cells is an enzyme from the family of subtilisin/kexin proprotein convertases (PCSKs) capable of cleaving the spike (S), dividing it into S1 and S2 units. The S1 unit houses the Receptor Ligand Domain (RBD) which will recognize the angiotensin-converting enzyme (ACE2) present on the surface of vascular endothelial and smooth muscle cells, while the S2 unit contains the fusion machinery having since it exposes the fusion peptide after site 2 is cleavaged by the human cell's transmembrane serine protease 2 (TMPRSS2) [13, 14].

Through the cleavage of the peptide, the spike promotes the fusion of the virus membrane with that of the host cell in a process of syncytia formation. The increase in SARS-CoV-2 infection is more associated with increased cell-cell fusion than with virus-cell fusion. Furthermore, furin can increase cell-to-cell fusion in SARS-CoV-2 infections by up to 200 percent, allowing the virus genome to spread independently of the virion, the viral particle. Furin performs salt bridge interactions between two aspartates of its S1 binding pocket, ASP 258 and ASP 306, the arginine P1 of the peptide and also hydrogen interactions between P1 and PRO 256 [11, 13, 14].

VII.. HUMAN RECEPTOR ECTODOMAIN

The ligand receptor domain (DRL) of the virus recognizes the angiotensin-converting enzyme 2 (ACE 2), which is expressed on the surface of epithelial cells in the oral cavity, with greater emphasis on lingual muscle tissue (Saadi et al, 2021). The study of the sequencing of single-stranded viral RNA and the ACE 2 enzyme was carried out using the public database (TCGA:The Cancer Genome Atlasand FANTOMS CAGE:Function Annotation of the Mammalian Genome Cap Analysis for Gene Expression) in 32 adjacent normal tissues, 13 of which were located in the oral lingual tissue, 2 in the lingual base, 3 in the floor of the oral cavity and 14 in the oral cavity as a whole. The expression of ACE2 was higher in oral tissues (lingual muscle) than in other 19 tissues from different organs, however due to sample size limitations the p value (p=0.062) was not significant. Despite the presence of ACE2 in oral tissues, the nasopharyngeal region and also in the endothelium of small veins and arteries, its highest

concentration is in the epithelial cells of the pulmonary alveoli and in the enterocytes of the midgut [4, 11, 14].

VII. INNATE IMMUNOGENICITY

During gastrulation and organogenesis, epithelial cells need to detach from the united structure and migrate through the layers of mesenchymal tissue, which is why cells in general have the ability to move, penetrate and decompose components of the extracellular matrix. The expression and activation of transcription factor-inducing mesenchymal cells occur in response to signaling pathways including those mediated by: transforming growth factor beta (TGF-beta), platelet-derived growth factor (PDGF). English) among others [15].

The nasal atrium structurally presents cells from the keratinized multi-layer of the squamous epithelium as well as cells from the sebaceous glands, while the internal nasal valve is made up of cylindrical epithelium, with goblet cells, presenting mucus. Therefore called transitional mesenchymal epithelium due to biochemical changes in the epithelial cells that lead them to configure mesenchymal characteristics, the nasal epithelium presents its innate immunogenicity through the recognition of pathogens (Pattern Recognition Receptors PRRs) which can be divided into three broad subunits: Toll Receptors (TLRs), Retinoic Acid Inducing Gene Type 1 Receptors [(RIG)I-like] and the Nucleotide-Bound Oligomeric Domain [(NOD)-like receptor – NLR in English [16].

VIII. TOLL-RECEPTORS

Toll receptors are transmembrane proteins with extra- and intracellular domains. The extracellular domain is responsible for recording pathogen-associated molecular patterns (PAMPS) and the cytoplasmic domain indicates the process and signaling. TRL-8, TLR 1 and TLR-10 are Toll receptors that have been observed recognizing viral microorganisms (viruses) mainly those containing single-stranded RNA genome, miRNA and imidazoquilone anti-viral components [16].

The intracellular membrane Toll-like receptor 4 is capable of interacting with the soluble subunit of the CD14 glycoprotein (sCD14ST) mediating a gene 88 response to primary myeloid differentiation (MyD88) which mediates a protein complex in response to lipopolysaccharides. Furthermore, they can regulate the activity of the NF-Kappa B transcription factor, positively regulate the proliferation of smooth muscle cells and also the RNA polymerase 2 promoter, managing to promote the viral response. By positively regulating the production of IL6 and tumor necrosis factor, Toll-like receptors 4 can trigger the JNK signaling cascade towards the cell nucleus (Fig.2) [17].

The Toll 4 receptor of macrophages, in the presence of LPS stimulation of *Escherichia coli* (strain 055:B55 in B21 cells containing the SARS-CoV-2 RBD peptide) under culture of murine RAW 264.7 and BV2 microglia cells in DMEM medium, triggered the MyD88-dependent signaling pathway through pattern recognition factors (PRRs) and disintegrated mitochondria biogenesis consuming O2. Antibodies against nitric oxide synthase (iNOS) and those against caspase 3 (Asp175) showed a significant decrease in cell viability as a whole, under stimulation of the DRL or RBD ligand domain compared to the control condition without the stimulus [6, 17].

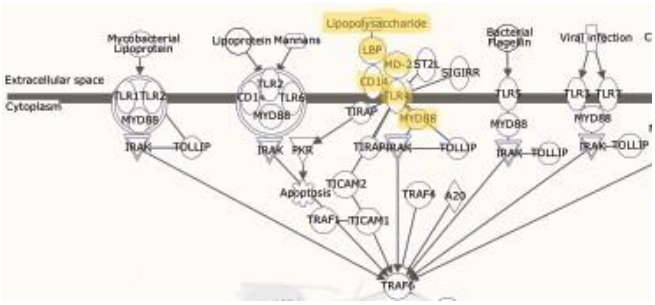

Figure 2: Microrganism antigens, membrane receptors and signaling pathways (*Quiagen.com/geneglobe/pathways*).

IX. CD14 MEMBRANE GLYCOPROTEIN

The membrane-anchored glycoprotein CD14 is present in vascular smooth muscle cells and also in cells of the immune system such as tissue macrophages and is capable of binding to the lipopolysaccharide (LPS) layer of Gram-negative bacteria and transferring it to the MD-complex. 2 of the Toll 4 receptor, dimerizing and activating the MD-2 signaling cascade. LPS is made up of lipid A, the core and the O antigen. The link between lipid A and oligosaccharides almost always occurs from a 3 deoxy D-manno-oct2-ulosonic acid unit KDO linked to the free C6 of glucosamine (GlcN) (Fig. 3) [7].

Some viruses such as the MMTV retrovirus have been observed expressing LPS-binding proteins and the poliovirus, which, like SARS-CoV-2, is an mRNAss virus (single stranded), had its infectivity increased after exposure *in vivo* to the intestinal microbiota of murine guinea pigs compared to a control group with suppressed microbiota (guinea pigs previously treated with antibiotics). Therefore, it was postulated that some viruses contain specific agents binding to the polysaccharides (LPS) of Gram-negative bacteria, considering that the LPS layer was the greatest enhancer of the viral effect, with only Nacetylglucosamine (GlcNAc) of the polysaccharides demonstrating activity [18].

The CD14 molecule and the Toll-like receptor 4 were expressed and quantified by immunological ELISA assay in coronary epithelial cells, as well as the cytokines IL8 and MCP1 and the CD14 glycomolecule. Endotoxin signaling in coronary epithelium is modulated by the interaction of two serum proteins: LBP and sCD14 (Fig.2). Healthy patients with low-grade infections present with serum molar concentrations of LBD and sCD14 less than or equal to 1. However, patients with chronic and high-grade infections present with chronically elevated levels of sCD14, with increases in sCD14 are correlated with high mortality from bacteremia. Considering that the levels of the glycoprotein subunit of soluble membrane CD14 (sCD14ST) increase discreetly and gradually during the inflammatory response, this has been tested as a biomarker to predict the prognosis of mortality from COVID-19 [10, 19].

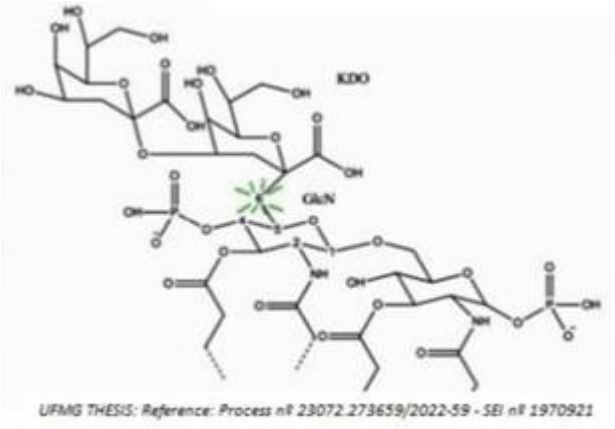

Figure 3: KPO unit of Lipid A linked to the free C6 of glucosamine (GlcN).

X. CELLULAR COMMUNICATION

Vascular smooth muscle cells (CVML) are among the most abundant in vascular walls, and in atherosclerotic lesions they are present in 30%. CVMLs show considerable plasticity and carry out cell-to-cell communication between endothelial cells and cells of the immune system during the inflammation process. They are sensitive and interactive cells that influence the behavior of other cellular components in the walls vascular. These smooth muscle cells can also produce and secrete cytokines such as interleukin 6, the monocytic cytokine chemotactic protein 1 (CCL2) and adhesion cytokines such as ICAM-1. Furthermore, CVMLs such as stromal cells can develop features reminiscent of fibroblasts, osteoblasts, and macrophage-like cells [15].

Macrophages regulate CVML clonality and apoptosis during arteriosclerosis. Clusters of macrophages have been found in arteriosclerosis plaques. Single-cell RNA sequencing (scRNA-seq) was used to identify the different cell subsets between the platelet portions of the proximal adjacent and atherosclerotic core. During the propagation of atherosclerosis RNA-seq revealed different cell populations during atherosclerotic progression. Three clusters highly expressed markers: TAGLN, ATAC2, MYH11, PDGFRB, myeloid cell markers: CD68, CD14, CY3CR1, LY2, others expressed CD45 and CD3, endothelial cell cluster: CD34 and PECAM1, dendritic cell cluster: CD1C and CD123. Grouping of B cells: CD79A and CD79B. The secretion of Il-1-beta was the main ligand of macrophages to CVML. TREM-2 was responsible for the change in macrophages. This membrane glycoprotein (TREM-2) consists of an extracellular domain, immunoglobulin ligand, a transmembrane domain, and a cytoplasmic tail that interacts with the ligand tyrosine kinase DAP 12 to form a receptor signaling complex [17, 20].

XI. PHOSPHOLIPID BILAYER

The basic element of cytoplasmic membranes is the phospholipid bilayer and they all share fundamental properties such as being impermeable to polar or charged compounds and permeable to nonpolar ones. Phospholipids form a bilayer in which the nonpolar regions of the lipid molecules in each layer are oriented toward the center of the bilayer and their polar groups oriented outward, interacting with the aqueous phase on each side. The outer layer of the bilayer generally consists of phosphatidylcholine and sphingomyelin, while the inner layer (cytoplasmic side) contains phosdatidylserine, phosphatidylethanolamine and phosphatidylinositols [5].

Plasma cell membranes maintain a different lipid composition in their exofacial and cytofacial layers. Phosphatidylserine, for example, is located exclusively in the inner leaflet of the plasma membrane and its exposure in the outer leaflet serves as a marker that prevents cell death in eukaryotes. Symmetrical membrane models lack some key structures of natural cell membranes, and these lacks can critically affect interactions with proteins and smaller molecules. There is a broad need to expand biophysical tools with systemic models that will mimic the asymmetric membrane environment [21, 22].

The presence of cholesterol in the cytoplasmic membrane of a living cell was verified through laboratory testing. Erythrocytes were isolated from healthy donors and a small fraction of cholesterol was replaced by the fluorescent analogue dehydroergosterol (DHE) with similar chemical and biophysical properties. The asymmetric distribution of DHE was revealed by energy transfer magnetic resonance imaging (TERM) until its extinction in the outer leaflet. Then the Di4 fluoropore (charged and without risk of escape between the leaflets) was added to the outer leaflet of the erythrocyte. Thus, the induction of Di4 until the quenching of DHE fluorescence provided a relatively broad reading of the outer leaflet in DHE elucidating the fact that approximately 50% of DHE is hidden between the symmetric liposomes. Computational predictions suggested that cholesterol is found in high quantities in the outer leaflet of plasma membranes [22].

The fibroblasts NIH 3T3 were 20% more compacted in the plasma membrane exolasma than in the cytoplasmic leaflet (40.9 +/- 2.31 $^{o}A^{2}$/lipid). For inference, the Di4 fluorophore was experimentally calibrated to simulate lipid packing (area per lipid) using synthetic bilayers spanning a wide range of lipid densities. These two measurements were highly correlated allowing translational analysis of Di4 half-life within the specific structural parameter of the membrane (area/lipid) [22].

XII. LIPID EXTRACTION

Neutral lipids are covalently linked and can be extracted from tissues by nonpolar solvents while polar lipids, which are linked by electrostatic forces and hydrogen bonds, require polar solvents capable of breaking such bonds and releasing them. A mixture of chloroform and methanol followed by the addition of potassium chloride solution aims for better phase separation according to the method developed by Folch and collaborators and which was later developed and modified by Bligh and Dyer who simplified the methodology. The method developed by Bligh and Dyer involves the formation of a two-phase system based on the proportions of solvents added during the extraction process. The formation of this

two-phase system is based on the theory of three-component liquid-liquid equilibrium (chloroform-methanol-water): ternary diagram of the solubility of two liquids partially miscible with each other (chloroform and water) with a third (completely miscible methanol) miscible in chloroform and/or water). The difference between the method of Folch and collaborators and that of Bligh and Dyer is in the proportion of volumes: chloroform and methanol in a proportion of 2 to 1 (2:1 – v/v) according to Folch et al.; chloroform, methanol and water in a ratio of 1 to 2 to 0.8 (1:2:0.8 – v/v) [23].

The flow of water molecules through the lipid bilayer can be calculated using the liquid chromatography technique. This technique for separating mixtures consists of a stationary phase and a solid phase. A bilipid membrane fragment can be mixed with a solution of chloroform, methanol and water (1:2:0.8). This mixture is then added to a silica gel column (stationary phase) that will retain the polar molecules. For the mobile phase, non-polar solvents will be used for elution, such as methanol (to elute charged polar molecules) and acetone (to elute uncharged ones). The more polar lipids will bind more to the silica and will migrate less through the silica column while the less polar molecules will migrate more because they will bind with less intensity. The elution product that will leave the column can be measured spectrophotometrically and thus we will have the lipid concentration of the initial membrane fragment [5, 23].

XIV. PROTEINS: INTEGRAL/ PERIPHERAL

The phospholipid bilayer is crossed by many membrane proteins. Integral proteins are covalently linked to a membrane lipid such as glycosylphosphatidylinositol (GPI). Treatment with the enzyme phospholipase C is capable of releasing GPI from the membrane if you want to study it. Peripheral proteins associate with the membrane through electrostatic interactions and hydrogen bonds with hydrophilic domains of integral proteins and with polar groups of membrane lipids. Changes in pH or ionic strength, the removal of calcium by chelating agents or the addition of urea and/or carbonate can release peripheral membrane proteins (Fig. 4) [5, 21].

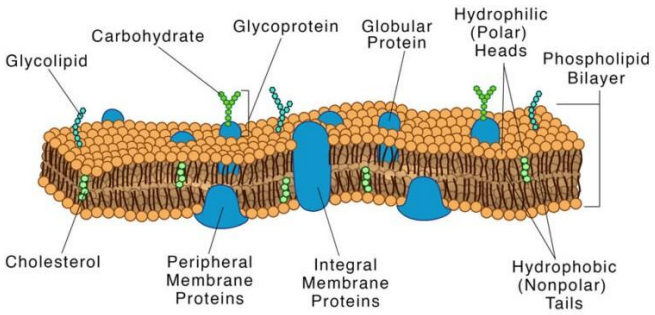

Figure 4: Fluid mosaic model for the structure of the cytoplasmic membrane. (*Sciencefacts.net/fluid-mosaic-model.html)*

The unilamellar vesicles of the lipid bilayer can be prepared to study their composition based on their interactions with a substrate. The generation of unilamellar vesicles has been carried out using 3 methods: application of a differential pH to vesicles containing anionic lipids, addition of enzymes that selectively modify the lipid groups of origin of the outer leaflet and external addition of lipid-carrying molecules that catalyze the intervesicular exchange of external lipid leaflets. The pH adjustment method exploits the fact that certain anionic phospholipids (phosphatidylglycerol and phosphatidic acid) rapidly diffuse between bilayer leaflets in their uncharged and protonated forms. Applying-However, a differential pH across the bilayer induces the redistribution of these lipids, with accumulation on the more alkaline side. The addition of the phospholipase D enzyme can enzymatically modify its original group (head), converting the outer phosphate leaflet to phosphatidylethanolamine using PS-decarboxylase or the outer phosphatidylcholine leaflet [5, 22].

A catalytic exchange of lamellar vesicles was performed with cyclodextrin-methylbeta as lipid carrier, the water-soluble cyclodextrin methyl-beta presenting a ring-shaped oligosaccharide with a hydrophilic outer surface and a central hydrophobic cavity wide enough to accommodate the lipid chain. The reversible formation of a methyl-beta cyclodextrin complex effectively displaced water from the cavity, replacing unfavorable interactions with favorable ones. At high concentrations of cyclodextrin-methyl-beta the lipid suspension of the vesicle dissolved completely, however at low concentrations, a dynamic equilibrium was created between the intact vesicles, the cyclodextrin-methyl-beta complexes and the free cyclodextrin-methyl-beta. A dense sucrose solution (25% wt/v) flooding the vesicle acceptor lumen separated the original acceptor pool (containing the single lamellar vesicles) from the lipid leaflets [22].

XV. CONCLUSION

This review presented the anatomical characteristics of the virus that causes COVID-19, detailing the mechanism of interaction of SARS-CoV-2 with the epithelial cells of the nasal and oropharyngeal mucosa, from the moment of its entry, relating a series of innate receptors responding to their viral antireceptors.

Given the high transmissibility [(approximately 17% of infected people in Brazil (2023 data)] through the dispersion of droplets (droplets of flugge or spittles) in the conjunctivae and/or mucous membranes of the nose and/or mouth, it was noted by the health teams at Hospital São Paulo (Brazil), the need to carry out quick, efficient and preferably low-cost diagnostic methods, taking into account the socio-economic conditions of our country.

This article proposes to use the surface glycoprotein CD14 and its receptor Toll-4 as biomarkers to predict the severity of COVID-19 in hospitalized patients. Moreover it proposes the creation of a simple and low-cost biochemical technique capable of extracting these biomarkers directly from naso- and oropharyngeal secretion samples.

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article.

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