UNIVERSITY OF DE SÃO PAULO

Faculty of Pharmaceutical Sciences

Department of Food and Experimental Nutrition

LADAF – LABORATORY OF FUNCIONAL FOODS DEVELOPMENT

Lígia Prestes Fernandes

Effect of baseline eicosapentaenoic omega 3/omega 6 fatty acids ratio on COVID-19 severity in non-immunized patients

São Paulo

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3º Examinator

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RESUMO

Fernandes, L.P. **Efeito da razão entre ácidos graxos ômega 3 e ômega 6 na severidade da COVID-19 em pacientes não imunizados.** 2023. 82f. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2023.

Desde o início da pandemia de SARS-CoV-2 observou-se uma forte associação entre a produção excessiva de citocinas pró-inflamatórias e a gravidade da COVID-19. Nesse contexto, anti-inflamatórios foram amplamente utilizados visando aliviar os sintomas e melhorar o prognóstico dos pacientes. Diversos estudos apontam que a maior proporção de ácidos graxos ômega 3, em especial, os ácidos eicosapentaenóico (EPA) e docosahexaenóico (DHA), em relação aos ácidos graxos ômega 6, como o ácido araquidônico (AA), contribui para a resolução de processos inflamatórios através de alteração do perfil de oxilipinas, e redução da síntese da prostaglandina E_2 (PGE_2). Partindo dessa premissa, o objetivo deste estudo foi avaliar se a maior relação EPA+DHA/AA no momento da infecção viral, poderia estar associada a menor produção de citocinas pró-inflamatórias via redução de PGE_2 e, em consequência, um melhor prognóstico dos pacientes. Para avaliar essa hipótese, amostras de sangue foram coletadas em 180 pacientes não vacinados e diagnosticados com COVID-19, admitidos entre 5 de julho de 2020 a 17 de setembro de 2020 em hospitais públicos da cidade de São Paulo. Os pacientes foram classificados em categorias de 1 a 5, aumentando progressivamente de acordo com a gravidade da doença durante a internação hospitalar. Os ácidos graxos e oxilipinas foram quantificados por cromatografia acoplada à espectrometria de massas. Os resultados mostraram que o Grupo 1 apresentou uma razão EPA/AA maior que o Grupo 5 ($p = 0.010$), confirmando a hipótese, porém em termos de EPA e não de ômega 3 ($p = 0.276$). Observou-se também que esse resultado foi acompanhado pela maior concentração de citocinas pró-inflamatórias, em especial IL-6 ($p=0.002$) e Proteína C-Reativa ($p<0.001$). Entretanto, esse efeito anti-inflamatório não foi associado com a concentração de PGE_2. Pacientes do Grupo 1 também apresentaram uma menor concentração de F_2-Isoprostanos, um importante biomarcador de estresse oxidativo, comparado ao Grupo 4 ($p=0.009$). Esse resultado parece estar mais associado a um provável efeito da redução da inflamação. Pode-se concluir que a maior razão EPA/AA no momento da infecção viral foi associada com a menor concentração de citocinas inflamatórias e a menor severidade da doença. Entretanto, mais estudos são necessários para identificar o mecanismo desse efeito, ressaltando que a razão EPA/AA é um fator modificável na dieta, e outras infecções que implicam na produção excessiva de citocinas inflamatórias podem ocorrer novamente.

Palavras-chave: COVID-19, eicosapentaenóico, oxilipinas, prostaglandina, inflamação

ABSTRACT

Fernandes, L.P. **Effect of baseline eicosapentaenoic omega3/omega6 fatty acids ratio on COVID-19 severity in non-vaccinated patients.** 2023. 82f. Dissertation (Master) – Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2023.

Since the beginning of the SARS-CoV-2 pandemic, a strong association has been observed between excessive production of pro-inflammatory cytokines and the severity of COVID-19. In this context, anti-inflammatory drugs have been widely used to alleviate symptoms and improve the prognosis of patients. Several studies indicate that a higher proportion of omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), compared to omega-6 fatty acids such as arachidonic acid (AA), contributes to the resolution of inflammatory processes by altering the profile of oxylipins and reducing the synthesis of prostaglandin E_2 (PGE_2). Based on this premise, the objective of this study was to evaluate whether a higher EPA+DHA/AA ratio at the time of viral infection could be associated with lower production of pro-inflammatory cytokines via a reduction in PGE2 and, consequently, a better prognosis for patients.To test this hypothesis, blood samples were collected from 180 unvaccinated patients diagnosed with COVID-19, admitted to public hospitals in the city of São Paulo between July 5, 2020, and September 17, 2020. The patients were classified into categories 1 to 5, progressively increasing according to the severity of the disease during hospitalization. Fatty acids and oxylipins were quantified using chromatography coupled with mass spectrometry. The results showed that Group 1 had a higher EPA/AA ratio than Group 5 ($p = 0.010$), confirming the hypothesis, although in terms of EPA and not omega-3 ($p = 0.276$). It was also observed that this result was accompanied by a higher concentration of pro-inflammatory cytokines, especially IL-6 ($p=0.002$) and C-reactive protein ($p<0.001$). However, this anti-inflammatory effect was not associated with the concentration of PGE2. Patients in Group 1 also had a lower concentration of F2-isoprostanes, an important biomarker of oxidative stress, compared to Group 4 ($p=0.009$). This result appears to be more associated with a probable effect of inflammation reduction. It can be concluded that a higher EPA/AA ratio at the time of viral infection was associated with a lower concentration of inflammatory cytokines and a milder severity of the disease. However, further studies are needed to identify the mechanism of this effect, emphasizing that the EPA/AA ratio is a modifiable factor in the diet, and other infections that involve excessive production of pro-inflammatory cytokines may occur again.

Keywords: COVID-19, eicosapentaenoic acid, oxylipins, prostaglandin, inflammation

ABBREVIATIONS

4-HHE: 4-hydroxyhexenal

4-HNE: 4-hydroxynonenal

AA: arachidonic acid

ACE2: angiotensin-converting enzyme 2

ALA: alpha-linolenic acid

ALI: acute lung injury

APCs: antigen-presenting cells

ARDS: acute respiratory distress syndrome

BHT: Butylhydroxytoluene

BTK inhibitors: Bruton Tyrosine Kinase inhibitors

cAMP: Cyclic adenosine monophosphate

COX: cicloxygenases

cPLA$_2$: cytosolic PLA$_2$

CRP: C-reactive protein

Cytp450: cytochrome P450

DAMPS: damage-associated molecular patterns

DHA: docosahexaenoic acid

EETs: epoxyeicosatrienoic acids

EPA: eicosapentaenoic acid

ER: endoplasmatic reticulum

GM-CSF: as granulocyte-macrophage colony-stimulating factor

GPR120: G protein-coupled receptor 120

HETEs: hydroxyeicosatetraenoic acids

ICAM-1: intercellular adhesion molecule-1

ICU: intensive care units

IKK: inhibitor of nuclear factor-κB kinase

IL-1: interleukin 1

IL-6: interleukin 6

INF-γ: interferon-gamma

iPLA$_2$: Ca2+-independent PLA$_2$

IRAK4: interleukin-1 receptor-associated kinase 4;

IRF: interferon-regulator factor

LOX: lipoxygenases

LPS: lipopolysaccharide

LTs: leukotrienes

MCP-1: monocyte chemoattractant protein 1

MDA: malondialdehyde

Myd88: myeloid differentiation primary response 88 protein

NEMO: NF-kB essential modulator

NF-kB: nuclear factor kappa B

$NLRP_3$: NOD-, LRR- and pyrin domain-containing protein 3

NSAIDs: nonsteroidal anti-inflammatory drugs

OTI: orotracheal intubation

PAF-AH: platelet-activating factor acetylhydrolases

PAMP: pathogen-associated molecular patterns

PCR: polymerase chain reaction

PGE_2: Prostaglandin E_2

PGH_2: Prostaglandin H_2

PI: proteasome inhibitors

PI3K: phosphoinositide 3-kinase

PLA_2: phospholipase A_2

PPARγ: peroxisome proliferator-activated receptor gamma

PUFAs: polyunsaturated fatty acids

RIP: receptor-interacting protein

ROS: reactive species

RXR: retinoid receptor

$sPLA_2$: secretory PLA_2s

SPMs: specialized pro-resolving mediators

SRAA: renin-angiotensin-aldosterone system

ssRNA: single-stranded RNA

TBA: 2 thiobarbituric acid

TEP: 1,1,3,3 Tetraethoxypropane

TLR: toll like reception

TMPRSS2: transmembrane serine protease 2

TNF-α: tumor necrosis factor-α

TRADD: tumor necrosis factor receptor type-1 associated DEATH domain protein

TRAF: TNF receptor-associated factor

VCAM- 1: vascular cell adhesion molecule-1

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SUMMARY

1.INTRODUTION

On March 11th, 2020, the World Health Organization (WHO) classified severe acute respiratory syndrome caused by the new coronavirus (SARS-CoV-2) as pandemic disease. At this point, the world had already confirmed 35 millions of cases and reached 1 million of deaths, just a few months after its beginning in the city of Wuhan - central China - on December 12th, 2019.

Since the beginning of the SARS-Cov-2 infection on December 2020, the knowledge about how the virus is transmitted and infects human cells allowed to rapidly develop vaccines and drugs, abruptly reducing the mortality rate[1,2]. However, the clinical response of vaccinated or not vaccinated patients infected with SARS-CoV-2 virus differs among the individuals due to factors that are still being investigated[3]. Unfortunately, Sars-Cov-2 is not the last threat for human health, and to understand how some factors present in the individuals, before the virus infiltration, could be associated to the severity of the disease, is essential to better protection for the next infections.

During these last years it was observed that while some patients present only mild symptoms or are even asymptomatic, others need to be treated in the intensive care units (ICU) and survive with different types of limitations, that have been known as “long COVID”, characterized by multiple symptoms for weeks or months after initial SARS-CoV-2 infection, even showing negative polymerase chain reaction (PCR) [4,5]. Among the factors involved in the critical diseases progression, the high concentration of pro-inflammatory cytokines has been associated to the incapability of the immune system to prevent the spreading of viruses to the lungs, in a condition known as “cytokine storm” [6,7]. This fact explains the relative success of dexamethasone prescription for hospitalized patients, when little was known about how to deal with the disease[8]. Based on this experience, it was concluded that excessive inflammation promoted damage to the cells, apoptosis and loss of tissues integrity, leading to more severe cases and death[9,10].

The viral single-stranded RNA (ssRNA) in the cytoplasm or even the activation of different receptors on cell membrane induces the migration of the nuclear factor kappa B (NF-kB) to the nucleus, where it will promote the expression of genes that encode to pro-inflammatory molecules, such as

interleukin 1 (IL-1), interleukin (IL-6), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor-α (*TNF-α),* monocyte chemoattractant protein 1 *(*MCP-1*),* interferon-gamma (INF-γ) and others[11]. These molecules attract immune cells to the site of the infection, as part of the normal defense, amplifying the inflammatory condition. This process causes stress in the endoplasmatic reticulum, that in turn, activates hydrolases, such as phospholipase A_2 (PLA_2) among others, to remove the fatty acids that are esterifying the phospholipids in the membrane. These fatty acids are now substrate to oxidative reactions, that can occur directly by reactive species (ROS) and also catalyzed by enzymes, including cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 (Cytp450), that can themselves be activated by cytokines, such as TNFα, resulting in several types of oxylipins[12,13]. Depending on the fatty acid precursor and oxidative pathway, these oxylipins can induce pro-inflammatory or anti-inflammatory responses. For example, influenza A virus activates the synthesis of Prostaglandin E_2 (PGE_2) formed from arachidonic acid (AA) oxidation, that binds to E2/E4 receptors, migrates to the cell surface and actives cyclic adenosine monophosphate (cAMP), phosphoinositide 3-kinase (PI3K) and β-arrestin, decreasing INF-γ expression[14]. This INF-γ decrease is associated to a higher viral replication in the host cells[14,15]. Other oxylipins synthesized from AA are known to increase the inflammatory response by promoting vasoconstriction and platelet aggregation, leading to the increase of temperature, pain and edema[16].

In general, it has been reported that compounds formed from enzymatic and non-enzymatic oxidation of omega 6 fatty acids (n-6 FA), such as AA, would be more inflammatory than those formed from omega 3 fatty acids (n-3 FA), as for example eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [17]. In addition, n-3 FA could also exert an anti-inflammatory activity by other mechanisms, such as inhibiting inflammasome pathway, binding to the peroxisome proliferator-activated receptor gamma (PPARγ) hindering the migration of NF-kB to the nucleus, and also promoting the inflammation resolution, being substrate to the synthesis of specialized pro-resolving mediators (SPMs) [18,19]. On the other side, EPA and DHA are more unsaturated than n-6 FA, being much more susceptible to oxidation. Several studies have shown that some secondary products of polyunsaturated fatty acids oxidation, such as

malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), 4-hydroxyhexenal (4-HHE) and isoprostanes, that can be potentially cytotoxic and their concentration have been positively correlated to acute and chronic inflammatory conditions[20].

Therefore, it can be suggested that the proportion between omega 3/omega 6 fatty acids (n-3/n-6 FA) ratio at the moment of virus infection, could influence the pro-inflammatory cytokine production, and in consequence, the prognostic of the patient during his hospitalization, highlighting that the n-3/n-6 FA ratio is a changeable factor that depends on the diet or supplementation. Despite the importance of this information, only few studies have been published towards n-3 FA and SARS-Cov-2 infection, probably due to the high risk involved in this type of investigation before the vaccines. In this sense, our objective was to investigate if baseline n-3/n-6 FA ratio would be associated to inflammation and oxidative stress of non-vaccinated patients with COVID-19, classified according to the severity of the disease during the hospital internment.

2.REVIEW OF LITERATURE

2.1 SARS-CoV-2 infection

The COVID-19 pandemic, triggered by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has exerted a substantial worldwide influence on public health, economies, and societal welfare. Nearly 7 million fatalities have been recorded, with over 700 million individuals having contracted the virus. Among the infected population, around 80% presented mild to moderate clinical manifestations, and the advance from more severe symptoms to a critical condition of illness happened to about 5% of them[21]. Evidence supports the causality of the immunological phenomena known as “cytokine storm” in acute respiratory distress syndrome (ARDS), a condition also presents in patients with severe COVID-19[6,22]. The excessive release of cytokines triggers an uncontrolled immune response, leading to lung tissue damage and, consequently, organ failure[23]. Therefore, suppressing this extreme cytokine production is a crucial aspect in reducing mortality in patients. At the onset of the pandemic, prior to the development and approval of vaccines[24], immediate treatment strategies were primarily focused on drugs aimed to combating viral

replication, blocking viral entry, administering anti-inflammatory steroids, and selective cytokine blockers[25,26].

The mechanism of SARS-CoV-2 entry into host cells is mediated by the binding of the spike (S) protein to its receptor, angiotensin-converting enzyme 2 (ACE2), and subsequent membrane fusion. Facilitated by the transmembrane serine protease 2 (TMPRSS2) protein, the viral spike protein (S) on the surface of SARS-CoV-2 binds to ACE2 and initiates the endocytosis process of the virus within the host's respiratory epithelial cell[3,27]. Viral RNA released from the endosome is replicated as partial and complete copies of the genome and translated in the endoplasmatic reticulum (ER) to form new SARS-CoV-2 viruses. Within the endosome, double-stranded RNA and ssRNA activate toll-like receptors (TLR 3 and TLR7/8) [3,28]. This process triggers a series of events involving the release of NF-kB, which migrates to the nucleus and initiates the transcription of genes encoding cytokines, chemokines, adhesion molecules, and growth factors[28]. These proteins attract immune cells to the site of infection, promoting a pro-inflammatory feedback loop[3,28,29] (**Figure 1**)

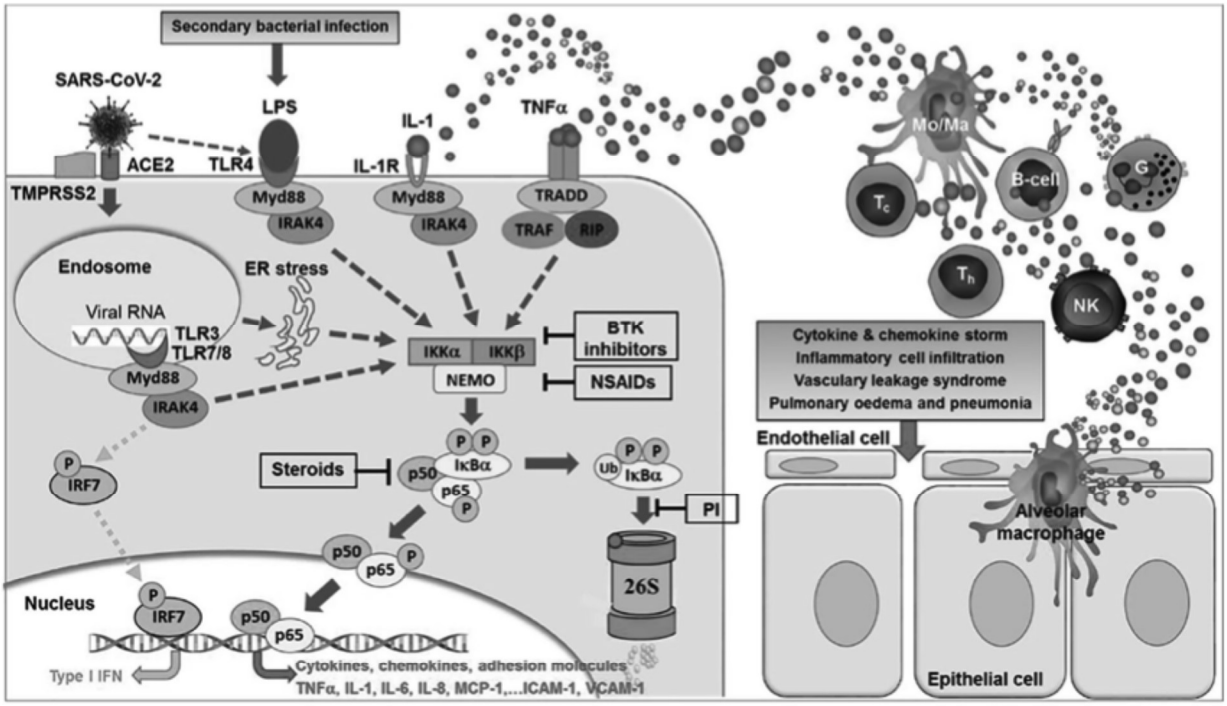

Figure 1: Representation of the entrance of the SARS-CoV-2 virus binding in ACE2, its replication until reaching the activation of inflammation and promoting an inflammatory loop. Adapted from Kircheis *et al.* (2020)[28]

The viral replication and subsequent release induce pyroptosis in the host cell, leading to the liberation of nucleic acids[30], damage-associated molecular patterns (DAMPs), and viral pathogen-associated molecular patterns (PAMPs). DAMPs e PAMPs are recognized by alveolar macrophages, which produce more pro-inflammatory cytokines and chemokines. PAMPs and DAMPs attract monocytes, which subsequently differentiate into macrophages, as well as T cells[3], thereby intensifying the inflammatory response.

Particularly, SARS-CoV-2 has the ability to rapidly activate Th1 cells, leading to the secretion of inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), which activates inflammatory monocytes CD14+ and CD16+ to produce higher quantities of IL-6 and TNF-α [31]. Additionally, it should be noted that a minimal level of type I interferon (IFN) has been observed in the bloodstream of individuals infected with SARS-CoV-2, which is in contrast to the findings in patients with influenza virus[29]. Type I IFN plays a crucial role in the immediate antiviral response, being activated through autocrine signaling of the IFN type I receptor[32]. Several studies have also observed the presence of anti-type I interferon antibodies in COVID-19 patients, and this fact has been associated with disease severity and mortality [33,34]. Collectively, uncontrolled immune responses result in poor resolution of inflammation, thereby contributing to the role of “cytokine storm” in aggravation of the disease outcome.

2.2 Relationship between fatty acids, oxylipins and inflammation

Inflammation is a natural process in the body's defense against pathogens[35]. Throughout its course, significant changes occur in the individual's metabolism, including immune responses that engage the production of a large number of chemical mediators[36,37]. At the onset of inflammation, increased blood flow to the affected area and increased vascular wall permeability result in the delivery of chemotactic substances to the affected site, attracting leukocytes that release specific chemical mediators to control and resolve the inflammation[36,37]. Among these mediators, prostaglandins and leukotrienes may be present, which

are derived from arachidonic acid (AA), an omega-6 fatty acid[17]. This fact suggests that the intake of fatty acids can play a positive or negative role in the inflammatory process.

The phospholipid membrane consists of various components, including polyunsaturated fatty acids. For this reason, the composition of fatty acids strongly influences cellular responses during an ongoing inflammatory condition[17]. Most cells contain relatively large amount of AA in their cell membranes, compared to other polyunsaturated fatty acids[17,38]. AA can be released from the cell membrane by phospholipase A_2 (**Figure 2**) to be metabolized into various pro-inflammatory oxylipins by the action of enzymes such as lipoxygenase (LOX), cyclooxygenase (COX), and P-450 epoxygenase[16].

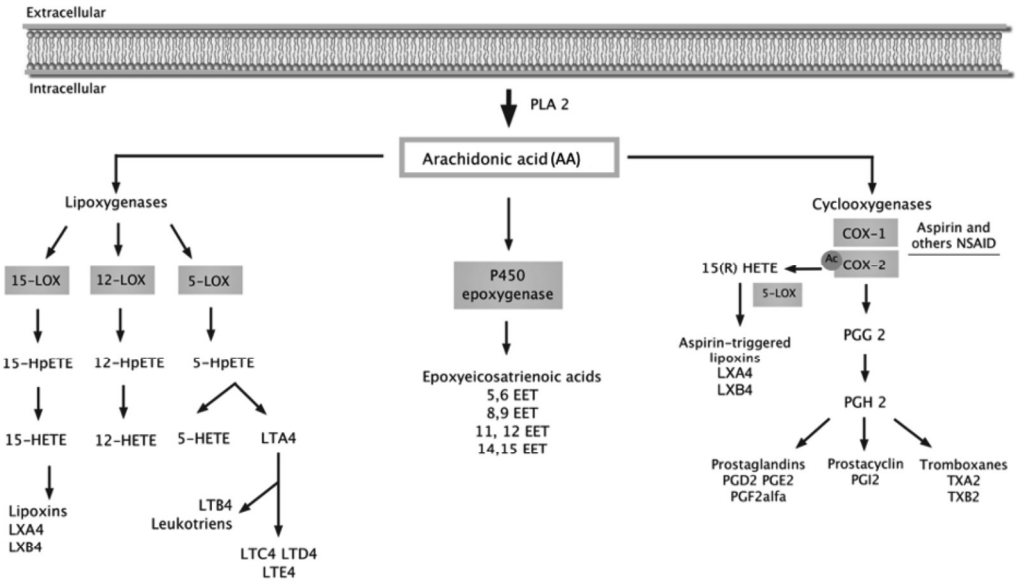

Figure 2: Eicosanoids biosynthesis from AA. Upon exposure to a pathogenic stimulus, phospholipase A_2 (PLA_2) hydrolyzes arachidonic acid (AA) from the cell membrane. AA is then metabolized through one of three pathways: P-450 epoxygenase, lipoxygenase (LOX), or cyclooxygenase (COX), which generates prostanoids such as prostaglandins, thromboxanes, and prostacyclin. Ingestion of aspirin leads to the acetylation of COX-2. Lipoxins are formed from 15-HEPTE by the action of either 5-LOX or 12-LOX. Adapted from Dennis & Norris[16] (2015) and Harizi[39] *et al.* (2008)

For example, COX catalyzes the conversion of AA into prostaglandin G2, which is further converted into prostaglandin H_2, a substrate for the production of other prostaglandins (prostaglandin D_2, prostaglandin E_2, and prostaglandin $F_{2\alpha}$), thromboxanes (thromboxane A_2 and thromboxane B_2), and prostacyclin[12]. Each

molecule attach on specific receptors to mediate and regulate inflammatory processes[40,41]. PGE_2, for example, leads to pro-inflammatory effects such as fever, increased vasodilation, vascular permeability, increased pain, among others[42]. Moreover, PGE_2 induces the production of the pro-inflammatory cytokine IL-6[42]. The role of PGE_2 extends from the innate to adaptive immune system, mediating the interaction between antigen-presenting cells (APCs) and T lymphocytes[43,44]. Another well-known function is its contribution to the influx of neutrophils, macrophages, and mast cells[43]. It is worth to highlight that the synthesis of PGE_2 occurs in macrophages, dendritic cells, fibroblasts, and endothelial cells[17]. PGE_2 is considered a potent pro-inflammatory mediator and an important pharmacological target in inflammation, but it also can inhibit other inflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha)[25, 45].

EPA and DHA compete with AA for the same metabolic enzymes, and being preferred substrates, lead to the production of less pro-inflammatory or anti-inflammatory metabolites[42]. Therefore, increasing the intake of α-linolenic acid (ALA) and, consequently content of EPA and DHA in cell membranes, would contribute to reduce inflammatory responses[46].

2.3 Omega 3 fatty acids and resolution of inflammatory cycle

The previously described inflammatory process is orchestrated to be detrimental to the pathogen without causing harm to the host. Inflammation is typically self-limiting and resolves itself, often rapidly. This is attributed to several regulatory processes involving the secretion of anti-inflammatory cytokines and pro-resolution lipid mediators that inhibit pro-inflammatory signaling, as well as the activation of regulatory cells that decrease the activity of pro-inflammatory cells. [17] Loss of these regulatory processes could result in excessive, inappropriate or ongoing inflammation that can cause irreparable damage to host tissues[45]. When this self-regulatory process fails (**Figure 3**), the risk of tissue damage and chronic inflammation increases, such as in rheumatoid arthritis, inflammatory bowel diseases, and, in the case of COVID-19 patients, compromised lung tissue leading to death[17,31].

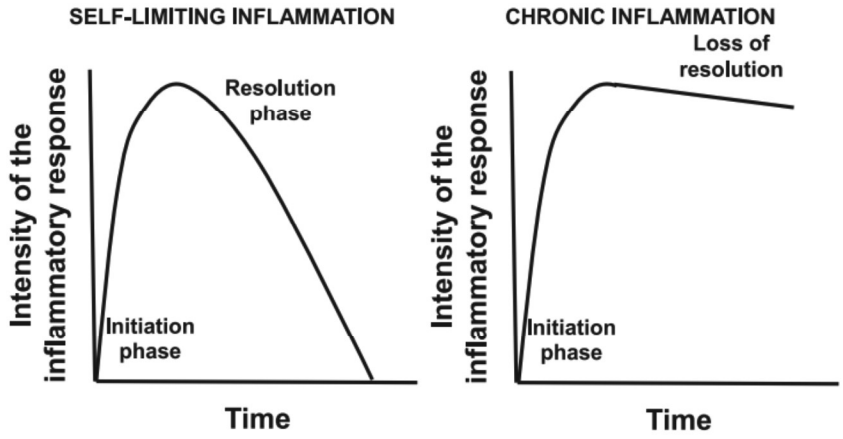

Figure 3: Graphic representation of the course of controlled inflammation and failed resolution, depicting the vectors of inflammation intensity versus time. Adapted from Innes and Calder (2018)[17]

Resolvins, protectins, and maresins metabolized through their conversion from EPA and DHA are examples of pro-resolution lipid mediators (SPMs) (**Figure 4**), and their effects are well described in the literature[17,47,48]. This conversion occurs via the enzymatic reaction of lipoxygenase in the presence of an ongoing inflammatory state[49].

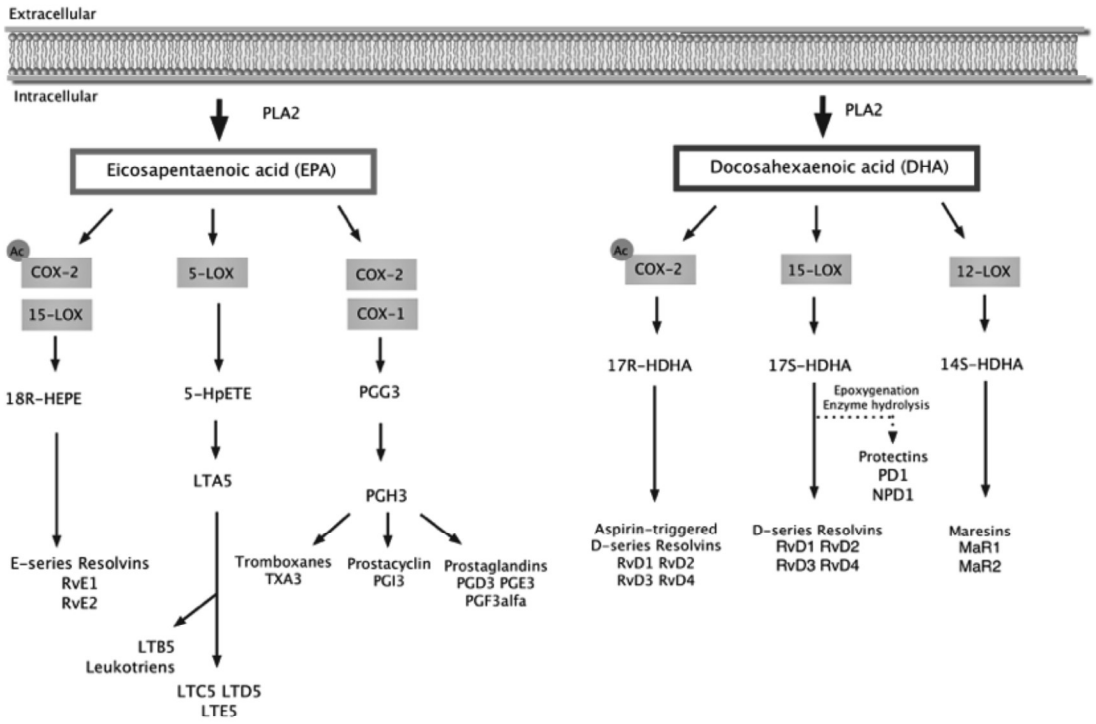

Figure 4: Eicosanoids biosynthesis from eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. Specialized pro-resolution mediators (SPMs) are produced by LOX and COX enzymes. Adapted from Basil & Levy (2015)[41] and Serhan (2020)[7]

Due to these effects, lipid mediators have attracted significant attention as potential pharmacological targets for the treatment of chronic inflammation. Studies with EPA and DHA have shown that their incorporation into cellular membranes is dose-dependent, and these fatty acids can replace AA in the phospholipid bilayer[50], through competitive metabolism. In presence of EPA and DHA in the membrane, there is a preference for the metabolism of lipid mediators derived from EPA and DHA over the metabolism of pro-inflammatory eicosanoids derived from AA[17]. It has been demonstrated that after 1 to 4 weeks of EPA and DHA supplementation, an increase in circulating n-3 FA levels can be observed[51]. However, in mononuclear cells, these changes were only observed after 6-12 mounths[51,52]. In the same study, but involving young individuals supplemented with fish oil, an elevation of EPA and DHA levels in mitochondrial membranes and muscles was detected after 12 weeks[51].

EPA and DHA can attenuate the activation of the NF-kB transcription factor pathway induced by various agonists such as IL-1, IL-2, and TNF-α, as well as modulating the inflammatory response by binding to the G protein-coupled receptor 120 (GPR120) [53]. Activation of GPR120 by EPA or DHA (**Figure 5**) occurs through the binding of β-arrestin 2, forming a complex that prevents phosphorylation of the protein IKK, thereby inhibiting the NF-kB inflammatory pathway[53,54].

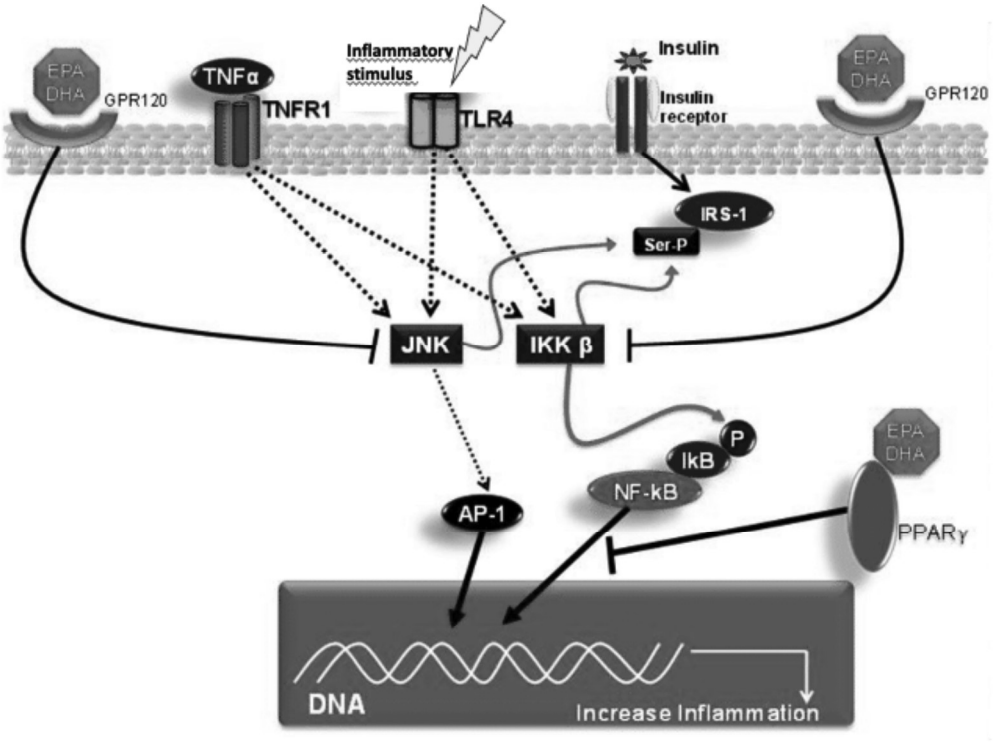

Figure 5: The mechanisms of GPR120 when associated with polyunsaturated fatty acids and its anti-inflammatory functions[54]. By binding to the GPR120 receptor on the cell membrane, EPA and DHA weaken the IKK cascade, which in turn reduces the activity of the NF-kB and AP-1 signaling pathways, leading to a decrease in the expression of pro-inflammatory genes, such as TNF-α and IL-6. Additionally, EPA and DHA act on PPARγ, which reduces the activity of the NF-kB pathway. Adapted from Rogero and Calder (2018)[54]

It is also important to note that EPA and DHA have the ability to bind to peroxisome proliferator-activated receptors (PPARs), a group of nuclear receptors that form heterodimers with the retinoid X receptor (RXR) and bind to the target gene region responsible for promoting lipid metabolism and inflammatory responses[55,56]. When activated, PPAR-alpha and PPAR-gamma isoforms reduce the expression of pro-inflammatory genes, thereby inhibiting NF-kB activation[57].

Clinically severe patients infected with SARS-CoV-2 often present intense inflammatory responses and an ongoing "cytokine storm", as a result of eicosanoid mediator production from lipids, jointly with extreme oxidative stress[58]. Recent studies involving EPA and DHA supplementation of patients that present chronic inflammatory conditions (e.g., arthritis, diabetes, obesity, and coronary diseases) have shown that EPA and DHA can modulate the resolution of

inflammatory responses[45]. Studies involving the supplementation of EPA and DHA in the inflammation of critically ill and chronic patients have been repeatedly conducted. Morita *et al*. [59] investigated the effects of protectin D1 on the course of H5N1 influenza virus infection in mice. The authors found that protectin D1 suppressed the replication of the influenza virus, and its administration improved the animal survival. Another study demonstrated that specialized pro-resolving mediators (SPMs), particularly resolvins derived from EPA (E-series) and DHA (D-series), can inhibit neutrophils, suppress lymphocyte proliferation and natural killer cell activity, and inhibit the production of TNF-α, IL-1, IL-6, IL-2, and interferon[60].

The efficacy of EPA and DHA supplementation in patients with acute respiratory distress syndrome (ARDS) has also been demonstrated. A meta-analysis showed that the intake of these compounds can reduce the duration of mechanical ventilation and ICU stay in critically ill patients with ARDS, due to improved blood oxygenation[61].

Bistrian *et al*. (2020) suggested that seriously ill patients with SARS-CoV-2 could benefit from increased fish oil supplementation in parenteral nutrition, indicating that these patients would respond more rapidly to the anti-inflammatory effects of lipid mediators such as resolvins, maresins, and protectins[62]. Similarly, the combination of fish oil in parenteral diet, along with low-dose aspirin, has been shown to activate E-series resolvins (EPA) and D-series resolvins (DHA) [63], suggesting that n-3 FA supplementation could be a strategy to treat hospitalized patients.

2.4 Relation between PUFAs and oxidative stress

It is important to highlight that PUFA can suffer non-enzymatic oxidative stress, especially when the reactive oxygen species (ROS)[20] are present in a higher concentration. Non-enzymatic lipid oxidation of PUFA in the membrane forms important metabolites such as the as isoprostanes (IsoPs) and neuroprostanes (NeuroPs)[34,64]. These bioactive lipid mediators are active in inflammatory modulation and infection. Of all these compounds, the F_2-IsoPs series 5 and 15 are formed from AA oxidation by ROS[65]. These molecules have been reported to be abundant *in vivo* and are recognized as oxidative stress

biomarkers[66]. Among all of these compounds, the 5- and 15-series of F_2-IsoPs formed through ROS mediated oxidation of AA, F_3-IsoPs from EPA and 4- and 20- series NeuroPs from DHA, have been reported as the most abundant *in vivo*[67,68]. F_2-IsoPs have also been recognized as biomarker of oxidative stress, because it has been associated with several chronic inflammatory diseases[69,70].

The hypoxia caused by pneumonia decreases the energy supply from cell metabolism, activating the anaerobic pathway for energy production, leading to acidosis and the increase of ROS. This oxidative stress, which also restrains the immune system and depletes the antioxidant system, leads to severe tissue damage[71]. Therefore, considering the "cytokine storm" during SARS-CoV-2 infection and the increased production of reactive oxygen species (ROS), there is a concern regarding whether replacing AA with EPA+DHA in the lipid membrane would be beneficial. A study conducted by Mori *et al.* (1999) examined diabetic type 2 patients who included fish meals in their diet for two months and found a 20% reduction in urinary F_2-isoprostanes levels[72]. Similar results were observed in a study involving hospitalized elderly patients with COVID-19 who received intravenous infusions of approximately 6.0 g of EPA and DHA for five days, resulting in a decrease in 15-F_{2r}-IsoPs levels[73]. Thus, it is plausible that n-3 FA has the potential to reduce oxidative stress[66,73].

2.5 PUFAs intake and COVID-19 mortality

The intake of EPA and DHA has shown beneficial effects not only on cardiovascular health but also on other conditions such as inflammatory disorders, among others diseases[74]. While humans have the ability to synthesize EPA and DHA from dietary ALA, this conversion (**Figure 6)** tends to be low, with metabolic variation between individuals[74,75]. Therefore, the intake of PUFAs, whether through dietary sources or supplementation, becomes essential[74].

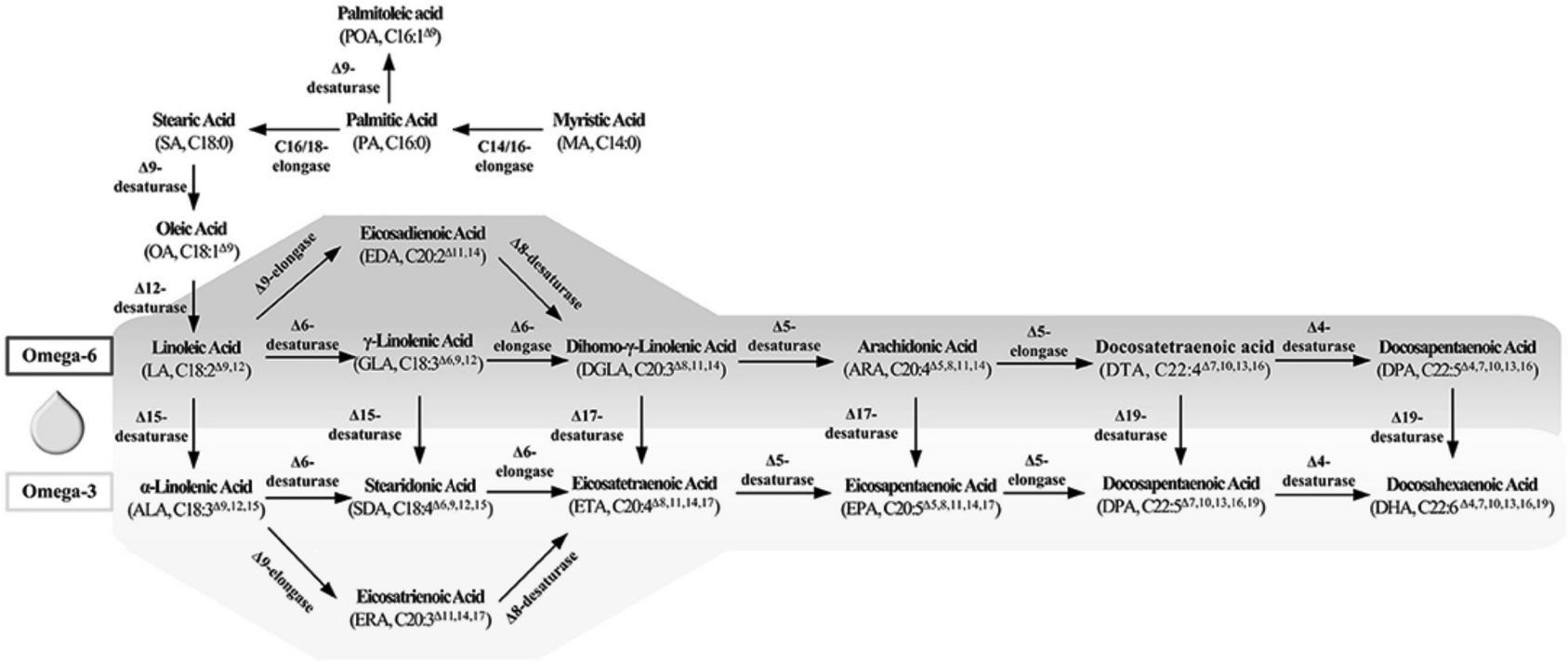

Figure 6: Schematic steps of desaturation and elongation of PUFA. The desaturation and elongation pathway of n-3 and n-6 FA involves enzymatic processes mediated by Δ6 and Δ5 desaturases, which are encoded by the FADS2 and FADS1 genes, respectively. This pathway encompasses the conversion of various fatty acid substrates. Linoleic acid (LA) serves as the precursor for the synthesis of dihomo-gamma-linolenic acid (DGLA), which can further undergo desaturation to produce AA. On the other hand, ALA is converted to EPA through successive desaturation and elongation steps. EPA can then be further elongated to form docosapentaenoic acid (DPA) and eventually undergoes further desaturation to generate DHA. Adapted from Zhuang *et. al* (2022) [76]

According to the 2010 Global Burden of Metabolic Risk Factors of Chronic Diseases (GBD) [77] study conducted by the Nutrition and Chronic Diseases Expert Group (NutriCoDE), the global average intake of n-3 FA was 163 mg/day, while the recommended minimum daily intake is 200-500 mg/day[78,79]. Interestingly, countries with high consumption of EPA and DHA exhibit a lower population risk for chronic inflammatory diseases[80].

Thus, the dietary habits of specific populations can serve as predictors of health status or disease susceptibility[81]. Asher *et al.* (2021) conducted a study revealing an association between n-3 FA levels and patient mortality. The results indicated that individuals with higher levels of n-3 FA had a decreased likelihood of death compared to those with lower levels ($p = 0.071$). Specifically, the study found that patients with an n-3 FA index exceeding 5.7% experienced a remarkable 75% reduction in the risk of mortality[82]. Another intriguing ecological study compared different regions of the world based on their n-3 FA consumption and the mortality rate of COVID-19 patients[80]. The results, demonstrate that countries within the Eastern Mediterranean region have the lowest omega-3 intake (45.14 mg/day) and experienced the highest mortality rates during the pandemic. Conversely, the Southeast Asian region, with an intake of 634 mg/day, exhibited the best outcomes for COVID-19 patients.

Zapata *et al*. (2021) demonstrated that patients with mild and severe COVID-19 who had a low n-3 FA profile were three times more likely to die compared to patients in other quartiles[83]. Similarly, another study conducted with severely ill hospitalized elderly patients infected with SARS-CoV-2 found that individuals with higher levels of omega-3 had a lower likelihood of mortality[84].

3. HYPOTHESIS

Non-vaccinated patients infected with SARS-CoV-2 who presented higher baseline EPA/AA acids ratio had a lower concentration of inflammatory cytokines due a lower concentration of the oxylipin PGE_2, and consequently, they had a better prognostic during the hospital internment.

4.OBJECTIVES

The objective of this study was to evaluate the association between baseline omega 3/omega 6 fatty acids ratio in non-vaccinated patients infected with SARS-CoV-2 virus and the severity of the disease during their hospital internment.

4.1 Specific objectives

(1) Standard the fatty acids method using GC/MS to a reduce volume of plasma (50 µL);
(2) Identify difference in the baseline n-3/n-6 FA ratio among the 180 patients;
(3) Determine the oxylipns, cytokines and oxidative stress biomarkers in the blood samples;
(4) Identify difference in the evaluated biomarkers among the patients classified according to the disease severity;
(5) Apply multivariate statistical approach to build an algorithm able to predict the disease severity according to a set of variables.

5. METHODOLOGY

5.1. Malonaldehyde (MDA) concentration determined for smaller plasma sample

Malondialdehyde (MDA) is a product of lipid peroxidation that occurs as a result of the oxidative degradation of polyunsaturated fatty acids (**Figure 7**), catalyzed by physiological enzymes or through non-enzymatic reactions (under pathological conditions) [85]. Lipids are susceptible to oxidation by reactive oxygen species (ROS), particularly in disease conditions, and MDA is considered one of the major biomarkers of oxidative stress.

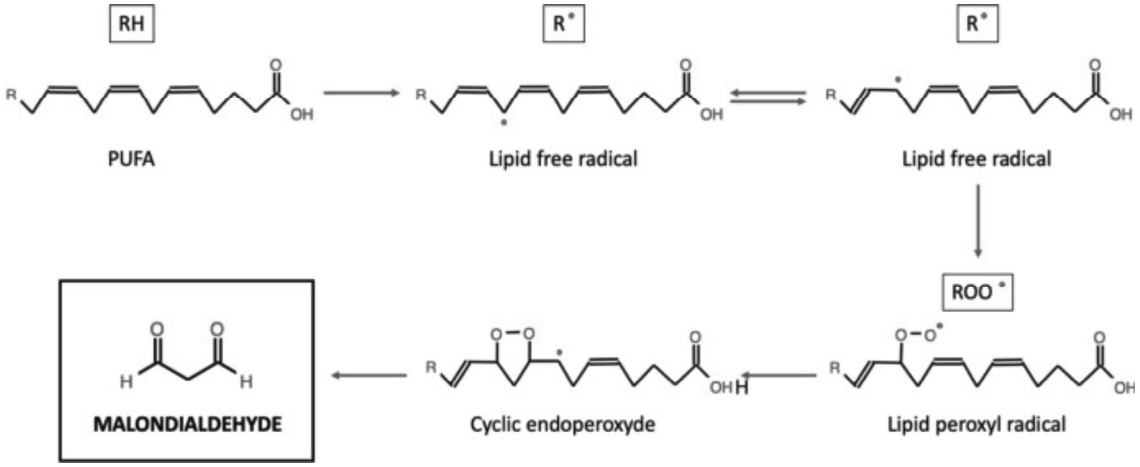

Figure 7: Steps to the formation of MDA through lipid oxidation. Adapted from Mas-Bargues *et al.* (2021)[85]

Overall, the formation of MDA through lipid peroxidation represents a key process in the assessment of oxidative stress, and its impact on cellular health and disease.

Methodology

Malonaldehyde (MDA) concentration was determined by reverse phase HPLC[86]. In clinical studies, it is frequently observed as one of the most commonly reported indicators of lipid peroxidation. A commonly employed assay method involves the reaction between thiobarbituric acid (TBA) and MDA, which produces a pink chromogen known as the MDA-TBA adduct. A standard curve was prepared using 1,1,3,3 Tetraethoxypropane(TEP): 20 to 100 µM MDA.

Chemical and reagents

TEP, sodium hydroxide, Trichloroacetic acid (TCA), 2 Thiobarbituric acid (TBA), Butylhydroxytoluene (BHT) and iodate potassium were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, United States).

Sample preparation

About 0.05 mL of human plasma was mixed with 12.5 µL of a 0.2% solution of butylated hydroxytoluene (BHT) and 6.25 µL of 10 N sodium hydroxide (NaOH). The

10 N NaOH solution was prepared by dissolving 20 g of sodium hydroxide in 50 mL of MilliQ water. Additionally, 0.2 g of BHT was diluted in 100 mL of ethanol to prepare the solution used in the sample preparation. The resulting mixture was subjected to heating in a water bath at 60°C for a duration of 30 minutes. Subsequently, 750 µL of a solution prepared by dissolving 36 g of trichloroacetic acid (TCA) in 500 mL of MilliQ water was added. The prepared mixture was then centrifuged at 12,000 rpm for 10 minutes to separate the precipitate. After centrifugation, 500 µL of the supernatant was combined with 250 µL of a 0.6% solution of thiobarbituric acid (TBA) in water and subjected to vortexing. The samples were once again heated in a water bath, this time at 95 °C, for 30 minutes. Following the heating step, 750 µL of butanol was added to each sample and stirred for 15 seconds. The samples were then subjected to centrifugation at 12,000 rpm for 10 minutes.Subsequently, 650 µL of the resulting supernatant was filtered and transferred to a vial for subsequent analysis using high-performance liquid chromatography (HPLC).

High-performance Liquid Chromatographic analysis and instrumental conditions

The TBA–MDA conjugate derivative was injected in a Phenomenex reverse-phase C18 analytical column (250 mm × 4.6 mm; 5 mm; Phenomenex) with an LC8-D8 pre-column (Phenomenex AJ0-1287) coupled to a HPLC (Agilent Technologies 1200 Series). Samples were quantified by fluorometry at an excitation of 515 nm and emission of 553 nm. The HPLC pump delivered the isocratic mobile phase: 40% PBS (A) (10 mmol, pH 7.1) and 60% methanol (B) at a flow rate of 1 mL/min. The gradient applied was: 0 – 4 min, 40% of solvent A and 60% of solvent B; 4 – 6 min, 45% of solvent A and 55% of solvent B, and then, the initial conditions were reestablished after 5 min.

Method Validation

Linearity can be observed by the calibration curve (**Figure 8**), showing a determination coefficient R= 0.9922 and a correlation coefficient r^2= 0.9845.

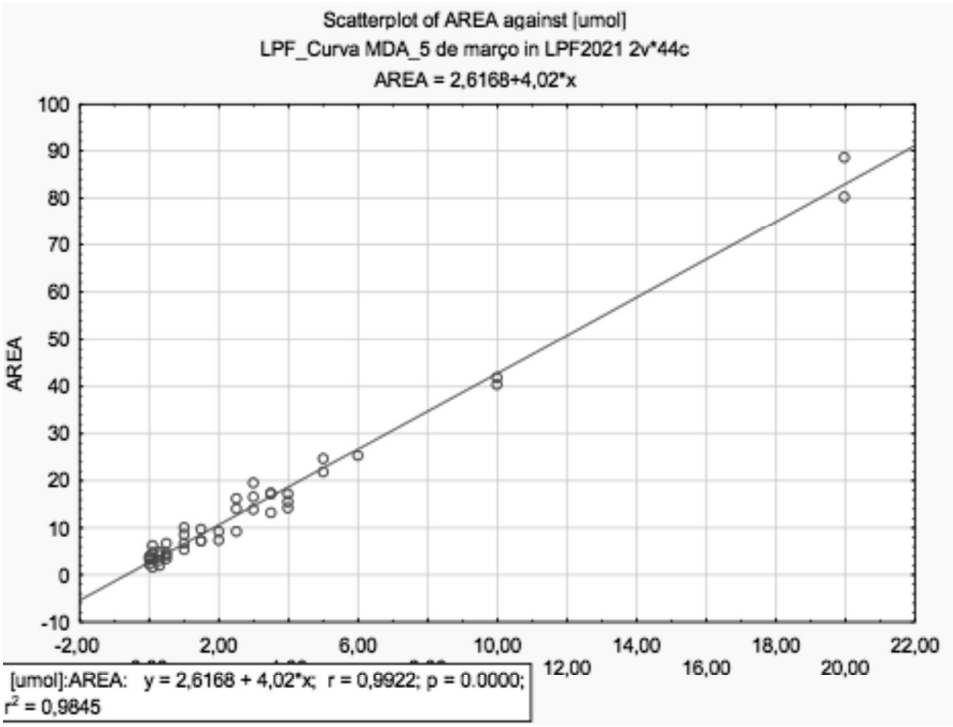

Figure 8: Curve of calibration using TEP for malondialdehyde (MDA)

The LOQ was expressed as the mass of analyte which gives a signal 10 times above the mean blank signal, characterized as the standard deviation of the blank signal), while the LOD was expressed as the mass of analyte which gives a signal tree times above the mean blank signal. LOD values obtained to 0.1961 µMol and LOQ ranged to 0.6538 µMol.

Figure 9 and **10** presents an example of chromatogram obtained from a patient sample and from the TEP at concentration of 7.78µMol/L and 19.97µMol/L, respectively.

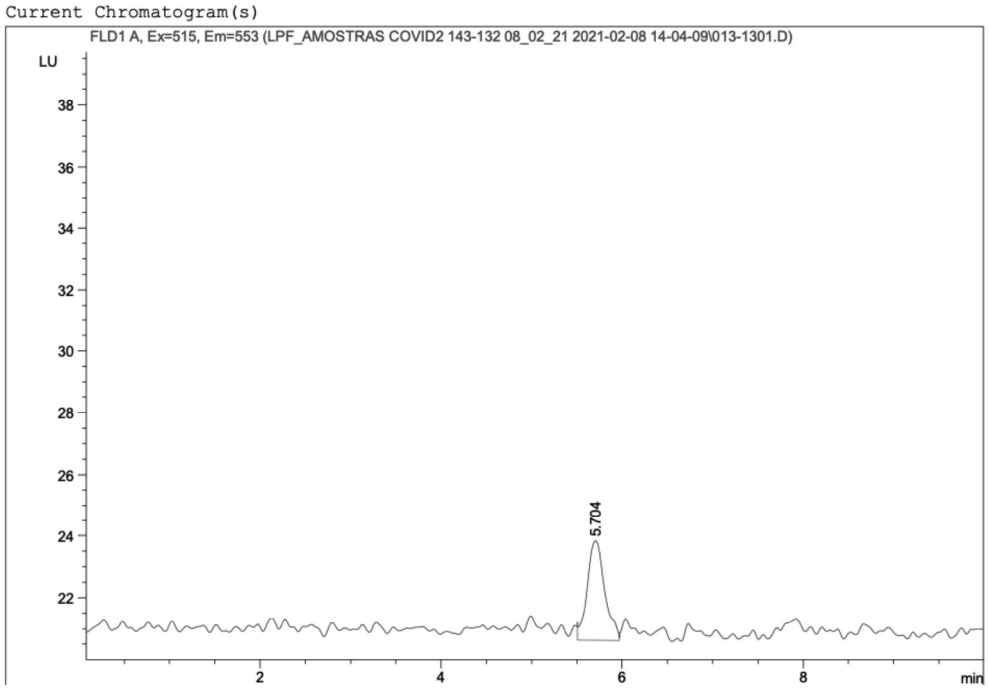

Figure 9: Example of the chromatogram obtained from the patient analysis **(**Eg: Patient n.135**)**

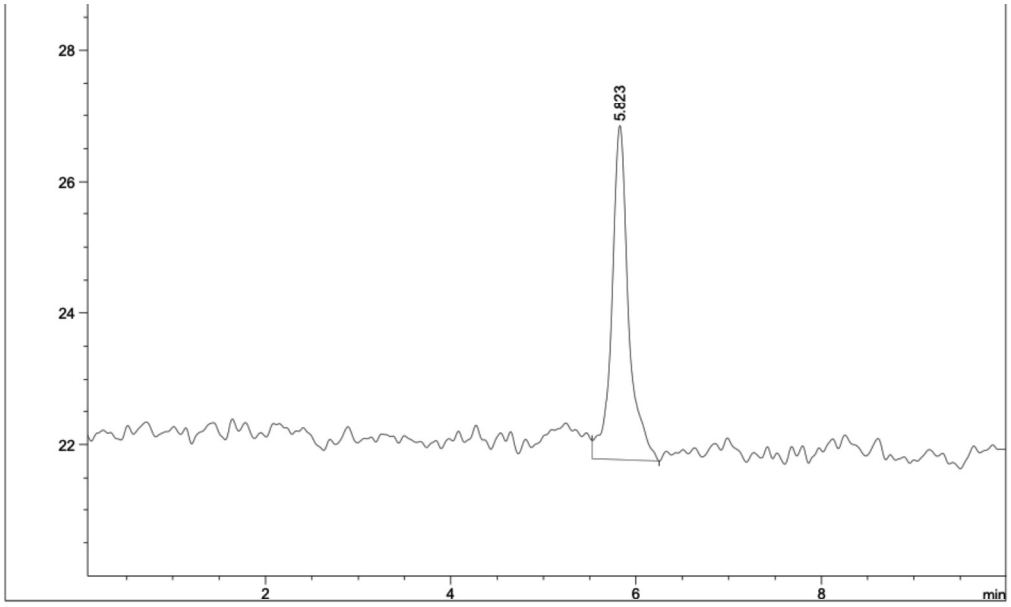

Figure 10: Example of the chromatogram obtained to the standard 1,1,3,3 Tetraethoxypropane (TEP) analysis**.**

5.2. Fatty acids concentration determined for smaller plasma sample

Fatty acids have several metabolic functions in humans and exist integrated into more complex lipids or in free forms. It constitutes essential structural elements of biological membranes and have important role as signaling molecules. Free fatty acids have important functions as potent signaling molecules taking part in physiological process[87]. Therefore, it becomes relevant to determine their amount in human plasma. Gas chromatography (GC) has become widely used as a reliable tool for the quantitative analysis of fatty acids[88]. The method is used since 1950 when it was discovered that gas chromatography could separate short-chain fatty acids[89]. GC coupled mass spectrometry (GC/MS) has become a routine procedure with broad application to biochemical and biomedical research[88].

Methodology

The method for determination of esterified fatty acids was adapted to use a smaller amount of sample: from 150 µL to 50 µL. The original method described by Shirai *et al*[90] applied 0.5 mL of hexane to dilute de analyte. As the amount of sample in our study was reduced, 0.25 mL of hexane was applied instead of the original volume. The amount of C23:0 fatty acid was also reduced in order to adequate its peck size. The change was from 50 µL to 10 µL. BHT, which aims to protect fatty acids from oxidation during the preparation, also changed from 50 µL to 30 µL.

Chemical and reagents

Internal standard C23:0 (Fluka 91478), Fatty Acids Methyl Ester Component Mix (Supelco 47885-U), BF_3-Metanol 14% (Sigma B1252) and gaseous nitrogen. Isooctane, methanol and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, United States).

Sample preparation and extraction

Fatty acid samples were esterified following the method described by Shirai *et al*[90]. Plasma samples (50 µl) were added to tubes containing 10 µl of internal standard (Tricosanoic Acid Methyl Ester - C23:0), 20 µl of 0.5% BHT, and 1 ml of 0.5 M methanolic NaOH. The solution was vortexed for 15 seconds and heated in a water bath at 100°C for 5 minutes. After cooling, the samples were mixed with 2 ml of 14% BF3 in methanol, vortexed for 15 seconds, and heated in a water bath at 100°C for 5 minutes. After cooling, 1 ml of isooctane was added. The tubes were vigorously shaken for 30 seconds, and 5 ml of a saturated NaCl solution was added. The tubes were gently homogenized to enhance the extraction efficiency of suspended samples using a salting-out agent. This agent increased the concentration of apolar analytes by increasing the ionic strength of the solution, resulting in decreased solubility of these compounds[91]. After centrifugation at 9,000 x g for 3 minutes, 450 µl of the organic phase was transferred to a new vial and dried under a nitrogen stream. The recovered lipids were reconstituted in 0.25 ml of hexane.

Gas Chromatographic analysis and instrumental conditions of the mass spectrometer

In this study, GC-MS analysis of the fatty acids methyl ester (FAMEs) was conducted using an Agilent 7890A gas chromatograph equipped with an autosampler connected to an Agilent 5975C MS with a Triple Axis Detector. This detector configuration reduces noise from secondary particles and enhances detection sensitivity.For separation of the FAMEs, a highly apolar fused silica capillary column (J&W DB-23 Agilent 122-236) was employed. This column is coated with a stabilized cyanopropyl phase, allowing for the separation of positional and geometric isomers. The injection volume was set at 10 µL, and the GC inlet was operated in pulsed split mode at 250 °C using a glass-packed liner.The temperature program for the GC oven was as follows: the initial temperature of 70 °C was maintained for 2 minutes, followed by a ramp of 20 °C/min up to 230 °C, which was held for 51.67 minutes. Helium gas was used as the carrier gas at a constant linear velocity of 1.3 mL/min. The inlet and transfer line temperatures were set at 250 °C. The MS ion source temperature and

both quadrupoles were maintained at 230 °C and 150 °C, respectively. The MS operated at 70 eV, and the mass spectra were acquired in the range of m/z 40-500.

Examples of the chromatograms

Figure 11 and **12** presents an example of chromatogram obtained from a patient sample and from the fatty acids methyl ester standard, respectively.

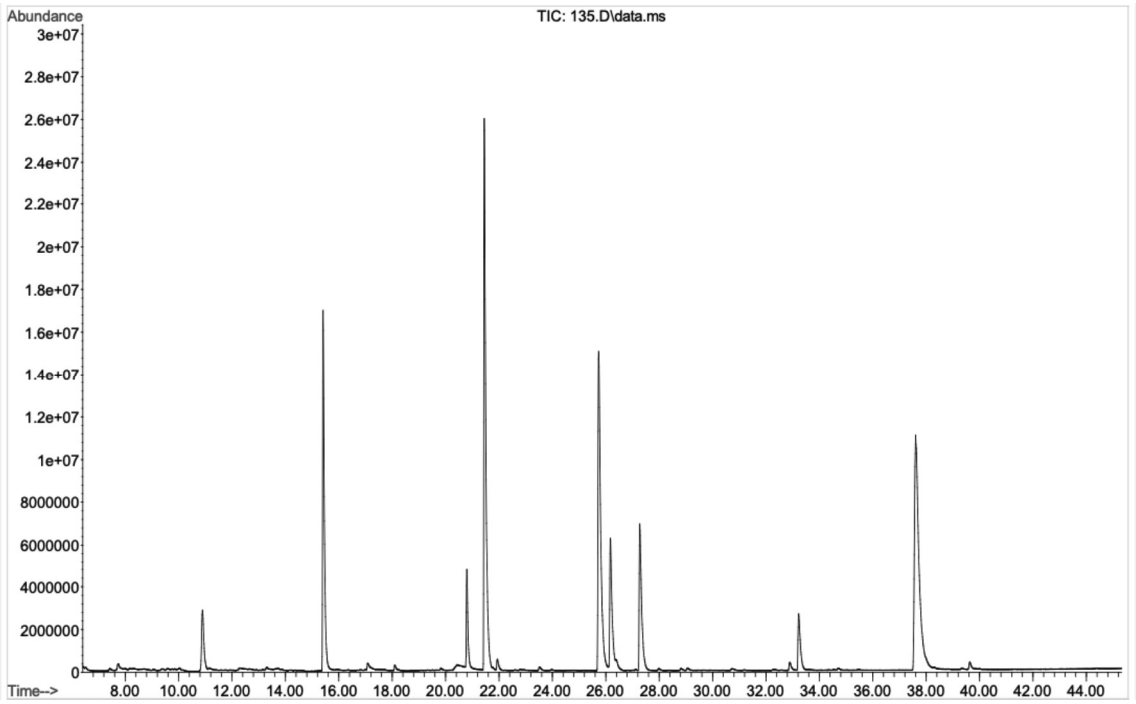

Figure 11*:* Example of the chromatogram obtained from the patient sample **(**Eg: Patient n.135**)**

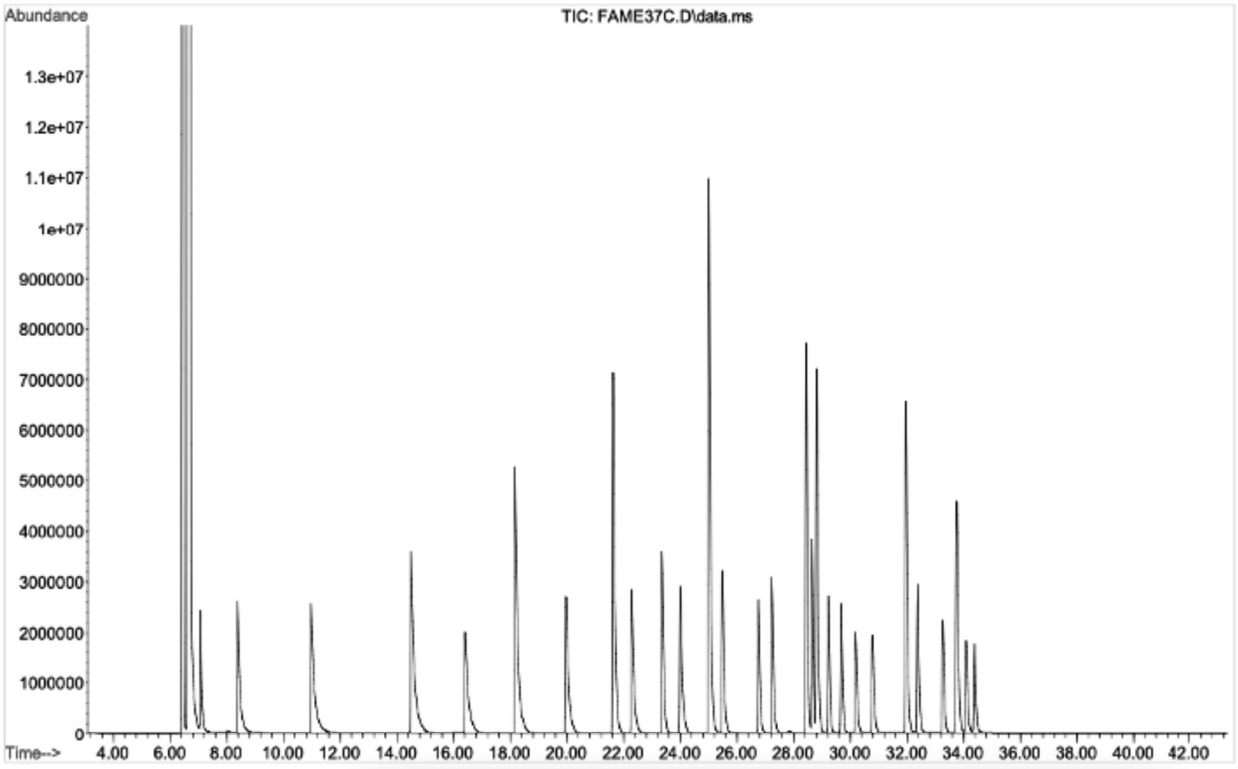

Figure 12: Example of the chromatogram obtained from the fatty acids methyl ester standard (FAME)

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ARTICLE: Eicosapentaenoic/Arachidonic acid baseline ratio was inverse associated to the severity of COVID-19 in non-immunized patients[1]

[1]Submitted

Association between the omega-3/omega-6 acid ratio and the severity of COVID-19 in hospitalized patients

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Contributors

LPF carried out the chromatography and wrote the manuscript. IHM, ALF and LPS contributed equally to collect the samples of the infected patients and organized the clinical data. MMR and BG contributed to discuss the results and edit the manuscript.

LPB supported the statistical analysis. GLM was responsible for the oxylipins determination. RMRP was responsible for the supervision of the trial. IAC led the funding acquisition, methodology supervision, project administration, and contributed to the conceptualization, statistical analysis and writing of the manuscript. All authors read and approved the final version of the manuscript.

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ABSTRACT

IMPORTANCE: A higher proportion of omega 6 than omega 3 fatty acids has been associated to inflammation and oxidative stress. In the context of COVID-19, an excessive production of pro-inflammatory cytokines was associated with disease severity and poor outcomes.

OBJECTIVE: to investigate whether the baseline omega 3/omega 6 fatty acids ratio and their oxylipins were associated with inflammation and oxidative stress in unvaccinated patients with COVID-19, classified according to the severity of the disease during hospitalization.

DESIGN, SETTING, AND PARTICIPANTS: A randomized multicenter trial included 180 hospitalized patients with COVID-19 enrolled from June 5, 2020 to September 17, 2020. There were no approved vaccines or specific treatments for COVID-19 at this time.

INTERVENTION: Blood samples and clinical data were obtained from patients during admission to the hospital.

MAIN OUTCOMES AND MEASURES: The patients in the trial were classified into five groups according to the severity of their disease. Group 1 was the least severe and Group 5 was the most severe. Three specific types of fatty acids - eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA) - as well as their enzymatic and non-enzymatic oxylipins were determined using chromatography coupled mass spectrometry.

RESULTS: There was no difference in the ratio of omega-3 to omega-6 fatty acids between the groups (*p=0.276*). However, the EPA/AA ratio was lower in Group 5 compared to Group 1 (*p=0.010*). This finding was correlated with an increase in both C-Reactive Protein (*p<0.001*) and interleukin-6 (*p=0.002*). Furthermore, the concentration of F_2-Isoprostanes was higher in Group 4 than in Group 1 (*p=0.009*), while no significant changes were observed for other oxylipins among groups with

varying degrees of disease severity. Multivariate analysis did not present any standard of biomarkers, suggesting the high complexity of factors involved in the disease severity.

CONCLUSIONS AND RELEVANCE: A higher EPA/AA ratio upon hospital admission was found to be associated with lower concentration of C-Reactive Protein and interleukin-6, leading to a better prognosis of hospitalized SARS-CoV-2 patients. However, the mechanisms behind this association are still not fully understood and require further investigation.

TRIAL REGISTRATION: ClinicalTrials.gov Identifier: NCT04449718

Introduction

Since the onset of the SARS-CoV-2 outbreak in 2020, it has been observed that while some patients only exhibited mild clinical symptoms or were even asymptomatic, others required treatment in intensive care units (ICUs).[1-4] Furthermore, the prognosis of COVID-19 patients has been associated with a multitude of factors, with a high concentration of pro-inflammatory cytokines being one of the main features in the adverse response, resulting in a poorer outcome and/or increased mortality.[4,5]

Viral ssRNA(+) in the cytoplasm induces stress in the endoplasmic reticulum, leading to the release of fatty acids that esterify the phospholipids in the cell membrane. These fatty acids can be substrates for oxidative reactions, giving rise to various oxylipins.[6] The type of oxylipin formed, whether pro- or anti-inflammatory, depends on the fatty acid precursor and the oxidative pathway involved. Generally, oxylipins derived from enzymatic and non-enzymatic oxidation of omega-6 fatty acids, such as arachidonic acid (AA), have been found to be more inflammatory compared to those derived from omega-3 fatty acids (n-3 FA) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). [5,7-8] On the other hand, EPA and DHA are more unsaturated than omega-6 fatty acids (n-6 FA), making them more susceptible to oxidation and potentially giving rise to other cytotoxic oxylipins.[9]

Therefore, this study aimed to investigate whether the baseline ratio of omega-3/omega-6 fatty acids is associated with the inflammation and oxidative stress in non-

immunized patients with COVID-19, classified according to the severity of the disease during hospitalization.

Methods

Patients

Randomized patients diagnosed with COVID-19 by polymerase chain reaction (PCR) test or by serology assay (ELISA) to detect IgG against SARS-CoV-2 at hospital admission were recruited from the Clinical Hospital of the School of Medicine of the University of São Paulo and the Ibirapuera Field Hospital, São Paulo, Brazil. Patients were enrolled in this multicenter study from June 5, 2020, to September 17, 2020. This study was included as an additional part of another research, thus following the same inclusion and exclusion criteria previously reported.[10] The patients provided written informed consent before participation according to the Ethics Committee of the Clinical Hospital of the School of Medicine of the University of São Paulo and the Ethics Committee of the Ibirapuera Field Hospital (CAAE 38237320.3.0000.0068). Blood samples were collected by venipuncture into EDTA Vacutainer® tubes. Plasma samples were kept at -80 °C until the final analysis.

Patient stratification according to disease severity during hospitalization

The criteria adopted to stratify patients according to disease severity were: length of hospital stay in days from the date of randomization until hospital discharge or death, need for supplemental oxygen, non-invasive mechanical ventilation, need for orotracheal intubation (OTI) in intensive care units (ICUs), and death. Based on these criteria, patients were stratified into five groups. **Group 1** included patients hospitalized for less than the median, calculated as 5 days, without supplemental oxygen. **Group 2** comprised those discharged until 5 days but received supplemental oxygen and/or non-invasive mechanical ventilation. **Group 3** comprised patients who remained hospitalized for over a median (5 days) and needed supplemental oxygen and/or non-invasive mechanical ventilation. Finally, **Group 4** included patients who

were treated in intensive care units (ICUs) and received orotracheal intubation (OTI), while **Group 5** was composed of patients who died during hospitalization. Thus, the severity of the disease increased from Group 1 to Group 5.

Outcome Measures

According to our hypothesis, the primary outcome was defined as the severity of the disease. The secondary outcome was the laboratory data obtained from each patient's plasma, especially on fatty acids and oxylipins. Detailed methodology procedures are described in **Supplement 1**.

Sample Size Calculation

According to our hypothesis, the concentration of the most important oxylipin prostaglandin E_2 (PGE_2) was chosen for the sample size calculation. Thus, in this context, a sample size of 180 patients was estimated to be enough to have 90% power to detect a difference of 10%, considering a 2-sided alpha of 0.05, based on PGE_2 mean and deviation reported in the other study.[11]

Statistical Analysis

Two statistical approaches were applied to treat these data. Firstly, the characteristics and biomarkers were compared between the five groups using one-way ANOVA, followed by the Tukey test when the values presented a normal distribution (Anderson–Darling test) and homogeneity of variances (Hartley test). Non-parametric Kruskal–Wallis test was followed by controlled comparisons using Bonferroni analysis when the normality and homogeneity assumptions were not verified. One patient was excluded from the sampling because information about his discharge could not be found. In the second approach, a multivariate analysis was employed as an exploratory tool to identify standards-based characteristics associated with the disease's severity. From the original continuous variables, 22 were selected to be included as active variables in the multivariate analysis. The criteria applied to this selection was to change according to the five groups. The principal component

analysis (PCA) was based on correlation. Adopting Ward's method and Euclidean distance, cluster analysis was carried out to group variables and patients. The significance was set at a p-value of 0.05. All analyses were performed using Statistica v. 13.4 (TIBCO Software Inc, Round Rode, Texas, USA) and R v. 4.0.4 (R Development Core Team, 2021).

Results

Patient Characteristics

The general patient characteristics at hospital admission (**Table 1**) showed a wide range of age and BMI. The biomarkers determined in the patients at hospital admission are shown in **Table 2.** The fatty acids' proportion agrees with the most observed profile in occidental human studies[12], in which the presence of EPA and DHA is usually low due to the lower consumption of marine foods. Furthermore, the oxidative stress biomarkers indicated large variability between the patients. Drugs prescribed to less than 5% of the patients during the hospital stay were described in **eTable 1** in **Supplement 2**.

Biomarkers according to disease severity during hospitalization

Anthropometric data, immune cells, oxylipins and cytokines concentration, and fatty acids proportion determined in the patient's plasma classified according to the five disease severity groups are shown in **eTable 2** in **Supplement 2**. From these values, the most relevant results associated with our hypothesis that showed different values between Groups 4 and/or 5 and Groups 1, 2, and 3 are presented in **Figure 1.** Although no difference has been observed to the n-3FA/n-6FA ratio among the groups, the EPA/AA ratio decreased from Group 1 to Group 5 (*p = 0.010;* **Figure 1A**). Subsequently, this result was followed by two inflammatory markers in COVID-19 patients: C-reactive protein (**Figure 1B**) and Interleukin-6 (**Figure 1C**). Concerning F_2-Isop concentration, Group 4 had higher values in comparison to Group 1 (**Figure 1D**).

Biomarkers according to the cluster analysis

A multivariate analysis that considered 22 biomarkers was performed using another statistical approach. The original matrix was standardized according to the mean and deviation and applied to the cluster analysis using Ward's method and Euclidean distance. **Figure 2** shows the patients' heatmap. All levels of severity were distributed among the clusters, suggesting that there was no standard of reclassification of the patients. This result was confirmed through a discriminant analysis (**eTable3** in **Supplement 2**), in which the rate of success of the patient's classification based on the 10 major PCs was only 42.80%.

Discussion

Initially, the general characteristics of the patients, shown in **Table 1,** were in agreement with most data on other SARS-CoV-2 patients hospitalized in 2020, considering that no vaccines were viable, and drugs were administered only to alleviate the symptoms and avoid other infections .[4,6,13-14] However, the BMI of Group 5 was unexpected, since the most severe conditions were associated with overweight and obesity.[15] In this sense, the intense catabolic state induced by infection in SARS-Cov-2 patients leads to significant body weight loss, which is largely associated with a reduction in lean mass. Thus, it is possible to consider the higher prevalence of sarcopenia in patients of Group 5.

This study's first challenge was finding a difference in the n-3 FA/n-6 FA ratio among the patients since n-3 FA supplements or fish consumption in the Western diet has been deficient.[16] Thus, the difference observed in the EPA concentration could have been due to the endogenous conversion from α-linolenic acid (ALA), which was also low.[2,17] Although the cellular incorporation of EPA and DHA occurs mainly at the expense of AA[18], this replacement seems to depend on the fatty acid pool.[19] This could be why no changes were observed in the AA proportion among the patients ($p = 0.664$), besides that no n-3 FA was supplemented. DHA is found to have a higher concentration in plasma than EPA[20], but its variability is lower than EPA[8] as its synthesis demands the transfer of EPA to the peroxisomes, where it is β-oxidized to form DHA.[17,21] Moreover, DHA is rapidly driven to phospholipids and cholesterol

esterification.[22] The EPA/AA ratio observed in this study was also in agreement with the ratio presented by the patients at the baseline of the clinical trials.[6,23-25]

The main hypothesis of this study is summarized in **Figure 3**. It is supposed that after SARS-CoV-2 infection, the innate immune system cells promote the synthesis of cytokines, chemokines, growth factors, and adhesion molecules, trying to counteract virus replication and spreadability. In addition, PLA_2, among other hydrolases, releases fatty acids from the phospholipid chain to be used as a substrate for oxidant enzymes, such as lipoxygenases (LOX), cyclooxygenases (COX), and cytochrome P450 (CytP450), leading to the formation of many oxylipins involved in the immune response and the resolution of inflammation. [21,26] It has been reported that EPA- and DHA-derived oxylipins have a less potent inflammatory action than AA-derived oxylipins.[21] PGE_2 formed from AA (**Figure 3**) has been associated with increased cytokine expression, although this effect depends on several other conditions.[8]

Our results clearly showed that a lower EPA/AA ratio, mainly observed in Groups 4–5 (**Figure 1A**), was associated with a higher concentration of IL-6 and CRP, typically elevated in critically ill COVID-19 patients [3,5,27-29], bringing a worse prognostic to these patients. Other studies carried out on patients with sepsis or multiple inflammatory and respiratory problems have shown a reduction of CRP and IL-6 concentrations after n-3 FA supplementation.[27,30] Diverging from our hypothesis, the study data suggested that this beneficial effect was not associated with changes in PGE_2 concentration.

PGE_2 is an immunomodulatory eicosanoid generated by COX that crosstalks with cytokines through several mechanisms.[31] PGE_2 increases arterial dilation and microvascular permeability, increasing the blood flow into inflamed tissue and regulating cytokine expression in immune cells[32], such as IL-6. [8] It has been reported that during influenza-A virus infection, PGE_2 was upregulated, leading to inhibition type I interferon production, and suppressing apoptosis through EP_2 and EP_4 receptors, causing an increase in virus replication. [29,33] PGE_2 activates the Nuclear factor-kappa B (NF-κB) in macrophages synergistically with TNFα through EP2 receptors, thereby inducing the expression of pro-inflammatory genes that codify COX-2 and Monocyte chemoattractant protein 1 (MCP-1).[29] It has been proposed that lowering PGE_2 concentrations by inhibiting Microsomal prostaglandin E synthase-1 (mPGES-1) could enhance the host immune response against SARS-

CoV-2.[34] Our clinical trial did not identify any difference in PGE_2 concentrations among groups with varying disease severity. Thus, it is possible that a higher EPA and DHA concentration is necessary to change plasma PGE_2 concentrations. Moreover, PGE_2 in plasma is rapidly metabolized and excreted in the urine as 11-α-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid.[35,36] Consequently, urine PGE_2 may better reflect its synthesis from AA than PGE_2 in plasma.[35]

A reduction of an important marker of non-enzymatic lipid peroxidation, F_2-IsoPs (**Figure 1D**), was observed in Group 1 (mild) compared with Group 4 (critical). In another study, urinary 15-F_{2r}-IsoPs was lower in older subjects hospitalized for COVID-19 who were receiving daily IV infusions containing about 6 g EPA+DHA/ day for 5 days, leading to the conclusion that n3-FA treatment promoted the reduction of oxidative stress in COVID-19).[6] The high amount of F_2-IsoPs observed in Group 4 compared with Group 1 can also be due to the excessive secretion of reactive oxygen species (ROS) by the immune cells[29], since n-3 FA-derived specialized pro-resolving lipid mediators (SPMs) can blunt ROS production from neutrophils.[27]

EPA, DHA, and their oxylipins formed by enzymatic and non-enzymatic oxidative reactions can exert an anti-inflammatory effect through other mechanisms not thoroughly investigated in our study, given that our focus was on PGE_2. Actually, the fact that SPMs were not detected analysis does not discard the "resolution hypothesis"[5], based on the association between SPMs derived from n-3 FA and the severity of COVID-19 [37], considering that SPMs blunt polymorphonuclear cells infiltration[38] and have an essential role in efferocytosis improvement, reducing the massive infiltration of necrotic cell debris observed *post-mortem* in the lungs of deceased COVID-19 patients.[39] In addition, viral-induced cell debris causes endoplasmic reticulum stress (ER stress), intensifying the inflammatory cycle [28] and emphasizing the role of efferocytosis by macrophages in reducing the cytokine storm. Moreover, deficient concentrations of SPMs have been identified in the setting of common human inflammatory lung diseases.[28] The efficacy of dexamethasone in COVID-19 could be partly due to its ability to induce pro-resolving lipid mediators.[40]

As summarized in **Figure 3**, EPA may act as a peroxisome proliferator-activated receptor-γ (PPARγ) agonist, inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) translocation to the nucleus, leading to a lower expression of genes that codify the pro-inflammatory cytokines, such as IL-1β and IL-6. It has been reported that the inhibition of NFκB reduces inflammation and increases

the survival of mice infected with Sars-CoV. [41] Thus, if n-3 FA can inhibit NFκB, as postulated by other studies [18], it can be suggested that the lower concentration of CRP and IL-6 is a result of NFκB inhibition promoted by n-3 FA (**Figure 3**).

Clinical and routine laboratory data at hospital admission were applied to build an algorithm that could predict non-worsening patients during the first two weeks, showing a success rate higher than 99%.[4] However, a multivariate analysis applied to our data showed that no standard of biomarkers could be identified to predict the severity of the disease (**Figure 2**), achieving a success rate of classification of only 43%, suggesting the complexity of the SARS-CoV-2 infection in terms of factors associated with the disease prognostic in the conditions evaluated in our study.

The immune response demands the inflammatory condition to control the viral infection. That is, inflammation is an essential tool of the immune cells.[42] EPA and DHA do not primarily act as immune suppressors.[18] Instead, EPA and DHA selectively stimulate the pro-resolving cytokines. For example, SARS-CoV-2 induces an inflammatory cytokine storm, which is strongly correlated with adverse clinical outcomes.[39] Omega-3 fatty acids and anti-inflammatory drugs can show different results according to the moment of their intake. While most studies have supplemented patients during treatment in the hospital [5], in our study, this protection was present at the time of infection, since blood samples were collected at the hospital admission and no intervention with n-3 FA was made during hospitalization. This aspect can be essential in infection control and must be further investigated to achieve a better protection system against future pathogens infections.

In addition, other factors not evaluated in our study, such as the initial charge of viral infection, could have contributed to the severity of the disease. Finally, our study has some limitations. Due to the low amount of sample and their rapid metabolism in plasma, some oxylipins were below the detection limit. As all patients were hospitalized, no comparison was made with patients who presented mild symptoms and were not treated in the hospitals. It is worth noting that a higher EPA/AA can also be associated with other factors, including a more diverse and healthy diet [43], indirectly contributing to a better prognosis of patients during hospitalization. For these reasons, extensive research is needed to confirm our results.

Conclusion

A higher EPA/AA ratio prior to infection was found to be associated with lower concentration of C-Reactive Protein and interleukin-6, leading to a better prognosis of hospitalized SARS-CoV-2 patients. However, the physiological mechanism of this effect must be further investigated, since a higher EPA/AA ratio was not associated to a lower concentration of oxylipins derived from enzymatic oxidation as PGE_2, but rather to a lower concentration F_2-IsoP formed through non-enzymatic oxidation.

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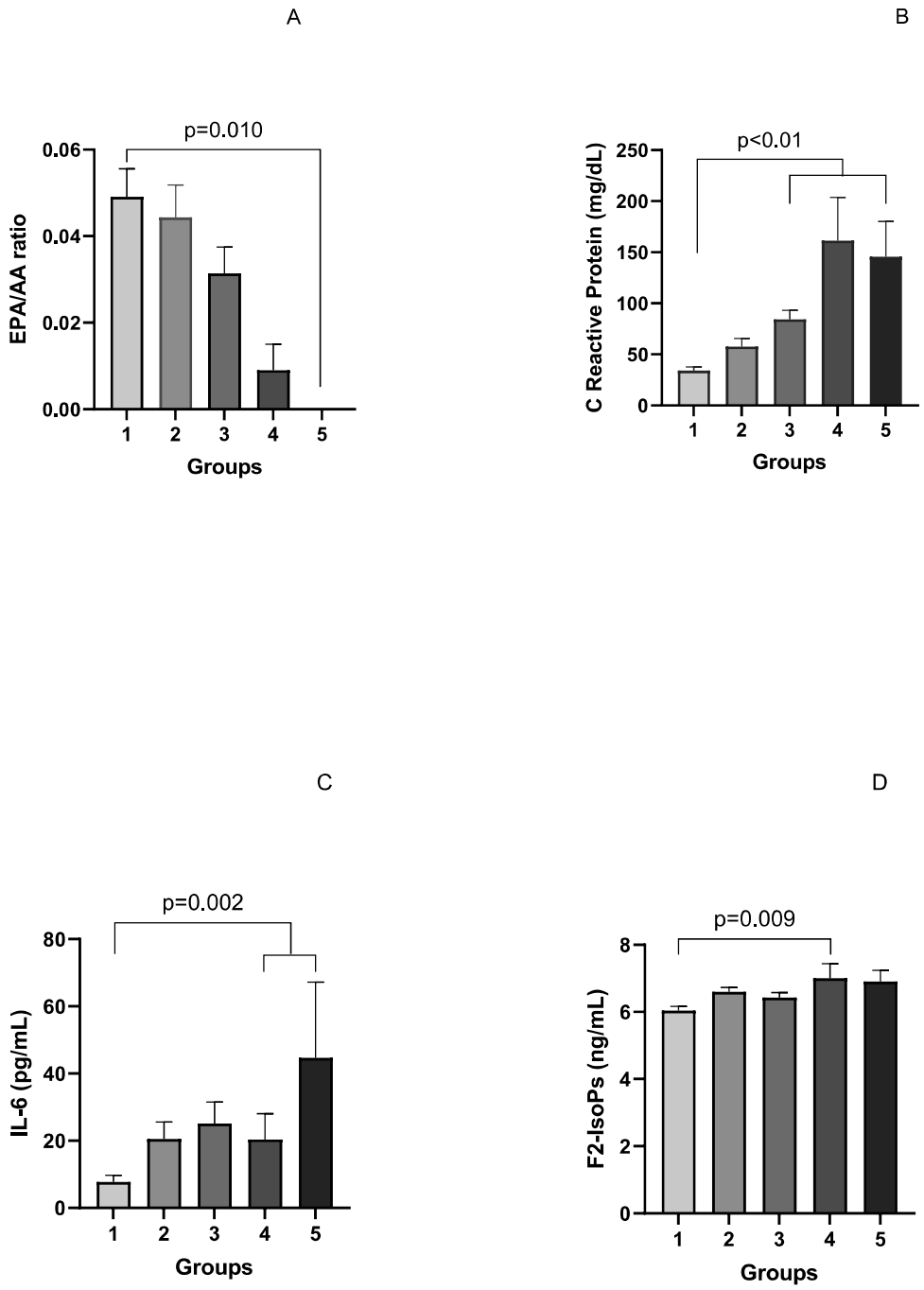

Figure 1. Mass spectrometry analysis of plasma fatty acids, cytokines and oxylipin according to the disease severity that increased from Group 1 (less severe) to Group 5 (the most severe). **A**, EPA/AA ratio; **B**, C-reactive protein (CRP) (mg/dL); **C**, Interleukin-6 (IL-6) (pg/ml); **D**, 5-series-F_2-IsoP concentration (ng/ml). Boxes and whiskers represent mean and SEM respectively. P-values were obtained by One-way ANOVA followed by Tukey HSD test or equivalent non-parametric Kruskal Wallis Test followed by Bonferroni corrections, both for independent groups (n: 177-179).

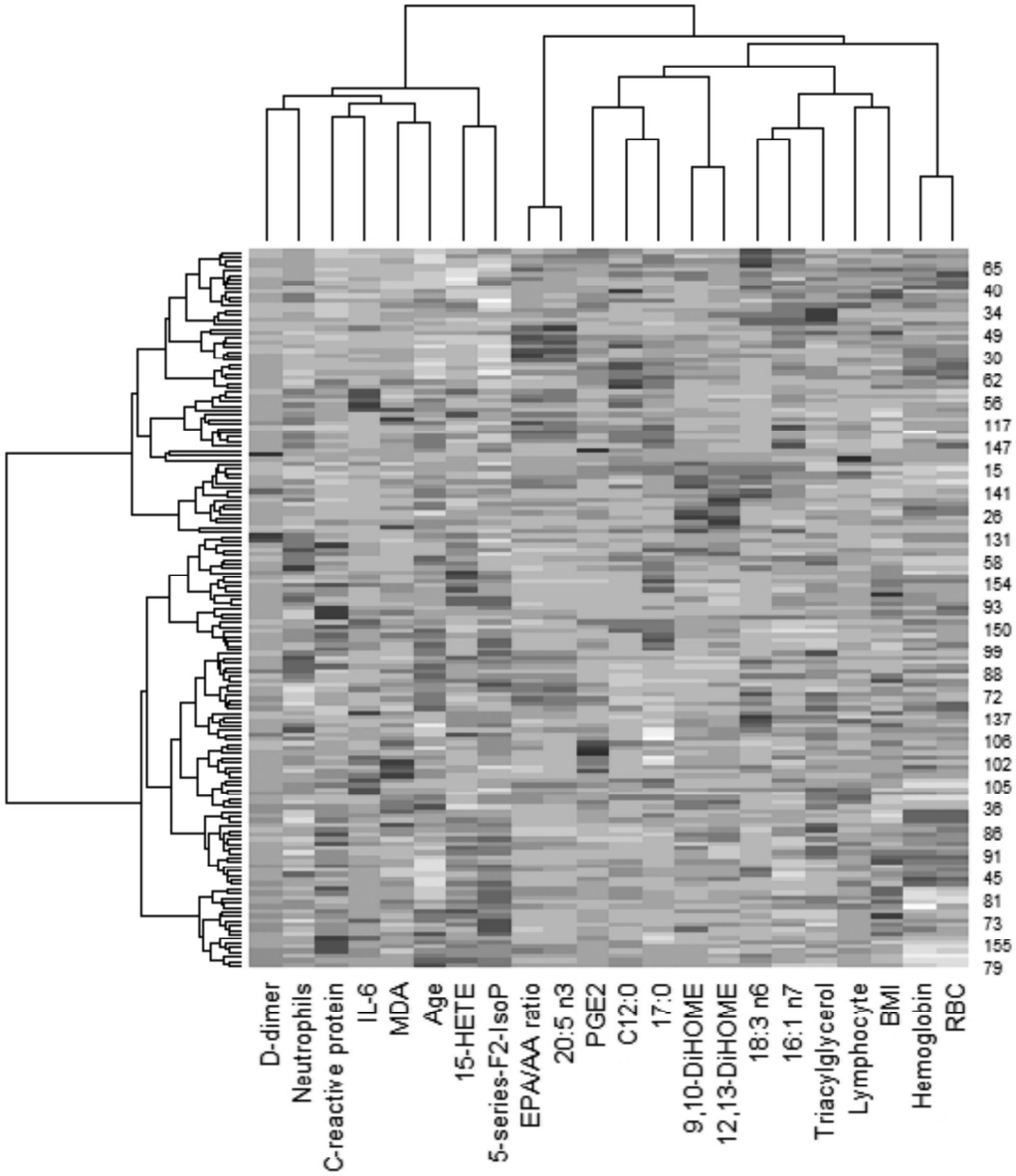

Figure 2. Heatmap representing the patients (159) according to the cluster analysis using Ward's method (22 variables). Rows represent some of individual value observed for each variable (columns), including the dendrogram obtained to patients and variables.

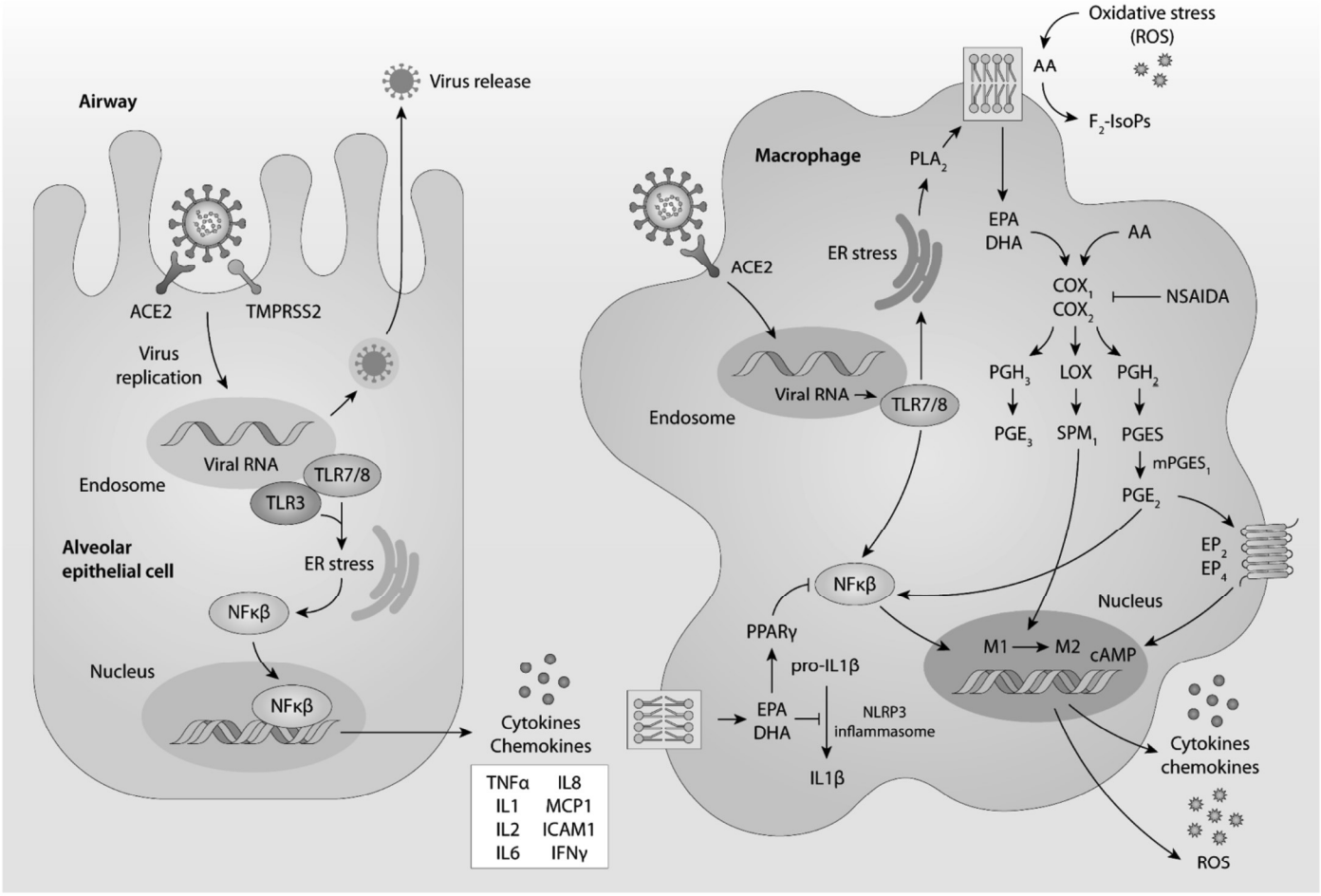

Figure 3. Coronavirus 2 (SARS-CoV-2) present in the airway infects cells that express the surface receptors angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2), such as the alveolar epithelial cells. The viral spike protein (S) on the surface of the virus binds to ACE2 and suffer endocytosis mediated by TMPRSS2. The acidification of the endosome leads to viral and cellular membrane fusion and release of viral single-stranded RNA (ssRNA) into the cytosol. The virus is replicated and released back to the airway. The viral RNA within the endosomes activates the Toll-like receptors (TLR3, TLR7/8), promotes ER stress, leading to the release of NFkB, that translocate to the nucleus and initiate transcription of various genes coding for inflammatory cytokines, chemokines, adhesion molecules, and growth factors. These proteins attract monocytes and T cells to the site of infection, promoting further inflammation, establishing a pro-inflammatory feedback loop. As part of the natural response to the ER stress, PLA_2 hydrolyze the fatty acids that are esterifying the phospholipids in the membranes, that will be the substrates to oxidative enzymes to produce different oxilypins. When AA is viable, COX produce PGH_2, that will be converted to PGE_2 and will bind to EP_2/EP_4 receptors present in the macrophages

membranes, activating cAMP, causing more inflammation, pain, immunoregulation, mitogenesis and cell injury. On the other side, if EPA and DHA are released from phospholipids, instead PGH_2, PGH_3 is synthesized. This mechanism explains the use of NSAIDs to treat COVID-19 patients. In addition, the excessive ROS produced by the macrophages, can directly oxide the AA as part of the phospholipids, increasing the F_2-IsoPs concentration. It can speculate that EPA and DHA could reduce the pro-inflammatory molecules by activating PPARγ and, consequently, reducing NFkB migration to the nucleus. Other mechanisms include the capacity of EPA and DHA to inhibit the NLRP3 inflammasome and consequent IL-1β maturation, besides the SPMs effect on changing the macrophage phenotype, leading to the inflammation resolution. **Abbreviations:** angiotensin-converting enzyme 2 (ACE2); transmembrane serine protease 2 (TMPRSS2); Toll-like receptors (TLR3, TLR7/8); Endoplasmic reticulum (ER); Nuclear factor-kappa B (NF-κB); Phospholipase A2 (PLA2); arachidonic acid (AA); Prostaglandin E2 (PGE2); cyclooxygenase (COX); lipoxygenase (LOX): cyclic adenosin monophopshate (cAMP); Non-steroidal anti-inflammatory drugs (NSAIDs) ; F2- Isoprostanes (F2-IsoPs); Peroxissome proliferator-activated receptor gamma (PPARγ), NLR Family Pyrin Domain Containing 3 (NLRP3); Inteleukin 1β (IL-1β); specialized pro-resolving mediators (SPMs); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA).

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Table 1. General Patient Characteristics at hospital admission

Characteristics	n	Mean (±SEM)	Median	Range (min-max)
Anthropometric data				
Age (years)	180	55.34 (± 1.09)	56.50	20.00 – 87.00
Weight (kg)	173	85.57 (± 1.55)	85.00	42.00 – 175.00
Height (m)	167	1.67 (± 0.01)	1.66	1.45 – 2.00
BMI (kg/m^2)	167	30.94 (± 0.51)	30.12	17.91 – 62.50
Hospital Admission				
Days from the first symptoms (days)	180	10.12 (± 0.28)	10.00	2.00 – 23.00
Hospital length of stay (days)	174	7.02 (± 0.56)	5.00	0.00 – 49.00
Orotracheal intubation (days)	175	1.03 (± 0.33)	0.00	0.00 – 30.00
Laboratory data				
Urea nitrogen (mg/dL)	179	38.84 (± 0.97)	38.00	13.00 – 87.00
Creatinine (mg/dL)	180	0.84 (± 0.02)	0.82	0.42 – 1.72
C-reactive protein (mg/dL)	180	68.88 (± 5.21)	51.00	1.60 – 397.20
RBC (10^{12}/L)	180	4.66 (± 0.05)	4.67	2.39 – 6.71
Hemoglobin (g/dL)	180	13.44 (± 0.14)	13.50	7.40 – 17.70
Platelet (10^3/µL)	180	292.47 (± 9.05)	276.50	38.00 – 723.00
D-dimer (mg/L)	178	1,605.72 (±356.50)	758.50	190.00 – 57,811.00
Leukocytes (10^3/µL)	180	9.18 (± 0.26)	8.56	1.80 – 20.87
Lymphocyte (10^3/µL)	180	1.27 (± 0.09)	1.02	0.19 – 15.00
Neutrophils (10^3/µL)	180	7.29 (± 0.24)	6.85	1.06 – 17.49
Eosinophils (10^3/µL)	179	0.04 (± 0.01)	0.00	0.00 – 0.56
Erythrocyte sedimentation rate (mm/h)	176	48.46 (± 2.80)	46.50	1.00 – 140.00
Total cholesterol (mg/dL)	177	171.16 (± 3.33)	169.00	65.00 – 301.00
High density lipoprotein (mg/dL)	176	36.66 (± 0.82)	36.00	18.00 – 93.00
Low density lipoprotein (mg/dL)	176	104.59 (± 2.72)	102.00	25.00 – 206.00
Triacylglycerol (mg/dL)	176	187.30 (± 6.09)	179.50	49.00 – 526.00
Sociodemographic data				**%**
Sex				
Female				46.67
Male				53.33
Ethinicity				
White				44.78
Pardo[a]				38.33
Black				13.89
Comorbidities				**%**
Obesity				53.29
Hypertension				46.67
Chronic heart disease				11.67
Chronic Obstructive Pulmonary Disease (COPD)				3.89
Asthma				5.56
Diabetes				25.56

Diarrhea	47.22
Rheumatic diseases	8.33
Others	41.11
Main pharmacological treatment during the hospital stay	**%**
Acetylsalicylicacid (100 mg)	7.22
Albuterol (100 mg)	18.33
Amlodipine (5 mg)	12.22
Atenolol (50 mg)	8.33
Azithromycin (500 g)	57.22
Captopril (25 mg)	10.56
Ceftriaxone (1g)	84.44
C*lonazepam (2.5 mg/mL)*	8.89
Codeine Sulfate (5 mg)	10.00
Dexamethasone (6 mg)	75.0
Dipyrone (1 g)	5.00
Dipyrone (500 mg)	58.33
Enalapril (20 mg)	5.00
Enoxaparin (40 mg)	87.78
Hydrochlorothiazide (25 mg)	6.11
Hydrochlorothiazide (50 mg)	6.67
Lactulose (667 mg)	9.44
Levothyroxine (50 µg)	5.00
Losartan (50 mg)	30.56
Metformin (850 mg)	9.44
Metoclopramide (5 mg)	51.11
NPH insulin (25 25 25)	8.89
Omeprazole (20 mg)	56.11
Omeprazole (40 mg)	5.56
Ondansetron (2mg)	16.67
Simvastatin (40 mg)	11.67
Hospital Admission	**%**
Oxygensupply	60.00
High-flow nasal canula (HFNC) oxygen, noninvasive ventilation (NIV)	10.00
Orotracheal intubation + invasive ventilation (NIV)	9.44
Intensive care unit (ICU)	14.44
Clinical end-points (discharge, death, dropout)	93.33, 3.89, 2.78

SI conversion factors: To convert creatinine to µmol/L, multiply values by 88.4; D-dimer to nmol/L, multiply values by 5.476.

[a] Pardo is the exact term used in Brazilian Portuguese, meaning “mixed ethnicity,” according to the Brazilian Institute of Geography and Statistics.

[b]Drugs that were prescribed to less than 1% of the patients during the hospital stay were described in **eTable1 in Supplement 2**

Table 2. Fatty acids, Oxidative Stress and Inflammatory Biomarkers of the Patients at Hospital Admission

Fatty Acids (%)	n	Mean (±SEM)	Median	Range (min-max)
C12:0 (lauric acid)	180	0.10 (± 0.01)	0.00	0.00 – 0.68
C14:0 (myristic acid)	180	0.40 (± 0.01)	0,39	0,00–0.98
C16:0 (palmitic acid)	180	37.87 (± 0.24)	37.97	20.62 – 47.22
16:1 n7(palmitoleic acid)	180	1.06 (± 0.03)	0.96	0.32 – 3.08
17:0 (heptadecanoic acid)	180	0.36 (± 0,01)	0.32	0.00 – 0.68
18:0 (stearic acid)	180	27.65 (± 0.44)	28.13	0.00 – 40.54
18:1 n9 (oleicacid)	180	11.96 (± 0.25)	11.23	1.47 – 24.20
18:2 n6 (linoleicacid)	180	15.11 (± 0.39)	14.95	0,00 – 29.81
18:3 n6 (γ-linolenic acid)	180	0,10 (± 0,01)	0,00	0,00 – 0.73
18:3 n3 (α-linolenic acid)	180	0.15 (± 0.01)	0.00	0.00 – 0.75
20:0 (arachidic acid)	180	0.06 (± 0.01)	0.00	0.00 – 0.43
20:2 n6 (eicosadienoic acid)	180	0.06 (± 0.01)	0.00	0.00 – 0.51
20:3 n6(dihomo-γ-linolenicacid)	180	0.56 (± 0.02)	0.54	0.00 – 1.68
20:4 n6 (arachidonic acid)	180	3.79 (± 0.09)	3.69	1.58 – 8.45
20:5 n3 (eicosapentaenoic acid)	180	0.15 (± 0.02)	0.10	0.00 – 1.19
22:6 n3 (docosahexaenoic acid)	180	0.54 (± 0.02)	0.51	0.00 – 1.43
SFA	180	66.45 (± 0.62)	67.08	39.29 – 85.27
MUFA	180	13.01 (± 0.27)	12.30	2.92 – 27.29
PUFA	180	20.55 (± 0.44)	20.34	3.33 – 36.78
Omega 3 FA	180	0.93 (± 0.03)	0.90	0.00 – 2.24
Omega 6 FA	180	19.61 (± 0.43)	19.28	2.95 – 35.82
n-3/n-6 FA ratio	180	0.05 (± 0.003)	0.05	0.00 – 0.32
(EPA+DHA)/AA ratio	180	0.18 (± 0.01)	0.17	0.00 – 0.56
EPA/AA ratio	180	0.04 (± 0.004)	0.03	0.00 – 0.22
Oxidative stress				
MDA (μMol)	180	4.78 (± 0.20)	4.14	1.39 – 19.76
PGE_2 (ng/ml)	180	0.68 (± 0.06)	0.47	0.00 – 8.02
15-HETE (ng/ml)	180	3.16 (± 0.14)	2.82	0.00 – 11.64
12-HETE (ng/ml)	180	12.04 (± 6.47)	1.34	0.00 – 870.78
5-HETE (ng/ml)	180	2.02 (± 0.14)	1.60	0.00 – 12.89
12,13-DiHOME (ng/ml)	180	4.34 (± 0.29)	3.70	0.00 – 25.86
9,10-DiHOME (ng/ml)	180	1.96 (± 0.13)	1.49	0.00 – 13.27
12,13-EpOME (ng/ml)	180	13.78 (± 0.65)	11.63	0.00 – 65.60
9,10-EpOME (ng/ml)	180	31.57 (± 1.65)	23.88	7.81 – 174.36
13-HODE (ng/ml)	180	9.37 (± 0.47)	7.22	2.10 – 46.53
14,15-EET (ng/ml)	180	2.55 (± 0.14)	2.02	0.00 – 12.94
11,12-EET (ng/ml)	180	18.58 (± 1.22)	14.42	2.92 – 178.33
5-series-F_2-IsoP (ng/ml)	180	6.41 (± 0.08)	6.40	3.98– 9.91
Cytokines				
IL-1β(pg/ml)	178	2.00 (± 0.24)	0.94	0.01 – 23.56
IL-6(pg/ml)	178	19.05 (± 2.80)	4.53	0.19 – 276.00
TNFα(pg/ml)	178	9.62 (± 0.64)	7.29	0.86 – 54.84

Abbreviations:

Saturated fatty acids (SFA); Monoinsaturated fatty acids (MUFA); Polyunsaturated fatty acids (PUFA); 5-Hydroxyeicosatetraenoic acid (5-HETE); 5-series F_2-isoprostanes (5-series-F_2-IsoP); 12-Hydroxyeicosatetraenoic acid (12-HETE); *9,10*-Dihydroxy-12-octadecenoic acid

(9,10-diHOME); 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME); 9,10-epoxy-12Z-octadecenoic acid (9,10 EpOME); 12,13-Epoxy-9(Z)-octadecenoic acid (12,13 EpOME); 13-Hydroxyoctadecadienoic acid (13-HODE); 11,12-epoxyeicosatrienoic acid (11,12-EET); 14,15-Epoxyeicosa-5,8,11-trienoic Acid (14,15-EET); 15-Hydroxyeicosatetraenoic acid (15-HETE); Arachidonic Acid (AA); Docosahexaenoic acid (DHA), Eicosapentaenoic acid (EPA); Interleukin 1β (IL-1β); Interleukin 6 (IL-6); Malondialdehyde (MDA); Prostaglandin E_2 (PGE_2) and Tumor Necrosis Factor α (TNF α).

Supplemental Online Content

Fernandes LP, Murai IH, Fernandes AL, et al. **Association between eicosapentaenoic acid/arachidonic acid ratio upon hospital admission and COVID-19 severity in unvaccinated hospitalized patients.**

eMethods.

eMethod 1. Determination of oxylipins.

Plasma (100µL) was placed in a microcentrifuge tube containing 500 µL 25% methanol in water and internal standard mix (1ng each deuterated oxylipin). The sample was vortexed and spun to pellet protein. The supernatant was then extracted on an Oasis MAX uElution plate (Waters Corp., Milford, MA) as follows. Sample wells were first washed methanol (200 µL) followed by 25% methanol in water (200 µL). The sample was then loaded into the well and washed with 600 µL 25% methanol. Eicosanoids were eluted from the plate with 30 µL 2-propanol/acetonitrile (50/50, v/v) containing 5% formic acid into a 96-well elution plate containing 30 µL water in each well. Samples were analyzed on a Waters Xevo TQ-XS triple quadrupole mass spectrometer connected to a Waters Acquity I-Class UPLC (Waters Corp., Milford, MA USA). Separation of analytes was obtained using an Acquity PFP column (2.1 x 100mm) with mobile phase A being 0.01% formic acid in water and mobile phase B acetonitrile. Eicosanoids were separated using a gradient elution beginning with 30% B going to 95% B over 8 minutes at a flow rate of 0.250 mL/min. One analysis was carried out/patient and the concentration was expressed as ng/mL plasma.

eMethod 2. Determination of the fatty acids.

Determination of fatty acids composition was carried according to Shirai et al. (2005) with some modifications. Plasma (50 µL) were transferred to test tubes containing 0.1 mg of tricosanoic acid methyl ester as internal standard (IS) (C23:0; Fluka 91478), 20 µL of a 0.5 % butylated hydroxytoluene (BHT) solution and 1 mL of a methanolic NaOH solution (0.5 M). Then, samples were placed in a boiling water bath for 5min, followed by addition of 2 mL of boron trifluoride diethyl etherate (BF_3) and boiling for 5 min. After cooling, 1 mL of isooctane was added and the mixture was vigorously homogenized. Then, 5 mL of a saturated NaCl solution was added and the samples were gently homogenized and centrifuged at 3,000 x g for 3min The organic phase was extracted, dried, re-suspended in 250 µL of hexane, and injected into a gas chromatography coupled with a triple quadrupole mass spectrometer (GC-MS Agilent 7890A GC System, Agilent Technologies Inc., Santa Clara, USA). Fatty acids were separated on a fused silica capillary column (J&W DB-23 Agilent Inc. Santa Clara, USA) with 60 m × 0.250 mm dimensions. Injection volume was 1 µL in the split mode (1:50) and the GC inlet temperature was 250ºC. High-purity Helium was used as the carrier gas at a flow rate of 1.3 ml/min. The oven temperature was programmed to rise from 80ºC to 175 °C at a rate of 5°C/min, followed by another gradient of 3°C/min until 230°C, which was maintained for 5 min. The transfer line temperature was 280°C. All mass spectra were obtained by electron impact (70 eV), in the scan mode (40 – 500 m/z). Compounds were identified by comparing of the retention time of fatty acids in the samples with the retention time of standards (FAME 37 Component Mix Supelco 47885), and also based on a comparison of their mass spectra with those given in the spectral database of the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). One analysis was carried out/patient and the ratio of the fatty acid/IS area was applied to calculate the percentage of each fatty acid. The peroxidability index (PI) was calculated using the equation reported by Hsu, Lee, and Chen (2001): PI = (% dienoic x 1) + (% trienoic x 2) + (% tetraenoic x 3) + (%pentaenoic x 4) + (% hexaenoic x 5).

Reference

Shirai, N.; Suzuki, H.; Wada, S. Direct methylation from mouse plasma and from liver and brain homogenates. *Analytical Biochemistry*, 343: 48-53, 2005.

H.C. Hsu, Y.T. Lee, M.F. Chen. Effects of fish oil and vitamin E on the antioxidant defense system in diet-induced hypercholesterolemic rabbits. *Prostaglandins & Other Lipid Mediators*, 66 (2001), pp. 99-108

eMethod 3. Determination of malondialdehyde (MDA).

MDA concentration was determined by reverse phase HPLC according to Hong et al. (2000) with some modifications Briefly, 50 µL of human plasma were mixed with 12.5 µL of 0.2% BHT and 6.25 µL of 10 N NaOH. The TBA–MDA conjugate derivative was injected in a Phenomenex reverse-phase C18 analytical column (250 mm × 4.6 mm; 5 mm; Phenomenex) with an LC8-D8 pre-column (Phenomenex AJ0- 1287) coupled to a HPLC (Agilent Technologies 1200 Series). Samples were quantified by fluorometry at an excitation of 515 nm and emission of 553 nm. The HPLC pump delivered the isocratic mobile phase: 40% PBS (A) (10 mmol, pH 7.1) and 60% methanol (B) at a flow rate of 1 mL/min. The gradient applied was: 0 – 4 min, 40% of solvent A and 60% of solvent B; 4 – 6 min, 45% of solvent A and 55% of solvent B, and then, the initial conditions were reestablished after 5 min. A standard curve was prepared using 1,1,3,3-Tetraethoxypropane (TEP, T9889 Sigma-Aldrich) (0.10 to 19.97 µmol/L MDA). One analysis was carried out/patient and the concentration was expressed as µmol/L plasma.

Reference

Hong YL, Yeh SL, Chang CY, Hu ML. Total plasma malondialdehyde levels in 16 Taiwanese college students determined by various thiobarbituric acid tests and an improved high-performance liquid chromatography-based method. *Clin Biochem* 2000;**33**:619-625. doi: 10.1016/s0009-9120(00)00177-6

Supplemental Online Content

Fernandes LP, Murai IH, Fernandes AL, et al. **Association between eicosapentaenoic acid/arachidonic acid ratio upon hospital admission and COVID-19 severity in unvaccinated hospitalized patients.**

eTable 1. Drugs prescribed to less than 5% of the patients during the hospital stay.

Pharmacological treatment during the hospital stay	% of the patients
Acetylcysteine (200 mg)	1.67
Alenia (Budesonide,Formoterol) (12/400 µg)	0.56
Amiodarone (200 mg)	0.56
Atorvastatin (20 mg)	1.67
Bactrim (Trimethoprim and Sulfamethoxazole) (400/80 mg)	0.56
Cabergoline (0.25 mg)	0.56
Calcium gluconate 10% (10 mL)	0.56
Carvedilol (3.125 mg)	3.33
Ceftriaxone (1 g)	1.11
Chlorthalidone (50 mg)	1.11
Ciprofibrate (100 mg)	0.56
Clarithromycin (500 mg)	0.56
Clonidine (0.1 mg)	0.56
cloperastine fendizoate *(3.54 mg)*	1.67
Clopidogrel (75 mg)	0.56
Colistin (150 mg)	0.56
Cyclobenzaprine (20 mg)	0.56
Dexchlorpheniramine (2 mg)	1.11
Diazepam (5 mg)	1.67
Digoxin (0.28 mg)	0.56
Dimethicone (40 mg)	0.56
Dornase alfa	0.56
Ezetimibe (10 mg)	0.56
Fenoterol + *Atrovent*	0.56
Finasteride (5.0 mg)	1.11
Fluoxetine (20 mg)	0.56
Fluticasone Propionate nasal spray (250 µg)	1.11
Formoterol fumarate (12 µg) + *budesonide (400 µg)*	0.56
Furosemide (40 mg)	1.67
Glifage (500 mg)	0.56
Heparin (5000 UI)	0.56
Hydralazine (25 mg)	1.11
Hydrocortisone (100 mg)	1.11
Hydroxyzine (10 mg)	0.56
Ketoprofen (100 mg)	1.67
Levofloxacin (500 mg)	0.56
Lidocaine (20 mg/mL)	0.56
Lithiumcarbonate (300 mg)	0.56
Loratadine (10 mg)	0.56
Meropenem (1 mg)	0.56
Methylprednisolone (70 mg)	1,67
Methylprednisolone (80 mg)	0.56
Metronidazole (500 mg)	0.56
Morphine	0.56
Oseltamivir (75 mg)	1.67
Oxacillin (2 mg)	0.56
Pantoprazole (40 mg)	0.56
Piperacillin	0.56
Piperacillin (4 g) and Tazobactam (0.5 g)	0.56
Prednisone (20 mg)	1.67
Propranolol (40 mg)	1.11
Pyridoxine	1.67
Scopolamine (20 mg)	1.11
Sertraline (50 mg)	2.22
Spironolactone (25 mg)	1.67
Tacrolimus (1 mg)	0.56
Tamsulosin (0.4 mg)	0.56
Tazobactam	0.56
Terbutaline (500 µg)	0.56
Thiamine (100 mg)	0.56
Tiotropium (2.5 mg)	0.56
Tramadol (50 mg)	2.78
Warfarin	0.56

eTable 2. Characteristics of the patients determined at hospital admission (baseline) according to the stratification based on the disease severity.

	Groups of patients (n=179*)					
Characteristics	**GROUP 1** (n=53)	**GROUP 2** (n=51)	**GROUP 3 (**n=58)	**GROUP 4** (n=10)	**GROUP 5** (n=7**)**	**P-value**[a]
Anthropometric data						
Age (years)	49.94 (± 1.86) [a]	53.98 (± 2.02) [ab]	59.57 (± 1.88)[b]	57.20 (± 4.56) [ab]	66.43 (± 5.39) [b]	*0.002*
BMI (kg/m^2)	31.00 (± 0.85) [a]	31.38 (± 0.72) [a]	30.95 (± 1.13)[a]	34.23 (± 2.03)[a]	22.75 (± 0.66)[b]	*0.005*
Laboratory data						
Ureanitrogen (mg/dL)	38.34 (± 1.42)	35.61 (± 1.84)	41.00 (±1.75)	38.78 (± 2.32)	45.29 (± 9.13)	*0.089*
Creatinine (mg/dL)	0.85 (± 0.03)	0.82 (± 0.03)	0.84 (± 0.03)	0.87 (± 0.11)	0.87 (± 0.11)	*0.908*
C-reactive protein (mg/dL)	33.98 (± 3.60) [a]	57.65 (± 7.86) [ab]	84.25 (± 8.74) [bc]	161.33 (± 42.31) [bc]	145.44 (± 34.63) [c]	*<0.001*
RBC (10^{12}/L)	4.86 (± 0.08) [a]	4.45 (± 0.10) [b]	4.59 (± 0.07)[ab]	5.12 (± 0.23) [a]	4.63 (± 0.23)[ab]	*0.002*
Hemoglobin (g/dL)	14.03 (± 0.23) [a]	12.81 (± 0.29) [b]	13.37 (± 0.20)[ab]	14.18 (± 0.58) [ab]	13.23 (± 0.65)[ab]	*0.020*
Platelet (10^3/µL)	308.15 (± 15.15)	298.90 (± 19.78)	291.40 (± 15.47)	226.30 (± 26.60)	248.57 (± 38.62)	*0.172*
D-dimer (mg/L)	1999.06 (± 1079.31) [ab]	1035.1 (± 141.69) [ab]	1974.91 (± 466.25) [a]	452.56 (± 75.39) [b]	1382.71 (± 247.00) [a]	*0.007*
Leukocytes (10^3/µL)	8.72 (± 0.35)	8.59 (± 0.50)	9.90 (± 0.54)	10.17 (± 1.05)	9.32 (± 1.25)	*0.231*
Lymphocyte (10^3/µL)	1.50 (± 0.11) [a]	1.43 (± 0.28) [ab]	0.97 (± 0.05) [b]	1.51 (± 0.59)[ab]	0.67 (± 0.14) [b]	*<0.001*
Neutrophils (10^3/µL)	6.54 (± 0.34) [a]	6.45 (± 0.40) [a]	8.42 (± 0.52) [b]	8.13 (± 0.75)[ab]	8.17 (± 1.25)[ab]	*0.014*
Eosinophils (10^3/µL)	0.04 (± 0.01)	0.05 (± 0.01)	0.03 (± 0.01)	0.05 (± 0.05)	0.01 (± 0.01)	*0.099*
Erythrocyte sedimentation rate (mm/h)	41.95 (± 4.97)	45.20 (± 5.66)	55.66 (± 4.58)	50.70 (± 11.40)	57.90 (± 18.50)	*0.228*
Total cholesterol (mg/dL)	183.38 (± 5.42)	172.22 (± 5.92)	161.19 (± 6.73)	161.30 (± 11.84)	174.17 (± 12.13)	*0.112*
High density lipoprotein (mg/dL)	37.00 (± 1.18)	36.64 (± 1.26)	36.56 (± 1.94)	37.78 (± 2.81)	33.67 (± 3.52)	*0.643*
Low density lipoprotein (mg/dL)	113.87 (± 4.38)	105.96 (± 4.96)	96.56 (± 5.38)	92.11 (± 9.02)	112.67 (± 12.71)	*0.099*

Triacylglycerol (mg/dL)	204.45 (± 11.13) [a]	197.46 (± 10.90) [ab]	173.46 (± 11.40) [ab]	142.33 (± 19.13) [b]	163.50 (± 15.25) [ab]	*0.013*
Fattyacids (%)						
C12:0 (lauric acid)	0.14 (± 0.02) [a]	0.09 (± 0.02) [ab]	0.09 (± 0.02) [b]	0.07 (± 0.05) [ab]	0.02 (± 0.02) [ab]	*0.039*
C14:0 (myristic acid)	0.46 (± 0.03)	0.37 (± 0.03)	0.38 (± 0.02)	0.43 (± 0.04)	0.40 (± 0.04)	*0.138*
C16:0 (palmitic acid)	37.69 (± 0.46)	37.72 (± 0.33)	37.93 (± 0.50)	39.37 (± 0.97)	37.28 (± 0.63)	*0.629*
16:1 n7 (palmitoleic acid)	1.16 (± 0.07) [a]	1.05 (± 0.05) [ab]	1.05 (± 0.06) [ab]	0.78 (± 0.07) [b]	0.74 (± 0.09) [ab]	*0.033*
17:0 (margaric acid)	0.35 (± 0.02) [a]	0.34 (± 0.02) [a]	0.35 (± 0.02) [a]	0.50 (± 0.04) [b]	0.43 (± 0.06)[ab]	*0.021*
18:0 (stearic acid)	27.17 (± 0.97)	27.92 (± 0.77)	27.76 (± 0.73)	28.36 (± 1.26)	26.71 (± 1.19)	*0.970*
18:1 n9 (oleic acid)	12.30 (± 0.51)	11.63 (± 0.42)	12.06 (± 0.46)	11.42 (± 0.66)	12.24 (± 0.88)	*0.908*
18:2 n6 (linoleic acid)	15.08 (± 0.86)	15.53 (± 0.54)	14.93 (± 0.72)	13.37 (± 1.69)	16.71 (± 1.25)	*0.759*
18:3 n6 (γ linolenic acid)	0.13 (± 0.02) [a]	0.12 (± 0.02) [ab]	0.08 (± 0.02) [ab]	0.00 (± 0.00) [b]	0.03 (± 0.03) [ab]	*0.008*
18:3 n3 (α-linolenic acid)	0.21 (± 0.03)	0.16 (± 0.03)	0.10 (± 0.02)	0.11 (± 0.04)	0.16 (± 0.06)	*0.132*
20:0 (arachidic acid)	0.07 (± 0.02)	0.05 (± 0.01)	0.06 (± 0.01)	0.02 (± 0.02)	0.05 (± 0.04)	*0.577*
20:2 n6 (eicosadienoicacid)	0.07 (± 0.02)	0.05 (± 0.01)	0.06 (± 0.01)	0.06 (± 0.04)	0.03 (± 0.03)	*0.841*
20:3 n6 (dihomo-γ-linolenicacid)	0.63 (± 0.04)	0.51 (± 0.04)	0.53 (± 0.05)	0.74 (± 0.08)	0.59 (± 0.08)	*0.064*
20:3 n3	0.12 (± 0.02)	0.08 (± 0.02)	0.09 (± 0.02)	0.08 (± 0.04)	0.04 (± 0.04)	*0.064*
20:4 n6 (arachidonic acid)	3.70 (± 0.17)	3.70 (± 0.14)	3.89 (± 0.17)	4.00 (± 0.21)	3.92 (± 0.22)	*0.664*
20:5 n3 (eicosapentaenoicacid)	0.19 (± 0.03)[a]	0.18 (± 0.03)[ab]	0.13 (± 0.02)[ab]	0.04 (± 0.02)[ab]	0.00 (± 0.00) [b]	*0.004*
22:6 n3 (docosahexaenoic acid)	0.54 (± 0.04)	0.50 (± 0.03)	0.53 (± 0.03)	0.66 (± 0.07)	0.65 (± 0.14)	*0.301*
SAFA	65.87 (± 1.27)	66.50 (± 1.05)	66.56 (± 1.16)	68.75 (± 2.10)	64.89 (± 1.70)	*0.869*
MUFA	13.46 (± 0.57)	12.68 (± 0.46)	13.11 (± 0.49)	12.20 (± 0.72)	12.98 (± 0.96)	*0.859*
PUFA	20.67 (± 0.92)	20.83 (± 0.67)	20.32 (± 0.81)	19.05 (± 1.88)	22.13 (± 1.33)	*0.850*
Omega 3 FA	1.06 (± 0.07)	0.92 (± 0.05)	0.85 (± 0.06)	0.89 (± 0.10)	0.85 (± 0.11)	*0.171*
Omega 6 FA	19.62 (± 0.91)	19.91 (± 0.64)	19.48 (± 0.79)	18.17 (± 1.86)	21.29 (± 1.34)	*0.845*

Omega 3/omega 6 ratio	0.06 (± 0.01)	0.05 (± 0.002)	0.05 (± 0.003)	0.07 (± 0.02)	0.04 (± 0.01)	*0.276*
EPA+DHA/AA	0.20 (± 0.01)	0.18 (± 0.01)	0.17 (± 0.01)	0.18 (± 0.02)	0.16 (± 0.03)	*0.327*
EPA/AA ratio	0.05 (± 0.01) [a]	0.04 (± 0.01) [ab]	0.03 (± 0.01) [ab]	0.01 (± 0.01) [ab]	0.00 (± 0.00) [b]	*0.010*
Perioxidability Index	31.90 (± 1.23)	31.64 (± 1.01)	31.39 (± 1.13)	30.74 (± 2.33)	33.40 (± 1.71)	*0.968*
Oxylipins and oxidative stress						
MDA (µMol)	4.08 (± 0.18) [a]	4.08 (± 0.23) [a]	5.81 (± 0.45) [b]	4.79 (± 0.82) [ab]	6.11 (± 1.88) [ab]	*0.009*
PGE2 (ng/ml)	0.96 (± 0.09) [a]	0.46 (± 0.06) [b]	0.69 (± 0.15) [ab]	0.41 (± 0.08) [b]	0.63 (± 0.17) [ab]	*<0.001*
15-HETE (ng/ml)	2.46 (± 0.18) [a]	3.42 (± 0.27) [b]	3.68 (± 0.28) [b]	2.17 (± 0.34) [ab]	3.60 (± 0.67) [b]	*0.002*
12-HETE (ng/ml)	3.36 (± 1.17)	4.44 (± 2.70)	29.95 (± 19.81)	1.12 (± 0.29)	2.08 (± 0.55)	*0.483*
5-HETE (ng/ml)	1.93 (± 0.29)	2.14 (± 0.23)	2.20 (± 0.28)	1.28 (± 0.25)	1.38 (± 0.47)	*0.489*
12,13-DiHOME (ng/ml)	5.73 (± 0.52) [a]	3.43 (± 0.41) [b]	4.18 (± 0.64) [b]	2.81 (± 0.61) [b]	4.22 (± 0.93) [ab]	*<0.001*
9.10-DiHOME (ng/ml)	2.62 (± 0.33) [a]	1.62 (± 0.14) [ab]	1.81 (± 0.23) [b]	1.25 (± 0.27) [ab]	1.73 (± 0.45) [ab]	*0.033*
12,13-EpOME (ng/ml)	12.89 (± 0.79)	14.39 (± 1.04)	14.84 (± 1.48)	7.91 (± 1.28)	16.36 (± 5.40)	*0.095*
9,10-EpOME (ng/ml)	27.38 (± 1.73)	32.20 (± 2.45)	35.76 (± 3.89)	20.97 (± 2.26)	41.55 (± 15.52)	*0.428*
13-HODE (ng/ml)	8.63 (± 0.53)	9.70 (± 0.74)	10.21 (± 1.08)	5.55 (± 0.60)	11.47 (± 4.11)	*0.067*
14,15-EET (ng/ml)	2.21 (± 0.21)	2.85 (± 0.25)	2.52 (± 0.22)	2.09 (± 0.26)	3.96 (± 1.59)	*0.273*
11,12-EET (ng/ml)	16.88 (± 1.13)	19.17 (± 1.53)	20.75 (± 3.13)	10.84 (± 1.90)	21.69 (± 10.79)	*0.058*
5-series-F_2-IsoP (ng/ml)	6.04 (± 0.13) [a]	6.59 (± 0.13) [b]	6.43 (± 0.14) [ab]	7.00 (± 0.43) [b]	6.90 (± 0.34) [ab]	*0.009*
Cytokines						
IL-1β (pg/mL)	1.78 (± 0.33)	2.14 (± 0.49)	2.13 (± 0.46)	2.74 (± 1.49)	0.74 (± 0.15)	*0.330*
IL-6 (pg/mL)	7.73 (± 2.00) [a]	20.47 (± 5.08) [ab]	25.13 (± 6.35) [ab]	20.39 (± 7.62) [ab]	44.65 (± 22.58) [b]	*0.002*
TNF-α (pg/mL)	9.02 (± 1.03)	9.18 (± 1.11)	10.54 (± 1.32)	11.54 (± 2.85)	7.38 (± 2.47)	*0.674*
Hospital Admission						
Days from the firsts symptoms (days)	10.62 (± 0.53)	11.29 (± 0.41)	9.22 (± 0.49)	8.30 (± 1.44)	8.57 (± 1.11)	-

Hospital length of stay (days)	3.38 (± 0.37)	3.98 (± 0.13)	8.18 (± 0.43)	31.14 (± 3.97)	22.57 (± 4.05)	-
Orotracheal intubation (days)	-	-	-	9.86 (± 1.44)	18.50 (± 4.40)	-
Sociodemographic data						
Sex						
Male (%)	57	39	59	50	86	*0.095*
Ethinicity (%)						*0.501*
White	45	51	52	40	29	
Pardo[a]	43	37	31	30	71	
Black	12	12	17	30	-	
Comorbidities						
Obesity	53	58	51	88	-	*0.021*
Hypertension	45	39	55	30	57	*0.375*
Chronic heart disease	6	12	12	20	29	*0.215*
COPD	-	2	10	-	-	*0.075*
Asthma	2	8	7	10	-	*0.477*
Diabetes	21	18	34	30	29	*0.269*
Diarrhea	43	51	47	50	43	*0.949*
Rheumatic diseases	11	10	5	10	-	*0.695*

[a]Values were expressed as mean (±SEM). P-values were obtained by ANOVA or Kruskal Wallis Test for quantitative variables and by Fisher Test for categorized variables. Values followed by the same letter are not different ($p<0.05$).[b] The number of sample/group can change according to the analyzed parameter*.**Abbreviations:** 5-Hydroxyeicosatetraenoic acid (5-HETE); 5-series F_2-isoprostanes (5-series-F_2-IsoP); 12-Hydroxyeicosatetraenoic acid (12-HETE); *9,10*-Dihydroxy-12-octadecenoic acid (9,10-diHOME); 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME); 9,10-epoxy-12Z-octadecenoic acid (9,10 EpOME);12,13-Epoxy-9(Z)-octadecenoic acid (12,13 EpOME); 13-Hydroxyoctadecadienoic acid (13-HODE); 11,12-epoxyeicosatrienoic acid (11,12-EET); 14,15-Epoxyeicosa-5.8.11-trienoic Acid (14,15-EET); 15-Hydroxyeicosatetraenoic acid (15-HETE); Chronic Obstructive Pulmonary Disease (COPD)

eTable 3. Eigenvectors (n=159 x 22 variables)

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
RBC	0.125	-0.530	0.177	0.049	0.303	0.126	0.018	0.065	0.221	-0.096
Hemoglobin	0.166	-0.518	0.153	0.100	0.333	0.063	-0.014	0.010	0.146	-0.027
Neutrophils	0.039	0.208	-0.162	-0.001	0.222	0.380	-0.265	0.288	0.109	0.207
Lymphocyte	0.148	0.038	0.178	0.012	-0.286	0.100	0.198	-0.073	-0.169	-0.625
C-reactive protein	-0.252	-0.110	-0.041	-0.219	-0.031	0.256	0.029	-0.065	-0.254	0.080
D-dimer	0.013	-0.071	-0.075	0.045	-0.093	0.158	-0.654	-0.152	-0.105	0.130
Triacylglycerol	0.134	0.256	0.207	0.311	0.294	-0.084	-0.087	0.083	-0.127	-0.138
IL-6	-0.039	-0.024	-0.094	-0.416	0.334	0.022	-0.015	0.084	-0.390	-0.063
MDA	-0.009	-0.094	-0.302	-0.105	0.358	-0.157	0.111	-0.065	-0.354	-0.230
PGE_2	0.118	-0.110	-0.086	0.015	-0.080	-0.516	-0.058	0.534	0.042	0.109
15-HETE	-0.168	0.233	-0.068	0.073	0.167	0.450	0.287	0.085	0.290	-0.037
12,13-DiHOME	0.261	-0.083	-0.363	0.302	0.019	0.028	0.261	-0.222	-0.156	0.189
9,10-DiHOME	0.289	-0.062	-0.342	0.266	-0.100	0.064	0.278	-0.079	-0.076	0.290
5-series-F_2-IsoP	-0.280	0.188	-0.083	0.089	0.153	-0.202	0.231	0.268	0.219	-0.045
C12:0	0.292	-0.098	-0.104	-0.266	-0.338	0.080	-0.005	0.344	-0.100	0.048
16:1 n7	0.355	0.231	0.018	0.068	0.150	0.056	-0.129	0.255	-0.058	-0.160
17:0	0.203	-0.096	-0.249	-0.229	-0.139	0.327	0.092	0.265	0.164	-0.238
18:3 n6	0.261	0.162	0.022	0.323	0.019	0.089	-0.208	-0.023	-0.126	-0.215
20:5 n3	0.372	0.203	0.160	-0.339	0.130	-0.064	0.098	-0.213	0.148	0.148
Age	-0.025	0.116	-0.475	-0.101	0.240	-0.143	-0.146	-0.118	0.099	-0.206
BMI (kg/m^2)	-0.021	0.060	0.334	0.067	0.121	0.168	0.228	0.268	-0.489	0.308
EPA/AA ratio	0.344	0.210	0.181	-0.345	0.127	-0.108	0.081	-0.239	0.171	0.180

Discriminant Analysis: SEVERITY versus PC1- PC10, using Cross validation

	True classification				
Groups	**1**	**2**	**3**	**4**	**5**
1	29	10	6	1	0
2	13	22	12	0	1
3	5	10	13	1	1
4	4	2	9	3	2
5	0	4	9	1	1
Total	51	48	49	6	5
Success	29	22	13	3	1
Success rate (%)	0.569	0.458	0.265	0.500	0.200

General success rate: 42.80%

FUTURE STUDIES

Inflammatory diseases are currently the major concern in the healthcare field, representing a significant challenge for medicine. The anti-inflammatory properties of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have attracted attention, and have already been extensively studied for their effects under different conditions. However, a better understanding about the use of EPA and DHA for hospitalized patients under parenteral nutrition care, may provide a strategy to treat other types of non-resolving inflammatory conditions.

Aspects relative to dosage, type of causal agent and the time of intervention must also be evaluated under the immune modulation context, since these factors can have an important influence on the effect of n-3 FA on acute and chronic inflammatory diseases prognosis. In addition, other studies have suggested that EPA and DHA and their oxylipins could contribute to reduce the continuous intake of anti-inflammatory drugs and, consequently, their known adverse effects. All these facts together stimulate our group to keep investigating the contribution of n-3 FA for human health.

ATTACHAMENTS

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Programa:	Ciência dos Alimentos
Área:	Nutrição Experimental
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Início da Contagem de Prazo:	21/12/2020
Data Limite para o Depósito:	21/06/2023
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Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 21/12/2020

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Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
DPG5006-1/1	A Crise das Pandemias e as Oportunidades para a Construção de um Mundo mais Seguro, Menos Desigual e Sustentável - II (Pró-Reitoria de Pós-Graduação - Universidade de São Paulo)	22/03/2021	30/05/2021	30	2	100	A	N	Concluída
MCM5900-5/1	Interações entre Metabolismo e Inflamação (Faculdade de Medicina - Universidade de São Paulo)	01/04/2021	09/06/2021	60	4	100	A	N	Concluída
FBA5905-2/4	Planejamento Experimental e Análise Multivariada	13/04/2021	26/07/2021	60	0	-	-	N	Matrícula cancelada
NUT5706-1/3	Antioxidantes Dietéticos (Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo)	05/05/2021	08/06/2021	60	4	100	A	N	Concluída
MCM5797-6/5	Obesidade (Faculdade de Medicina - Universidade de São Paulo)	08/06/2021	28/06/2021	60	4	100	A	N	Concluída
FBA5741-4/3	Química e Bioquímica de Alimentos I	31/08/2021	07/10/2021	60	4	100	B	N	Concluída
QBQ5733-9/1	Radicais Livres em Sistemas Biológicos (Instituto de Química - Universidade de São Paulo)	06/09/2021	28/11/2021	180	0	-	-	N	Matrícula cancelada
RFA5760-5/2	Farmacogenética (Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo)	16/11/2021	29/11/2021	60	4	100	A	N	Concluída
FBA5908-1/2	Nutrição Humana	03/04/2022	07/05/2022	45	3	100	A	N	Concluída
FBA5909-1/2	Microbiologia de Alimentos	09/05/2022	12/06/2022	45	3	100	A	N	Concluída

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COMPLEMENTARY WORK

Co-authorships: de Mello Barros Pimentel MV, Bertolami A, **Fernandes LP,** Barroso LP, Castro IA. Could a lipid oxidative biomarker be applied to improve risk stratification in the prevention of cardiovascular disease? Biomed Pharmacother. Apr 2023;160:114345. doi:10.1016/j.biopha.2023.114345

Co-authorshopis: Mailho-Fontana PL, Antoniazzi M, Coelho G, Pimenta D, **Fernandes L,** Kupfer A, Brodie E, Jared C. Milk provisioning in oviparous caecilian amphibians. Submitted to Science in 28th May, 2023.

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Manuscript Title: Milk provisioning in oviparous caecilian amphibians

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Journal: Science

Article Type: Research Article

Assigned To: Sacha Vignieri

Title: Milk provisioning in oviparous caecilian amphibians

Short Title:

Abstract: In most animals, the yolk is the only form of nutritional investment offered by the female to the embryo. Some species, however, have developed parental care behaviors which may be associated with parental specialized food provisioning essential for offspring survival, such as milk in mammalians. Here we document that females of the oviparous amphibian caecilian Siphonops annulatus provide milk to altricial nestlings during parental care. Daily for two months, young ingest milk released through the maternal cloaca stimulated by their begging behavior. Milk originates from the maternal oviduct epithelium's hypertrophied acinar glands and mainly comprises lipids and carbohydrates. Our data reveal a distinct modality of lactation in non-mammal species and expand the knowledge of parental care and communication in caecilians.

Field Codes:

Primary

Ecology

Secondary

Evolutionary biology