UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Biociências
Câmpus do Litoral Paulista

EVALUATION OF ACUTE HEPATIC TOXICITY AND INFLAMMATION INDUCED BY NITROSAMINES *IN Mus musculus* AND *Danio rerio*

ISABELLY ANNUNCIATO

SÃO VICENTE - SP
2024

Instituto de Biociências - Câmpus do Litoral Paulista
Praça Infante D. Henrique s/nº - CEP 11330-900 - São Vicente (SP) - Brasil
Tel. (13) 3569-7100 - Fax (13) 3569-7146 - diretor.clp@unesp.br

UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

INSTITUTO DE BIOCIÊNCIAS
CÂMPUS DO LITORAL PAULISTA

EVALUATION OF ACUTE HEPATIC TOXICITY AND INFLAMMATION INDUCED BY NITROSAMINES *IN Mus musculus* AND *Danio rerio*

CANDIDATE: ISABELLY ANNUNCIATO

ADVISOR: MARCOS HIKARI TOYAMA

CO-ADVISOR: SÉRGIO F. MAIA DE SOUSA

Dissertation presented to the São Paulo State University (UNESP), Institute of Biosciences, São Vicente, to obtain the title of Master in the Postgraduate Program in Biodiversity of Coastal Environments.

SÃO VICENTE - SP
2024

A615e

Annunciato, Isabelly

Evaluation of acute hepatic toxicity and inflammation induced by nitrosamines in Mus musculus and Danio rerio / Isabelly Annunciato. -- São Vicente, 2024

77 p. : il., tabs., fotos

Dissertação (mestrado) - Universidade Estadual Paulista (UNESP), Instituto de Biociências, São Vicente

Orientador: Marcos Hikari Toyama

Coorientador: Sérgio Filipe Maia de Sousa

1. Biochemistry. 2. Enzymatic analysis. 3. Molecular dynamics. 4. Histopathology. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Universidade Estadual Paulista (UNESP), Instituto de Biociências, São Vicente. Dados fornecidos pelo autor(a).

Essa ficha não pode ser modificada.

Evaluation of acute hepatic toxicity and inflammation induced by nitrosamines in *Mus musculus* and *Danio rerio*

Abstract

Nitrosamines are emerging contaminants known for their carcinogenic and oxidative properties. This study investigates the hepatotoxic and acute inflammatory effects of two nitrosamines, N-Nitrosodiethylamine (NDEA) and N-Nitrosodimethylamine (NDMA), on *Mus musculus* (mice) and *Danio rerio* (fish). Mice were exposed to 0,10 mg/kg and 0,20 mg/kg of NDEA and NDMA. Subsequent biochemical, histological, and hematological analyses were conducted to detect potential liver damage induced by these contaminants. Blood samples were collected from exposed and non-exposed groups, and liver tissues were evaluated for histological evaluation. Blood aliquots were used for hematological analysis, employing smear techniques and cell counting in a Neubauer chamber. Biochemical monitoring of liver function markers such as lactate dehydrogenase (LDH), oxalacetic transaminase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and C-reactive protein (CRP) was performed. Additionally, levels of interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), malondialdehyde (MDA), and lipoprotein-associated phospholipase A2 (Lp-PLA2), also known as platelet-activating factor acetylhydrolase (PAF-AH), were evaluated. PAF-AH is crucial in controlling inflammation, as it regulates plasma levels of PAF, a key lipid factor in the progression and development of inflammation. Our project aimed to demonstrate that nitrosamines can induce hepatocyte dysfunction, affect energy metabolism, and highlight the role of PAF in causing hepatotoxicity. In addition to *in vivo* and biochemical investigations, molecular docking, and molecular dynamics simulations were performed at BioSIM (University of Porto) to explore the potential of nitrosamines as non-selective inhibitors of Land's Cycle important enzymes (lysophosphatidylcholine acyltransferase 3 (LPCAT3), Cytosolic Phospholipase A2 (cPLA2) and PAF-AH). Considering nitrosamines are commonly found in water environments, we also monitored hematological and hepatic histological modifications induced by these compounds in *Danio rerio* (zebrafish), the hematological analysis was related to nuclear abnormalities in erythrocytes. This comprehensive study integrates biochemical, histological, and computational approaches to elucidate the hepatotoxic effects of nitrosamines and underscores the significance of PAF in mediating these effects. Through these investigations, we aim to provide deeper insights into nitrosamine-induced liver damage and inflammation mechanisms. Our results demonstrated that the nitrosamines evaluated caused increased liver damage in mice during a 24-hour exposure. Biochemical analysis of liver function markers, lipid peroxidation, and cytokines indicated elevated levels related to compound metabolism during both 4-hour and 24-hour exposures. In fish, damage was observed in liver tissue, gills, and nuclear abnormalities in erythrocytes; *In vitro* studies on PAF-AH inhibition by nitrosamines confirmed the results obtained from molecular simulation assays, demonstrating that the compounds have low affinity for the enzyme. The Molecular Dynamics results showed affinity between NDMA and cPLA2 and NDEA and LPCAT3.

Keywords: Nitrosamines, acute inflammation, liver function, *Mus musculus*, *Danio rerio*.

Table of Contents (TOC)

1.Introduction

1.1. N-nitrosamines, oxidative stress and mechanisms of inflammation

Nitrosamines became the focus of toxicological studies in 1954, when Magee and Barnes linked these compounds to liver damage in rats exposed to N-nitrosodimethylamine (NDMA). Subsequently, in 1956, the same researchers observed that rats fed NDMA developed liver tumors. The hepatotoxicity of nitrosamines was further confirmed in 1978, when Cassens et al. demonstrated liver damage in animals treated with nitrite. Nitrosamines, characterized by a nitroso group attached to an amine, encompass at least 300 documented types of hydrophilic molecules (Gushgari and Halden, 2018).

Classified as probably carcinogenic (group 2A) by the International Agency for Research on Cancer (IARC) and the United States Environmental Protection Agency (US EPA), N-nitrosamines are formed through various mechanisms involving nitrogen oxide and secondary amines in chemical and fermentative processes (IARC 1978; Park et al. 2015). These compounds can be synthesized endogenously and are found in processed foods, cigarette smoke, and as metabolites of certain drugs. Drinking water treatment is a significant source of nitrosamine production, particularly during the chloramination process, where chlorine and ammonia react to form chloramine, subsequently leading to the formation of NDMA and NDEA (Mitch et al. 2003). According to the US EPA and the Integrated Risk Information System (IRIS), concentrations of 0.7 ng/L of NDMA or 0.2 ng/L of NDEA in drinking water are associated with a lifetime cancer risk of 10^{-6} (US EPA 1987a, b). This highlights the significant health risks posed by these contaminants even at very low concentrations, underscoring the importance of monitoring and regulating their presence in drinking water.

Table 1: source of nitrosamines formation

Amine	Nitrosating source	Contaminant
Triethylamine	Sodium nitrite in acid mediumdiethylnitrosamine - NDEA (dealkylation)	
Amida	Nitrosating source	Contaminant
Dimethylformamide (DMF) undergoes hydrolysis in (H^+ or OH^-) generating DMA	Sodium nitrite (NaNO2)	dimethylnitrosamine - NDMA

Source: adapted from Paiva and Paiva, 2022

Nitrosamines became the focus of toxicological studies in 1954 when Magee and Barnes linked these compounds to liver damage in rats exposed to N-nitrosodimethylamine (NDMA). By 1956, they further discovered that rats fed NDMA developed liver tumors. This hepatotoxicity was corroborated in 1978 by Cassens et al., who observed similar liver damage

in animals treated with nitrite. Characterized by a nitroso group attached to an amine, nitrosamines are a family of hydrophilic compounds consisting of at least 300 different molecules (Gushgari and Halden, 2018).

The International Agency for Research on Cancer (IARC) and the United States Environmental Protection Agency (US EPA) classify N-nitrosamines as probably carcinogenic (group 2A) (IARC 1978). These compounds can form via mechanisms involving nitrogen oxide and secondary amines in chemical and fermentative processes (Park et al. 2015). Nitrosamines can be synthesized endogenously and are found in processed foods, cigarette smoke, and as metabolites of certain drugs. Drinking water treatment is a significant source of nitrosamine production, particularly during chloramination, where chlorine and ammonia react to form chloramine, leading to the formation of NDMA and NDEA (Mitch et al. 2003). According to the US EPA and the Integrated Risk Information System (IRIS), concentrations of 0.7 ng/L of NDMA or 0.2 ng/L of NDEA in drinking water are associated with a lifetime cancer risk of 10^{-6} (US EPA 1987a, b). This underscores the significant health risks posed by these contaminants even at very low concentrations, emphasizing the need for strict monitoring and regulation.

Economic development has exacerbated the contamination of water sources with emerging contaminants, both chemical and biological (Hespanhol, 2015). Studies have shown that the presence of NDMA and NDEA in water is often correlated with high levels of organic matter, typically in areas affected by sewage, nitrogen-based fertilizers, and industrial waste (Krasner et al., 2012). A recent study by the State University of Campinas analyzed eighteen drinking water samples from thirteen cities in the Campinas metropolitan region, São Paulo, Brazil. Seven types of nitrosamines were quantified, with NDMA detected in 89% of the samples and at the highest concentration of 67 ng/L (Vizioli, Hantao & Montagner, 2021). The Brazilian Ministry of Health stipulates a maximum allowed NDMA concentration in water of 0.0001 mg/L (Ordinance GM/MS No. 888, May 4, 2021).

NDMA and NDEA, being polar compounds, do not bioaccumulate but are resistant to biodegradation (Wilczak et al. 2003; Charrois et al. 2007). These compounds have garnered increasing attention due to their significant potential for inducing tumor development in humans (Gushgari and Halden, 2018). Studies have demonstrated that nitrosamines can cause various biochemical and genotoxic changes, including the production of reactive oxygen species (ROS), lipid peroxidation, increased nitric oxide release, decreased total antioxidant capacity, oxidative DNA damage, and inhibition of antioxidant enzymes such as catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase. They also exhibit pronounced

pro-inflammatory activity (Waly et al., 2018). Nitrosamines induce oxidative stress in a dose-dependent manner, affecting membrane integrity and antioxidant enzyme activity, leading to increased lipid peroxidation (Ahotupa et al., 1987; Bilal et al., 2017; Sheweita et al., 2017). Lipid peroxidation occurs when membrane phospholipids encounter ROS, resulting in hydroperoxidized lipids and alkyl radicals. This process alters membrane fluidity and integrity (Juan et al., 2021).

Polyunsaturated fatty acids (PUFAs) in phospholipids undergo metabolic recycling via the "deacylation-reacylation cycle" or "Lands Cycle," involving phospholipases A2 (PLA2s) and lysophospholipid acyltransferases (LPATs). Changes in membrane phospholipids activate this cycle (Juan et al., 2021). Cytosolic phospholipase A2 (cPLA2) releases PUFAs, forming platelet-activating factor (PAF) and arachidonic acid (AA). This cycle can be intensified by ROS, increasing the production of PAF, AA, eicosanoids, and pro-inflammatory mediators (Wu et al., 2016; Adebiyi et al., 2019). PAF, a lipid mediator, contributes to inflammatory and immune responses and is regulated by the enzyme PAF-AH (Ulambayar et al., 2019).

PAF is involved in various physiological processes, but excessive amounts can cause inflammation and oxidative stress (Papakonstantinou et al., 2017; Travers et al., 2021). PAF biosynthesis occurs via remodeling, initiated by cPLA2 acting on phosphatidylcholine, producing arachidonic acid, eicosanoids, and lysophosphatidylcholine (LPC), which is then converted to PAF by an acetyltransferase (Lys-PAFAT) (Wykle et al., 1980). Under normal conditions, PAF synthesis is minimal but increases during inflammation via the remodeling pathway. Various agents can stimulate PAF synthesis, including antigen-antibody interactions and inflammatory mediators (Owen et al., 2007; Kono et al., 2019; Ashraf et al., 2022). This project aims to investigate nitrosamines' potential to increase lipid peroxidation and ROS, thereby enhancing cPLA2 and LPCAT activity, leading to elevated arachidonic acid production and oxidative stress (Gawel et al., 2004; Su et al., 2019; Ferrara et al., 2021). Although less studied, PAF is a potent pro-inflammatory agent generated during cPLA2 action by the remodeling pathway (Linkous and Yazloviskaya, 2010; Stanca et al., 2013; Oehler et al., 2021). Monitoring PAF concentration is crucial for controlling inflammation progression. Inhibition of extracellular PAF-AH is a key point in the inflammatory process (Toyama et al., 2022). Therefore, this project will evaluate nitrosamines' ability to interact with extracellular PAF-AH.

1.2. Liver system in Swiss mice

Mice were introduced as animal models in laboratories in the 19th century and have since become a classical choice for in vivo testing. Swiss mice (Mus musculus) are particularly favored due to their ease of care, minimal space requirements, and short life cycles. Additionally, as mammals, mice share significant physiological and genetic similarities with humans (Harkness and Wagner, 1993). The liver in mice performs critical metabolic functions, absorbing, transforming, and storing both nutrients and toxic substances, which are later released into the bloodstream. This makes the liver essential for biotransformation processes. Moreover, it is the organ responsible for bile production and storage (Guillouzo, 1998).

Nitrites used in food preservation can transform into nitrosamines under the influence of heat and gastric acid. Once absorbed in the intestine, nitrosamines enter hepatocytes via the portal venous system, disrupting the liver's detoxification processes by interfering with cytochrome P450 enzymes. This interference can lead to cirrhosis and liver cancer (Magee and Barnes, 1956). The biotransformation of nitrosamines occurs within the microsomal system of liver P450. Initially, nitrosamines undergo hydroxylation of the alpha carbon of the alkyl group, catalyzed by P450, resulting in the formation of an aldehyde or ketone and an unstable primary N-nitrosamine. In the subsequent stage, unstable carbocations are produced that can bind to or modify genetic material (Paiva and Paiva, 2022). In this project, mice will be utilized for in vivo testing to ensure a comprehensive assessment of plasma liver markers following exposure to nitrosamines. This approach is designed to better understand the hepatotoxic effects and the biotransformation mechanisms involved.

1.3. Liver system in teleost fish

The literature describes the liver as a multifunctional organ, capable of acting in digestion, storage, detoxification, serum protein synthesis and vitellogenin production. In addition, the liver is fundamental for performing the deposition and metabolization of fats and carbohydrates and is also responsible for maintaining the metabolic balance of the body (Sales, C.F. et al., 2017). Most teleost fish have a liver that is reddish-brown in color and divided into lobules, located cranially and ventrally. In the digestion process, liver cells are responsible for secreting bile into the duodenum (Shtewi and Abusre, 2000, Sales, C.F. et al., 2017).

In many teleosts the liver has the so-called melanomacrophage centers (MMC), formed by aggregates of macrophages. Melanomacrophage centers can be formed in the kidneys, liver and spleen of fish when they are exposed within the aquatic ecosystem to various chemical agents,

such as contaminants, pollutants and biological agents. MMCs contain lipogenic pigment, lipofucsin, melanin, and hemosiderin, which may assist in the defense mechanism of teleosts (Roberts, 2012). In fish, after filtration of blood by the gills, the liver is responsible for performing the elimination and detoxification of various substances that have been absorbed by the body, which is extremely important for the maintenance of life of these animals (Girón-Pérez et al., 2007 and Covantes-Rosales et al., 2019).

The slower blood flow in fish relative to cardiac output causes the bile flow to circulate fifty times slower, which slows the clearance of toxic products, resulting in a longer time of action of chemicals in the body (Goessling and Sadler, 2015). Studies have suggested the hypothesis that toxic substances at lower concentrations can cause severe liver disorders when compared to mammals (Campos et al., 2008 and Goessling and Sadler, 2015). Exposure of liver tissue to chemical agents constitutes an important health risk, causing injury by the production of free radicals that initiate the process of lipoperoxidation (Faheem and Lone, 2017 and Xu et al., 2021). As in mammals, the fish liver is capable of producing humoral compounds, such as acute phase proteins (APPs) of the inflammatory response, among them inflammatory mediators and c-reactive protein (CRP). APPs are synthesized by various cells, among them hepatocytes. It is considered that these proteins have a universal character in animals, because their structure and acute phase response are very similar in all animals (Hoole et al., 2003, Roy et al., 2017 e Charlie-Silva et al., 2019).

1.4. The zebra fish as water polution bioindicator of toxicity

As a freshwater fish that can grow to around five centimeters in size as an adult, zebrafish (*Danio rerio*) are more cost-effective than rodents commonly used in research. Additionally, zebrafish require less space compared to rodents (Gheno, 2015). The zebrafish genome shares a 70-80% homology with the human genome, which is a significant similarity to mammals, making them valuable for various research applications, including behavioral, toxicological, and genetic studies (Lieschke & Currie, 2007). The use of *Danio rerio* in this project is further justified by the National Council for the Control of Animal Experimentation (CONCEA), which, through Normative Resolution No. 34, considers this species suitable for scientific studies. Numerous clinical and toxicological studies in the literature have successfully employed this teleost fish as an animal model, highlighting its efficacy and relevance in research. This approach ensures that the zebrafish can serve as an effective model for studying

the effects of nitrosamines, given their genetic similarities to humans and practical advantages in laboratory settings.

1.5. Molecular Anchoring and Molecular Dynamics of Nitrosamines with Extracellular PAF-AH (Lipoprotein-associated PLA2)

1.5.1. Molecular docking

Molecular docking, also known as anchoring, is a well-established in silico technique primarily aimed at drug discovery and understanding the interaction between drugs and macromolecules. This technique is instrumental in identifying new therapeutic compounds, predicting target-ligand interactions at the molecular level, and delineating the relationship between chemical structures (Pinzi & Rastelli, 2019). Additionally, docking can predict the effects of emerging contaminants in aquatic environments by considering the homology between human enzymes and those of aquatic animals. In studies involving various aquatic vertebrates, researchers have demonstrated interactions between anti-inflammatory drugs and their respective enzymes in these animals (Walker & McEldowney, 2013; Kumar et al., 2019).

The docking process involves two fundamental steps: predicting the conformation of the ligand, including its position and orientation within the molecular structure, and assessing the binding affinity. These steps are closely related to the data acquisition methods and their scoring schemes. Understanding the location of catalytic sites significantly enhances the efficiency of docking. Often, the catalytic site is known beforehand, allowing researchers to obtain information by comparing the target protein with a protein family or established protein structures (Meng et al., 2011). This prior knowledge helps in accurately predicting the interactions and potential impacts of the ligands. By leveraging molecular docking techniques, researchers can effectively explore the biochemical interactions of new drugs and contaminants, thereby advancing both pharmaceutical development and environmental toxicology studies.

1.5.2. *Molecular Dynamics Simulations*

Molecular dynamics (MD) is a technique used to provide detailed data on the movement of individual particles over time, describing their interactions empirically through a potential energy function (Karplus & McCammon, 2002). Several programs are widely used for MD simulations, including AMBER (Assisted Model Building with Energy Refinement) (Case et al., 2005), CHARMM (Chemistry at Harvard Macromolecular Mechanics) (Brooks et al., 2009), GROMOS (GROningen Molecular Simulation) and GROMACS (GROningen Machine for Chemical Simulation) (Van Der Spoel et al., 2005). In this project, we hypothesize that the

studied nitrosamines can induce acute liver inflammation and toxicity in Swiss mice. To monitor potential liver changes, we will conduct biochemical and histological investigations of the mice following exposure to NDMA and NDEA. Additionally, molecular dynamics studies were employed to evaluate the interaction between nitrosamines and extracellular PAF-AH, aiming to uncover potential mechanisms of enzyme inhibition. These MD simulations were carried out in collaboration with Prof. Dr. Sergio Filipe Sousa's laboratory, BioSIM, at the University of Porto in Portugal. By integrating these advanced techniques, we aim to gain a comprehensive understanding of the biochemical and molecular impacts of nitrosamines, providing insights into their hepatotoxic effects and interactions with key enzymes.

1.5.3. *In silico* analysis contextualization

The initial aim of this research project was to analyze the enzyme PAF-AH and the compounds NDEA and NDMA to assess the impact of these nitrosamines on the regulation of PAF levels. However, the initial results did not show a significant interaction, suggesting a possible absence or weak interaction between the nitrosamines and PAF-AH. This finding necessitated an expansion of the analyses after the initial phase of molecular dynamics, which included both PAF-AH and the main compounds, NDMA and NDEA. Advancing the investigation, the research entered a more detailed phase, incorporating docking studies and molecular dynamics for the metabolites of these nitrosamines. The goal was to investigate the previously observed lack of a direct relationship between nitrosamines and PAF-AH. The new findings raised questions about the possible interaction of nitrosamines and their metabolites with other enzymes linked to PAF, as well as the potential of these compounds to influence membrane remodeling under oxidative stress. Given the ability of nitrosamines, such as NDEA and NDMA, to act as pro-oxidants, increasing the production of reactive oxygen species (ROS) or reducing the cell's antioxidant capacity, a new dimension of analysis emerged. This aspect is particularly relevant because the metabolism of these substances in the liver, through cytochrome P450 enzymes, results in the formation of free radicals and other reactive species capable of damaging essential cellular components. This process may contribute to both carcinogenesis and other pathologies associated with oxidative stress. It raises the hypothesis that, in addition to possible direct interaction with PAF-AH or other enzymes related to PAF, nitrosamines may exert significant effects through the induction of oxidative stress. Therefore, the research expanded to include molecular dynamics assays involving key enzymes in the control and generation of PAF, as well as in membrane remodeling, considering the potential

pro-oxidant role of nitrosamines. This holistic and adaptive approach, guided by the acquisition of results and the evolution of research hypotheses, is essential for a deep and comprehensive understanding of the mechanisms involved. It also explores all possible implications of these interactions in the fields of biochemistry and pharmacology.

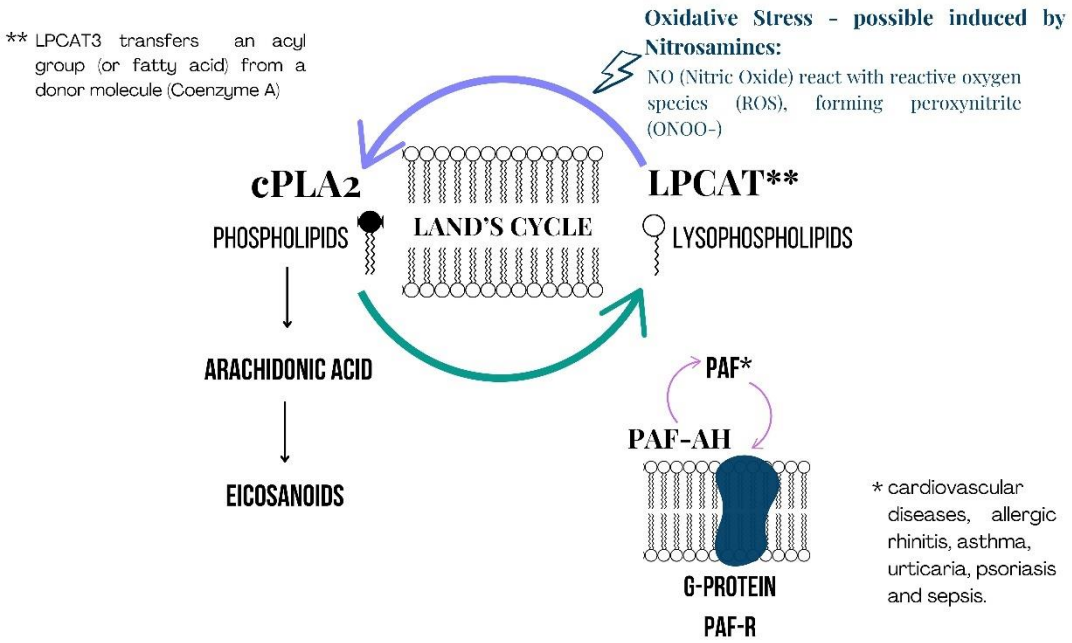

Figure1: The Land's cycle is a process that responds to plasma membrane damage by promoting membrane remodeling. Cytosolic phospholipase A2 (cPLA2) cleaves membrane phospholipids at the sn2 position, releasing arachidonic acid (a precursor to inflammatory processes) and lysophospholipids. Lysophospholipids lead to the production of platelet-activating factor (PAF), which is associated with cardiovascular diseases and allergic reactions. The enzyme platelet-activating factor acetylhydrolase regulates PAF levels. Membrane remodeling is facilitated by lysophosphatidylcholine acyltransferase 3, which reintroduces a fatty acid into the sn2 position of the membrane phospholipid. Source: own authorship.

1.6. Presentation of the Ligand and Target Molecule Files.

1.6.1. PAF-AH.

PAF-AH (lipoprotein-associated phospholipase A2) is a blood plasma enzyme that degrades oxidized and modified phospholipids, including Platelet Activating Factor (PAF), a mediator involved in inflammation from tissue injury, infection, and ischemia (Travers et al., 2021). PAF-AH binds to circulating lipoproteins like LDL and HDL. It consists of two subunits, with the alpha subunit containing the catalytic site (Samanta & Bahnson, 2008). The enzyme features an active site microenvironment that interacts with various inflammatory molecules, such as C-reactive protein and protein S, underscoring its biological significance (Travers et al., 2021; Kono & Arai, 2019). The histidine residue in the active site is crucial for its function and is highly conserved (Kono & Arai, 2019). PAF-AH, a 44 kDa protein, includes a catalytic triad

(serine, aspartic acid, and histidine) typical of αβ-hydrolases and a GXSXG motif characteristic of neutral lipases (Samanta & Bahnson, 2008). The enzyme's structure features an alpha/beta-hydrolase lipase fold with the catalytic triad in a hydrophobic pocket. The residual catalytic site comprises amino acids Phe110, Leu153, Phe274, Trp298, His272, Tyr160, Gln352, and Phe322, with Phe274 and Leu153 stabilizing the tetrahedral intermediate's negative charge, acting as the oxyanion hole (Samanta & Bahnson, 2008). This site stabilizes the catalytic triad. Their crystallography study (PDB:3D5E) showed a covalent interaction between PAF-AH and the ligand paraoxon (DEP), used as a reference to determine the catalytic site's coordinates and compare results with other ligands.

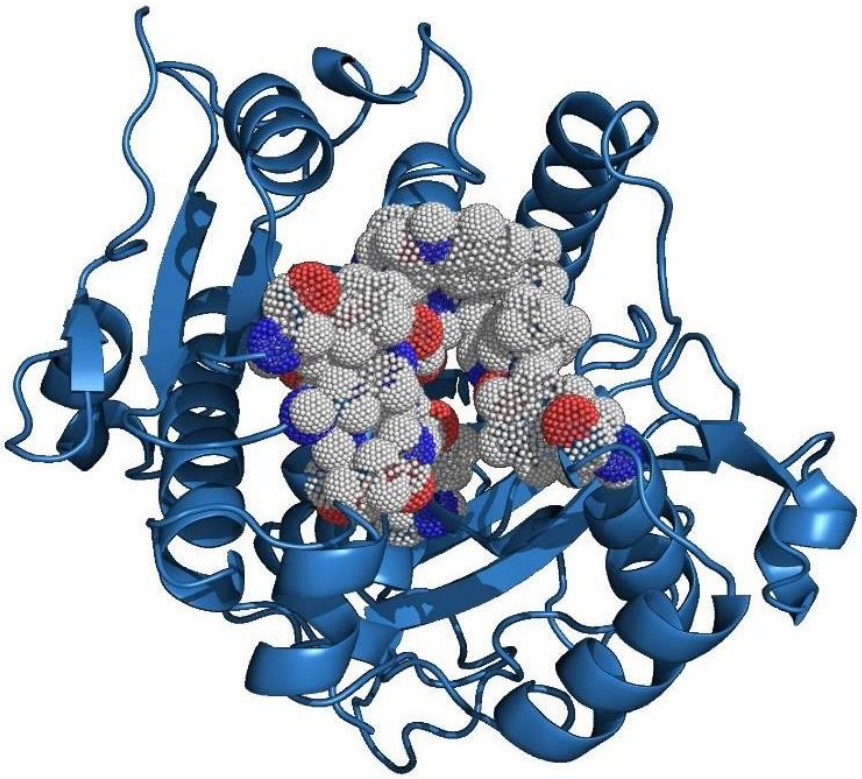

Figure 2:PAF-AH enzyme (chain A). The active site cavity is visualized using PyMOL software, with dots representing the cavity. Different colors indicate various elements: white for carbon, red for oxygen, and blue for nitrogen (This color coding is only applied to the dots, not to the cartoon representation of the rest of the enzyme structure). The enzyme is displayed in cartoon representation using PyMOL. The structure of PAF-AH (PDB ID: 3D5E) was obtained from the RCSB Protein Data Bank (http://www.rcsb.org).

1.6.2. cPLA2

Cytosolic phospholipase A2 (cPLA2) is crucial for regulating inflammatory responses by releasing arachidonic acid from membrane phospholipids. Arachidonic acid is a precursor to bioactive lipid mediators like prostaglandins and leukotrienes, which are essential for inflammation and immune responses (Nojima, 1991). cPLA2 removes polyunsaturated fatty

acids from the sn-2 position of phospholipids, forming platelet-activating factor (PAF) and arachidonic acid (AA). Reactive oxygen species and oxidative stress can intensify cPLA2 activity, increasing PAF, arachidonic acid, eicosanoids, and pro-inflammatory mediators (Adebiyi et al., 2019). The enzyme's catalytic activity is centered in the α/β hydrolase domain, specifically involving Ser228 and Asp549. cPLA2 activity is also regulated by calcium ions, which bind to the enzyme, causing a conformational change that enhances its affinity for phospholipids (Dessen et al., 1999). Understanding cPLA2's molecular mechanisms and regulation is key for developing targeted therapies to modulate inflammation. Giripladib, a selective cPLA2α inhibitor, has been used as a reference ligand in studies (Duvernay, 2015). This knowledge supports designing therapies to precisely control inflammation and associated diseases.

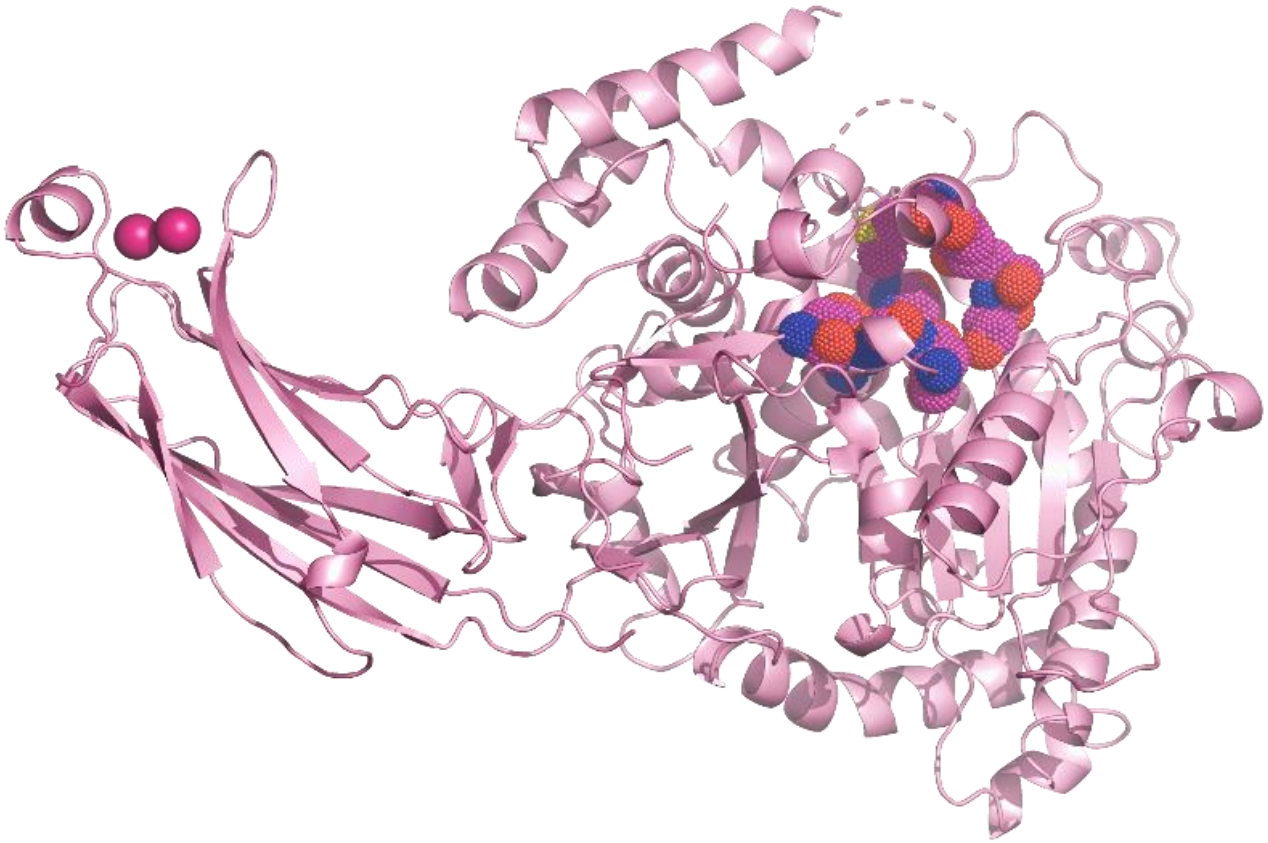

Figure 3: cPLA2 enzyme. The dots represent the active site cavity, and the two purple spheres depict calcium ions. The active site cavity is visualized using PyMOL software, with dots representing the cavity. Different colors indicate various elements: pink for carbon, red for oxygen, and blue for nitrogen (This color coding is only applied to the dots, not to the cartoon representation of the rest of the enzyme structure). The enzyme is displayed in cartoon representation using PyMOL. The structure of cPLA2 (PDB ID: 1CJY) was obtained from the RCSB Protein Data Bank.

1.6.3. LPCAT3.

Phosphatidylcholine (PC) is the primary constituent of cell membranes, synthesized de novo through the Kennedy pathway and extensively remodeled via the Land's cycle, which involves significant deacylation-reacylation. The enzyme lysophosphatidylcholine acyltransferase (LPCAT) catalyzes the re-acylation step. Among the four LPCAT members in humans, LPCAT3 specifically incorporates polyunsaturated acyl groups onto the sn-2 position of lysophosphatidylcholine. This activity modulates membrane fluidity and influences membrane protein functions (Zhang et al., 2021). LPCAT3 is crucial in lipid metabolism, particularly in the biosynthesis of phosphatidylcholine, a major phospholipid in cell membranes. Belonging to the lysophospholipid acyltransferase (LPLAT) family, LPCAT3 catalyzes the acylation of lysophospholipids to produce phospholipids. The enzyme's catalytic site consists of His388 and Asn352, with a tunnel formed by tyrosines (Tyr394, Tyr298, Tyr143, and Tyr151), all of which play important roles in the catalytic process (Zhang et al., 2021). By facilitating the incorporation of polyunsaturated fatty acids, LPCAT3 helps maintain the proper function and flexibility of cellular membranes, highlighting its essential role in cellular physiology and lipid metabolism. Lysophosphatidylcholine (LPC), a substrate of LPCAT3, was crystallized in complex with this enzyme (Zhang, et. al. 2021). Therefore, LPC was employed as the reference ligand for LPCAT3.

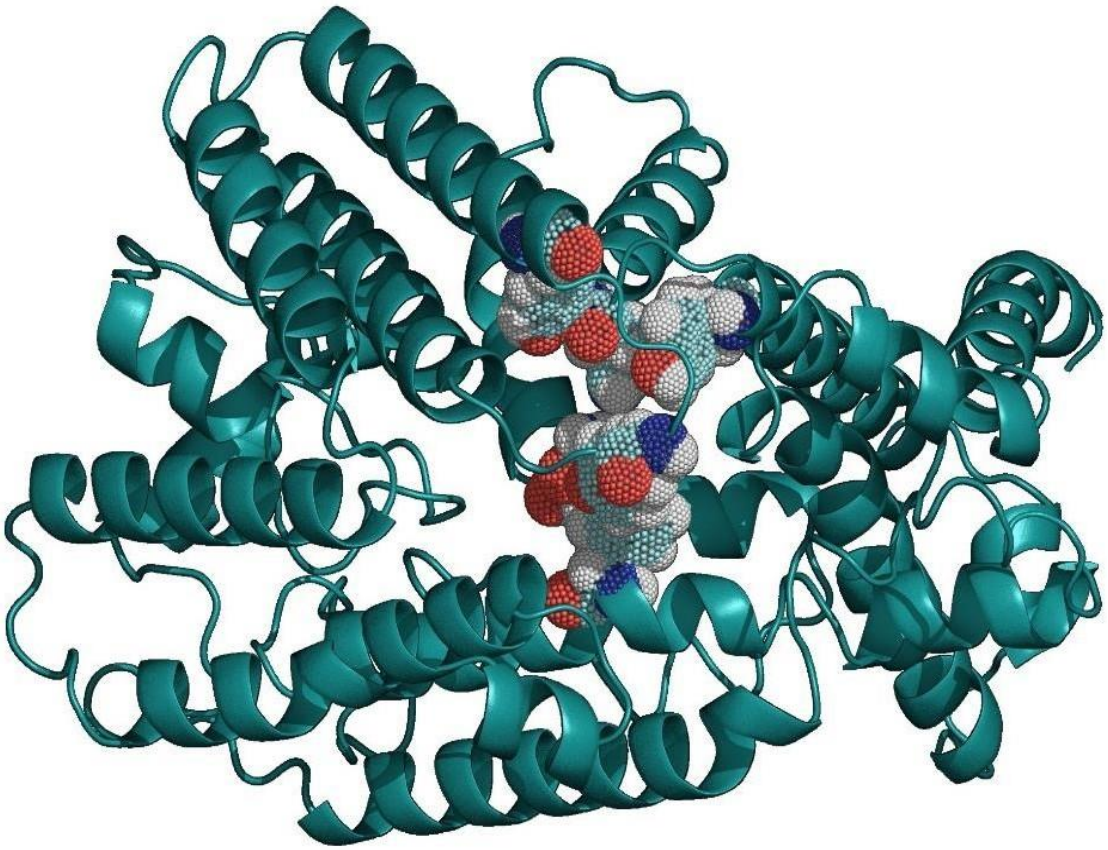

Figure 4: LPCAT3 enzyme. The circles show the active site cavity and the important tyrosines. The active site cavity is visualized using PyMOL software, with dots representing the cavity. Different colors indicate various elements: white for carbon, red for oxygen, and blue for nitrogen (This color coding is only applied to the dots, not to the cartoon representation of the rest of the enzyme structure). The enzyme is displayed in cartoon representation using PyMOL. The structure of LPCAT3 (PDB ID: 7F3X) was obtained from the RCSB Protein Data Bank.

1.7. Ligands (NDEA, NDMA and Metabolites).

N-nitrosamines, identified by the International Agency for Research on Cancer (IARC) in 1978 as probable carcinogens (Group 2A), are produced through various mechanisms involving the interaction of nitrogen oxides with secondary amines in industrial chemical processes and natural fermentations (Park et al., 2015). These compounds can form endogenously in the body, be present in processed foods such as meats and cheeses, be emitted by cigarette smoke, and result from the metabolism of certain drugs. They are also frequently detected in drinking water (Mitch et al., 2003). The persistence of N-nitrosamines in aquatic environments is particularly concerning. Unlike their rapid degradation in outdoor air through solar photolysis and other atmospheric reactions, nitrosamines such as NDMA and NDEA are more resistant in water. Photolysis can degrade these substances in the surface layers of bodies of water exposed to

sunlight, but this process is often limited by the absorption of light by dissolved substances (Papakonstantinou et al., 2017; Park et al., 2015). The primary catalyst for the bioactivation of NDMA to reactive intermediates in human liver microsomes is P450 2E1. The oxidation of the methyl group, known as α-methyl hydroxylation, results in the formation of α-hydroxy-NDMA, an unstable and mutagenic intermediate. This intermediate decomposes spontaneously, producing formaldehyde and methyl diazohydroxide. Formaldehyde can undergo further oxidation to formic acid and CO2, while methyl diazohydroxide forms the highly electrophilic methyldiazonium ion, which can alkylate DNA or undergo solvolysis to produce methanol (Li and Stephen, 2022).

The mutagenicity and genotoxicity of NDMA are well-established. NDMA induces alkylation of DNA and protein through two reactive intermediates: methyldiazonium ion and formaldehyde. Methyl DNA adducts formed by the methyldiazonium ion play a significant role in NDMA-induced carcinogenesis. Additionally, formaldehyde can generate DNA adducts, including cross-links or hydroxymethylene adducts (Li and Stephen, 2022). This comprehensive understanding of NDMA's pathways and impacts underscores the importance of monitoring and mitigating nitrosamine contamination in various environments to protect public health (Figure 5). The metabolic activation of N-nitrosodiethylamine (NDEA) for carcinogenicity is primarily facilitated by cytochrome P450 enzymes, specifically P450 2E1 and P450 2A6. During the P450-catalyzed hydroxylation at the α-carbon of the ethyl group in NDEA, an unstable intermediate, ethyl diazohydroxide, decomposes to generate the electrophilic ethyldiazonium ion (Erkekoglu and Baydar, 2010; Li and Stephen, 2022). This reaction also produces acetaldehyde as a byproduct through α-hydroxylation of the ethyl group. Moreover, when the β-carbon of NDEA undergoes hydroxylation, the reactive intermediate 2-hydroxyethyldiazonium ion can be formed through secondary α-hydroxylation on the other ethyl group of NDEA. This process adds to the complexity of NDEA metabolism, where denitrosation reactions can compete with bioactivation, indicating a dual role played by P450 enzymes in the biotransformation of NDEA (Li and Stephen, 2022). Understanding these metabolic pathways is crucial for developing strategies to mitigate the carcinogenic risks associated with NDEA exposure, highlighting the significant role of P450 enzymes in both activating and detoxifying this compound. The figure illustrates the mechanisms of biotransformation of N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) mediated by cytochrome P450 enzymes (P450s), resulting in the formation of different metabolites and final products.

For NDMA, the process begins with oxidation by P450s, forming the hydroxy-nitrosamine intermediate. This intermediate can decompose to form formaldehyde (HCHO), which is further oxidized to formic acid (HCOOH) and finally to carbon dioxide (CO2). Alternatively, the intermediate can decompose to form the methyldiazonium ion (CH3N2+), which decomposes into methanol (MeOH). The methyldiazonium ion can react with DNA, forming methyl DNA adducts, which are genotoxic lesions capable of causing mutations and potentially contributing to cancer development (Hemminki, 1993; Shuker & Margison, 1997). For NDEA, the biotransformation process also involves oxidation by P450s, which can occur at either the alpha or beta position. Alpha-hydroxylation results in the formation of a hydroxy-nitrosamine intermediate, which can decompose into aldehyde or ethyldiazonium ion. The latter can convert into ethyl-carbocation, a highly reactive species that can interact with DNA, proteins, and other biological macromolecules. Beta-hydroxylation follows a similar path, resulting in the formation of an intermediate that can decompose into 2-hydroxyethyldiazonium ion (Verna et al., 1996) Figure 5.

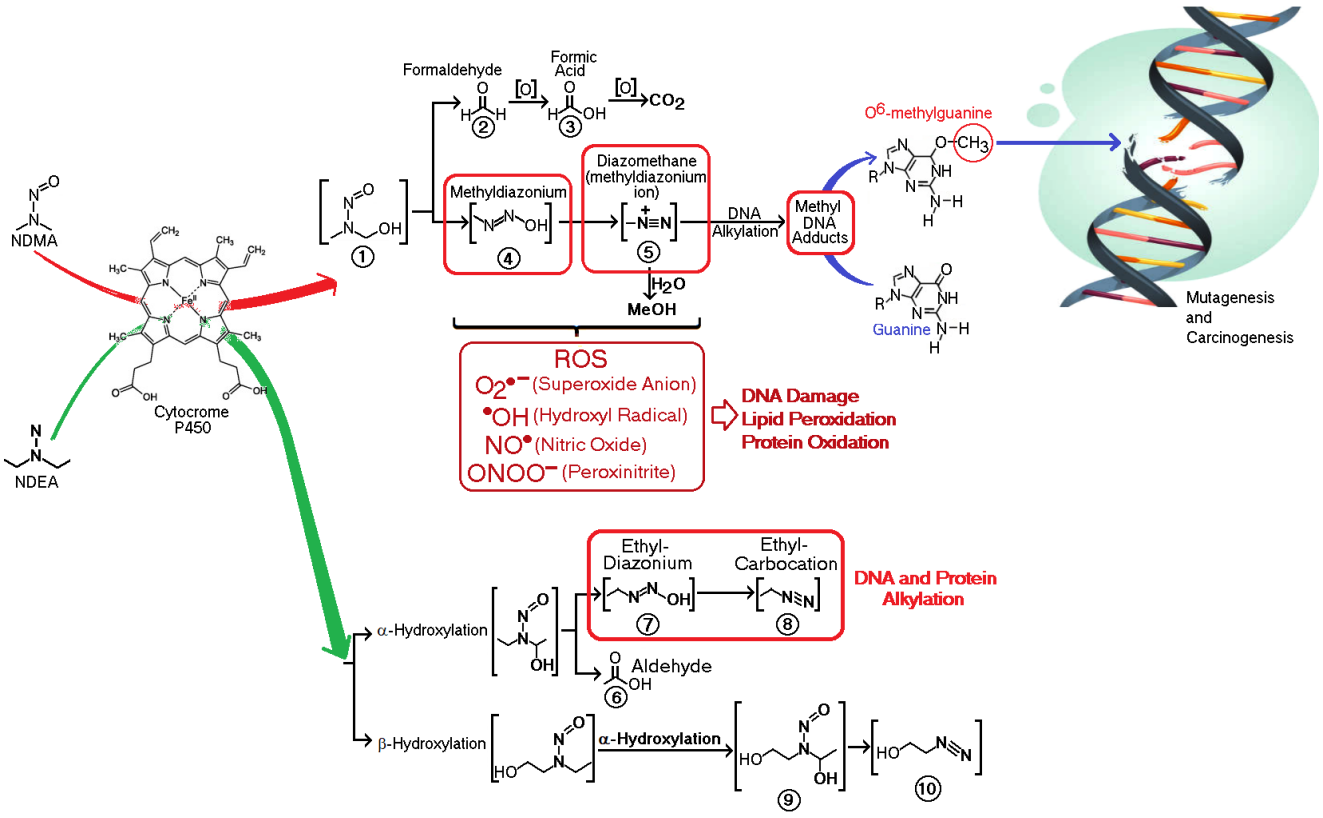

Figura 5. The figure illustrates the biotransformation mechanisms of two nitrosamine compounds, N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA), mediated by cytochrome P450 enzymes (P450s), resulting in the formation of different metabolites and end products. Numbers not indicated in the figure: 1- α-hydroxyNDMA; 9: α- hydroxylation on the other ethyl group of NDEA; 10: 2-hydroxyethyldiazonium ion. **Source:** Own autorship

These reactive products, especially diazonium ions, can interact with DNA, forming adducts that can cause massive DNA damage. Additionally, these compounds can increase cellular oxidative stress, promoting the production of reactive oxygen species (ROS) and potentially inducing the Fenton reaction. This process generates highly reactive hydroxyl radicals that can cause severe damage to DNA, proteins, and cell membranes (Klaunig & Kamendulis, 2004; Valko et al., 2006; Marnett, 2000; Halliwell & Gutteridge, 2015). The presence of these free radicals can lead to genetic mutations and, eventually, the development of chronic diseases, including cancer. In summary, the figure highlights the complexity of the biotransformation of nitrosamines such as NDMA and NDEA, elucidating their metabolic pathways and the potential toxic and carcinogenic effects associated with their reactive metabolites. This understanding is crucial for developing effective strategies to monitor and mitigate nitrosamine contamination, thereby protecting public health.

This work analyzed the interactions between NDMA and NDEA and some of the most known and stable metabolites with the enzymes cPLA2, PAF-AH and LPCAT3. These metabolites structures were obtained from the PubChem platform (Table 2 and Figure 6).

Table 2: Ligands used in molecular Docking and MD

Compound Name	**Chemical Formula**	**Molecular weight**	**PubChem ID**
NDMA	$C_2H_6N_2O$	74.08 g/mol	6124
NDEA	$C_4H_{10}N2O$	102.14g/mol	5921
2-Hydroxyethanediazonium	$C_2H_5N2O^+$	73.07g/mol	91426341
Methanol	CH_4O	32.042g/mol	887
Formaldehyde	CH_2O	30.026g/mol	712
Methyldiazonium Ion	$CH_3N_2^+$	43.048g/mol	115287
Ethanediazonium	$C_2H_5N_2^+$	57.07g/mol	158606
Acethaldehyde.	C_2H_4O	44.05g/mol	177
Giripladib*	$C_{14}H_{36}ClF_3N_2O_4S$	745.2g/mol	11721842
Lysophosphatidylcholine*	$C_{10}H_{22}NO_7P$	299.26g/mol	5311264
DEP (paraoxon)*	$C_4H_{10}O_3P^+$	137.09g/mol	6327654

***Reference Ligands**

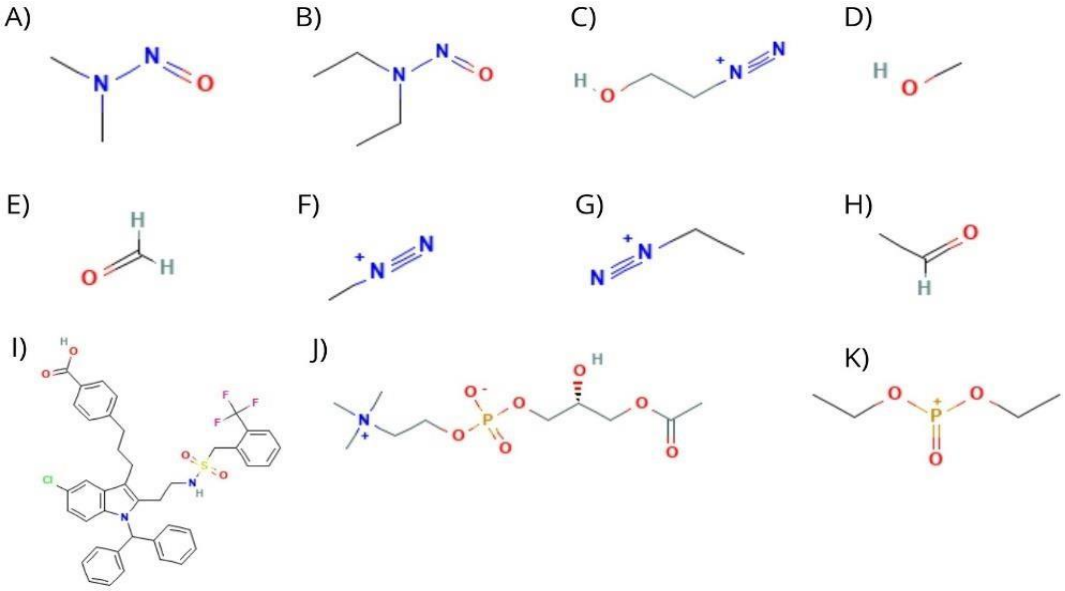

Figure 6. 2D structure available on the PubChem platform of the ligands.: A) NDMA; B) NDEA; C) 2-Hydroxyethanediazonium; D) Methanol; E) Formaldehyde; F) Methyldiazonium Ion; G) Ethanediazonium; H) Acethaldehyde; I) Giripladib; J) Lysophosphatidylcholine; K) DEP.

2. Material and Methods.

2.1. Danio rerio experiments.

2.1.1. Fish Acclimatization.

The cultivation and maintenance techniques adhered to the procedures outlined by the Brazilian Association of Technical Standards (ABNT, 2004) and the Organization for Economic Cooperation and Development (OECD, 2013). Specimens were acquired from authorized and registered suppliers for breeding or selling Danio rerio fish. The animals underwent a 90-day quarantine and acclimation period in 68-liter stock tanks (Dammski et al., 2011). The aquariums were equipped with hang-on filters (Grech CBG-800) providing chemical, mechanical, and biological filtration. During the quarantine, key parameters such as temperature, ammonia, nitrite, pH, dissolved oxygen, and water hardness were monitored and adjusted daily using aquarium test kits (Alcon Labcon: toxic ammonia, tropical pH, nitrite, dissolved O2, GH total hardness kits) to maintain recommended levels (Dammski et al., 2011). Water temperature was measured daily with a high-precision aquarium thermometer, ranging from 0 to 50 ºC. The fish were subjected to a 12-hour photoperiod. A mineral content restorer (Equilibrium - Seachem) was used to adjust water hardness. To eliminate toxic ammonia, nitrite, nitrate, heavy metals, chlorine, and chloramine, Seachem Prime was employed. Additionally, Seachem Stability, containing aerobic and facultative bacteria, was used to accelerate and stabilize biological filtration. The fish were fed commercial feed (JBL Novobel) once daily. All procedures were authorized by the Animal Ethics Committee of UNESP (IB/CLP-São Vicente). The study primarily focused on the effects of NDEA exposure on the fish due to the unavailability of NDMA in the commercial market within the stipulated testing period. The absence of NDMA hindered the intended assays, despite the available infrastructure for testing with *Danio rerio*.

2.1.2. Behavioral Analysis.

In 2-liter tanks, NDEA was diluted separately into different concentrations (2, 4, 8, 16 mg/L of the compound). This experiment was performed in triplicate, using 5 fish for each concentration. The fish remained in contact with the solutions for 96 hours, with the compound being administered every 24 hours due to the volatility of nitrosamines. Throughout this period, the animals were observed for behavioral changes and mortality. The fish were fasted for 24 hours prior to the experiment to ensure constant exposure concentrations. Behavioral analysis was conducted over the 96-hour period. The behavior of the fish was monitored by a human

observer and filmed at 0, 24, 48, and 96 hours of exposure. The behavioral reactions were categorized into stages, which were further divided into steps (Souza, 2015):

Table 3. Behavioral Characterization of *Danio rerio* After 96 Hours of Exposure to Different Concentrations of NDMA and NDEA.

Stage	1st Stage	2nd Stage	3rd Stage
Internship I	Increase in swimming activity	Tremors in the tail shaft	
Internship II	Circular swimming	Loss of posture	
Stage III	Loss of motility	Deposition of the animal at the bottom of the aquarium	Death*

*****Characterized by the absence of operculum movement and loss of response to mechanical stimuli. Source: adapted from Souza, 2015.

After the experiment, the remaining fish were euthanized (Souza, 2015) to collect biological material for histopathological and micronucleus analysis. This text outlines a behavioral experimental protocol designed to assess the effects of various concentrations of NDEA on Danio rerio over a 96-hour period. The study involves careful monitoring of fish behavior and mortality, with subsequent collection of biological samples for further analysis.

2.1.3. Genotoxicity Assessment – Micronucleus test.

The micronucleus (MN) test is a method used to detect genetic damage in cells caused by chemical, physical, or biological agents. This test identifies the formation of micronuclei, which are small, additional nuclei that appear when fragments or entire chromosomes are not correctly incorporated into the main nucleus during cell division. Widely used in genotoxicity assessment, the MN test is essential for ensuring the safety of new chemical compounds, monitoring environmental contamination, researching carcinogenesis mechanisms, evaluating the safety of food and pharmaceuticals, monitoring occupational exposure to hazardous substances, and studying the impact of pollutants in ecotoxicology. Upon arrival at the laboratory, blood aliquots for the MN test were prepared on microscope slides using heparinized glass capillaries. Ten blood smears were made for each fish. After air-drying at room temperature, the slides were stained with Giemsa. They were then analyzed at 100x magnification under a binocular microscope. Micronuclei were identified based on the criteria of Moron et al. (2006): genetic material not connected to the main nucleus, coloration and intensity similar to the main nucleus, and size up to one-third of the main nucleus. To determine the frequency of micronuclei, 1,000 cells per individual were counted. Additional cellular abnormalities, such as lobed nuclei, notched nuclei, blebbed nuclei, and binucleated cells, were also recorded (Carrasco et al., 1990).

2.2. *Mus musculus* Experiments.

Twenty-five female Swiss mice (approximately 30 g each, n = 5 per group) were used in this study. The mice were obtained from the Multidisciplinary Centre for Biological Research (CEMIB) at the State University of Campinas (UNICAMP) and housed in cages under controlled environmental conditions: a constant temperature of 25 ºC, a 12-hour light/dark cycle, with food and water available ad libitum. This experiment was conducted with the authorization of the Animal Ethics Committee of the Paulista Coast Campus (10/2022).

The mice were divided into five equal groups, each consisting of 5 mice:

Control group (treated only with 0.9% saline solution),

Group exposed to 0.10 mg/kg of NDMA,

Group exposed to 0.10 mg/kg of NDEA,

Group exposed to 0.20 mg/kg of NDMA,

Group exposed to 0.20 mg/kg of NDEA.

The *in vivo* experiments were conducted in accordance with the institutional guidelines of the ethics committee of UNESP, to which the project was submitted.

2.2.1. Acute Toxicity Tests and hematological assessment.

The assays were conducted over 4 and 24-hour periods, with the drug administered via peritoneal injection. The control group received no drug, while the treated group received the drug. Blood samples were collected by puncturing the caudal artery using a 27g scalp needle, and the obtained material was stored in collection tubes containing EDTA (ethylenediaminetetraacetic acid, BD Vacutainer EDTA tube). The samples were then centrifuged to separate the serum for subsequent biochemical analysis. Following blood collection, the rats were euthanized by cervical dislocation to remove their livers for histological analysis. For the red series analysis of Mus musculus, blood samples were centrifuged to determine the plasma to cellular element ratio using the microhematocrit assay with heparinized capillaries. Erythrocyte counts were performed using the Neubauer chamber cell estimation technique. For leukocyte counting and differentiation, blood smears were prepared and stained with Leishman stain (Cicero et al., 2020; de Paiva et al., 2013).

2.3. Biochemical Analysis

To assess hepatic alterations, liver biochemical analyses were performed to measure levels of lactate dehydrogenase, AST, ALT, Gamma GT, and C-reactive protein. These analyses utilized commercial kits handled according to the manufacturer's specifications and procedures. In addition to liver function tests, biochemical analyses of cytokines and lipid peroxidation were

conducted. Specifically, the levels of IL-6, TNF alpha (markers of acute inflammation), and MDA (malondialdehyde, a marker of lipid peroxidation) were measured using specific kits in accordance with the manufacturer's instructions. Furthermore, the levels of the enzyme lipoprotein-associated phospholipase A2 (PAF-AH) were monitored using a PAF Acetylhydrolase Inhibitor Screening Assay Kit (Item No. 10004380). All tests adhered to the usage instructions established by the kit manufacturer. For these analyses, nitrosamines were dissolved in DMSO and used at concentrations of 100 µg/ml, 10 µg/ml, and 1 µg/ml, with each concentration tested in triplicate. This comprehensive approach ensured a thorough evaluation of hepatic function, inflammatory markers, lipid peroxidation, and enzyme activity, providing detailed insights into the biochemical effects of the tested compounds. Data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) was performed, followed by the Tukey test to compare the various groups using GraphPad Prism 5 software. P-values of less than 0.05 were considered statistically significant.

2.4. Histological and Histopathological Analysis of *Danio rerio* and *Mus musculus*

2.4.1. Histological Processing

The zebrafish (*Danio rerio*) were fixed using Bouin's solution, while liver samples from Swiss mice (*Mus musculus*) were fixed using 10% buffered formalin. Samples from both species were fixed for 24 hours and then transferred to 70% alcohol. Subsequently, the samples were gradually dehydrated in ethanol, cleared in xylene, processed, and embedded in paraffin. Tissue sections of 5 µm were obtained using a microtome and stained with hematoxylin and eosin (H&E). Histopathological examinations were performed under a light microscope equipped with a digital camera and analyzed using a Bell Photonics biological microscope. This histological analysis was conducted in collaboration with the Group of Morphology of Aquatic Organisms at the Animal Morphophysiology Laboratory (LABMA) at UNESP IB/CLP, the project’s host institution.

2.4.2. Histopathological Analysis of Mus musculus Liver Samples

Data from the histopathological samples were expressed as mean ± SEM. A one-way analysis of variance (ANOVA) was performed to assess the results. Liver lesions were evaluated based on multiple parameters, including hepatic vacuolization, necrosis, nuclear hypertrophy, lymphocyte infiltration, vascular obstruction, and cell retraction (de Menezes et al., 2019; Noor et al., 2022). The severity of these lesions was scored on a scale from 0 to 4 (0: absent, 1: minimal, 2: mild, 3: moderate, 4: marked), as detailed in Table 4.

Table 4. The term "foci" is the plural of "focus" and, in the context of pathology, refers to small groups or localized areas of abnormal cells or lesions within a tissue. In histology and histopathology, "foci" are used to describe specific sites where pathological alterations occur, such as inflammation, necrosis, or abnormal cell proliferation. These foci can be counted and analyzed to determine the extent and severity of pathology in a tissue sample. **Source**: de Menezes et al. 2019

Severity	Proportion of Liver Affected	Scores	Quantifiable Finding
Minimal	Very small amount	1	1–2 foci
Mild or Slight	Small amount	2	3–6 foci
Moderate	Medium amount	3	7–12 foci
Marked or Many	Large amount	4	>12 foci, coalescing

2.4.3. Histopathological Analysis of *Danio rerio* Samples

Lesions in *Danio rerio* samples were identified and assessed according to a pre-established importance factor (w), which reflects the severity of the lesion: 1 for lesions with minimal pathological importance, 2 for lesions with moderate pathological importance, and 3 for lesions with high pathological importance. The identified lesions were then assigned a score (a) ranging from 0 to 6, where 0 indicates the absence of the lesion and 6 represents a frequent occurrence. To calculate an alteration index for each lesion, we multiplied the importance factor (w) by the score (a). The total alterations in an organ were determined by summing the products of the importance factor and the score for each lesion (total alterations = $\sum(w * a)$) (Bernet et al., 1999; Marinsek et al., 2024). This method provides a quantifiable measure of the severity and extent of histopathological lesions, allowing for a comprehensive assessment of the impact on the organs of *Danio rerio*.

2.5. *In Silico* Studies.

2.5.1. Molecular Docking.

Molecular docking is an in silico method widely used in drug discovery to predict the binding of molecules to specific biological targets. It is also applied in toxicology to develop classification models for predicting endocrine-disrupting potentials of chemicals (Trisciuzzi et al., 2018). Docking experiments were performed using the GOLD software, with Data Warrior and Pymol for preparing molecular libraries and analyzing results. For the simulations, Lp-PLA2 (PDB ID: 3D5E), cPLA2 (PDB ID: 1CJY), and LPCAT3 (PDB ID: 7F3X) were used as targets, with the indicated PDB references for preparing each target protein.

2.5.2. Preparation of Ligand and Target Molecule Files.

Ligands were obtained from the PubChem platform in 3D format (.SDF). DataWarrior software was used to prepare the ligands, generate conformers, and calculate properties. Open Babel added the correct protonation and pH to the ligand files. The target proteins were obtained from the RCSB Protein Data Bank (.PDB). Model coordinates for docking were prepared using GOLD, which also added hydrogens to the protein targets. The grid box size was chosen to encompass all amino acid residues in the catalytic site's microenvironment, based on reference ligands for the respective enzymes - PAF-AH: DEP; cPLA2: Giripladib; LPCAT3: LPC.

2.5.3. Evaluation of Molecular Docking Results.

Docking results were analyzed using Pymol, assessing binding energies, distances, and orientations of molecules in the PAF-AH active site. Three different GOLD score functions were applied: PLP normal, ChemScore, and GOLD score, with PLP normal generating the most consistent results. PLP functions model the steric complementarity between protein and ligand (GOLD User Guide – CCDC).

2.5.4. Covalent Docking.

DEP (Paraoxon) is described as a covalent inhibitor of PAF-AH. The GOLD covalent protocol for Setting Up Covalently Bound Ligands (GOLD User Guide) was followed to evaluate the interaction between DEP and PAF-AH.

2.5.5. Webina.

Due to difficulties encountered by GOLD in docking small molecules like formaldehyde and Methyldiazonium Ion, Webina (https://durrantlab.pitt.edu/webina/) was used for these compounds with all enzymes. Docking simulations were performed for each reference ligand with its respective enzyme, and binding free energy values were obtained. This approach allowed comprehensive analysis despite the small size of these molecules.

2.5.6. Molecular Dynamics.

For protein treatment, the AMBER14SB force field was used, while organic molecules were parameterized using ANTECHAMBER based on GAFF, with RESP charges determined through Gaussian16 at the HF/6-31G(d) level. Models were solvated in a rectangular box with TIP3P water, maintaining a minimum distance of 15 Å between the protein and the box edge. Na+ and Cl- ions were added to neutralize the system's charge and ensure ionic strength. Energy minimization of docked complexes was performed in four steps: (1) water molecules, (2) hydrogen atoms, (3) side chains of protein amino acids, and (4) the entire system, each for 10,000 steps. System equilibration used NVT and NPT ensembles with progressive heating over 2000 ps, using the Langevin thermostat at 310 K. Production molecular dynamics ran for 100 ns,

followed by subsequent analyses. CPPTRAJ calculated the protein's RMSD and RMSF, and Xmgrace plotted the graphs. Protein-ligand binding free energy was calculated using MM-GBSA methods, utilizing at least 500 conformations from molecular dynamics to assess predictions from docking, incorporating protein and molecule flexibility. These methods also determined the energetic contributions of different amino acids in the active site for coordinating the molecules.

In summary, the Docking and MD simulation protocols are outlined in the schemebelow (Figure 7).

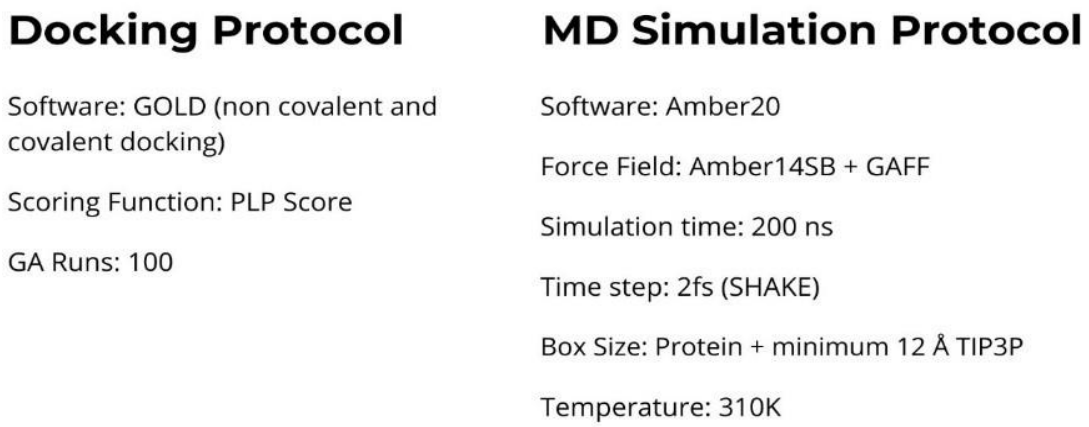

Figure 7: Summary of protocols used for docking and Molecular Dynamics.

3. Results and Discussion.

3.1. Danio rerio Behavior Analysis

No mortality was observed during the experimental assay under any of the tested conditions (controls or nitrosamine-exposed fish). Additionally, no significant behavioral alterations were detected between the exposed animals and the control group. The study conducted by Zheng et al. (2018) extensively described the toxicity of nitrosamines, including N-nitrosodiethylamine (NDEA), in adult zebrafish during the drinking water treatment process.

This study provided valuable insights into the adverse effects of nitrosamines on zebrafish, including data on acute toxicity and organism survival. Given that Zheng et al. (2018) have already conducted a comprehensive analysis of nitrosamine toxicity in zebrafish, including lethal effects at different concentrations, there is no need to repeat the experiment to determine the LC50.

Utilizing the available data in the literature is important because it allows for resource savings and avoids redundancy in scientific research. Additionally, by properly citing the

previous work, we ensure integrity and transparency in research conduct, contributing to the advancement of scientific knowledge ethically and efficiently.

The results of fish exposure to nitrosamines are strongly related to NDEA, due to the difficulty in finding NDMA on the market within the period stipulated for tests intended for use in fish, as the compound went through a period of shortage on the market.

3.2. Genotoxicity Assessment – Micronucleus test.

Arielle et al. (2021) describe that the micronucleus (MN) test and the analysis of nuclear abnormalities have high sensitivity and are therefore widely applied in studies to analyze the mutagenic effects of exposure to contaminants. In summary, the MN test seeks to identify chemical agents capable of causing cytogenetic damage. The abnormalities observed include the formation of micronuclei, blebbed nuclei, notched nuclei, and bud nuclei. Micronuclei (MN) are small extranuclear bodies that can originate from various causes, such as acentric chromosomes or chromatid fragments during anaphase.

These fragments of acentric chromosomes do not attach to the spindle fibers during telophase and thus do not become part of the main nucleus, instead being surrounded by another membrane to form micronuclei. Blebbed nuclei are characterized by the presence of small protrusions on the nuclear membrane, which may indicate disruptions in nuclear envelope integrity.

Notched nuclei have irregular deformities or notches along their perimeter. Bud nuclei occur when a small part of the nucleus is separated from the main nucleus by a thin nuclear membrane, potentially resulting from chromosome breakage or lagging chromosomes during cell division (Figure 8) (Arielle et al., 2021).

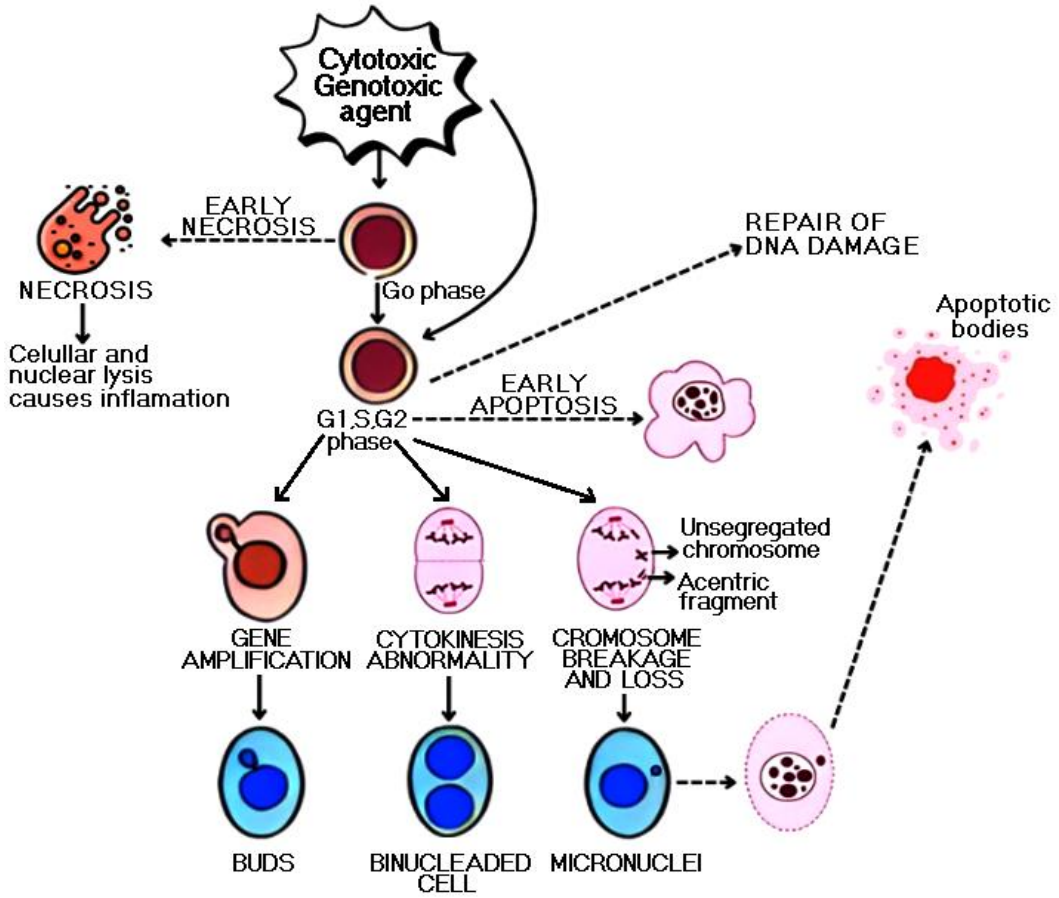

Figure 8. Scheme of micronucleus (MN) and some other nuclear abnormalities (NA) formation in fish. Clastogenic agents induce DNA strand breaks, which, if misrepaired or unrepaired, lead to the appearance of acentric chromatids or chromosome fragments, resulting in various chromosomal rearrangements, including the formation of micronuclei and other nuclear abnormalities in zebrafish. Addapted from: Arielle et al. 2021.

The MN analysis followed the methodology described by Smorodinskaya et al. (2023), Moron et al. (2006), and Carrasco et al. (1990). Cell counting adhered to the standard presented in a set of studies analyzed by Canedo et al. (2021), where the highest percentage of studies involving zebrafish for micronucleus and nuclear abnormalities (NA) analysis counted 1,000 cells. Our results show an increase in nuclear abnormalities corresponding to the dose increase, with the most frequently observed abnormality being binucleated cells. This was followed by the predominance of notched nuclei, blebbed nuclei, micronuclei, and finally nuclear buds (Figure 9).

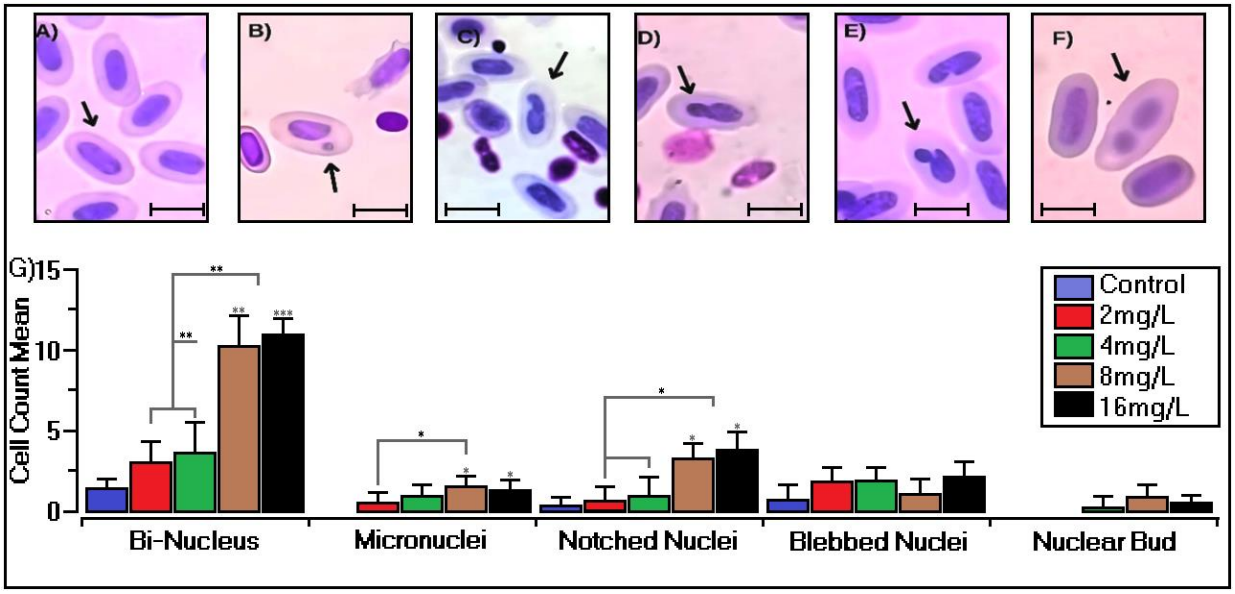

Figure 9: Characterization of Nuclear Abnormalities Found. Images (a-f) illustrate the nuclear abnormalities observed: erythrocyte (Er), micronucleus (Mn), notched nuclei, blebbed nuclei, nuclear bud, and binucleated cell. The graph shows the predominance of binucleated cells in exposed groups. Statistical significance ($p < 0.05$) was determined using the Kruskal–Wallis test, with blue asterisks indicating significant differences from the control group. Comparisons between other groups are shown with a line scheme; groups without asterisks were not significant (ns). The bar indicates 5 microns in length.

In this study, hematological analysis of *Danio rerio* specimens revealed a potential increase in fibrin formation in blood samples from groups exposed to 8 and 16 mg/L of the test substance (Figure 10).

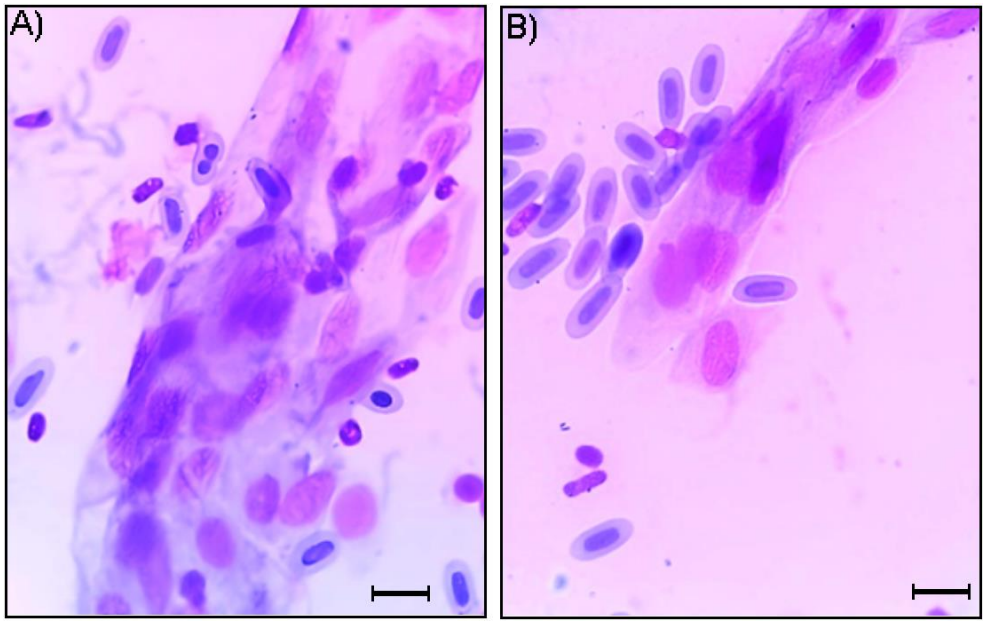

Figure 10: Both images probably illustrate some of the findings of fibrin encountered in the blood smears. The bar indicates 5 microns in length.

In the article by Brown et al., fibrin deposition in pancreatic adenocarcinomas was observed in female Syrian hamsters exposed to the chemical carcinogen N-nitroso-bis(2-

oxopropyl)amine (BOP). This compound is a nitrosamine known to be a potent carcinogenic agent capable of inducing tumor development in the pancreas. Exposure to BOP resulted in the formation of pancreatic adenocarcinomas in hamsters, and analysis of the tumor tissue revealed fibrin deposition in the tumor stroma. This suggests that the presence of nitrosamines, such as BOP, may trigger inflammatory and coagulation responses that contribute to the formation and progression of pancreatic tumors. In this study, hematological analysis conducted with specimens of *Danio rerio* revealed a significant increase in fibrin formation in blood samples. These findings suggest that exposure to nitrosamines can induce inflammatory and coagulation responses, as evidenced by the fibrin deposition observed in both pancreatic adenocarcinomas in Syrian hamsters and in the hematological analysis of *Danio rerio*. This highlights the potential role of fibrin formation in tumor progression and underscores the need for further research into the mechanisms by which nitrosamines contribute to coagulation responses.

3.3. *Danio reri*o Histology of Liver and Gills.

For the histological analysis of *Danio rerio* liver, semi-serial sections of 5 µm thickness were obtained from each animal and stained with Hematoxylin-Eosin. The analyses were conducted as described by Bernet et al. (1999) and Marinsek et al. (2024). These studies identified lesions according to a pre-established importance factor (w), which refers to the severity of the lesion: 1 for minimal pathological importance, 2 for moderate pathological importance, and 3 for high pathological importance. The lesions found in the organs were assigned a score (a) ranging from 0 to 6, with 0 indicating the absence of the lesion and 6 indicating frequent occurrence. To obtain an alteration index, the importance factor (w) was multiplied by the score (a). The total alterations found in the organ were calculated by summing the products of the importance factor and the score for each lesion (total alterations = $\sum(w * a)$) (Bernet et al., 1999; Marinsek et al., 2024). **Table 5** - Factors of importance of the histological lesion (Bernet, et. al., 1999).

Table 5. Classification of Lesion Based on Importance Factor (w).

Importance Factor (w)	Classification	Specification
w = 1	Minimal	Lesions with minimal pathological importance; the lesion is generally reversible
w = 2	Moderate	Lesions with moderate pathological importance; the lesion is reversible in most cases
w = 3	Marked	Lesions with high pathological importance; the lesion is generally irreversible, causing partial or total loss of the organ

Histological analyses of the lower concentrations (2 mg/L and 4 mg/L) did not reveal any histopathological alterations (Figure 11). Therefore, the alteration index (hepatosomatic index) was calculated only for the concentrations of 8 mg/L and 16 mg/L.

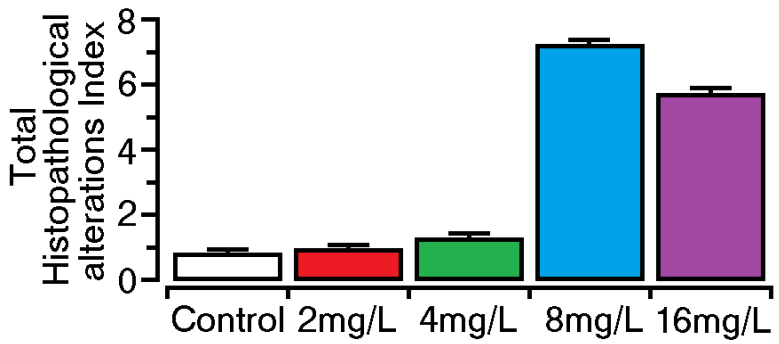

Figure 11: The graph represents the average index of total histopathological alterations in Danio rerio exposed to NDEA for 96 hours. Histological analyses of the lower concentrations (2 mg/L and 4 mg/L) did not reveal any histopathological alterations. Consequently, the alteration index (hepatosomatic index) was calculated only for the concentrations of 8 mg/L and 16 mg/L.

A visual examination of the slides revealed a predominance of hemorrhages and melanomacrophage centers in the livers of animals exposed to higher concentrations of the compound. Melanomacrophages have various functions, including engulfing resistant pathogens like parasite spores and bacteria (Agius, 1981), processing antigens during immune responses (Agius, 1981), breaking down, detoxifying, or recycling both endogenous and exogenous materials (Ferguson, 1976), storing metabolites from dead cells, including erythrocytes, and reacting to foreign bodies, including infectious agents (Agius; Roberts, 2003). The histopathological analysis showed that hemorrhages and vascular congestion suggest a possible link between the compound and circulatory disturbances. The presence of melanomacrophage centers at higher concentrations appears to be associated with the hemorrhagic process, as the increase in these centers coincides with the increase in hemorrhagic foci. It is also likely that their appearance is related to an inflammatory response caused by exposure to the contaminant and/or the hemorrhage itself, which can trigger an inflammatory response resulting in the formation of melanomacrophage centers. It is important to highlight that melanomacrophage centers play a crucial role in the degradation of hemoglobin and the recycling of iron (Campos et al., 2008).

The presence of hemosiderin (an iron storage complex) in these centers indicates their function in iron management following the phagocytosis of damaged erythrocytes.

Furthermore, the analysis of these centers is widely used in ecotoxicological studies, as changes in their quantity and size can indicate environmental stress, exposure to pollutants, and the presence of infectious diseases (Campos et al., 2008). Additionally, foci of necrosis, vascular obstruction (congestion), cellular hypertrophy, steatosis, and nuclear alterations were observed (Figure 12).

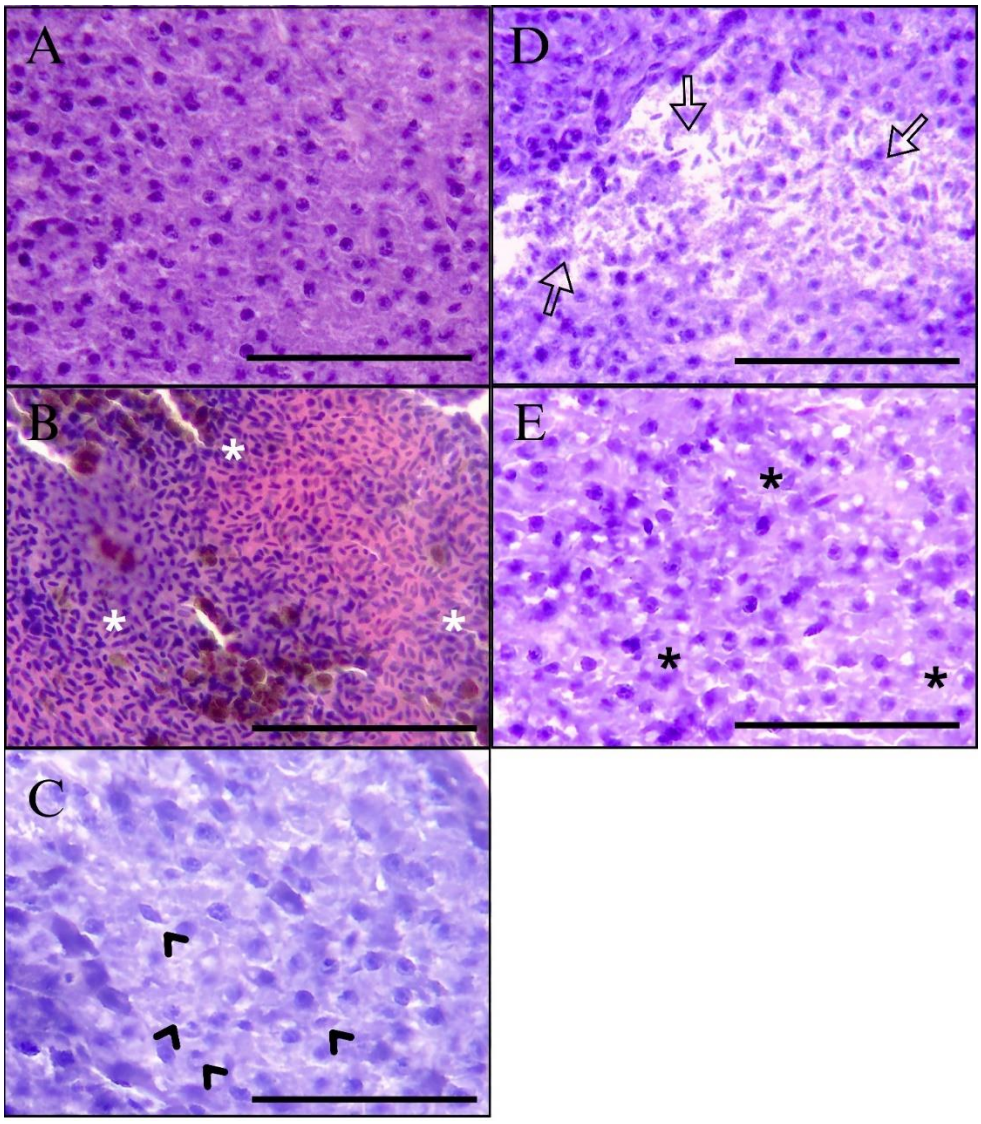

Figure 12. Histopathological Analysis of Fish Exposed to NDEA. The images in Figure 12 illustrate the histopathological changes in fish liver tissue exposed to NDEA. Image A shows the control group with no significant alterations. Image B shows hemorrhagic foci marked by asterisks, while melanomacrophage centers are identified as brownish spots. In Image C, demonstrates nuclear alterations, indicated by changes in the shape of the nuclei. Image D highlights areas of necrosis, marked by brackets, indicating regions of cell death. Image E. shows cell vacuolization (possible steatosis), characterized by the displacement of nuclei to the cell periphery, visible around the asterisks These images visually represent the significant histopathological effects of NDEA exposure. The highlighted bar in each figure indicates a distance of 400 micrometers.

According to the classification by Bernet et al. (1999), this study identified both progressive and regressive alterations in the liver tissues of *Danio rerio* exposed to NDEA. These alterations included necrosis (w = 3), circulatory disturbances such as hemorrhage (w = 1) and vascular congestion (w = 1), nuclear alterations (w = 2), and cellular hypertrophy (w = 1). Hepatocytes in fish typically have spherical nuclei, as observed in Danio rerio, but can exhibit variations in shape and size. This nuclear polymorphism may be linked to different stages of the cell cycle, environmental stress responses, or the presence of toxic substances. For instance, studies like that of Santos et al. (2004), which provided a histopathological description of *Nile tilapia* livers, underscore the importance of understanding nuclear variations in hepatocytes to assess fish health and environmental impacts.

The presence of cytoplasmic vacuolations, which increase the volume of hepatocytes, suggests probable regions of lipid and glycogen concentration or interactions between toxic agents and intracellular lipids (Santos et al., 2004). The accumulation of lipids and the reduction of glycogen in the hepatocyte cytoplasm can impair metabolic activities. In this study, foci of necrosis, vascular obstruction (congestion), cellular hypertrophy, steatosis, and nuclear alterations were observed at NDEA concentrations of 8 and 16 mg/L. Notably, hepatocyte nuclear hypertrophy was observed only at the 16 mg/L concentration (figure 13).

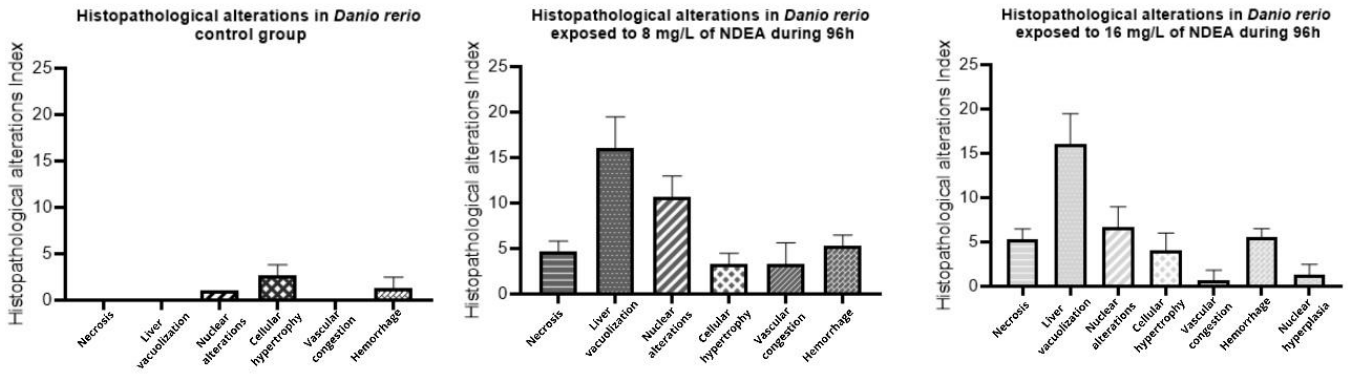

Figure 13: Histopathological alterations Index showing the main alterations between the control group and groups that showed histopathological alterations during the assay (see figure 10) (8 and 16 mg/L). Nuclear hyperplasia was one of the alterations found just in the higher concentration, it was observed that almost all the other alterations evaluated increased when compared to the control group. Data was expressed as mean ± SD.

The histological analysis of fish gills in toxicological assays is crucial because gills perform vital functions such as respiration, osmoregulation, and excretion. Being in direct contact with water, gills are highly sensitive to environmental contaminants, making them excellent indicators of water quality and the presence of pollutants. The cells and tissues of gills react quickly to changes in the chemical composition of water, allowing histological alterations such

as vasodilation, congestion, hyperplasia, and fusion of gill filaments to serve as biomarkers for evaluating exposure and the effects of specific contaminants. Additionally, the health of gills reflects the overall health of the aquatic ecosystem, with pathological changes potentially indicating broader environmental problems. Histological analysis provides a detailed and precise assessment of cellular and tissue alterations, essential for monitoring and evaluating the environmental impacts of pollutants on aquatic organisms, thereby contributing to the protection and management of aquatic ecosystems.

As described by Yancheva et al. (2016), histopathology involves the microscopic examination of cells and tissues and the semiquantitative determination of histological abnormalities. These alterations, when observed in fish, can serve as biomarkers to assess the health of fish exposed to contaminants (Camargo et al., 2007; Segura, 2023). The analysis of biomarkers allows for the examination of specific organs, such as gills and liver, which perform vital functions like respiration, accumulation, and biotransformation of xenobiotics in fish (Gernhofer et al., 2001; Segura, 2023).

The analysis of gills is particularly important because they are responsible for essential functions such as respiration, osmoregulation, and excretion. Since gills are in direct contact with the external environment, they are highly sensitive to changes in water quality, often making them primary targets of contaminants (Camargo et al., 2007). To complement the findings and analyses conducted in this study, we performed a qualitative description of the gill histology of animals exposed to NDEA. Although few alterations were observed, the groups exposed to 4, 8, and 16 mg/L exhibited vasodilation and congestion in the blood vessels of the gills in at least 3 out of 5 individuals at these concentrations (Figure 14, letter B). These or other predominant alterations were not observed in the other groups studied.

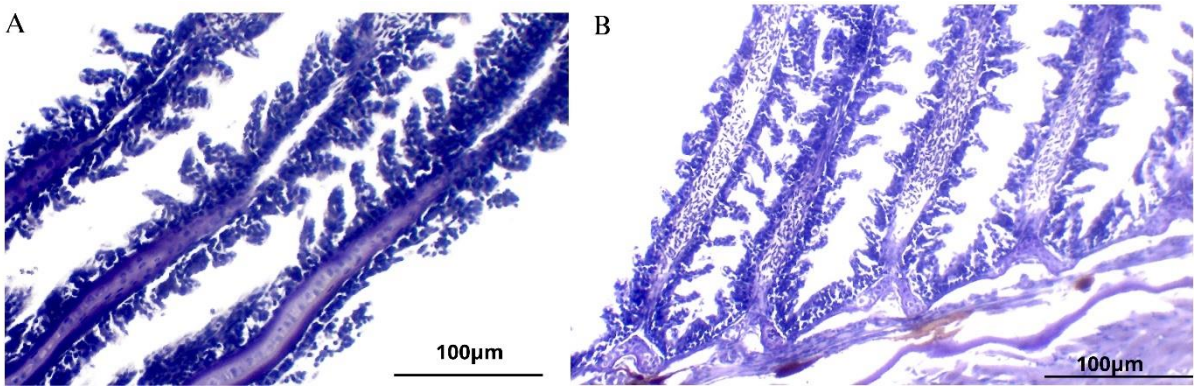

Figure 14: Both images show histological sections of *Danio rerio* gills. A) Control. B) Example of gills found in animals exposed to NDEA concentrations (4, 8, and 16 mg/L). Vasodilation and vessel congestion can be observed when compared to image A.

Studies conducted by Marinsek et al. (2024), Freitas et al. (2013), Camargo et al. (2007), and Segura (2023) have evaluated the histology of fish gills and elucidated that vasodilation can occur due to lesions and alterations in the gill epithelium, such as necrosis and mucus hypersecretion. Toxic substances in the water can cause these alterations, damaging epithelial cells and obstructing gas exchange pathways. It is important to note that vasodilation itself is not necessarily pathological. Under normal conditions, vasodilation is an essential process for gill respiration, allowing blood to flow through the gills and facilitating gas exchange. However, it can be classified as pathological when alterations in the gill epithelium and vasodilation compromise the fish's respiratory function, leading to health problems and premature death (Freitas et al., 2013). Therefore, although vasodilation in zebrafish gills can be considered a normal physiological process, its alteration can be an indicator of health problems caused by exposure to pollutants or other adverse conditions. In this study, a correlation was observed between the occurrence of vasodilation and exposure to the studied compound, as evidenced by the increased frequency of this characteristic with higher concentrations of the compound.

3.4. The toxicological analysis in mice (Hematology)

Our results indicate that exposure to nitrosamines can cause changes in mean corpuscular volume (MCV) levels in some groups. Specifically, NDMA at 0.20 mg/kg and 0.10 mg/kg resulted in significant increases in MCV to 183.3 fL and 112.0 fL, respectively. In contrast, groups exposed to NDEA showed values similar to the control, except for NDEA at 0.20 mg/kg, which resulted in a significant reduction to 66.0 fL. The NDMA-treated groups exhibited a reduction in erythrocyte count, with levels dropping to 2.4 million/mm³. Similarly, the NDEA-treated groups also showed lower erythrocyte counts, particularly the NDEA 0.10 mg/kg group. The observation of reduced RBC values in the NDMA-treated group suggests the presence of hemolysis. Hemolysis was observed during blood flow and may have contributed to the increased oxidative stress and hepatotoxicity. Common changes across all groups included polychromasia, lymphocytes with loose chromatin and evident nucleoli, and basophilic cytoplasm in lymphocytes (Figure 15). These findings suggest that exposure to NDMA and NDEA can differentially affect blood parameters, particularly MCV and erythrocyte count, in the studied subjects.

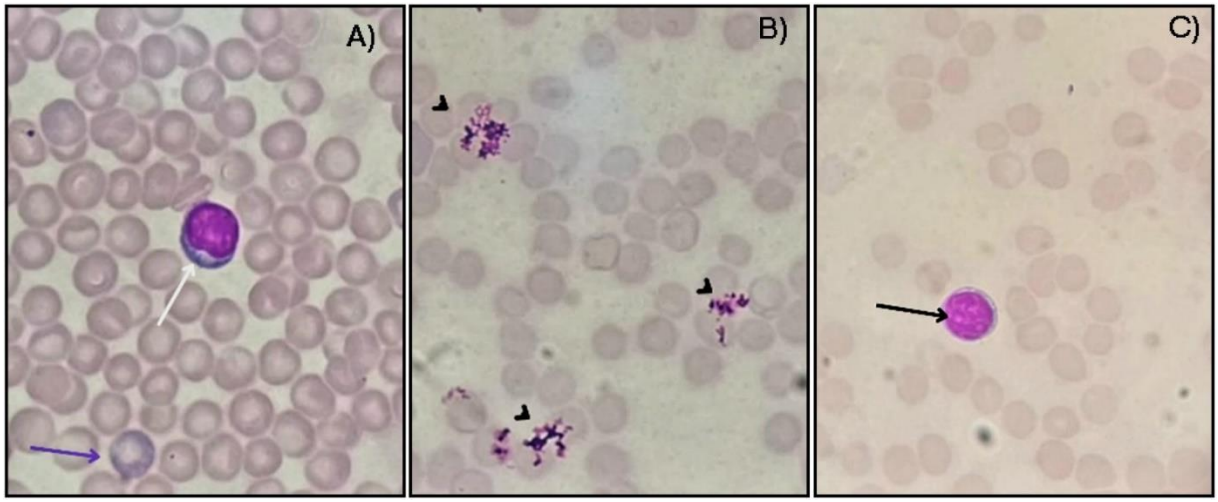

Figure 15: Some changes common to all groups A) purple arrow: polychromasia, white arrow: lymphocyte with basophilic cytoplasm; B) thrombi; C) lymphocyte with evident nucleolus. Magnification: 1000X. Coloring: Leishman.

Table 6: Hematological Analysis. The table evaluates the hematological changes observed in groups of mice exposed to NDMA and NDEA at different concentrations. The columns in light green, without lists, represent the gross value referring to the percentages that are on the side.

	Control		**NDMA 0,10***		**NDMA 0,20***		**NDEA 0,10***		**NDEA 0,20***	
RBC (mm^3)	3,2		3,2		2,4		2,8		5,6	
HB (g/dl)	11,6		12,0		14,6		10,0		12,3	
HT (%)	35,0		36,0		44,0		30,0		37,0	
VCM (fl)	109,3		112,0		183,3		107,1		66,0	
HCM (pg)	36,2		37,5		60,8		35,7		21,9	
CHCM (%)	33,1		33,3%		33,1%		33,3		33,2	
WBC	11,0%	11.000mm³	14,0%	14.000mm³	11,0%	11.000mm³	12,0%	12.000mm³	16,0%	16.000mm³
NE	10%	1.100mm³	6%	840mm³	19%	2.090mm³	5%	600mm³	3%	480mm³
EO	3%	330mm³	1%	140mm³	2%	220mm³	1%	120mm³	1%	160mm³
BA	0%	0mm³	0%	0mm³	0%	0mm³	0%	0mm³	0%	0mm³
MO	4%	440mm³	1%	140mm³	3%	330³	0%	0mm³	1%	160mm³
LY	81%	8.910mm³	87%	12.180mm³	69%	7.590mm³	88%	10.560mm³	88%	14.080mm³
LYAT	2%	220mm³	5%	700mm³	7%	770mm³	6%	720mm³	7%	1.120mm³
% Cells	100%		100%		100%		100%		100%	
PLQ	435.200mm³		THROMBS		THROMBS		THROMBS		THROMBS	

*Notes: polychromasia ++, lymphocytes with loose chromatin and evident nucleolus, basophilic cytoplasm in lymphocytes.

RBC(Red Blood Cells**), HB (**Hemoglobin**); HT (**Hematocrit**); VCM (**Mean Corpuscular Volume); **HCM** (Mean Corpuscular Hemoglobin); **CHCM** (Mean Corpuscular Hemoglobin Concentration); **WBC** (White Blood Cells); **NE** (Neutrophils); **EO** (Eosinophils); **BA** (Basophils); **MO** (Monocytes); **LY** (Lymphocytes); **LYAT** (Activated Lymphocytes); **% Cells** (Percentage of Cells); **PLQ** (Platelets)

3.4.1. Acute Toxicity Tests

Our hepatotoxicity analyses utilized Gamma GT (Gamma-Glutamyl Transferase - GGT) as a marker because it is produced in the liver and shows an increase when there is bile duct obstruction. Aronson et al. (1965) suggest that a combined analysis of GGT and alanine aminotransferase (ALT) can be essential to describe obstructive jaundice: lower ALT levels and mid-high GGT characterize obstruction, while high ALT levels and mid-low GGT characterize hepatitis. This latter characteristic was observed in the NDMA exposed groups (Figure 16), while NDEA showed the opposite, with lower ALT levels and mid-high GGT. Regarding alanine and aspartate aminotransferase, many studies describe these markers as effective in diagnosing drug-induced liver injury (Costa et al., 2023).

ALT has a half-life of 24 hours in mice and is released by hepatocytes into the bloodstream when there is hepatocellular injury. On the other hand, AST is not considered a liver-specific marker as its levels can be altered in the presence of muscle, cardiac, or skeletal damage (Yang et al., 2014). The AST enzyme is predominantly found in mitochondria, representing about 80% of the total, and is not released as quickly as ALT, which is a purely cytosolic enzyme. AST is present in high concentrations in various tissues, including the heart, liver, skeletal muscles, kidneys, and pancreas. In contrast, ALT is primarily found in the cytosol of hepatocytes and is considered a highly sensitive indicator of hepatocellular damage, capable, within certain limits, of providing a quantitative measure of the degree of liver injury (Al-Quraishy, 2002). In this context, it is possible to assume that, despite the observed increase in both transaminases, the specificity of NDMA action on the liver is greater than NDEA at its highest studied concentration.

The evaluation of lactate dehydrogenase (LDH) is justified by its presence in almost all tissues (Schumann et al., 2022). Anemias, hepatitis, fractures, and cancer, among other conditions, can be indicated by an increase in LDH, making its evaluation nonspecific. For this reason, its evaluation should be conducted along with other liver markers (Farhana et al., 2022; Drent et al., 1996). In this study, an increase in AST and ALT was observed in organisms exposed to nitrosamines, corroborating the findings related to LDH. Evaluations of C-reactive protein (CRP) indicate the presence of an inflammatory or infectious process (Nehring et al., 2022; Costa et al., 2023). Some studies, such as those by JR Mitchell and collaborators (1973) and Costa et al. (2023), demonstrate the use of acetaminophen (APAP) as the gold standard for hepatotoxicity. In this study, acetaminophen was used as a positive control.

Our findings revealed that mice subjected to these concentrations tested for 24 hours exhibited notably elevated AST levels compared to the control group (saline) at the concentration of NDEA 0.20 mg/kg. In the analysis of other hepatotoxic assays, the levels of ALT, CRP, LDH, and GGT were also higher than those in the control group, albeit to a lesser extent. These concentrations of NDEA indicated hepatotoxic effects possibly attributable to the compound (Figure 17). In a comparison relating the observed levels of biochemical markers analyzed in mice exposed to NDEA for 4 and 24 hours, it is possible to observe their progression and increase.

Nonetheless, trials involving the NDMA compound at the identical concentrations of 0.10 mg/kg and 0.20 mg/kg proved to be highly toxic, leading to the demise of all exposed animals within less than 24 hours. This outcome hindered the collection of blood and tissue samples, given that necrosis had already commenced in the tissues, and blood had already undergone coagulation. For this reason, the results obtained during the 24-hour experiment, both in vivo and in vitro, are solely associated with the NDEA compound at concentrations of 0.10 mg/kg and 0.20 mg/kg. The histological analysis of animals exposed to NDMA was conducted for characterization purposes.

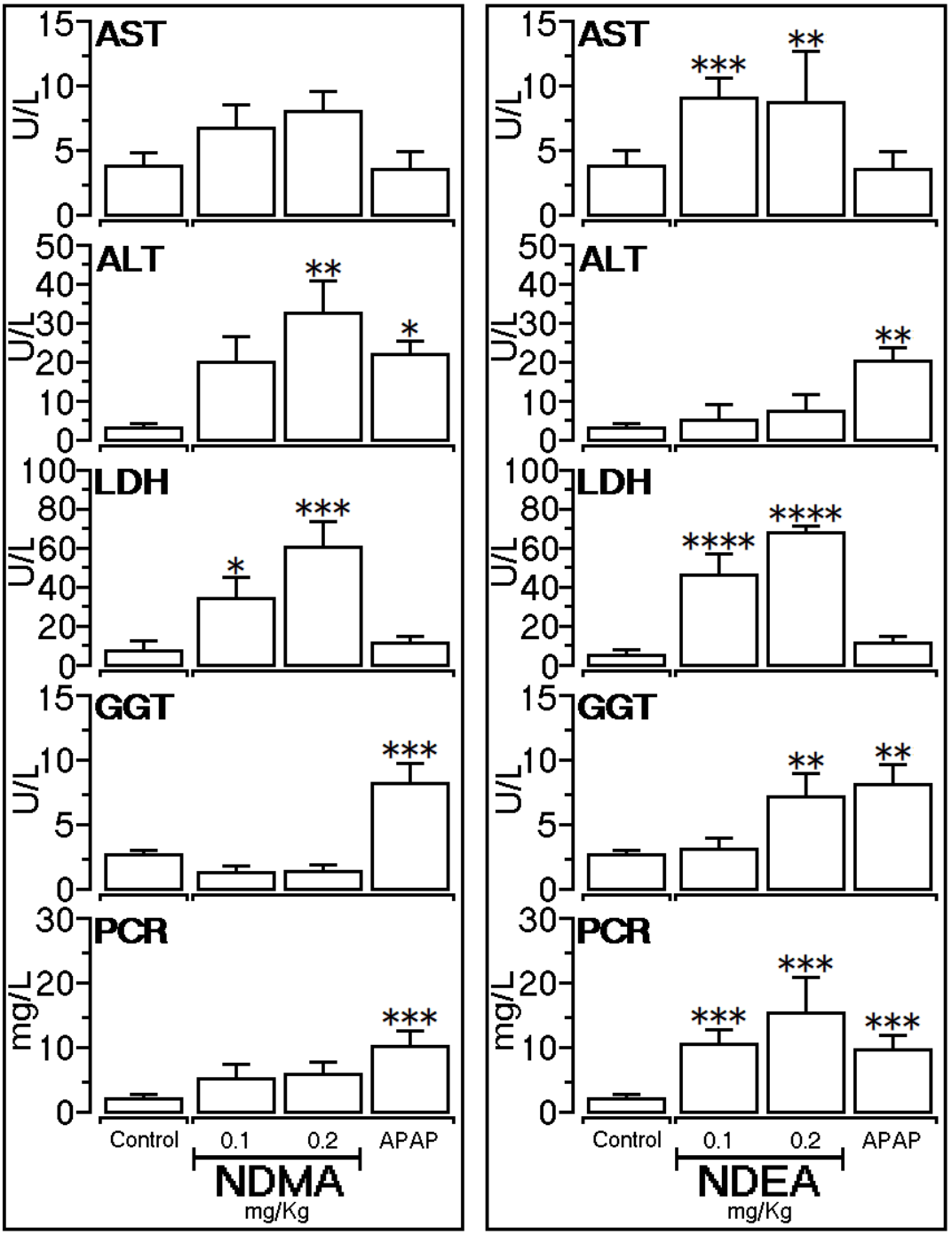

Figure 16: Biochemistry analysis to evaluate the hepatotoxicity of NDEA compound at 0.10 and 0.20 mg/kg applied in swiss mice specimes during 4 hours. Signifcant diferences were observed between non-infected and infected mice groups with *p<0.05, ***p<0.01 and ****p<0.0001, using the Tukey's test multiple comparison test.

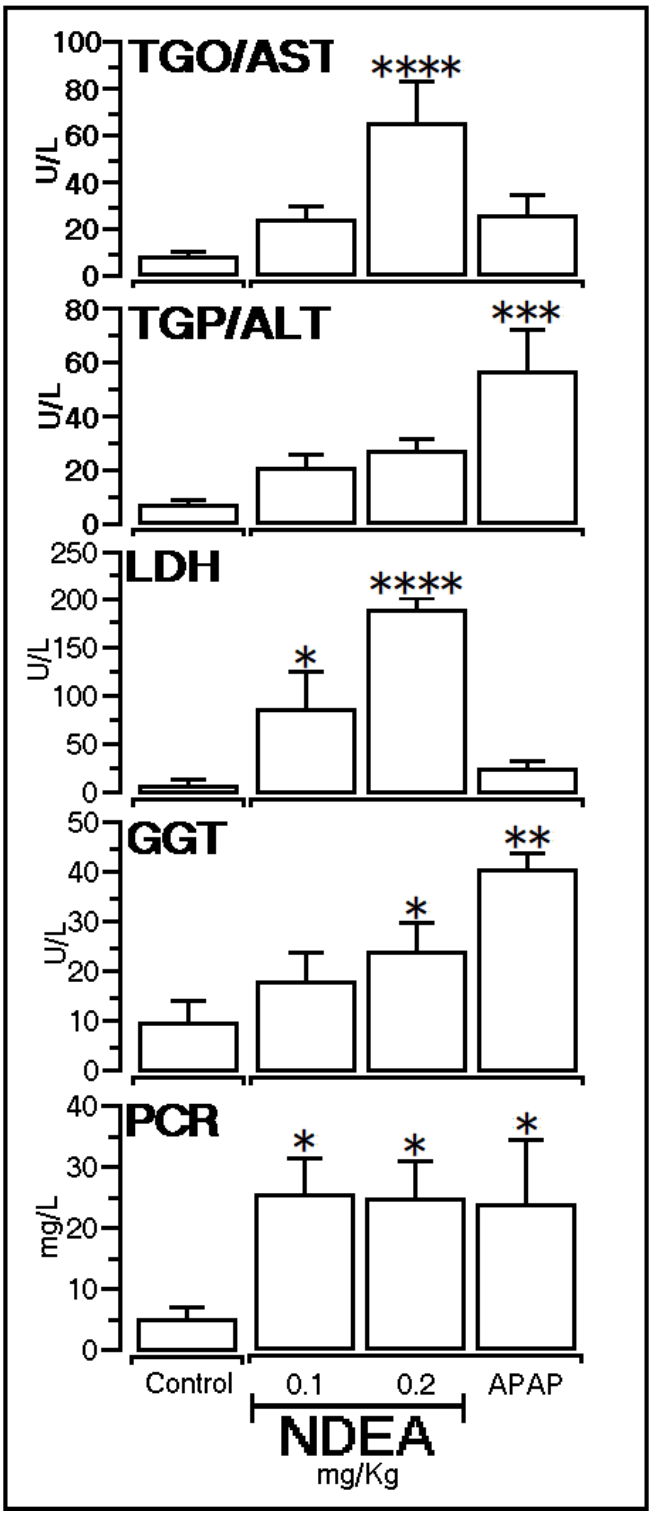

Figure 17: Biochemistry analysis to evaluate the hepatotoxicity of NDEA compound at 0.10 and 0.20 mg/kg applied in swiss mice specimes during 24 hours. Signifcant diferences were observed between non-infected and infected mouse groups with *p<0.05, ***p<0.01 and ****p<0.0001, using the Tukey's test multiple comparison test.

Before the death of the animals, respiratory symptoms were observed 2 hours after intraperitoneal injection. Respiratory symptoms related to exposure or poisoning have already been reported in the literature, in studies such as those by Jönsson et al. (2009); Hidajat et al. (2019a); Freund (1937); Kimbrough (1982), which evaluated changes in the respiratory system of human workers exposed daily to NDMA. Khanna and Puri (1966) described macroscopic congestion in the lungs of rats exposed to 3.75 mg/kg/day in the diet for 1–12 weeks. In 1980, Manduagwu and Bassir used a concentration of 0.20 mg/kg of NDMA orally in rats and observed an increase in AST and ALT, as well as liver necrosis, like the present study. Garland et al. (1988) also exposed rats orally to a concentration of 0.10 mg/kg of NDMA, describing an increase in ALT in their work.

Straif et al. (1999) demonstrated that 2,875 German women occupationally exposed to nitrosamines during their work in a rubber factory in Germany had an increased mortality rate related to non-alcoholic liver cirrhosis compared to the general population rate of German women. All 10 cases of death from non-alcoholic cirrhosis reported were among women employed in the production of technical rubber goods. It was also observed that the risk of death from non-alcoholic liver cirrhosis increased with earlier hiring dates and longer employment duration, suggesting a correlation between prolonged exposure to nitrosamines and the development of non-alcoholic liver cirrhosis.

3.4.2. Biochemical analysis of cytokines and lipid peroxidation

Our findings suggest that NDMA exposure caused a visible increase in lipid peroxidation and oxidative tissue damage, which may be an indicator of cellular and tissue damage. We hypothesize that exposure to the NDMA compound may justify liver necrosis, based on the analysis that indicated an increase in TBARS/MDA levels, which may be an indicator of oxidative damage and lipid peroxidation in liver tissues. It is known that liver necrosis can occur due to a variety of factors, including hypoxia, ischemia, and chemical toxicity (Guicciardi et al, 2023). In the case of exposure to NDMA, it is possible that the substance caused an intensified oxidative response in hepatocytes, leading to tissue damage and subsequent necrosis.

The serum levels of TNF alpha and IL-6 were also higher during the 4-hour exposure period for both nitrosamines at the studied concentrations, however, NDEA possibly had more influence on TNF alpha levels, while NDMA caused greater changes in IL-6 levels. A study

developed by Ponce-Lopez et al., 2023, showed similar data related to IL-6 and TNF alfa when studying the effects of NDEA in the hippocampus of rats (Figure 18).

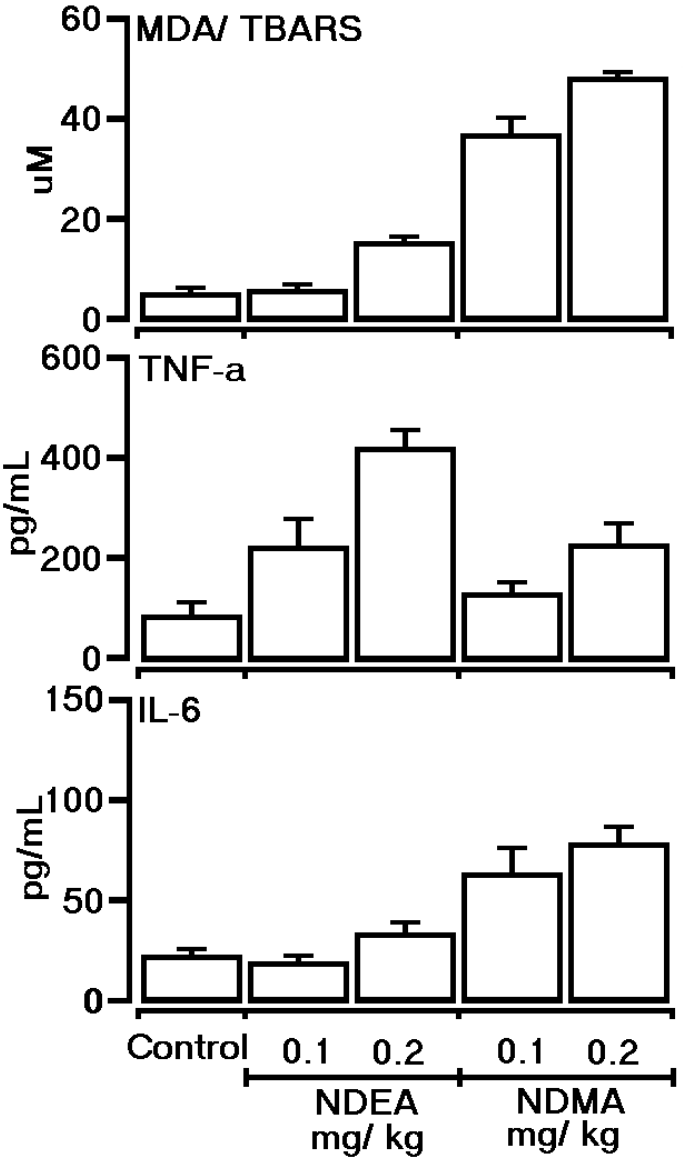

Figure 18: MDA/TBARS, TNF-alfa, and IL-6 levels in studied groups. The MDA/TBARS concentrations increased in treated groups, especially when NDMA was applied, indicating possible lipid peroxidation related to the compound. TNF-alfa increased in groups treated with NDEA, while, IL-6 increased in groups treated with NDMA.

An analysis of the results according to the literature published by Murphy, 2014 suggests that the systemic increase in TNF-α (tumor necrosis factor-alpha) can significantly impact anaphylaxis and vascular congestion, often leading to severe outcomes, including death. In

severe cases, the overwhelming inflammatory response and vascular leakage can lead to disseminated vascular congestion, where multiple organs become engorged with blood. This can cause multi-organ failure, contributing to the lethality of anaphylactic reactions. These findings may possibly justify the vascular congestion observed during histological analysis and even elucidate a process that possibly contributed to the lethality found in animals exposed to NDMA.

3.4.3. *Mus musculus* Histology

The initial experiments exposing Mus musculus to nitrosamines for 4 hours did not show any significant tissue-level damage. Therefore, to further characterize the potential hepatic effects related to exposure time, the study was repeated with an increased exposure duration of 24 hours. The extended 24-hour exposure period revealed that animals exposed to NDMA exhibited liver necrosis and mortality before completing the full 24-hour exposure. This suggests that the longer exposure duration was necessary to induce more severe hepatic damage and toxicity. The observation of liver necrosis and premature mortality in the NDMA-exposed group indicates that this nitrosamine compound can elicit acute hepatotoxic effects when exposure is prolonged. This is an important result because it allows the scientific community to better elucidate the time-dependent progression associated with this nitrosamine and liver injury. By extending the exposure duration, the study was able to uncover the more severe hepatic consequences of nitrosamine exposure that were not apparent with the initial 4-hour protocol. The histopathological study revealed vacuolization of the hepatocyte cytoplasm in the groups treated with NDEA, albeit to a lesser extent compared to the group treated with paracetamol. The histological results showed a predominance of vascular obstruction, hepatic vacuolization, and cell retraction. The severity of these characteristics was scored on a scale from 0 to 4 (0: absent, 1: minimal, 2: mild, 3: moderate, 4: marked) (Figure 19).

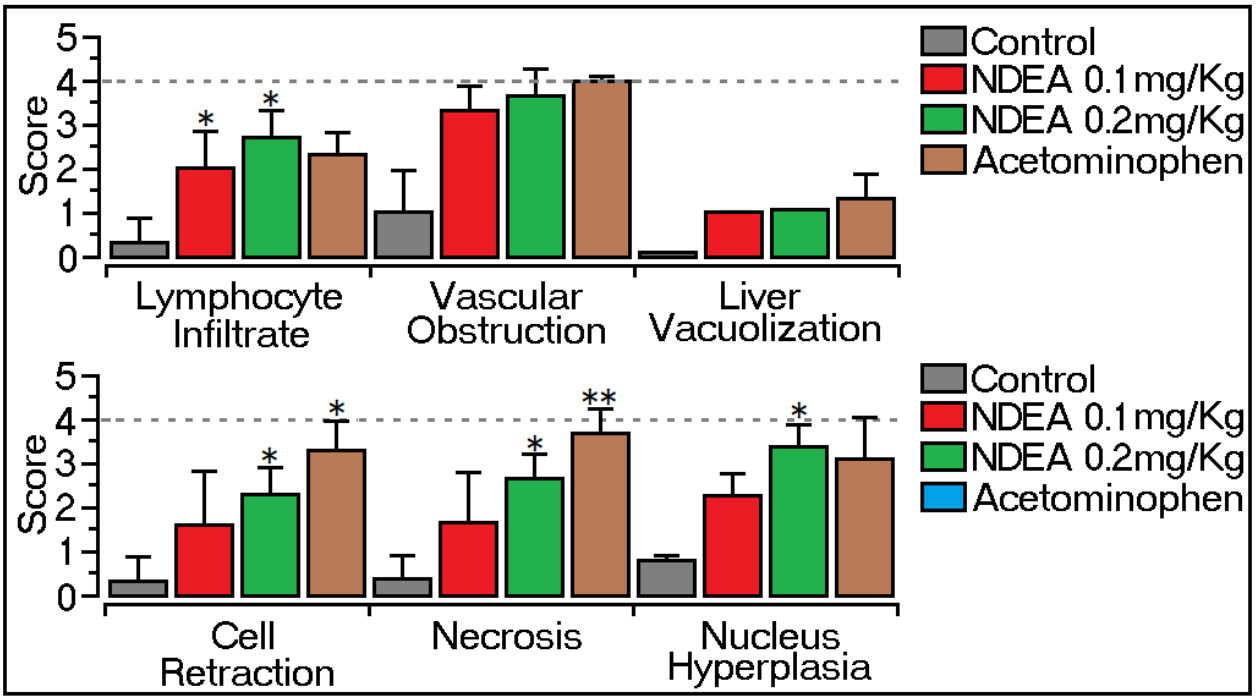

Figure 19: Semiquantitative histopathological scores of lymphocyty Infiltrate, vascular obstruction, liver vacuolization, cell retraction, necrosis and nucleus hyperplasia. The data are expressed as the means±SD. The Tukey test was used to analyze significant differences relative to the control group. The dotted line indicates the maximum score of 4. Asterisks (*) indicate significant differences relative to the control group, with *p<0.05, ***p<0.01, and ****p<0.0001.

According to the study conducted by Hosoki et al. (1989), obstruction of the hepatic veins is the most common pathophysiological mechanism in hepatic vascular diseases. These diseases form a heterogeneous group, with obstruction of the hepatic venous system caused by various factors such as thrombosis, atherosclerosis, embolism, or external vessel compression, often due to tumors. Hepatic diseases related to the obstruction of blood vessels in the liver can have profound consequences on the organ's function (Crawford, 2006; Ebert, 2006). The studies by Crawford (2006) and Ebert (2006) associate hepatic vascular obstruction with the occurrence of necrosis and an increase in hepatic markers, specifically transaminases. This is a relevant finding observed in the histopathological analysis conducted in this work. A qualitative analysis of liver histopathology revealed significantly more pronounced blood vessel obstruction in the groups exposed to nitrosamines (at both concentrations) and acetaminophen compared to the control group. Hemorrhagic foci were also observed at a concentration of 0.20 mg/kg (NDEA) and in NDMA at both concentrations. Figure 20 illustrates some of the alterations found and quantified by score.

The review conducted by Janmeda et al. (2024) elucidates that the oral or parenteral administration of NDEA, even at low doses, results in significant liver damage, as observed in the present study. The same study suggests that the bioactivation of nitrosamines by the CYP450 enzyme leads to oxidative stress and cellular damage due to the formation of reactive oxygen species, which alter the antioxidant system in tissues. This is closely related not only to the damage observed in the livers of exposed mice but also to the carcinogenic properties of these compounds and the damage described by other studies in various organs, notably the kidneys. Studies by Schoental et al. (1974), Rogers et al. (1975), Frank et al. (1988), and Verna et al. (1996) describe some properties of NDEA, including the analysis of its metabolism. In these studies, rats exposed to NDEA showed that the compound is metabolized and cleared from the bloodstream completely within 4 hours, with only a small portion being excreted in the urine. However, if the metabolism of this compound is inhibited, more than 10% of NDEA will likely be excreted in the urine, and the body's clearance time will extend to around 24 hours. It is therefore understood that liver damage can directly influence the metabolism of nitrosamines. As highlighted earlier in this study, the concentrations used for NDMA resulted in the death of the animals in less than 24 hours. Upon dissection, a significant difference in liver coloration was observed (Figure 21), indicating total organ necrosis. For this reason, the analyses for the NDMA compound are qualitative and are shown in Figure 22.

In the animals exposed to NDMA at both concentrations, there was a very pronounced cellular vacuolization, indicative of possible steatosis, common to all individuals analyzed. This characteristic was particularly notable when compared to the animals exposed to NDEA, acetaminophen, and the control group. Vacuolization, or degeneration of hepatocytes, occurs when an agent interferes with the cell's fatty acid metabolism, either by increasing its synthesis or by impairing its utilization, transport, or excretion (Brasileiro Filho, 2004). Kimbrough (1982) reported that five individuals poisoned with NDMA at unknown concentrations via oral ingestion through lemonade exhibited mild to severe thrombocytopenia. Two of these individuals died, and their autopsies revealed primary effects including hemorrhages, cirrhosis, and liver necrosis, as well as internal hemorrhages in other organs (Cooper and Kimbrough, 1980; Kimbrough, 1982).

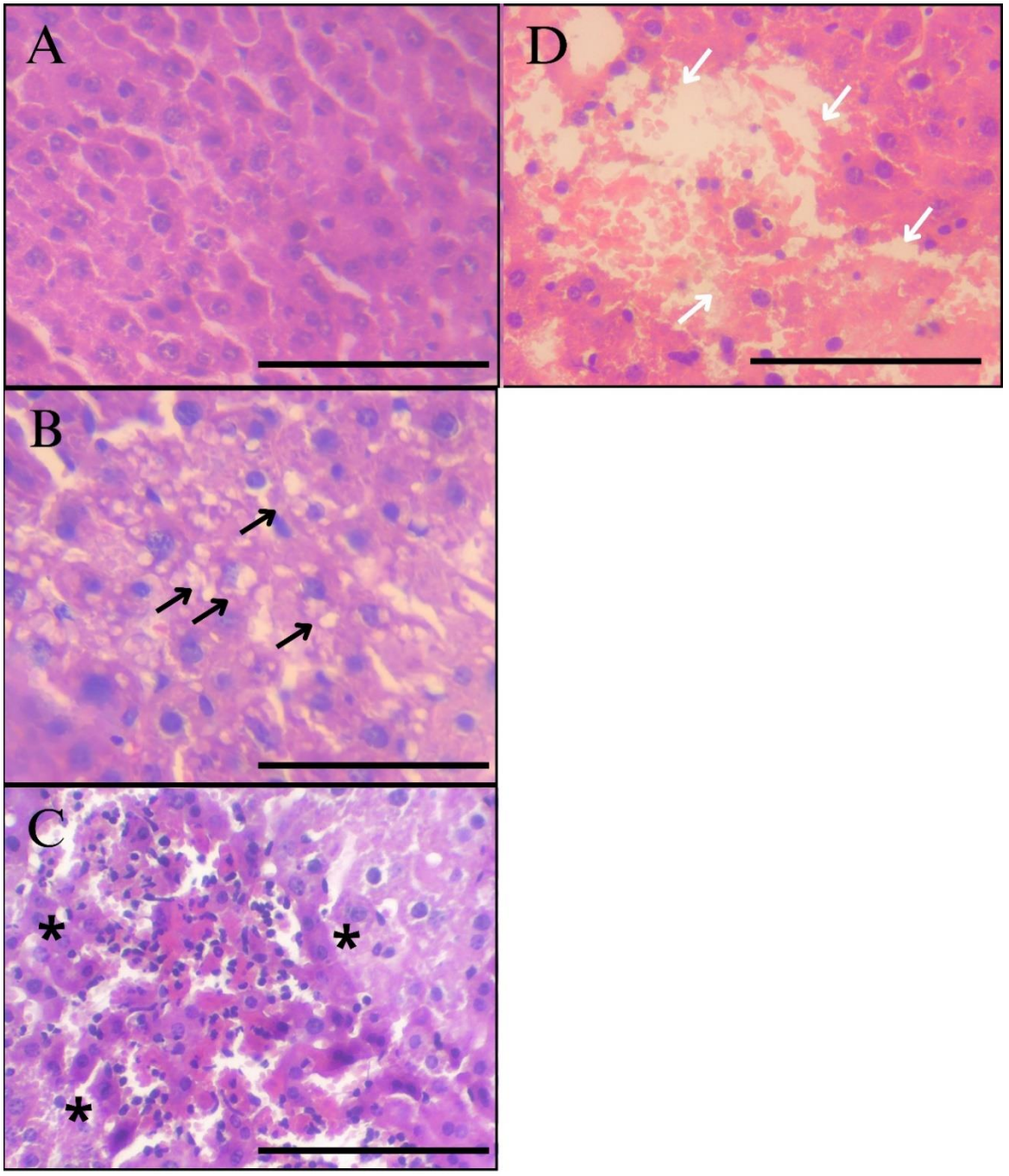

Figure 20: Histopathological evaluations of the liver were conducted qualitatively using a light microscope in a blinded trial, adhering to the guidelines provided by the Society for Toxicologic Pathology. A) Control B) Hepatic vacuolization, the entire region where white and round vacuos are seen corresponds to vacuolization, some of them are highlighted by black arrows, C) inflammatory infiltration, the most predominant region of infiltration is highlighted by asterisks, the lymphocytes correspond to small dots of intense purple color in the middle of the tissue; D) Necrosis, the visible region of necrosis is demarcated by white arrows, it is possible to see nuclei no longer surrounded by cell cytoplasm, as well as scattered cytoplasm stained in pink. The highlighted bar in each figure indicates a distance of 400 micrometers.

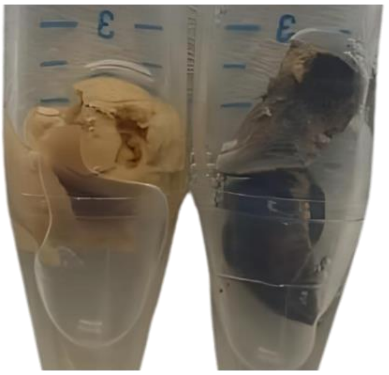

Figure 21: Differential liver coloration post-conservation in 70% ethanol for subsequent histological procedures. On the left, liver of a mouse exposed to NDEA 0.20 mg/kg; on the right, necrosis of the organ is observed after exposure to NDMA at the same concentration. Macroscopic view.

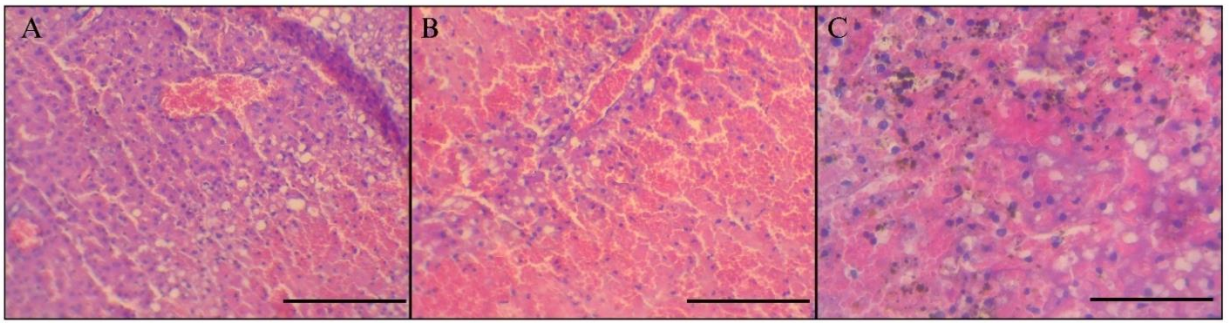

Figure 22: Mice exposed to NDMA exhibited organ necrosis with color alteration and indications of A and B: necrosis; A and C: marked hepatic steatosis. In image C, it is also possible to observe the potential presence of hemosiderin (brownish spots). These findings were common to all individuals exposed to NDMA for 24 hours (at both evaluated concentrations), and in all of them, the visualization of possible steatosis and necrosis was significant. The highlighted bar in each figure indicates 100 micrometers.

A study by Jacobson et al. (1955) demonstrated that exposing dogs to NDMA concentrations of 16 to 144 ppm for 4 hours resulted in prolonged coagulation time, extended prothrombin time, reduced plasma cholinesterase levels, and leukopenia. The dogs exposed to NDMA at all concentrations exhibited severe liver toxicity and mortality. It is believed that the described hematological effects, such as increased coagulation time, were a direct consequence of the hepatotoxicity induced by the compound. In rats given NDMA via intravenous injection, the concentrations of unmetabolized NDMA in the liver, spleen, kidney, lung, and brain were about 70% of the arterial blood concentrations. These concentrations decreased in parallel with blood levels to undetectable amounts within 4 hours post-exposure, indicating that these tissues do not accumulate NDMA in rats (Johnson et al. 1987). Numerous studies describe the severe

hematological and hepatic effects of NDMA exposure in both humans and animals. Much of the literature suggests that hepatotoxicity is the most prominent and characteristic systemic effect of NDMA and NDEA, leading to centrilobular necrosis, hemorrhage, fibrosis, cirrhosis, and ascites.

3.4.4. PAF-AH Inhibithor screening assay

The graph illustrates that there was no inhibition of enzymatic activity by the studied nitrosamines. Statistical analysis revealed no significant difference in the enzymatic activity of PAF-AH between the control (absence of compounds) and during exposure to the nitrosamines NDMA (Figure 23A) and NDEA (Figure 23B). This indicates that neither NDMA nor NDEA inhibited PAF-AH enzyme activity at any of the tested concentrations.

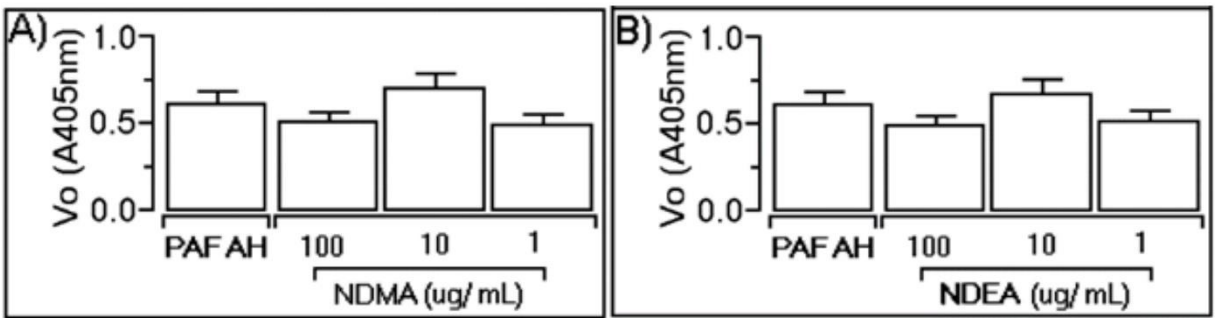

Figure 23: Analysis of PAF-AH Enzyme Inhibition in Interaction with Nitrosamines NDMA and NDEA at Different Concentrations. The graph shows that there was no inhibition of enzymatic activity by the studied nitrosamines. Statistical analysis revealed no significant difference between the enzymatic activity of PAF-AH in the absence of compounds and during exposure to the nitrosamines NDMA and NDEA.

3.5. *In Silico*

Literature has elucidated that compound with specific motifs, such as a sulfur or phosphate group, often establish covalent interactions with the OG of serine (Samanta andBahnson, 2008; Sarma and Liu, 2016). Analyzing the chemical structure of all molecules studied in this work, it was observed that only DEP (paraoxon) interacts in a covalent way with the serine that belongs to the catalytic site of PAF-AH. For this reason, the covalent docking protocol was applied only for this compound (Figure 24).

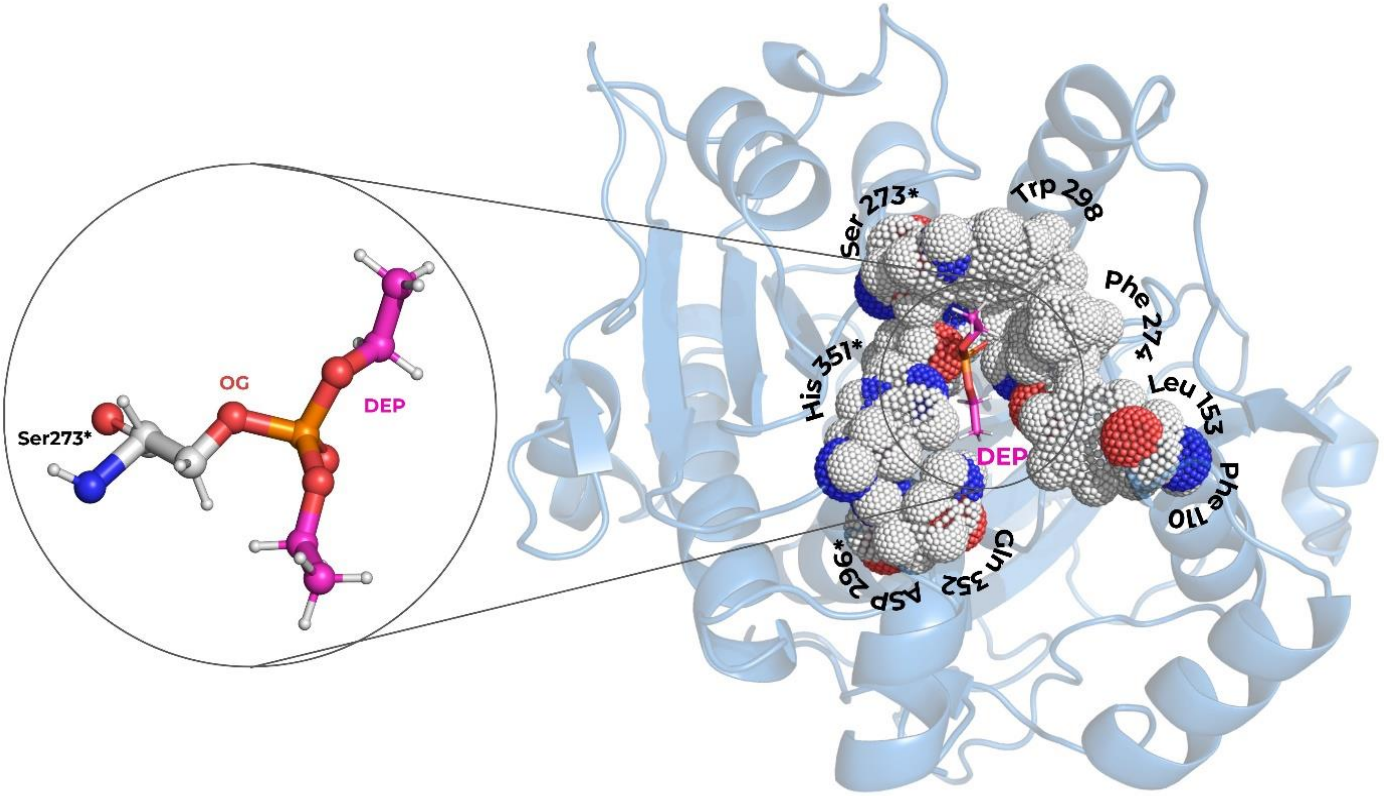

Figure 24. DEP has carbon atoms represented in pink, phosphorus in orange, oxygens in red, and hydrogens in white. The serine residue has carbon atoms represented in white, nitrogen in blue, oxygens in red, and hydrogens in white. The molecules exhibit a covalent bond at the OG atom of serine 273, which interacts with the phosphorus of the DEP compound. *Residues of catalytic site.

This study investigated the interactions of specific compounds with enzymes, applying covalent and non-covalent docking techniques to determine binding affinity,, measured by the Piecewise Linear Potential (PLP) score. DEP (paraoxon) wasidentified as the only compound to interact covalently with the serine in the catalytic site of PAF-AH (Samanta and Bahnson, 2008), justifying the exclusive use of covalentdocking for this molecule. Adjustments to the PLP scores, considering the number of heavy atoms, were made to ensure fair comparisons.

Both techniques for docking, the covalent and the non-covalent, were developed to evaluate which one could demonstrate the best PLP score (Figure 25) and the best pose compared to the crystallization model, this analysis brings more accuracy to theobtained data.

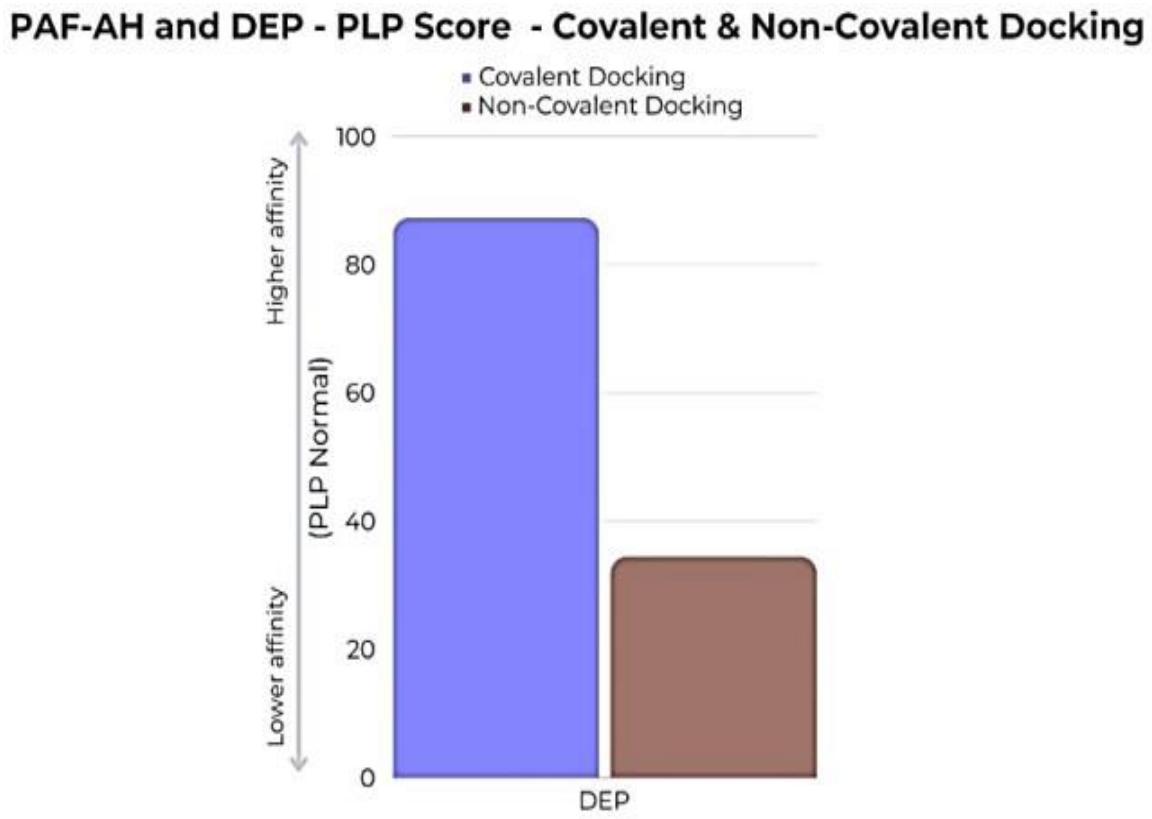

Figure 25. PLP Normal Score comparison between Non-Covalent Docking and Covalent Docking done with PAF-AH and the compound DEP (paraoxon).

Conceptually, it is crucial to emphasize the significance of examining Gold PLP norms to comprehend the obtained results. The Piecewise Linear Potential (PLP) serves as the default scoring function in GOLD (Table 8). PLP is employed to representthe steric complementarity between the protein and ligand, as detailed in the GOLD User Guide from CCDC. The methodology adopted in this study involved normalizing the PLP by the number of heavy atoms of the respective ligands, a strategy aimed at balancing the scores relative to the size of the molecules. This specific approach was employed to make comparisons fairer and more meaningful, especially when assessing steric fit between the protein and theligand. This method

was crucial to ensure robust and comparable analysis, contributingto a deeper understanding of ligand-protein interactions.

Table 8: PLP Normal Score divided for the Number of Heavy Atoms for all the compounds dockedutilizing GOLD and DataWarrior protocol.

PLP Normal Score/Number of Heavy Atoms			
Ligands	**Enzymes**		
	PAF-AH	*LPCAT3*	*cPLA2*
NDMA	4.32	8.01	6.23
NDEA	4.23	9.22	9.00
Acetaldehyde	7.46	7.22	6.41
Ethanediazonium	5.38	6.05	5.13
2-Hydroxyethanediazonium	5.17	11.08	8.48
Methanol	8.60	8.13	9.40
DEP (Covalent Docking)	10.92	-	-
LPC	-	2.94	-
Giripladib	-	-	2.57

To validate the binding poses, inhibitors and substrates with proven efficacy in the literature were employed, such as DEP for PAF-AH, lysophosphatidylcholine for LPCAT3, and Giripladib for cPLA2. Due to software limitations with small molecules like formaldehyde and methyl diazonium ion, Webina was utilized, allowing for more comprehensive analyses of these interactions. The results obtained from the *in silico* tests involving molecular docking illustrate the interaction between the ligand compounds and the target enzymes (PAF-AH, LPCAT3, and cPLA2). The GOLD software was unable to perform docking for the molecules formaldehyde and Methyldiazonium Ion, as both have a very limited numberof atoms, making it impossible to generate coordinates for docking. In an effort to address this limitation, the Webina software was utilized to conductdockings for both compounds with all enzymes (Kochnev et al, 2020). Additionally, docking simulations were performed for each reference ligand alongside its respectiveenzyme. The binding free energy values were extracted through this methodology. This approach aimed to overcome the challenges posed by the small size of formaldehyde and Methyldiazonium Ion, allowing for a comprehensive analysis of their interactions with the enzymes under investigation. (Table 9).

Table 9: Free Energy calculations for both small ligands, docked using Webina as a tool. Consistent coordinates and grid box sizes were maintained for each enzyme. The docking was performed using the respective reference ligand for each enzyme.

	Affinity (Kcal/mol)		
Ligands	**Enzymes**		
	PAF-AH	*LPCAT3*	*cPLA2*
Formol	-1,6	-1.5	-1.8
Methyldiazonium Ion	-2.7	-2.3	-2.7
DEP	-3.9	-	-
LPC	-	-6.5	-
Giripladib	-	-	-10.0

Interactions with the amino acids belonging to the catalytic site or the residual catalytic site can affect enzyme activity by increasing or decreasing it (Stafforini, 2009). After the Docking results, Molecular Dynamics was done to confirm the docking predictions, and evaluate the consistency of the pose predicted. The PLP scores and binding free energy indicate the compounds' low affinity for the proteins.

The analysis of molecular dynamics involving LPCAT3, PAF-AH, and cPLA2 with compounds NDMA, NDEA, and their metabolites did not reveal significant interactions. It was observed that only the compound NDEA interacted and remained within the enzymatic cavity of the cPLA2 enzyme throughout a 200 ns molecular dynamics simulation. With LPCAT3, only the compound NDMA showed interaction. Surprisingly, none of the compounds interacted with the PAF-AH enzyme during the simulation period. These results (Table 10) highlight the specificity of interactions between compounds and different enzymes, suggesting potential patterns of molecular recognition and selective affinity.

Table 10: Ligands that interacted or did not interact with the enzymes studied.

Ligands That Remained in The Active Site			
Ligands		**Enzymes**	
	PAF-AH	*LPCAT3*	*cPLA2*
NDMA	No	Yes	No
NDEA	No	No	Yes
Acetaldehyde	No	No	No
Ethanediazonium	No	No	No
2-Hydroxyethanediazonium	No	No	No

Formol	No	No	No
Methyldiazonium Ion	No	No	No
Methanol	No	No	No
DEP (Covalent Docking)	Yes	-	-
LPC	-	Yes	-
Giripladib	-	-	Yes

Another relevant analysis is the distance between the bonds established with the amino acid residues belonging to the active site. This distance is important because the catalytic residues in the enzyme need to be close enough to the substrate so thatthey can interact and catalyze the chemical reaction. If the distance is too large, the interaction will be weak, and the reaction will not be efficient. If the distance is too small, there may be electrostatic repulsion between the catalytic residues and the substrate, preventing the interaction (Kohen, A. & Klinman, 1999). According to scientific literature, the ideal distance between the catalytic residuesof the enzyme and the substrate can vary significantly depending on the specific enzyme and substrate involved in the reaction. The distance can be influenced by various factors, such as the spatial orientation of the active residues, the rigidity of thesubstrate, Van der Waals interactions, among others. It is therefore difficult to establisha single distance for enzyme activity. Although the range of 3 to 5 angstroms (Kohen, & Klinman, 1999; Kohen, et. al. 1999; Fakhar, 2016) is mentioned in somereferences, it is not an universal measure and can vary according to the enzyme and substrate in question.

Molecular Dynamics simulations corroborated the docking predictions, revealing low affinity of the compounds for the proteins and highlighting specific interactions of NDEA with cPLA2 and NDMA with LPCAT3. Analysis of the proximity between ligandsand catalytic residues underscored their importance for catalytic efficacy, with distancegraphs, illustrating the variation of these interactions over time. For the most noteworthy results we uncovered, distance graphics were created (Figures 26 and 27) toillustrate the movement of the compounds in relation to the closest catalytic residue.

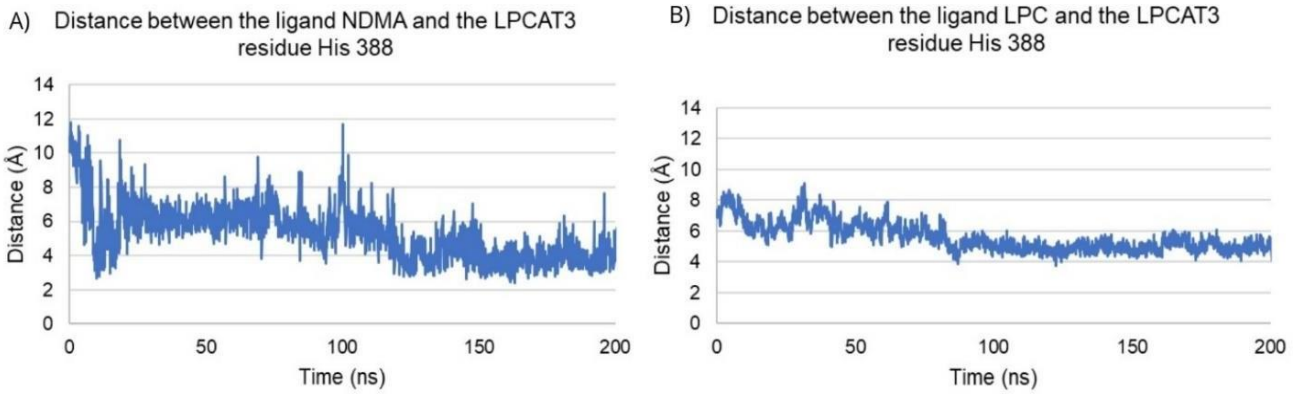

Figure 26: Distance between the residue Histidine 388 and the ligands NDMA and LPC (reference ligand). A) The graphic analysis generated by XMgrace software illustrates the approximation of the ligand and the residue during the Molecular Dynamics simulation. It can be observed that, probably due to the size of the ligand, NDMA exhibits more movement in relation to this residue than graph B) LPC. However, both graphics depict a similar interaction with an approximation of the ligands over time.

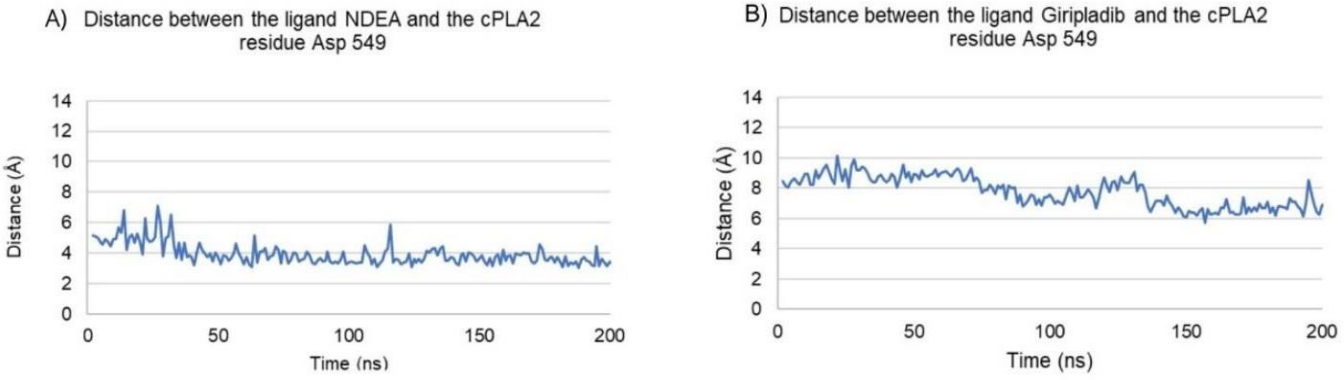

Figure 27: Distances between the compounds A) NDEA; B) Giripladib. Throughout all the MD simulations, the compounds remained stable or approached the focal amino acid.

In Molecular Dynamics simulations, it was observed that the ligands initiallypositioned favorably relative to the enzymes in the early stages of the simulation. However, this interaction was quickly lost, leading to a significant deviation of the ligands from their initial positions, as demonstrated in Figure 28. Specifically, compounds NDEA and NDMA maintained distances between 2 and 6 angstroms fromthe amino acid residues in the catalytic sites of the enzymes cPLA2 and LPCAT3, respectively.

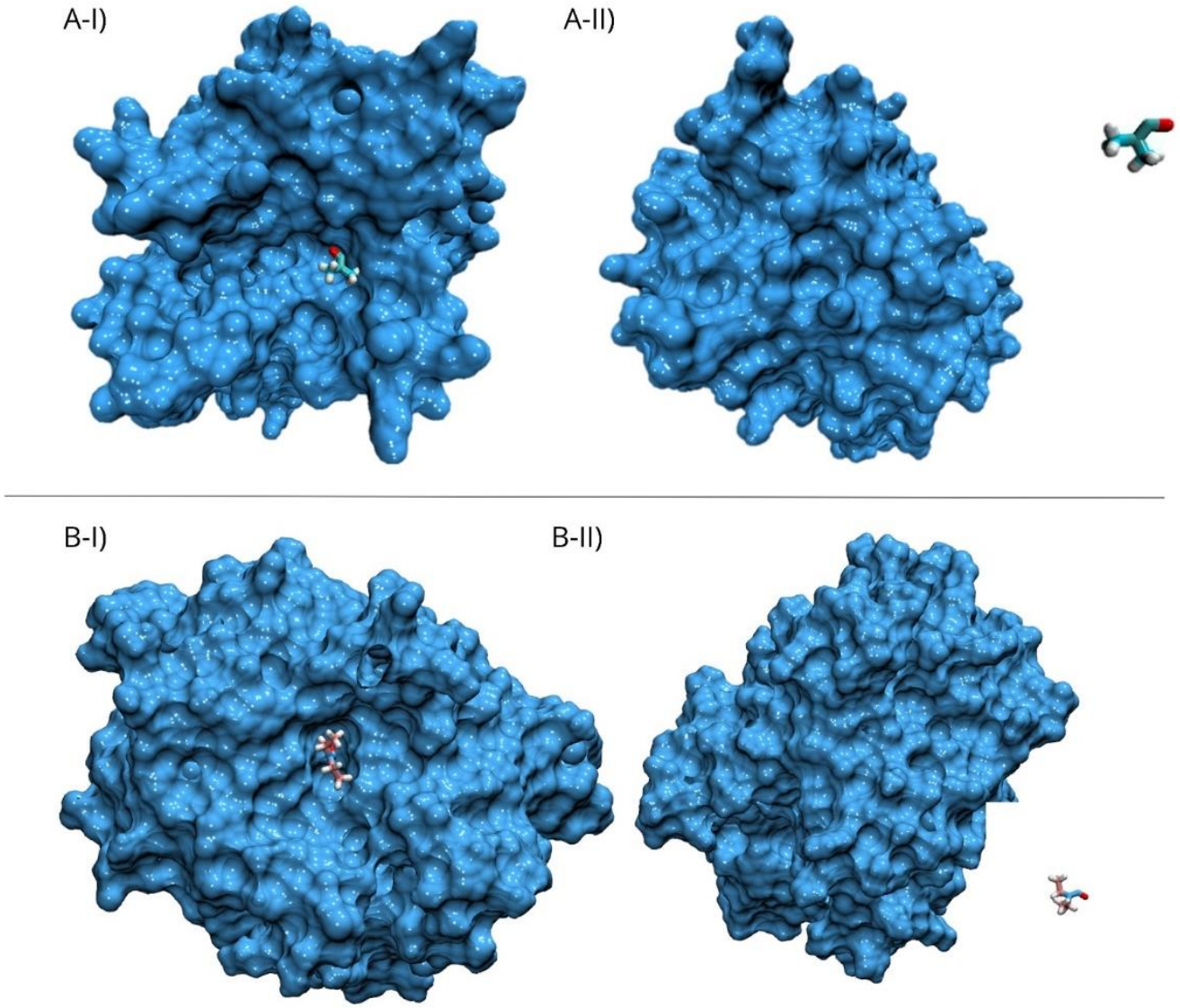

Figure 28: The images demonstrate A-I) PAF-AH enzyme and NDMA ligand at the beginning of the molecular dynamics simulation. A-II) PAF-AH enzyme and NDMA ligand at the end of the molecular dynamics simulation. It is noted that the ligand completely dissociated from the enzyme during the MD simulation. In B-I) the PAF-AH enzyme and NDEA ligand are observed at the beginning of the simulation, and in B-II) the ligand and the enzyme at the end of the simulation, where they completely lost interaction.

Contrastingly, ligands that interacted from the beginning to the end of the dynamics consistently remained within the pocket of the enzymatic active site throughout the entire period (Figure 29 and 30).

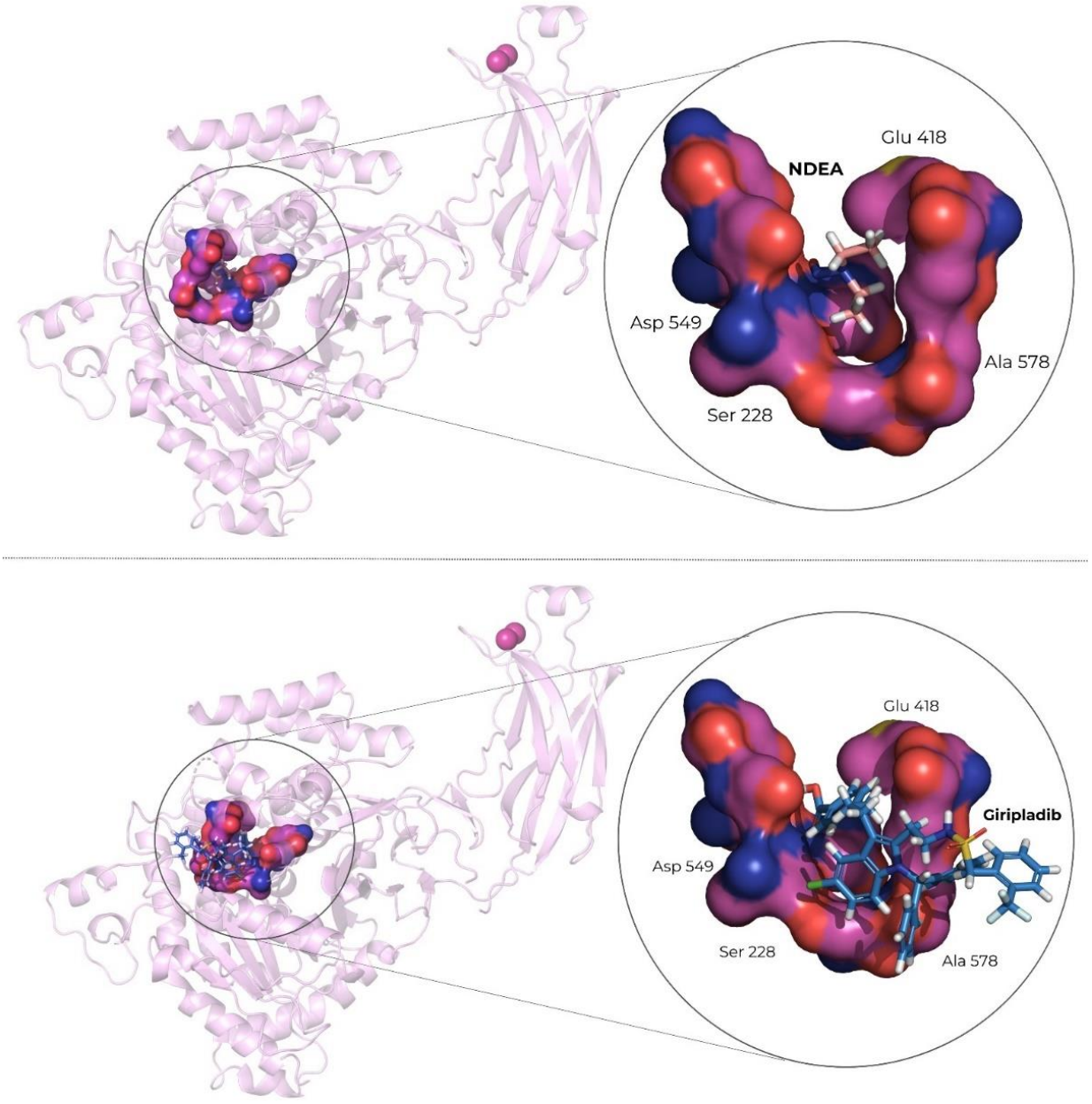

Figure 29: View of the interaction between the compounds NDEA, and the reference ligand Giripladib with the cPLA2 enzyme. The mentioned compounds remained in the same region of the active site during the molecular dynamics' simulation, from the beginning till the end.

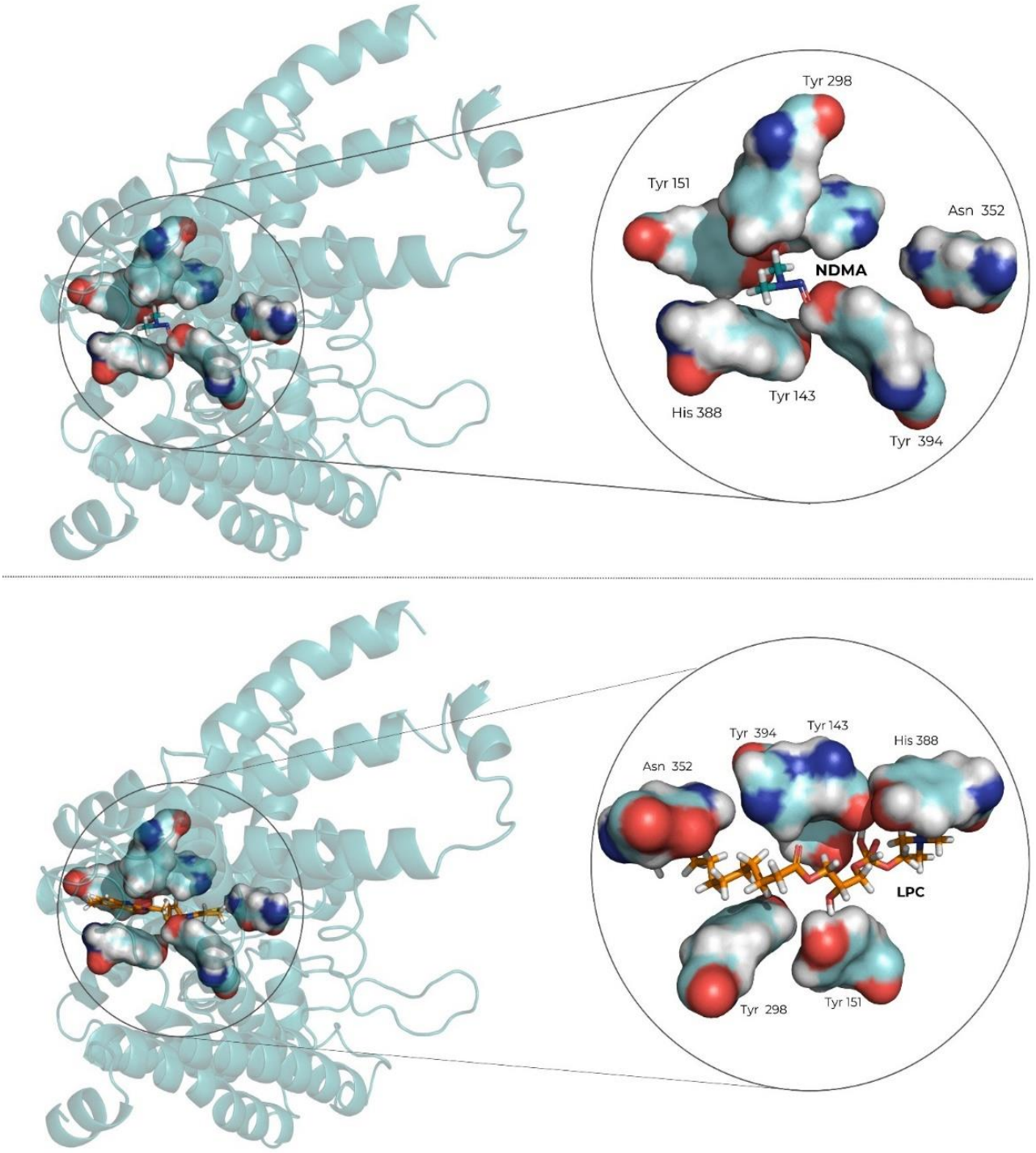

Figure 30: View of the interaction between the compounds NDMA, and the reference ligand LPC with the LPCAT3 enzyme. The mentioned compounds remained in the same region of the active site during the molecular dynamics' simulation.

Quantification of structural discrepancies was performed through RMSD analysis,while binding energy was estimated using MM/GBSA methodology, emphasizing vander Waals and electrostatic interactions. The studied compounds exhibited lower affinities compared to reference ligands.

The RMSD (Root Mean Square Deviation) is a measure that quantifies the root mean square of the discrepancies between atoms in different molecular structures or conformations. This metric is commonly used in the fields of molecular modelling, molecular dynamics, and molecular docking to assess the structural similarity or difference between a reference structure (such as an experimental crystallographic structure) and a predicted or simulated structure (Coutsias and Wester, 2019)

The RMSD calculates the square root of the mean of the squares of the differencesin spatial positions of corresponding atoms between two structures. The smaller the RMSD, the greater the structural similarity between the molecules. In other words, the RMSD provides a quantitative measure of how accurately a predicted or simulated structure aligns with the reference structure (Coutsias and Wester, 2019). RMSD analysis was used to quantify variations in ligand positions, showing that both NDEA and NDMA exhibited small fluctuations in their conformations during the simulation with the enzymes cPLA2 and LPCAT3, indicating stability in the interaction.These observations are represented in Figure 31.

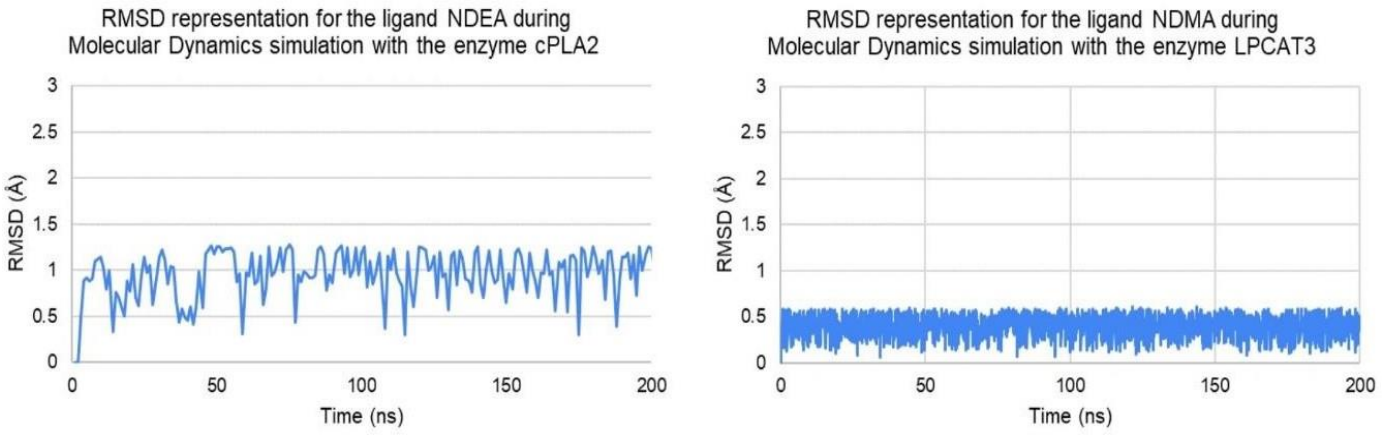

Figure 31: RMSD analysis for the interacting ligands and their respective enzymes.

To quantify the binding energy between the ligands and enzymes, the MM/GBSA method was employed, with results detailed in Table 5. MM/GBSA is a molecular modeling method that estimates

the ligand's free energy of binding through a combination of molecular mechanics, the Generalized Born solvent model, and surfacearea analysis. This methodology is particularly useful for providing detailed insights into ligand-receptor interactions, highlighting the energetic contributions vital to the formation of the ligand-enzyme complex. Additionally, MM/GBSA facilitates the prioritization of compounds for further development in drug discovery studies, due to its ability to analyze extensive datasets of ligand-receptor complexes (Ylilauri and Pentikäinen, 2013).

In MM/GBSA analysis (Molecular Mechanics/Generalized Born Surface Area), components such as EVWD (Van der Waals Energy), EEL (Electrostatic Energy), EGB(Generalized Born Energy), and ESURF (Surface Energy) are crucial for calculating the ligand-protein binding free energy. EVWD reflects Van der Waals forces, essentialfor understanding non-covalent interactions and determining ligand-protein complex stability. A negative EVWD indicates attraction, while a positive one suggests repulsion. EEL evaluates electrostatic interactions, and EGB represents solvation energy, calculated by the Born model, related to the solvent's effect on interactions. ESURF accounts for energy linked to the complex's surface area, influencing the binding free energy. (Ylilauri and Pentikäinen, 2013; Ahinko et al., 2019).

An accuracy of estimating binding energy utilizing the MM/GBSA method was donefor the compounds that interacted with the proteins (Table 11).

Table 11. Results for the MM/GBSA calculations and all components.

		MM		GBSA		ΔGbinding
Protein	**Ligand**	**E_{VDW} (kcal/mol)**	**E_{EL} (kcal/mol)**	**E_{SURF} (kcal/mol)**	**E_{GB} (kcal/mol)**	**MM/GBSA (kcal/mol)**
LPCAT3	NDMA	-14.3 ± 0.1	-4.9 ± 0.2	-2.3 ± 0.0	8.3 ± 0.0	-13.2 ± 0.1
	LPC	-57.8 ± 0.3	-75.5 ± 0.9	-8.6 ± 0.0	90.0 ± 0.7	-52.0 ± 0.3
cPLA2	NDEA	-19.6 ± 0.4	-4.3 ± 1.4	-2.9 ± 0.0	12.4 ± 1.4	-14.5 ± 0.5
	Giripladib	-53.9 ± 1.4	-16.6 ± 3.2	-7.0 ± 0.2	42.3 ± 3.0	-35.3 ± 1.1

These parameters are crucial for understanding molecular interactions and calculating the ligand's free energy of binding in MM/GBSA studies, offering detailed insights into the forces contributing to complex formation. The results indicated that ligands NDEA and NDMA have

lower affinity compared to reference ligands, with MM/GBSA values ranging between -13.2 ± 0.1 and -14.5 ± 0.5 kcal/mol, against -52.0 ± 0.3 and -35.3 ± 1.1 kcal/mol of reference ligands. Van der Waals interactions were decisive in the formation of the LPCAT3-NDMA and cPLA2-NDEA complexes, with significant energy values. Studies such as those by Neves et al. (2020) and Jamal andAlharbi (2022) also explored interactions between nitrosamines and enzymes, using similar methodological approaches, including Docking, Molecular Dynamics, MM/GBSA, PLP, and RMSD. These researches contribute to a deeper understandingof binding mechanisms and the potential biological effects of ligand-protein interactions, reinforcing the importance of these integrated analyses in the fields of biochemistry and drug discovery.

A study by Mouchlis and Dennis (2022) examined the inhibitory effects on the cPLA2 enzyme, highlighting two key factors influencing strong binding within itshydrophobic pocket: ligand size and aromaticity. Fluoroketone compounds with longeraromatic hydrophobic tails tend to exhibit potent inhibition of cPLA2. This analysis sheds light on the limited affinity of most compounds investigated in our study for cPLA2, while also revealing the modest affinity of the nitrosamine NDEA for the enzyme's active site.

Additionally, Mouchlis and Dennis (2022) observed that highly effective inhibitors of PAF-AH feature aromatic residues that selectively interact with aromatic residues inthe protein's active site. Comparing these findings with our results, it becomes evidentthat nitrosamines and their metabolites do not conform to the typical inhibitors of this enzyme.

Du et al. (2019), Zhang et al. (2021), and Reed et al. (2022) described various LPCAT3 substrates and inhibitors, noting their structural similarities with LPC. These comparisons suggest that these enzymes exhibit a particular affinity for inhibitors based on their structural and size characteristics.

4.Conclusions

4.1.Behavioral assays

The behavioral assays with zebrafish did not show any discernible behavioral changes, according to the criteria employed, when compared to the control group (which was not exposed to the drugs). When comparing this result with the other results obtained, such as the micronucleus test and histopathological analyses, the evidence collected during the research suggests that the studied compound does not have effects related to the nervous system and the equilibrium of fish at the studied concentrations. Thus, the observed damages were systematically related to hepatic damage and nuclear abnormalities in erythrocytes.

4.2. Micronucleo and Histological analysis in Danio rerio

The micronucleus and histopathological analyses suggest a strong relationship between exposure to NDEA, the analyzed concentrations, and the development of pathologies and liver lesions. The visualization of nuclear abnormalities in erythrocytes suggests the interaction of the compound or its metabolites with genetic material. In the histopathological analysis shows, mainly, the hepatocyte degerneration (necrosis and liver vacuolization).

4.3. In vivo – Mus musculus

The results show that exposure to nitrosamines caused an increase in white blood cells (leukocytes), especially neutrophils, possibly indicating a significant inflammatory response. The increase in atypical lymphocytes suggests that the nitrosamines affected the animals' immune function.

Our biochemical results demonstrated that mice exposed to nitrosamines during 4 and 24 hours showed significantly higher levels of AST, ALT, PCR, LDH, and GGT in almost all groups when compared to the control group (saline). the specificity of NDMA action on the liver is greater than NDEA at its highest studied concentration. In a comparison relating the observed levels of biochemical markers analyzed in mice exposed to NDEA for 4 and 24 hours, it is possible to observe their progression and increase.

However, experiments with the NDMA compound at the same concentration of (0.10 mg/kg and 0.20 mg/kg) demonstred to be so toxic that it resulted in the death of all exposed animals in less than 24 hours. This event prevented the collection of blood. But the liver was collected and it was necrosed with totally different colour than the livers collected from control group and NDEA exposed groups. Histopatological analysis suggests a several hepatic vacuolization, vascular obstruction, signs of inflammation and hemosiderin also were observed. Increases in MDA levels during NDMA exposures, show lipid peroxidation and can be related to tissue damage, resulting in necrosis. An analysis of the results according to the literature suggests that the systemic increase in TNF-α (tumor necrosis factor-alpha) can significantly impact anaphylaxis and vascular congestion, often leading to severe outcomes, including death. In severe cases, the overwhelming inflammatory response and vascular leakage can lead to disseminated vascular congestion, where multiple organs become engorged with blood. This can cause multi-organ failure, contributing to the lethality of anaphylactic reactions. These findings may possibly justify the vascular congestion observed during histological analysis and even elucidate a process that possibly contributed to the lethality found in animals exposed to NDMA.

4.4. In vitro

Our in vitro test for analyzing the inhibition of PAF-AH enzyme activity confirms the results obtained from the in silico molecular dynamics tests, as both suggest the absence of interaction between nitrosamines and the enzyme's active site. The lack of this interaction is an interesting result because it speaks to the influence of nitrosamines, which are contaminants present in our daily lives, on the regulation of PAF. This work suggests that the damage caused by nitrosamines in the body is linked to their metabolism, which occurs in the liver, and to the hepatic damage that would be caused and subsequently lead to possible damage in other areas of the body, as suggested by various works in the scientific literature.

4.5. In silico

This work emphasizes the selectivity of interactions between compounds and enzymes, suggesting distinct patterns of molecular recognition. Through an integrated approach of Docking, Molecular Dynamics, and MM/GBSA analyses, it contributes to a deeper

understanding of ligand-protein interactions and emphasizes the relevance of optimal distance between ligands and catalytic residues, varying according to the specific enzymatic system.

In summary, the data obtained after the *in silico* analyses carried out so far suggestthat the NDEA and NDMA compounds did not interact in a relevant way with the majority of the enzymes in focus. It is, also, evident from the results that the studied metabolites do not exert any influence on any of the enzymes.

This analysis considers PLP Normal Scores and MM/GBSA analyses. Otherwise, the compound NDMA possibly interact with the enzyme LPCAT3, while MD simulations suggest that NDEA interact with the enzyme cPLA2.

The results suggest that the nitrosamines studied do not have a significant impacton the regulation of PAF. Therefore, further studies need to be conducted to understand the strength of the interaction between NDEA and cPLA2, as well as LPCAT3 and NDMA. In both cases, these interactions can potentially alter the inflammation levels in the organism and the cell membrane remodeling.

These results not only underscore the importance of a more in-depth analysis of the complex effects of nitrosamines and related metabolites on human health but alsoemphasize the need for relevant mitigation strategies and regulations. Prolonged exposure to these compounds requires ongoing attention and risk assessment.

Our study offers valuable insights into the molecular interactions between nitrosamines, some of their metabolites, and key enzymes associated with the inflammatory process and membrane remodeling. The results underscore that nitrosamines possess a distinct toxic profile, broadening our comprehension of the array of biological responses elicited by these compounds.

4.6. General Conclusions

Based on the results presented, it is concluded that exposure to nitrosamines, even at low concentrations and for relatively short periods, can cause significant damage to both *Danio rerio* fish and *Mus musculus* mice. The observed alterations in erythrocytes and liver damage highlight the toxicity of this contaminant, which is present in significant quantities in water and food, posing a risk not only to humans but also to aquatic ecosystems. The metabolism of these compounds is identified as the main cause of damage, including hepatotoxicity, oxidative stress, and interactions with genetic material, emphasizing the need for monitoring and control of these compounds in the environment.

The study showed that there are likely no direct relationships between nitrosamines and PAF control, as both in vitro and in silico assays highlighted the lack of interaction of these compounds with the PAF-AH enzyme, responsible for the degradation and regulation of PAF. However, other relevant findings were reported, such as the influence of these compounds on hematological and genetic alterations, and especially hepatotoxic effects. Our study suggests that the biochemical and tissue changes caused are associated with the metabolization of the compounds. We suggest nitrosamines act as potential metabolic disruptors. During their metabolism by cytochrome P450, these compounds appear to increase oxidative stress by interfering with mitochondrial function. This interference possibly can lead to the generation of reactive oxygen species (ROS) and initiate the Fenton reaction.

5. Funding and Acknowledgments

This study was financed, in part, by the São Paulo Research Foundation (FAPESP), Brasil. Process number 2023/01973-4 and 2022/04772-7, São Paulo Research Foundation (FAPESP). My deepest thanks to FAPESP.

I immensely appreciate all the researchers at the BIOMOLPEP laboratory for their phenomenal teamwork, camaraderie, and, above all, friendship. You were essential in the development of this work. I especially thank Dr. Caroline Ramos da Cruz for always clarifying my most complicated questions, for always being an inspiration and a reference to me, both as a person and as a researcher. I appreciate each contribution you made to the completion of this work.

Gustavo Antonio Fernandez, Adeilso Junior Bispo dos Santos, Michelle Rafael, and Mariana Novo Belchor, I lack words to describe the importance of your roles in all the experiments conducted, with emphasis on in vivo tests and hematological analyses. Thank you for standing by my side during this master's degree, for always giving me hope, and for bringing much happiness to this entire period; you make everything worthwhile.

I thank the entire team at the LABMA laboratory for collaborating on the histological procedures. Special thanks to Dr. Renata de Britto Mari for opening the doors of her laboratory and enabling this very important contribution to this dissertation. My heartfelt thanks to your brilliant student Isabelly Cristina Oliveira for guiding me through the entire histological procedure with phenomenal skill and knowledge; your help was invaluable.

I immensely thank Dr. Sergio Filipe Maia de Sousa and his entire team at BioSIM, especially the doctoral student Fabio Martins, for providing me with unconditional support and for sharing much of the knowledge in Docking and Molecular Dynamics that I currently possess. Dr. Sergio deserves countless thanks for welcoming me to his laboratory at a foreign university and for enabling my introduction to the field of molecular simulations. The months in his laboratory were unforgettable and enriching; I sincerely thank and congratulate the entire team; you are inspiring.

I extend my sincerest thanks to Dr. Marcos Hikari Toyama, my advisor. Thank you for sharing your knowledge; it was a privilege to develop this work alongside someone with admirable intellect. I appreciate being part of all the wonderful opportunities that arose during this journey, for trusting me as a researcher, and for always contributing to the successful completion of this project.

Finally, I would like to thank my parents, my sister Yasmin Annunciato, my brother-in-law Dhereck Zavataro Rosa, and my boyfriend Bartlomiej Bierowka. To my parents, for being the main reason for who I am today, for supporting me even when I was far away, for always encouraging me to study, and for always being my greatest inspiration. To my twin sister, a brilliant researcher, thank you for embarking on this adventure with me, for always sharing the good and bad times, and especially for being a major factor in my achievements. Yasmin, you are the person I admire the most in the world, as a researcher, sister, and individual. I thank my brother-in-law Dhereck for his unconditional support, emotional support, company, and laughter. To my boyfriend, thank you for coming into my life at the best time of this process, for sharing an exchange with me, and for inspiring me to continue even on difficult days. Thank you for making everything lighter, for believing in me, and especially for supporting me in the final phase of my master's degree. Thank you for always being there for me.

6. References

ABNT Brazilian Association of Technical Standards. 2004. NBR 15088:04. Aquatic Ecotoxicology (Acute Toxicity) Test method with fish. 19p.

Adebiyi, M.G., et al., 2019. Metabolomic and molecular insights into sickle cell disease and innovative therapies.

Adebiyi, M.G., et al., 2019. Metabolomic and molecular insights into sickle cell disease and innovative therapies. Blood Adv. 23;3(8),1347-1355.

Agius, C., & Roberts, R. J. (1981). Effects of starvation on the melano-macrophage centres of fish. Journal of Fish Biology, 19(2), 161-169.

Agius, C., & Roberts, R. J. (2003). Melano-macrophage centres and their role in fish pathology. *Journal of fish diseases*, *26*(9), 499-509.

Ahinko, M., Niinivehmas, S., Jokinen, E., & Pentikäinen, O. T. (2019). Suitability of MMGBSA for the selection of correct ligand binding modes from docking results. Chemical Biology & Drug Design, 93(4), 522-538.

Ahotupa, M., Béréziat, J.C., Bussacchini-Griot, V., Camus, A.M., Bartsch, H. Lipid peroxidation induced by N- nitrosodimethylamine (NDMA) in rats in vivo and in isolated hepatocytes. Free Radic Res Commun. 1987, 3(1-5), 285-91.

AL-HABORI, M. et al. Toxicological evaluation of Catha edulis leaves: a long term feeding experiment in animals. Ethno-Pharmacology, v. 83, p. 209-217, 2002.

Al-Quraishy, S.; Dkhil, M.A.; Abdel Moneim, A.E. Hepatotoxicity and oxidative stress induced by Naja hajecrude venom. J.Venom. Anim. Toxins Incl. Trop. Dis. 2014, 20, 42.

Aronson, S.J.; Junge, N.; Trabelsi, M.; Kelmemi, W.; Hubert, A.; Brigatti, K.W.; Fox, M.D.; de Knegt, R.J.; Escher, J.C.; Ginocchio,V.M.; et al. Disease burden and management of Crigler-Najjar syndrome: Report of a world registry. Liver Int. 2022, 42, 1593–1604.

Ashraf MA, Nookala V. 2022. Biochemistry of Platelet Activating Factor. 2021 Apr 19. In: StatPearls [Internet].

B. F. (2022). LPCAT3 inhibitors remodel the polyunsaturated phospholipid content of human cells and protect from ferroptosis. ACS Chemical Biology, 17(6), 1607-1618.

Baker MA, Nandivada P, Mitchell PD, Fell GL, Pan A, Anez-Bustillos L, Dao DT, Gura KM, Nosé V, Puder M. Pretreatment with intravenous fish oil reduces hepatic ischemia reperfusion injury in a murine model. Surgery. 2018 May;163(5):1035-1039.

Barnes, J. M., & Magee, P. N. (1954). Some toxic properties of dimethylnitrosamine. British journal of industrial

Beard, Jessica C., and Timothy M. Swager. "An organic chemist's guide to N- nitrosamines: their structure, reactivity, and role as contaminants." The Journal of organic chemistry 86.3 (2021): 2037-2057.

Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P., & Wahli, T. (1999). Histopathology in fish: proposal for a protocol to assess aquatic pollution. *Journal of fish diseases*, *22*(1), 25-34.

Bilal N, Suhail N, Hasan S, Ashraf GM, Fatima S, Khan HY, Alharbi MS, Alexiou A, Banu N. Exacerbation of *N-nitrosodiethylamine* Induced Hepatotoxicity and DNA Damage in Mice Exposed to Chronic Unpredictable Stress. Front Pharmacol. 2017, 8, 360.

Blood Adv. 23;3(8),1347-1355.

BRASILEIRO FILHO, G. Bogliolo patologia geral. 7 ed. Rio de Janeiro: Guanabara Koogan, 2004. p. 53-59.

Brazil. Environ Sci Pollut Res 28, 32823-32830 (2021). https://doi.org/10.1007/s11356-021-12998-4 Walker, S. D.; Mceldowney, S. 2013. Molecular docking: A potential tool to aid ecotoxicity testing in

Brooks, B. R., Brooks III, C. L., Mackerell Jr, A. D., Nilsson, L., Petrella, R. J., Roux, B., ... & Karplus, M. (2009). CHARMM: the biomolecular simulation program. Journal of computational chemistry, 30(10), 1545-1614.

Brown, L. F., Chester, J. F., Malt, R. A., & Dvorak, H. F. (1987). Fibrin deposition in autochthonous Syrian hamster pancreatic adenocarcinomas induced by the chemical carcinogen N-nitroso-bis (2-oxopropyl) amine. *J Natl Cancer Inst*, *78*(05), 979-986.

Camargo, M. M., & Martinez, C. B. (2007). Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream. Neotropical Ichthyology, 5(3), 327-336

Campos, C. M. D., de Moraes, J. R., & Moraes, F. R. D. (2008). Histopathology of liver, kidney, and spleen of Piaractus mesopotamicus, Prochilodus lineatus, and Pseudoplatystoma fasciatum parasitized by myxosporeans, captured in the Aquidauana River, Mato Grosso do Sul, Brazil. Revista Brasileira de Parasitologia Veterinária, 17, 200-205.

Campos, C. M. D., de Moraes, J. R., & Moraes, F. R. D. (2008). Histopatologia de fígado, rim e baço de Piaractus mesopotamicus, Prochilodus lineatus e Pseudoplatystoma fasciatum parasitados por

myxosporídios, capturados no Rio Aquidauana, Mato Grosso do Sul, Brasil. *Revista Brasileira de Parasitologia Veterinária*, *17*, 200-205.

Campos, C. M.; Moraes, J. R. E.; Moraes, F. R. Histopathology of liver, kidney and spleen of *Piaractus*

Canedo, A., de Jesus, L. W. O., Bailão, E. F. L. C., & Rocha, T. L. (2021). Micronucleus test and nuclear abnormality assay in zebrafish (Danio rerio): Past, present, and future trends. *Environmental Pollution*, *290*, 118019.

Carrasco, Kenneth R.; Tilbury, Karen L.; Myers, Mark S. 1990. Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. Canadian Journal of Fisheries and Aquatic Sciences, v. 47, n. 11, p. 2123-2136.

Case, D. A., Cheatham III, T. E., Darden, T., Gohlke, H., Luo, R., Merz Jr, K. M., ... & Woods, R. J. (2005). The Amber biomolecular simulation programs. Journal of computational chemistry, 26(16), 1668-1688.

Cassens, R. G., Ito, T., Lee, M., & Buege, D. (1978). The use of nitrite in meat. Bioscience, 28(10), 633-637.

Charlie-Silva, I., Klein, A., Gomes, J.M.M., Prado, E.J.R., Moraes, A.C., Eto SF, Fernandes DC, Fagliari JJ, Junior JDC, Lima C, Lopes-Ferreira M, Conceição K, Manrique WG, Belo MAA. Acute-phase proteinsduring inflammatory reaction by bacterial infection: Fish-model. Sci Rep. 2019 Mar 18;9(1):4776.

Charrois, J.W.A. and Hrudey, S.E. 2007. Breakpoint chlorination and free-chlorine contact time: implications for drinking water N-nitrosodimethylamine concentrations, Water Res. 41(3), 674-682.

Cicero, Arrigo FG; D'addato, Sergio; Borghi, Claudio. 2020. A Randomized, Double-Blinded, Placebo-Controlled, Clinical Study of the Effects of a Nutraceutical Combination (LEVELIP DUO®) on LDL Cholesterol Levels and Lipid Pattern in Subjects with Sub-Optimal Blood Cholesterol Levels (NATCOL Study). Nutrients, v. 12, n. 10, p. 3127.

Cooper SW, Kimbrough RD. 1980. Acute dimethylnitrosamine poisoning outbreak. J Forensic Sci 25(4):874-882.

Costa, Caroline RC, et al. "The First Anti-Snakebite and Hepatoprotective Characterization of a Trypsin Kunitz-like Inhibitor (EcTI) from the Plant Enterolobium contortisiliquum; A Case of Two Soul Mates Meeting." Pharmaceuticals 16.4 (2023): 632.

Coutsias EA, Wester MJ. RMSD and Symmetry. J Comput Chem. 2019 Jun 5;40(15):1496-1508. doi: 10.1002/jcc.25802. Epub 2019 Mar 3. PMID: 30828834.

Covantes-Rosales, C.E., Trujillo-Lepe, A.M., Díaz-Reséndiz, K.J.G, Toledo-Ibarra, G.A., Ventura-Ramón, G.H., Ortiz-Lazareno, P.C., Girón-Pérez, M.I. Phagocytosis and ROS production as biomarkers in Nile tilapia (Oreochromis niloticus) leukocytes by exposure to organophosphorus pesticides. Fish Shellfish Immunol. 2019,84, 189-195.

Crawford JM. Vascular disorders of the liver. Clin Liver Dis. 2010 Nov;14(4):635-50. doi: 10.1016/j.cld.2010.08.002. PMID: 21055687. organization and insulin sensitivity in skeletal muscle. J Clin Invest. 2021 Apr 15;131(8):e135963.

Crawford, James M. Evidence-based interpretation of liver biopsies. Laboratory investigation, v. 86, n. 4, p. 326-334, 2006.

da Silva Santos N, Oliveira R, Lisboa CA, Mona E Pinto J, Sousa-Moura D, Camargo NS, Perillo V, Oliveira M, Grisolia CK, Domingues I. Chronic effects of carbamazepine on zebrafish: Behavioral, reproductive and biochemical endpoints. Ecotoxicol Environ Saf. 2018 Nov 30;164:297-304. doi: 10.1016/j.ecoenv.2018.08.015. Epub 2018 Aug 17. PMID: 30125776.

Dammski, A.P.; Müller, B.R.; Gaya, C.; Regonato, D. Zebrafish: Manual de Criação em Biotério. 1ed.Universidade Federal do Paraná. 107p. 2011.

de Menezes, M.N., Salles, É.M., Vieira, F. et al. IL-1α promotes liver inflammation and necrosis during blood-stage Plasmodium chabaudi malaria. Sci Rep 9, 7575 (2019). https://doi.org/10.1038/s41598-019-44125-2

de Paiva, M. J. T. R., de Pádua, S. B., Tavares-Dias, M., & Egami, M. I. (2013). Methods for hematological analysis in fish. Editora da Universidade Estadual de Maringá-EDUEM. dos Santos, L. L., & De-Carli, B. P. Behavior of zebrafish exposed to benzodiazepines.

Dessen A, Tang J, Schmidt H, Stahl M, Clark JD, Seehra J, Somers WS. Crystal structure of human cytosolic phospholipase A2 reveals a novel topology and catalytic

Drent, M.; Cobben, N.A.M.; Henderson, R.F.; Wouters, E.F.M.; Van Dieijen-Visser, M. Usefulness of Lactate Dehydrogenaseand Its Isoenzymes as Indicators of Lung Damage or Inflammation. Eur. Respir. J. 1996, 9, 1736–1742. Available online:https://erj.ersjournals.com/content/9/8/1736 (accessed on 14 March 2023).

Du X, Hu J, Zhang Q, Liu Q, Xiang X, Dong J, Lou B, He S, Gu X, Cao Y, Li Y, Ding T. A novel assay for measuring recombinant human lysophosphatidylcholine acyltransferase 3 activity. FEBS Open Bio. 2019 Oct;9(10):1734-1743. doi: 10.1002/2211-5463.12712. Epub 2019 Aug 30. PMID: 31376210; PMCID: PMC6768109.

Duvernay MT, Matafonov A, Lindsley CW, Hamm HE. Platelet Lipidomic Profiling: Novel Insight into Cytosolic Phospholipase A2α Activity and Its Role in Human Platelet Activation. Biochemistry. 2015 Sep 15;54(36):5578-88. doi: 10.1021/acs.biochem.5b00549. Epub 2015 Sep 1. PMID: 26295742; PMCID: PMC7748375.

Duvernay, Matthew T., et al. "Platelet lipidomic profiling: novel insight into cytosolic phospholipase A2α activity and its role in human platelet activation." Biochemistry 54.36 (2015): 5578-5588.

Ebert, Ellen C. Hypoxic liver injury. In: Mayo Clinic Proceedings. Elsevier, 2006. p. 1232-1236.

Emsley, Paul; Cowtan, Kevin. Coot: model-building tools for molecular graphics. Acta crystallographica section D: biological crystallography, v. 60, n. 12, p. 2126-2132, 2004.

environmental risk assessment of pharmaceuticals. Chemosphere, v. 93, n. 10, p. 2568-2577.

Erkekoglu, P. and Baydar, T. 2010. Evaluation of the protectiveeffect of ascorbic acid on nitrite- and nitrosamine- induced cytotoxicity and genotoxicity in human hepatoma line. Toxicol Mech Methods. 20(2), 45-52.

Erkekoglu, P. and Baydar, T. 2010. Evaluation of the protectiveeffect of ascorbic acid on nitrite- and nitrosamine- induced cytotoxicity and genotoxicity in human hepatoma line. Toxicol Mech Methods. 20(2), 45-52.

Faheem, M. and Lone, K.P. Oxidative stress and histopathologic biomarkers of exposure to bisphenol-A in the freshwater fish, *Ctenopharyngodon Idella*. Brazilian Journal of Pharmaceutical Sciences, 2017, 53(3):e17003

Fakhar Z, Naiker S, Alves CN, Govender T, Maguire GE, Lameira J, Lamichhane G, Kruger HG, Honarparvar B. A comparative modeling and molecular docking study on Mycobacterium tuberculosis targets involved in peptidoglycan biosynthesis. J Biomol Struct Dyn. 2016 Nov;34(11):2399-417. doi: 10.1080/07391102.2015.1117397. Epub 2016 Apr 4. PMID: 26612108.

Farhana, A.; Lappin, S.L. Biochemistry, Lactate Dehydrogenase. StatPearls 2022. Available online: https://www.ncbi.nlm.nih.gov/books/NBK557536/ (accessed on 14 March 2023).30.

Ferguson, H. W. (1976). The relationship between ellipsoids and melano-macrophage centres in the spleen of turbot (Scophthalmus maximus). *Journal of Comparative Pathology*, *86*(3), 377-380.

Ferrara, P.J., Rong, X., Maschek, J.A., Verkerke, A.R., Siripoksup, P., Song, H., Green, T.D., Krishnan, K.C., Johnson, J.M., Turk, J., Houmard, J.A., Lusis, A.J., Drummond, M.J., McClung, J.M., Cox, J.E., Shaikh, S.R., Tontonoz, P., Holland, W.L. and Funai, K. Lysophospholipid acylation modulates plasma membrane lipid

Frank N, Bertram B, Frei E, Hadjiolov D, Wiessler M. Influence of thiocompounds on the metabolism of N-nitrosodiethylamine. Carcinogenesis. 1988;9(7):1303-6.

Freitas, R. A., Correia, K. D. M., Tavares, M. G. D. O., Oliveira, G. M. C., Cintra, A., Riciole, H., ... & Antoniosi Filho, N. R. (2013). Avaliação das brânquias de danio rerio expostos a diferentes concentrações de gasolina e diesel. Pesticidas: r. ecotoxicol. e meio ambiente, 23, 59-66.

Freund HA. 1937. Clinical manifestations and studies in parenchymatous hepatitis. Ann Intern Med 10(8):1144-1155. http://doi.org/10.7326/0003-4819-10-8-1144.

GAWEŁ, Stefan et al. Malondialdehyde (MDA) as a lipid peroxidation marker. Wiadomosci lekarskie (Warsaw, Poland: 1960), v. 57, n. 9-10, p. 453-455, 2004.Wiadomosci Lekarskie (Warsaw, Poland: 1960). 2004; 57 (9-10): 453-455.PMID: 15765761.

Gernhofer, M., M. Pawet, M. Schramm, E. Müller & R. Triebskorn. (2001). Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. Journal of Aquatic Ecossystem, Stress and Recovery, v. 8: 241-260.

Gheno, E. M. The zebrafish in Brazilian science: scientific production, impact and collaboration. Federal University of Rio Grande do Sul. Institute of Basic Health Sciences. Graduate Program in Science Education: Life and Health Chemistry (352). 2015.

Girón-Pérez, M.I., Santerre, A., Gonzalez-Jaime F, Casas-Solis J, Hernández-Coronado M, Peregrina-Sandoval J, Takemura A, Zaitseva G. Immunotoxicity and hepatic function evaluation in Nile tilapia (Oreochromis niloticus) exposed to diazinon. Fish Shellfish Immunol. 2007, 23(4), 760-769.

Goessling, W. and Sadler, K.C. Zebrafish: an important tool for liver disease research. Gastroenterology. 2015, 149(6), 1361-177.

Groff, J. M., & Zinkl, J. G. (1999). Hematology and clinical chemistry of cyprinid fish: common carp and goldfish. Veterinary Clinics of North America: Exotic Animal Practice, 2(3), 741-776.

Guicciardi ME, Malhi H, Mott JL, Gores GJ. Apoptosis and necrosis in the liver. Compr Physiol. 2013 Apr;3(2):977-1010. doi: 10.1002/cphy.c120020. PMID: 23720337; PMCID: PMC3867948.

Guillouzo, A. (1998). Liver cell models in in vitro toxicology. Environmental health perspectives, 106(suppl 2), 511-532.

Gushgari AJ, Halden RU. 2018. Critical review of major sources of human exposure to N- nitrosamines.Chemosphere. 210, 1124-1136

Harkness SE, Wagner JE, Biologia e Clínica de coelhos e roedores, 3. ed., São Paulo: Livraria Roca Ltda, 1993, 238p

Hespanhol, I. Direct potable reuse and the challenge of emerging pollutants. Revista USP, n. 106, p. 79-94, 2015. Hoole, D., Lewis, J.W., Schuwerack, P.M., Chakravarthy, C., Shrive, A.K., Greenhough, T.J., Cartwright, J.R.

Hidajat M, McElvenny DM, Ritchie P, et al. 2019a. Lifetime exposure to rubber dusts, fumes and Nnitrosamines and cancer mortality in a cohort of British rubber workers with 49 years follow-up. Occup Environ Med 76(4):250-258. http://doi.org/10.1136/oemed-2018-105181.

Hosoki T, Kuroda C, Tokunaga K, Marukawa T, Masuike M, Kozuka T. Hepatic venous outflow obstruction: evaluation with pulsed duplex sonography. Radiology. 1989 Mar;170(3 Pt 1):733-7. doi: 10.1148/radiology.170.3.2644659. PMID: 2644659.

IARC (1978) Monographs on the evaluation of carcinogenic risks to humans, some N-nitroso compounds, vol 17. International Agency for Research on Cancer Publications, Lyon.

Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis. Oxid Med Cell Longev. 2019, 2019:5080843.

Inflammatory interactions in fish exposed to pollutants and parasites: a role for apoptosis and C reactive protein. Parasitology. 2003, 126 Suppl:S71-85.

Jacobson KH, Wheelwright HJ, Clem JH, et al. 1955. Studies on the toxicology of Nnitrosodimethylamine vapor. AMA Arch Ind Health 12(6):617-622.

Jamal QMS, Alharbi AH. Molecular docking and dynamics studies of cigarette smoke carcinogens interacting with acetylcholinesterase and butyrylcholinesterase enzymes of the central nervous system. Environ Sci Pollut Res Int. 2022 Sep;29(41):61972- 61992. doi: 10.1007/s11356-021-15269-4. Epub 2021 Aug 11. PMID: 34382170.

Janmeda P, Jain D, Chaudhary P, Meena M, Singh D. A systematic review on multipotent carcinogenic agent, N-nitrosodiethylamine (NDEA), its major risk assessment, and precautions. J Appl Toxicol. 2024 Jan 11. doi: 10.1002/jat.4574. Epub ahead of print. PMID: 38212177.

Janmeda, Pracheta et al. A systematic review on multipotent carcinogenic agent, N-nitrosodiethylamine (NDEA), its major risk assessment, and precautions. Journal of Applied Toxicology, 2024.

Johnson KW, Munson AE, Holsapple MP. 1987. Primary cellular target responsible for dimethylnitrosamine-induced immunosuppression in the mouse. Immunopharmacology 13(1):47-60. http://doi.org/10.1016/0162-3109(87)90026-9.

Juan, C.A. et al., 2021. The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. Int J Mol Sci. 22(9),4642.

Karplus, M., & McCammon, J. A. (2002). Molecular dynamics simulations of biomolecules. Nature structural biology, 9(9), 646-652.

Khanna SD, Puri D. 1966. The hepatotoxic effects of dimethylnitrosamine in the rat. J Pathol Bacteriol 91(2):605-608. http://doi.org/10.1002/path.1700910238.

Kimbrough RD. 1982. Pathological changes in human beings acutely poisoned by dimethylnitrosamine. In: Magee KD, ed. Nitrosamines and human cancer.

Kochnev Y, Hellemann E, Cassidy KC, Durrant JD. Webina: an open-source library and web app that runs AutoDock Vina entirely in the web browser. Bioinformatics. 2020 Aug 15;36(16):4513-4515. doi: 10.1093/bioinformatics/btaa579. PMID: 32559277; PMCID: PMC7575045.

Koga, Marianna Mainardi. 2015. PAF as an endogenous regulator of dendritic cell phenotype and function. PhD Thesis. University of São Paulo.

Kohen, Amnon, et al. "Enzyme dynamics and hydrogen tunnelling in a thermophilic alcohol dehydrogenase." Nature 399.6735 (1999): 496-499.

Kono, N. and Arai, H. 2019. Platelet-activating factor acetylhydrolases: An overview and update. Biochim Biophys Acta Mol Cell Biol Lipids. 1864(6), 922-931.

Kono, N. and Arai, H. 2019. Platelet-activating factor acetylhydrolases: An overview and update. Biochim Biophys Acta Mol Cell Biol Lipids. 1864(6), 922-931.

Krasner, SW, et al, 2013. Formation, precursors, control, and occurrence of nitrosamines in drinking water: a review. Water research , 47 (13), 4433-4450.

Kriczinski, R.; Romanello, L. Comparative molecularmodeling of the enzyme Adenylsuccinate Synthase(ADSS) from the parasite Schistosoma mansoni. Luminaria, União da Vitória, v. 22, n. 02, p. 31-39, 2020. Kumar, M.; Sodhi, K. KA; Singh, D. K. Bioremediation of Penicillin G by Serratia sp. R1, and enzymatic study through molecular docking. Environmental Nanotechnology, Monitoring & Management, v. 12,p. 100246, 2019.

Kumar, M.; Sodhi, K. KA; Singh, D. K. Bioremediation of Penicillin G by Serratia sp. R1, and enzymatic study through molecular docking. Environmental Nanotechnology, Monitoring & Management, v. 12,p. 100246, 2019.

Li, Yupeng, and Stephen S. Hecht. "Metabolic activation and DNA interactions of carcinogenic N-nitrosamines to which humans are commonly exposed." International journal of molecular sciences 23.9 (2022): 4559.

Lieschke, J.G.; CURRIE, P.D. "Animal models of human disease: zebrafish swim into view". Nature Reviews- Genetics, 8 (5). 2007. MARZA, E. et al. Developmental expression and nutritional regulation of a zebrafish gene homolugous to mammalian microsomal triglyceride transfer protein large subunit. Development Dynamics, New York, 232, (2), 506-518. 2005.

Linkous, A. and Yazlovitskaya, E. Cytosolic phospholipase A2 as a mediator of disease pathogenesis. Cellular Microbiology, 2010, 12(10), 1369-1377

Magee PN, Barnes JM. 1956. The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. Br J Cancer 10(1):114-122. http://doi.org/10.1038/bjc.1956.15.

Magee PN, Hultin T. 1962. Toxic liver injury and carcinogenesis. Methylation of proteins of rat-liver slices by dimethylnitrosamine in vitro. Biochem J 83:106-114. http://doi.org/10.1042/bj0830106.

Magee PN. 1956. Toxic liver injury; the metabolism of dimethylnitrosamine. Biochem J 64(4):676-682. http://doi.org/10.1042/bj0640676.

Magee, P. N., & Barnes, J. M. (1956). The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. British journal of cancer, 10(1), 114.

Marinsek, G. P., Ribeiro, C. C., Gusso-Choueri, P. K., Choueri, R. B., de Souza Abessa, D. M., & de Britto Mari, R. (2024). Multiple biomarkers in pufferfish as a proxy of environmental health in brazilian marine protected areas. *Science of The Total Environment*, *914*, 169742.

Martinez, C. B. R.; CÓLUS, I. M. S. Biomarcadores em peixes neotropicais para o monitoramento da poluição aquática na bacia do rio Tibagi In: Medri, M.E.; Bianchini, E.; Shibatta, O.A.; Pimenta, J.A. (Eds.) A bacia do rio Tibagi. Londrina: 2002. Chap. 29, p. 551 - 577.

mechanism. Cell. 1999 Apr 30;97(3):349-60. doi: 10.1016/s0092-8674(00)80744-8. PMID: 10319815.

medicine, 11(3), 167.

Meng, X. et al. 2011. Molecular docking: a powerful approach for structure-based drug discovery. Current computer-aided drug design, v. 7, n. 2, p. 146-157.

mesopotamicus, Prochilodus lineatus and *Pseudoplatystoma fasciatum* parasitized by myxosporidia, captured in Aquidauana River, Mato Grosso do sul, Brazil. Rev. bras. Parasitol. Vet., 2008, 17 (4), 200 -205.

Mitch, W.A., et al., 2003. N-nitrosodimethylamine (NDMA) as a contaminant of drinking water: a review.Environmental engineering science , 20 (5), 389-404.

Mitch, W.A., et al., 2003. N-nitrosodimethylamine (NDMA) as a contaminant of drinking water: a review. Environmental engineering science , 20 (5), 389-404.

Mitchell, J.R.; Jollow, D.J.; Potter, W.Z.; Davis, D.C.; Gillette, J.R.; Brodie, B.B. Acetaminophen-induced hepatic necrosis. I. Role ofdrug metabolism. J. Pharmacol. Exp. Ther. 1973, 187, 185–194

Moron, Sandro Estevan, 2006. et al. Study of changes in plasma ion concentration and the induction of micronuclei in Piaractus mesopotamicus exposed to the herbicide atrazine. J. Braz. Soc. Ecotoxicol, v. 1, n. 1, p. 27-30.

Mouchlis VD, Dennis EA. Membrane Association Allosterically Regulates Phospholipase A2 Enzymes and Their Specificity. Acc Chem Res. 2022 Dec 6;55(23):3303-3311. doi: 10.1021/acs.accounts.2c00497. Epub 2022 Oct 31. PMID: 36315840; PMCID: PMC9730854.

Murphy, Kenneth. Imunobiologia de Janeway. Disponível em: Minha Biblioteca, (8th edição). Grupo A, 2014.

Nehring, S.M.; Goyal, A.; Bansal, P.; Patel, B.C. C Reactive Protein. StatPearls 2017, 65, 237–244. Available online: http://europepmc.org/books/NBK441843. (accessed on 15 March 2023) Maduagwu EN, Bassir O. 1980. A comparative assessment of toxic effects of dimethylnitrosamine in six different species. Toxicol Appl Pharmacol 53(2):211-219. http://doi.org/10.1016/0041-008x(80)90421-4.

Neves Cruz J, Santana de Oliveira M, Gomes Silva S, Pedro da Silva Souza Filho A, Santiago Pereira D, Lima E Lima AH, de Aguiar Andrade EH. Insight into the Interaction Mechanism of Nicotine, NNK, and NNN with Cytochrome P450 2A13 Based on Molecular Dynamics Simulation. J Chem Inf Model. 2020 Feb 24;60(2):766-776. doi: 10.1021/acs.jcim.9b00741. Epub 2019 Oct 28. PMID: 31622091.

Nojima, S. 1991. Platelet-activating factor (PAF): an introduction. Lipids. 26(12), 965-966.

Nojima, S. 1991. Platelet-activating factor (PAF): an introduction. Lipids. 26(12), 965-966. Papakonstantinou, V.D., et al., 2017. A Review on Platelet Activating Factor Inhibitors: Could a New Class of Potent Metal-Based Anti-Inflammatory Drugs Induce Anticancer

Noor, Kiran Kousar, et al. "Hepatoprotective role of vitexin against cadmium-induced liver damage in male rats: A biochemical, inflammatory, apoptotic and histopathological investigation." Biomedicine & Pharmacotherapy 150 (2022): 112934.

OECD, 2013. Guideline for Testing of Chemicals, 236. Fish Embryo Acute Toxicity (FET) Test. OECD, Paris, France.

Oehler, B., Brack, A., Blum, R., Rittner, H.L. Pain Control by Targeting Oxidized Phospholipids: Functions, Mechanisms, Perspectives. Front Endocrinol (Lausanne). 2021, 11, 613868.

Owen, J.S., et al, 2007. Platelet-activating factor. Methods Enzymol. 434, 105-116.

p. 10256-10260.

Paiva, A. L. S., & Paiva, P. H. S. D. (2022). Contaminação de medicamentos por N-nitrosaminas: bases regulatórias para controle.
Papakonstantinou, V.D., et al., 2017. A Review on Platelet Activating Factor Inhibitors: Could a New Class of Potent Metal-Based Anti-Inflammatory Drugs Induce Anticancer Properties? Bioinorg Chem Appl. 2017, 6947034.
Park, Jong-eun et al. 2015. Distribution of seven N-nitrosamines in food. Toxicological research, v. 31, n. 3, p. 279-288.
Park, Jong-eun et al. 2015. Distribution of seven N-nitrosamines in food. Toxicological research, v. 31, n. 3, p. 279-288.
Pettersen, Eric F. et al. 2004. UCSF Chimera-a visualization system for exploratory research and analysis. Journal of computational chemistry, v. 25, n. 13, p. 1605-1612.
Pieri G, Theocharidou E, Burroughs AK. Liver in haematological disorders. Best Pract Res Clin Gastroenterol. 2013 Aug;27(4):513-30. doi: 10.1016/j.bpg.2013.06.012. PMID: 24090939.
Pinzi, Luca; Rastelli, Giulio. 2019. Moleculardocking: shifting paradigms in drug discovery. International journal of molecular sciences, v. 20, n. 18, p. 4331.
Properties? Bioinorg Chem Appl. 2017, 6947034.
Ranzani-Paiva, M.J.T., Pádua, S.B. and Tavares-Dias, M. 2013. Methods for hematological analysis in fish. 1st Ed. Eduem, Maringá, 135 p.
rats. Previous Nutr Food Sci. 23(4):288-293. doi:10.3746/pnf.2018.23.4.288.
Reed, A., Ichu, T. A., Milosevich, N., Melillo, B., Schafroth, M. A., Otsuka, Y., ... & Cravatt,
Roberts, H.E. 2010. Physical examination of fish. In: Roberts, HE (ed.). Fundamentals of ornamental fish health. John Wiley & Sons, Ames, p. 161-165.
Roberts, R. J. Fish pathology. 4th. ed. London: W. B. Saunders, 2012. 590 p.
Rogers AE, Wishnok JS, Archer MC. Effect of diet on DEN clearance and carcinogenesis in rats. British Journal of Cancer. 1975;31(6):693-5.
Roy, S., Kumar, V., Kumar, V., Behera, B.K. Acute Phase Proteins and their Potential Role as an Indicator for Fish Health and in Diagnosis of Fish Diseases. Protein Pept Lett. 2017, 24(1), 78-89.
Sales, C. F., Silva, R. F., Amaral, M. G., Domingos, F. F., Ribeiro, R. I., Thomé, R. G., & Santos, H. B. (2017). Comparative histology in the liver and spleen of three species of freshwater teleost. Neotropical Ichthyology, 15.
Sali, A.; Overington, J. P. 1994. Derivation of rules for comparative protein modeling from a database of protein structure alignments. Protein Sci., v. 3, p. 1582-1596.
Sali, A; Blundell, T. L. 1993. Comparative protein modelling by satisfaction of spatial restraints. J. Mol.Biol., v. 234, p. 779-815.
Samanta U, Bahnson BJ. Crystal structure of human plasma platelet-activating factor acetylhydrolase: structural implication to lipoprotein binding and catalysis. J Biol Chem. 2008 Nov 14;283(46):31617-24. doi: 10.1074/jbc.M804750200. Epub 2008 Sep 10. PMID: 18784071; PMCID: PMC2581546.
Santos, A.A.; Ranzani-Paiva, M.J.T.; Felizardo, N.N.; Rodrigues, E. De L. Histopathological analysis of the liver of Nile tilapia, Oreochromis niloticus, cultured in net-tanks in the Guarapiranga reservoir, São Paulo, SP, Brazil. Boletim do Instituto de Pesca, v.30, p.141-145, 2004.
Santos-Filho, O. A.; Alencastro, R. B. de. 2003. Protein modeling by homology. Química Nova, v. 26, n. 2, p. 253-259, Mar.
Sarma BK, Liu X, Kodadek T. Identification of selective covalent inhibitors of platelet activating factor acetylhydrolase 1B2 from the screening of an oxadiazolone-capped peptoid-azapeptoid hybrid library. Bioorg Med Chem. 2016 Sep 1;24(17):3953-3963. doi: 10.1016/j.bmc.2016.04.047. Epub 2016 Apr 23. PMID: 27160052; PMCID: PMC4972644.
Schoental R, Gough TA, Webb KS. Carcinogens in rat milk. Transfer of ingested diethylnitrosamine into milk by lactating rats. Br J Cancer. 1974;30(3):238-40.

Schumann, K.; Mauch, C.; Klespe, K.; Loquai, C.; Nikfarjam, U.; Schlaak, M.; Akçetin, L.; Kölblinger, P.; Hoellwerth, M.; Meissner,M.; et al. Real-World Outcomes Using PD-1 Antibodies and BRAF + MEK Inhibitors for Adjuvant Melanoma Treatment from 39Skin Cancer Centers in Germany, Austria

and Switzerland. J. Eur. Acad. Dermatol. Venereol. 2022, 37, 894–906. Available online:https://onlinelibrary.wiley.com/doi/full/10.1111/jdv.18779 (accessed on 14 March 2023).
Sci Rep. 6, 29637.
Segura, B. M. (2023). Histopathological biomarkers in zebrafish (Danio rerio) used for biomonitoring in urban streams of Campo Grande–MS (Doctoral dissertation, Federal University of Mato Grosso do Sul).
Sharma, P. and Chadha, P. 2021. Bisphenol A induced toxicity in blood cells of freshwater fish Channa punctatus after acute exposure. Saudi Journal of Biological Sciences. doi:10.1016/j.sjbs.2021.04.088 Shen, Y., et al., 2018. Fish red blood cells express immune genes and responses. Aquaculture and Fisheries 3(1), 14 -21.
Sheweita, S.A., El Banna, Y.Y., Balbaa, M., Abdullah, I.A., Hassan, H.E. N-nitrosamines induced infertility and hepatotoxicity in male rabbits. Environ Toxicol. 2017, 32(9), 2212-2220.
Shtewi, H. And Abusrer, S. Morphology and histology of the liver of Oblada melanura (Linnaeus, 1758) (Teleostei: Sparidae) in tripoli coast, western Libya. Bull. Inst. Natn. Scien. Tech. Mer de Salammbô, 2000, 47,1-9.
Smorodinskaya, S., Kochetkov, N., Gavrilin, K., Nikiforov-Nikishin, D., Reznikova, D., Vatlin, A., ... & Danilenko, V. (2023). The Effects of Acute Bisphenol A Toxicity on the Hematological Parameters, Hematopoiesis, and Kidney Histology of Zebrafish (Danio rerio). *Animals*, *13*(23), 3685.
Souza, G. C. Study of the toxicity of perillyl alcohol nanoemulsion (NPOH) on zebrafish (*Danio rerio* Hamilton, 1822). Macapá: Dissertation (Master's Degree), 2015.
Souza, João Pedro de Albuquerque . 2015. Molecular docking study of cinnamic acid derivatives against HIV-1 replicative cycle enzymes. Course Conclusion Paper. Universidade Tecnológica Federal do Paraná.
Stanca, E., Serviddio, G., Bellanti, F., Vendemiale, G., Siculella L, Giudetti AM. Down-regulation of LPCAT expression increases platelet-activating factor level in cirrhotic rat liver: potential anti-inflammatory effect of silybin. Biochim Biophys Acta., 2013, 1832(12), 2019-2026.
Straif K, Weiland S, Werner B, et al. 1999. Elevated mortality from nonalcohol-related chronic liver disease among female rubber workers: Is it associated with exposure to nitrosamines? Am J Ind Med 35(3):264-271. http://doi.org/10.1002/(sici)1097-0274(199903)35:33.0.co;2- x.
Su, L.J., Zhang, J.H., Gomez, H., Murugan, R., Hong, X., Xu, D., Jiang, F., Peng, Z.Y. Reactive Oxygen Species- Treasure Island (FL): StatPearls Publishing 32491324.
Teske, Luiza Pereira. Computational simulation analysis of theoretical sibutramine derivatives. BS thesis. Federal Technological University of Parana, 2019.
Toyama, M.H., Rogero, A., de Moraes, L.L.F, Fernandes, G.A., da Cruz Costa, C.R., Belchor, M.N., De Carli, A.M., de Oliveira, M.A. Gallic Acid as a Non-Selective Inhibitor of α/β-Hydrolase Fold Enzymes Involved in the Inflammatory Process: The Two Sides of the Same Coin. Pharmaceutics. 2022 Feb 6;14(2):368.
Travers JB, Rohan JG, Sahu RP. New Insights Into the Pathologic Roles of the Platelet- Activating Factor System. Front Endocrinol (Lausanne). 2021, 15;12:624132.
Travers, J.B., et al, 2021. New Insights into the Pathologic Roles of the Platelet-Activating Factor System. Front Endocrinol (Lausanne). 12, 624132.
Trisciuzzi D. et al. 2018. Molecular docking for predictive toxicology. In: Nicolotti O (ed) Computational toxicology. Springer New York, New York, pp 181-197.
Trisciuzzi D. et al. 2018. Molecular docking for predictive toxicology. In: Nicolotti O (ed) Computational toxicology. Springer New York, New York, pp 181-197.
Trott, O., & Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. Journal of computational chemistry, 31(2), 455 -461.
Ulambayar, B., et al., 2019. Increased platelet activating factor levels in chronic spontaneous urticaria predicts refractoriness to antihistamine treatment: an observational study. Clin Transl Allergy. 17, :33.

Ulambayar, B., et al., 2019. Increased platelet activating factor levels in chronic spontaneous urticaria predicts refractoriness to antihistamine treatment: an observational study. Clin Transl Allergy. 17, :33.

US EPA (1987a) IRIS Chemical Assessment Summary of NNitrosodimethylamine. U.S. Environmental Protection Agency, Washington, DC.

US EPA (1987b) IRIS Chemical Assessment Summary of NNitrosodiethylamine. U.S. Environmental Protection Agency, Washington, DC.

Van Der Spoel, D., Lindahl, E., Hess, B., Groenhof, G., Mark, A. E., & Berendsen, H. J. (2005). GROMACS: fast, flexible, and free. Journal of computational chemistry, 26(16), 1701-1718.

Verna L, Whysner J, Williams GM. N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacology & therapeutics. 1996;71(1-2):57-81.

Vizioli, BD, Hantao, LW & Montagner, CC Nitrosamines from drinking water in a large metropolitan region of

Wallace, Andrew C.; Laskowski, Roman A.; Thornton, Janet M. 1995. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. Protein engineering, design and selection, v. 8, n. 2, p. 127- 134.

Waly, M.I., et al., 2018. The protective effect of curcumin against nitrosamine-induced gastric oxidative stress in

Wilczak, A., et al., 2003. Formation of NDMA in chloraminated water coagulated with DAMAC cationic polymer, JAWWA 95 (9) (2003) 94-106.

Wu, H., et al., 2016. Hypoxia-mediated impaired erythrocyte Lands' Cycle is pathogenic for sickle cell disease.

Wykle, Robert L.; Malone, B.; Snyder, F. 1980. Enzymatic synthesis of 1 -alkyl-2-acetyl-sn-glycero-3-phosphocholine, a hypotensive and platelet-aggregating lipid. Journal of Biological Chemistry, v. 255, n. 21,

Xu, Z., Cao, J., Qin, X., Qiu, W., Mei, J., Xie, J. Toxic Effects on Bioaccumulation, Hematological Parameters, Oxidative Stress, Immune Responses and Tissue Structure in Fish Exposed to Ammonia Nitrogen: A Review. Animals (Basel). 2021, 11(11), 3304.

Yancheva, V., Velcheva, I., Stoyanova, S., & Georgieva, E. (2016). Histological biomarkers in fish as a tool in ecological risk assessment and monitoring programs: a review. Applied ecology and environmental research, 14(1), 47.

Yang, Y.; Pham, T.X.; Wegner, C.J.; Kim, B.; Ku, C.S.; Park, Y.K.; Lee, J.Y. Astaxanthin Lowers Plasma TAG Concentrations andIncreases Hepatic Antioxidant Gene Expression in Diet-Induced Obesity Mice. Br. J. Nutr. 2014, 112, 1797–1804. Availableonline: https://www.cambridge.org/core/journals/british-journal-of-nutrition/article/astaxanthin-lowers-plasma-tag-concentrations-and-increases-hepatic-antioxidant-gene-expression-in-dietinduced-obesity-mice/49F447C0D80B03FB07FAD952E805E990 (accessed on 15 March 2023)

Ylilauri, M., & Pentikäinen, O. T. (2013). MMGBSA as a tool to understand the binding affinities of filamin–peptide interactions. Journal of chemical information and modeling, 53(10), 2626-2633.

Zhang Q, Yao D, Rao B, Jian L, Chen Y, Hu K, Xia Y, Li S, Shen Y, Qin A, Zhao J, Zhou L, Lei M, Jiang XC, Cao Y. The structural basis for the phospholipid remodeling by lysophosphatidylcholine acyltransferase 3. Nat Commun. 2021 Nov 25;12(1):6869. doi: 10.1038/s41467-021-27244-1. PMID: 34824256; PMCID: PMC8617236.