ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

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ΤΟΞΙΚΟΛΟΓΙΑ

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΠΛΩΜΑΤΙΚΗ ΕΡΓΑΣΙΑ

Σε βάθος ανάλυση των βιοδεικτών του οξειδωτικού στρες που μεταβάλλονται σε αρουραίους μετά από επαγόμενη καρδιοτοξικότητα από την χορήγηση ανθρακικλινων, με στόχο τον προσδιορισμό τιμών κατωφλίου.

Παπαδημητρίου Γεωργία

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DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

MASTER PROGRAM IN

TOXICOLOGY

MASTER THESIS

In depth analysis of biomarkers of oxidative stress altered in rats after induced cardiotoxicity by administration, aiming to identify threshold values.

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Larisa,2024

ΤΡΙΜΕΛΗΣ ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ

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ABSTRACT

Anthracyclines are widely used in cancer treatment and are known to have significant cardiotoxic effects in humans. In clinical practice, there are well-established guidelines for assessing cardiotoxicity, which is typically diagnosed through echocardiography and objective measures of cardiac function. However, cardiotoxicity is not currently a primary focus in general toxicology and safety pharmacology preclinical studies, despite evidence that other drugs and chemicals can also harm the heart. Additionally, there is a lack of standardized criteria or reference values in the literature for characterizing cardiotoxic effects in animal models. This comprehensive review specifically evaluates five oxidative stress biomarkers, namely MDA, GSH, GPx , SOD, CAT, of myocardial function in animal studies related to anthracycline-induced cardiotoxicity. The analysis of these parameters, in conjunction with the most commonly histopathological lesions and inflammation biomarkers and echocardiographic indices, shows promising results and suggests the potential for establishing objective values for anthracycline-induced cardiotoxicity. The range of these indices values differentiate normal cardiac function from animals with pathological findings indicative of anthracycline cardiotoxicity. By analysing this data, there is potential to develop solid guidelines for assessing cardiotoxicity as a separate hazard category in preclinical animal studies for regulatory purposes.

ΠΕΡΙΛΗΨΗ

Οι ανθρακυκλίνες χρησιμοποιούνται ευρέως στη θεραπεία του καρκίνου και είναι γνωστό ότι έχουν σημαντικές καρδιοτοξικές επιδράσεις στον άνθρωπο. Στην κλινική πρακτική, υπάρχουν καθιερωμένες κατευθυντήριες γραμμές για την αξιολόγηση της καρδιοτοξικότητας, η οποία συνήθως διαγιγνώσκεται μέσω ηχοκαρδιογραφήματος και βασίζεται σε αντικειμενικές μετρήσεις της καρδιακής λειτουργίας. Ωστόσο, η καρδιοτοξικότητα δεν περιγράφεται ως χωριστή κατηγορία κινδύνου χημικών ουσιών μέσω των διαθέσιμων κανονισμών, παρά τις ενδείξεις ότι και άλλα φάρμακα και χημικές ουσίες μπορούν επίσης να βλάψουν την καρδιά. Επιπλέον, υπάρχει έλλειψη τυποποιημένων κριτηρίων ή τιμών αναφοράς στη βιβλιογραφία για τον χαρακτηρισμό των καρδιοτοξικών επιδράσεων σε ζωικά μοντέλα. Αυτή η ολοκληρωμένη ανασκόπηση αξιολογεί συγκεκριμένα πέντε βιοδείκτες οξειδωτικού στρες, δηλαδή MDA, GSH, GPx, SOD, CAT, της μυοκαρδιακής λειτουργίας σε μελέτες σε ζώα που σχετίζονται με την καρδιοτοξικότητα που προκαλείται από ανθρακυκλίνες. Η ανάλυση αυτών των παραμέτρων, σε συνδυασμό με τις συνηθέστερες ιστοπαθολογικές αλλοιώσεις και τους βιοδείκτες φλεγμονής και τους ηχοκαρδιογραφικούς δείκτες, δείχνει ελπιδοφόρα αποτελέσματα και υποδηλώνει τη δυνατότητα καθορισμού αντικειμενικών τιμών για την επαγόμενη από ανθρακυκλίνη καρδιοτοξικότητα. Το εύρος των τιμών αυτών των δεικτών διαφοροποιεί τη φυσιολογική καρδιακή λειτουργία από τα ζώα με παθολογικά ευρήματα ενδεικτικά της καρδιοτοξικότητας από ανθρακυκλίνες. Με την ανάλυση αυτών των δεδομένων, υπάρχει η δυνατότητα ανάπτυξης σταθερών κατευθυντήριων γραμμών για την αξιολόγηση της καρδιοτοξικότητας ως ξεχωριστής κατηγορίας κινδύνου σε προκλινικές μελέτες σε ζώα για κανονιστικούς σκοπούς.

CHAPTER 1

1.1 Introduction

Cardiovascular disease (CVD) continues to be a leading cause of morbidity and mortality despite advances in modern medicine.[1,2] Environmental exposures, particularly chemicals, are estimated to contribute to at least 23% of all cardiovascular pathologies.[3]
Chemotherapeutics cardiotoxicity is a major concern for clinicians treating different kinds of cancer, as it is seriously affects their treatment options and the survival of the patient.In experiments with animals, where new anticancer compounds are tested and different treatments are explored to reduce the heart related side effects of anticancer drugs, accurately determining the level of heart damage is crucial. This assessment is necessary to evaluate any potential benefits of the counteracting treatment under investigation. [4] Anthracyclines are strong chemotherapy agents extracted from Streptomyces spp. used to treat a range of blood-related and solid cancers. The different types available for treatment are daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone and valrubicin [5,6]. These compounds find application in the treatment of a wide range of cancers, including leukemia, lymphomas, as well as cancers affecting the breast, stomach, uterus, ovaries, lungs, and bladder.Anthracyclines, known for their significant cardiotoxicity and ability to cause myocardial suppression in many patients, are also employed in animal research as a simple and cost-effective method to create a model of dilated cardiomyopathy [7] ,as opposed to interventional research animal models of infarction and myocardial ischaemia (e.g. permanent ligation of the left anterior descending artery (LAD) or the application of a cryo-pen on the heart's surface results in cryo-scar ischemia).
In the literature, researchers have employed different animal species along with various dosing and administration protocols of anthracyclines to study the development of anthracycline-induced cardiotoxicity [8] and monitoring of the progress thereof, as well as testing different compounds/schemes for ameliorating myocardial damage. To monitor cardiotoxicity caused by anthracyclines, biochemical markers are used in addition to cardiac imaging. [9] Alongside anthracyclines, certain pharmaceutical compounds like anabolic steroids and everyday chemicals such as metals and pesticides have also been associated with negative impacts on cardiac health, resulting an impaired cardiac function.[10]
The European regulatory network has a well-established system for controlling the toxicity and potential risks to human health posed by chemicals. However, cardiotoxicity is not currently classified as a distinct hazard class, and there are no specific criteria available to

legally classify chemicals in advance as cardiotoxic and avoid potential long-term cardiovascular complications, which could significantly burden any national health system.[3] The evaluation of cardiotoxicity in anticancer agents, particularly anthracyclines, could involve monitoring biomarker indices of antioxidant stress and inflammation processes in animal studies, alongside histopathological findings. The uniformity in anthracycline cardiotoxicity induction across animal models and the consistency of myocardial function decline across studies are critical questions. Addressing these issues is beneficial for clinical medicine, guiding the selection of agents to manage cardiotoxic complications during oncology treatment. Additionally, it aids regulators in establishing echocardiographic reference values for chemical-induced cardiotoxicity in animals. An in-depth analysis was conducted by our team, concentrating on the most prevalent histopathological lesions and commonly used biomarkers of myocardial function in animal studies related to anthracycline-induced cardiotoxicity. Additionally, the analysis explored the range of these values, differentiating normal cardiac function from animals with pathological findings indicative of anthracycline cardiotoxicity as per author presentation. In the scope of this present thesis, our examination will delve into analyzing biomarkers of oxidative stress.

1.2. Globally Harmonized System of Classification and Labelling of Chemicals (GHS)

Chemicals, through their life cycle, from the production to their handling, transportation and usage, have the potential for adverse effects to people or to environment. Individuals of all ages, diverse linguistic backgrounds using various alphabets, and from different social backgrounds, including those who may be illiterate, are regularly exposed to hazardous substances. To protect human health and environment, countries and organizations have created laws or regulations that require information to be prepared and transmitted throught labels or safety data sheets (SDS) to those using chemicals.

The laws and regulations regarding chemical safety were similar in some aspects, their variations were significant enough to lead in different labels or SDS. Additionally, because of the extensive global trade in chemicals and the necessity of creating national systems to guarantee the safe use, transportation, and disposal of hazardous chemicals, it was acknowledged that a globally standardized method for classification and labeling would form the basis for such systems.[11]

The "Globally Harmonized System of Classification and Labelling of Chemicals (GHS)" is a new system for classifying chemicals based on their hazards, incorporating standardized elements for hazard communication such as labels and safety data sheets. The primary aim is

to ensure that information about the physical hazards and toxicity of chemicals is available to improve the protection of human health and the environment during the handling, transport, and use of these chemicals.The GHS also establishes a foundation for harmonizing chemical rules and regulations at national, regional, and global levels, which is a crucial factor for facilitating trade.

The primary audiences for the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) are governments, regional institutions, and international organizations. However, the system also provides enough context and guidance for industry professionals who will be responsible for implementing the adopted requirements.[11]

The Globally Harmonized System of Classification and Labelling of Chemicals (GHS) aims to enhance the protection of human health and the environment by providing a universally comprehensible system for hazard communication. It also serves as a recognized framework for countries that do not have an existing system in place. Additionally, the GHS works to reduce the need for extensive testing and evaluation of chemicals, while also facilitating international trade by ensuring that hazards are properly assessed and identified on an international level.[12]

The GHS provides harmonized criteria for classifying substances and mixtures based on their physical, health, and environmental hazards. The classification criteria for health and environmental hazards are independent of specific test methods. It also includes harmonized hazard communication elements, such as labeling and safety data sheet requirements. The GHS covers all hazardous chemicals, although the application of its hazard communication elements may vary depending on the product category or stage in the life cycle. The target audiences for the GHS include consumers, workers (including transport workers), and emergency responders. [12]

Physical hazards

- Explosives (Divisions 1.1-1.6 (with 1.1 being the most hazardous, 1.6 the least hazardous))
- Flammable gases (Categories 1 and 2)
- Flammable aerosols (Categories 1 and 2)
- Oxidizing gases (Category 1)
- Gases under pressure (4 Groups include: Compressed gas, Liquefied gas, Dissolved gas, and Refrigerated liquefied gas)
- Flammable liquids (Categories 1 – 4)
- Flammable solids (Categories 1 and 2)
- Self-reactive substances (Types A-G)

- Pyrophoric solids (Category 1)
- Pyrophoric liquids(Category 1)
- Self-heating substances (Categories 1 and 2)
- Substances which in contact with water emit flammable gases (Categories 1 – 3)
- Oxidizing liquids (Categories 1 – 3)
- Oxidizing solids (Categories 1 – 3)
- Organic peroxides (Types A-G)
- Substances corrosive to metal (Category 1)

Health hazards

- Acute toxicity (Categories 1, 2, 3 and 4)
- Skin corrosion/irritation (Categories 1A, 1B, 1C, and 2)
- Eye Effects (Categories 1, 2A, and 2B)
- Sensitization (Skin or Eye) (Category 1A and 1B)
- Germ cell mutagenicity (Categories 1A, 1B and 2)
- Carcinogenicity (Categories 1A, 1B and 2)
- Reproductive toxicity (Categories 1A, 1B and 2) and additional category for effects on or via lactation
- Target organ systemic toxicity: single and repeated exposure (Single: Categories 1-3 Repeated: Categories 1 and 2)
- Aspiration toxicity (Category 1 and 2)

Environmental hazards

- Acute Aquatic Toxicity (Categories 1 -3)
- Chronic Aquatic Toxicity (Categories 1 -4)

1.3 Classification, Labelling and Packaging Regulation (CLP)

The Classification, Labelling and Packaging (CLP) Regulation ((EC) No 1272/2008) is based on the United Nations' Globally Harmonised System (GHS) and its purpose is to ensure a high level of protection of health and the environment, as well as the free movement of substances, mixtures and articles. Since 1 June 2015, the CLP Regulation is the only legislation in force in the EU for classification and labelling of substances and mixtures. The CLP Regulation amended the Dangerous Substances Directive (67/548/EEC (DSD)), the

Dangerous Preparations Directive (1999/45/EC (DPD)) and Regulation (EC) No 1907/2006 (REACH). The CLP Regulation is legally binding throughout the Member States of the EU and is directly applicable to all industrial sectors. It mandates that manufacturers, importers, or downstream users of substances or mixtures must classify, label, and package their hazardous chemicals correctly before introducing them to the market. The main objective of CLP is to assess whether a substance or mixture exhibits properties that lead to a hazardous classification. This classification serves as the initial step in communicating the hazard. When relevant data, such as toxicological information, aligns with the classification criteria in the CLP Regulation, the hazards associated with a substance or mixture are determined by assigning specific hazard class and category. These hazard classes under CLP encompass physical, health, environmental, and other additional hazards. [13]

Once a substance or mixture undergoes classification, the identified hazards must be communicated to various actors in the supply chain, including consumers. Hazard labelling allows the hazard classification, with labels and safety data sheets, to be communicated to the user of a substance or mixture, to alert them about the presence of a hazard and the need to manage the associated risks. [13]

CLP establishes specific criteria for labelling elements such as pictograms, signal words, and standard statements covering hazards, prevention, response, storage, and disposal for each hazard class and category. It also outlines general packaging standards to ensure the safe handling of hazardous substances and mixtures. Apart from hazard communication through labelling requirements, CLP forms the basis for numerous legislative provisions related to the risk management of chemicals. [13]

Physical hazards

- Explosives (Unstable explosives, Divisions 1.1, 1.2, 1.3, 1.4, 1.5, and 1.6)
- Flammable gases (Categories 1A (including unstable gases(Categories A and B) and pyrophoric gases*) 1B and 2
- Aerosols (Categories 1, 2 and 3)
- Oxidising gases (Category 1)
- Gases under pressure (Compressed gas, liquefied gas, refrigerated liquefied gas, dissolved gas)
- Flammable liquids (Categories 1, 2 and 3)
- Flammable solids (Categories 1 and 2)
- Self-reactive substances and mixtures (Types A, B, C, D, E, F, & G)
- Pyrophoric liquids (Category 1)
- Pyrophoric solids (Category 1)
- Self-heating substances and mixtures (Categories 1 and 2)

- Substances and mixtures which in contact with water emit flammable gases (Categories 1, 2 and 3)
- Oxidising liquids (Categories 1, 2 and 3)
- Oxidising solids (Categories 1, 2 and 3) Organic peroxides (Types A, B,C,D, E,F&G)
- Corrosive to metals (Category 1)
- Desensitised explosives*

- **Health hazards**
- Acute toxicity (Categories 1, 2, 3 and 4)
- Skin corrosion/irritation (Categories 1, 1A, 1B, 1C and 2)
- Serious eye damage/eye irritation (Categories 1 and 2)
- Respiratory or skin sensitisation (Category 1, Sub-categories 1A and 1B)
- Germ cell mutagenicity (Categories 1A, 1B and 2)
- Carcinogenicity (Categories 1A, 1B and 2)
- Reproductive toxicity (Categories 1A, 1B and 2) plus additional category for effects on or via lactation
- Specific target organ toxicity (STOT) – single exposure ((Categories 1, 2) and Category 3 for narcotic effects and respiratory tract irritation, only)
- Specific target organ toxicity (STOT) – repeated exposure (Categories 1 and 2) Aspiration hazard (Category 1)

- **Environmental hazards**
- Hazardous to the aquatic environment (Category Acute 1, Categories Chronic 1, 2, 3, and 4)

- **Additional hazards**
- Hazardous to the ozone layer (Category 1)

1.4 Weight of Evidence

The weight of evidence approach involves combining information from multiple independent sources to provide enough evidence to fulfil an information requirement. [13]
This approach is beneficial when,

- the information from a single piece of evidence alone is not sufficient to fulfil an information requirement. This could be, for example, due to clear deficiencies in one of the existing studies.
- individual studies provide different or conflicting conclusions.

The value of evidence depends on factors like data quality, result consistency, the nature and severity of effects, and how relevant the information is. Using the weight of evidence approach involves scientific judgment, so it's crucial to have sufficient and trustworthy documentation. In general, the more information you offer, the more compelling your weight of evidence becomes. Ensure that you present the information in a structured and organised way and take into account the robustness and reliability of the different data sources to support your justification. For example, in vivo and in vitro data carry more weight in the decision than a QSAR or other computational methods. To begin constructing your weight of evidence argument, collect information from a variety of sources, such as: published literature, read-across from chemical analogues, (Q)SAR predictions, data from existing studies, in vitro studies, epidemiological data/human experience.[13]

The World Health Organization (WHO) describes weight of evidence assessment as a process where all relevant evidence for a risk assessment is evaluated and weighted.[14,15] A review by ANSES (French Agency for Food, Environmental and Occupational Health & Safety) defines weight of evidence assessment as a structured synthesis of different lines of evidence, which may vary in quality, to determine the level of support for hypotheses.[14,16] The core of most definitions is that weight of evidence assessment is a process for integrating evidence to arrive at conclusions.[14,17]

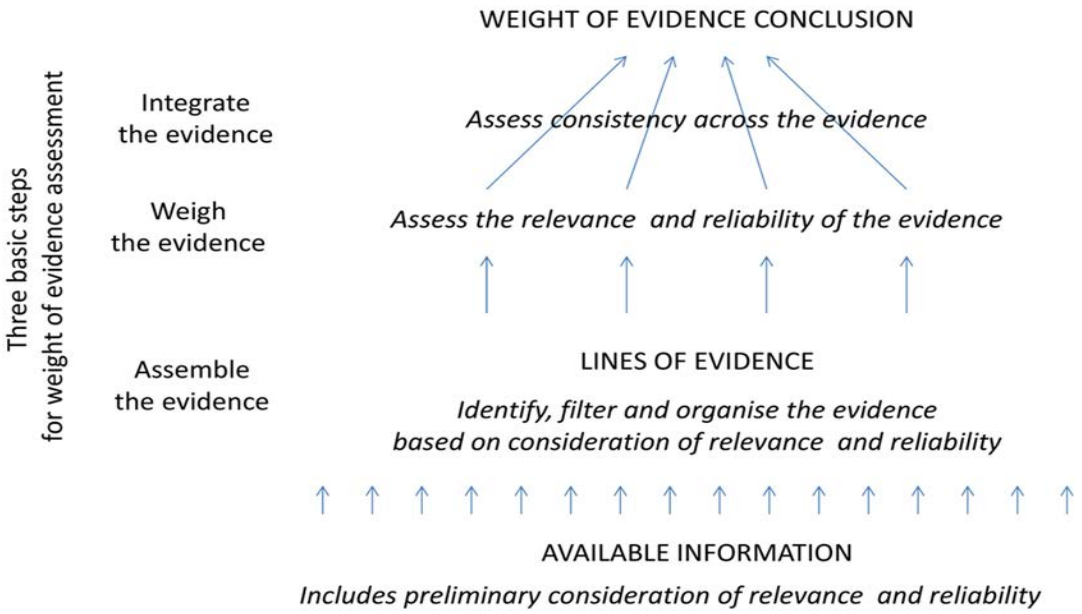

Figure 1: Relationship of relevance (including biological relevance), reliability and consistency to the three basic steps of weight of evidence assessment and to the conclusion for a weight of evidence question. [20]

According to the article 9 of CLP: «*Where the criteria cannot be applied directly to available identified information, manufacturers, importers and downstream users shall carry out an evaluation by applying a weight of evidence determination using expert judgement in accordance with section 1.1.1 of Annex I to this Regulation, weighing all available information having a bearing on the determination of the hazards of the substance or the mixture, and in accordance with section 1.2 of Annex XI to Regulation (EC) No 1907/2006.*»[18]

Annex I, paragraph 1.1.1.3.: «*A weight of evidence determination means that all available information bearing on the determination of hazard is considered together, such as the results of suitable in vitro tests, results of adequate non-mammalian embryo models such as aquatic eleutheroembryos as well as invertebrate species, relevant animal data, human experience such as occupational data and data from accident databases, epidemiological and clinical studies and well-documented case reports and observations. For substances, information from the application of the category approach (grouping, read-across), (Q)SAR, and omics' results are also considered. The quality and consistency of the data shall be given appropriate weight. Information on substances related to the substance being classified shall be considered, as appropriate. Information on substances or mixtures related to the mixture being classified shall be considered in accordance with Article 9(4). Information on the site of action and the mechanism or mode of action study results shall also be considered. Both positive and negative results shall be assembled together in a single weight of evidence determination;*» [19]

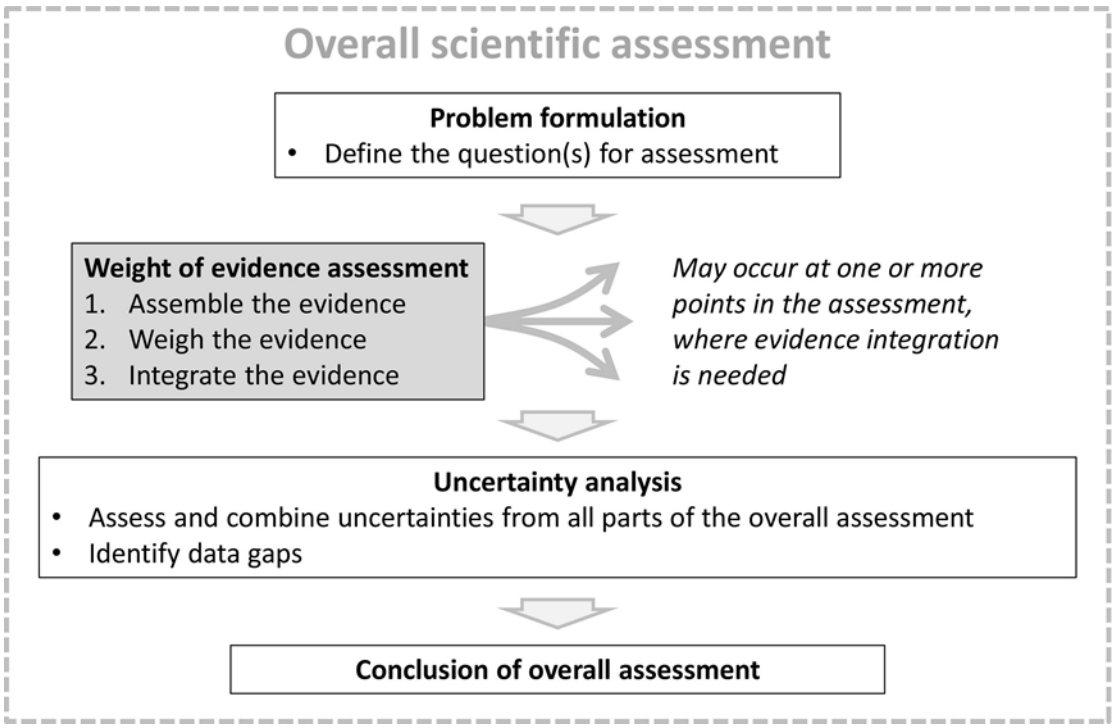

Figure 2: Diagrammatic illustration of weight of evidence assessment as a 3-step process which may occur at one or more points in the course of a scientific assessment. [20]

CHAPTER 2

2.1. Cardiotoxicity

Cardiotoxicity is defined as the inability of the heart to pump blood through the body effectively. [25]The term "cardiotoxicity" was initially coined in 1946 to describe the cardiac side effects of local anesthetics, mercurial diuretics, and digitalis. In the 1970s, it was also used to describe the cardiac complications associated with anthracyclines (such as daunorubicin and doxorubicin), combination therapy (doxorubicin and radiotherapy), and 5-fluorouracil. [22] Early observations of "cardiotoxicity" focused on the clinical syndrome of heart failure (HF).There isn't a universally agreed-upon definition of cardiotoxicity. Different organizations have established their own definitions of cardiotoxicity, often based on specific threshold changes in LVEF. [23] A fall below a certain level or an absolute change in LVEF is interpreted as an early indicator of cardiotoxicity. [22]

To sum up cardiotoxicity can be identified through various criteria, such as a decrease in left ventricular ejection fraction (LVEF), presence of heart failure (HF) symptoms/signs, LVEF reduction below 55% with HF symptoms, or a LVEF decrease of 10% greater than or equal to 10% or an LVEF lesser than 55% without signs or symptoms of HF. [27]

Types of Heart Problems Caused by Cardiotoxicity: [24]

- Arrhythmia or abnormal heart rhythm
- Cardiomyopathy: the heart pumps less efficiently, which decreases the amount of blood and oxygen sent to the organs and may increase risk for blood clot and heart attack
- Congestive heart failure: develops when cardiomyopathy gets to the point of causing symptoms because the heart can’t pump enough blood, which can damage your organs and cause fluid to build up in your lungs
- Coronary artery disease: narrowing or blocking of coronary arteries usually caused by plaque build up
- Hypertension or high blood pressure :when blood pressure is too high and left untreated it can lead to heart attack, stroke and other health problems
- Hypotension or low blood pressure :when blood pressure is too low, it can cause dizziness or lightheadedness, nausea, fainting and other symptoms
- Myocarditis: swelling or inflammation of the heart muscle, which causes an abnormal heartbeat, can weaken the heart muscle and lead to cardiomyopathy
- Peripheral vascular disease: narrowing or blocking caused by plaque build-up outside of the coronary arteries

- Valve disease: narrows or weakens the valves, which causes blood flow abnormalities through the valves

Various methods exist for detecting anthracycline-induced cardiotoxicity. One of the most successful approaches is endomyocardial biopsy. However this method has limitations such as invasiveness and uncertainty about sample quality and myocardial damage representation. [27]Due to these limitations, noninvasive methods are preferred, with echocardiography being widely used.[25] Using this technique, the left ventricular ejection fraction, which is an indicator of cardiac systolic function, can be quantified. Standard 2D echocardiography has limitations: it requires high image quality for accurate LVEF measurement, is affected by ventricular foreshortening, and relies on mathematical models for LV volume calculations. Enhancements include contrast agents for better border visualization and 3D echocardiography for faster analysis and reduced observer variability.[25]

2.2. Anthracyclines

Anthracyclines stands out to be the most effective anticancer drugs, isolated from a pigment-pruducing *Streptomyses spp* in the early 1960s.[24] Daunorubicin was the first identified anthracycline from *S.peucetius*, with doxorubicin, a derivative, discovered later as a more effective antitumor agent.[28] Anthracyclines are widely utilized in treating hematologic malignancies like leukemias and lymphomas, as well as solid-tumor malignancies such as carcinomas and sarcomas due to their potent anticancencer properties.[28,29]
Anthracyclines are characterized chemically by the presence of an aglycone ring coupled with an amino sugar component (figure 3).[31]There are also quinone and hydroquinone moieties present on adjacent rings, which permit the gain and loss of electrons.[25]Many analogs have been produced, but only a small number have developed into approved agents.[33]The different types of anthracyclines commonly used for cancer treatment are:

- Daunorubicin
- Doxorubicin
- Epirubicin
- Idarubicin
- Mitoxantrone
- Valrubicin

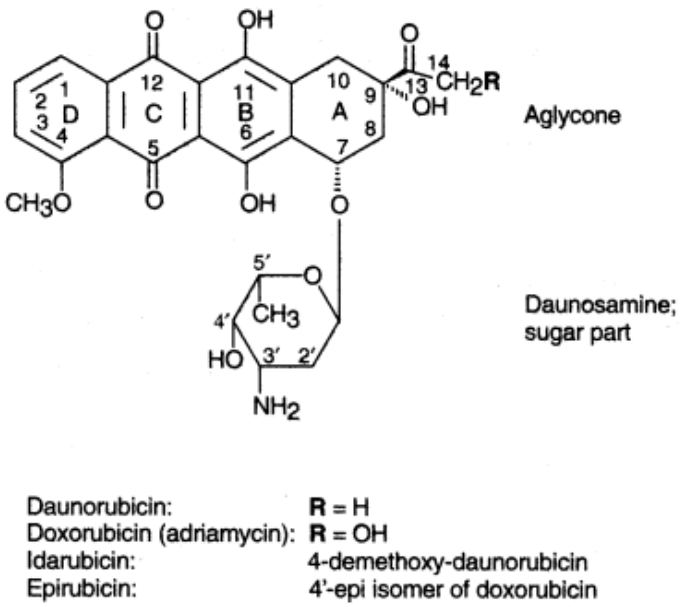

Figure 3: Anthracycline chemical structures. [35]

The extensive use of anthracyclines for over 40 years has greatly enhanced cancer survival rates.[28] Before the 1960s, children with leukemia had an overall 5-year survival rate of 30%. However, due to the expanded utilization of anthracycline-based chemotherapy and improved early detection methods, the 5-year survival rate for children has risen to 80%.[34]

2.2.1. Mechanism of Action

The mechanisms underlying the cytostatic and cytotoxic effects of anthracyclines involve several pathways, such as free radical generation, lipid peroxidation, direct impacts on cell membranes, and interactions with enzymes. [36]

Enzyme Interaction: The predominant explanation for how anthracyclines work involves their interaction with topoisomerase-II. This interaction leads to the formation of a ternary complex that inhibits the re-ligation of double-stranded DNA breaks. As a result, it induces growth arrest and apoptotic cell death. [36]

DNA Intercalation: Anthracyclines contain a chromophore moiety that acts as an intercalator, inserting itself between adjacent base pairs of DNA within the cell nucleus. This intercalation impedes DNA and RNA synthesis, particularly in rapidly in highly replicating cells blocking cell division.

ROS: Redox reactions generate reactive oxygen species in the presence of cytochrome P450 reductase, NADH dehydrogenase, and xanthine oxidase. When there's an excess of ROS that

cannot be effectively detoxified, it causes oxidative stress, DNA damage, and lipid peroxidation. These processes ultimately trigger apoptosis or programmed cell death.
DNA Adduct Formation: Anthracycline drugs are recognized for their ability to form adducts with DNA, a process facilitated by formaldehyde-releasing pro-drugs. These adducts act by obstructing specific transcription factors, ultimately triggering apoptosis or programmed cell death.
Even though anthracyclines are widely used in chemotherapy, their therapeutic effectiveness is limited due to their extensive adverse event profile, ranging from mild to severe. [36]

2.2.2. Acute and Chronic Adverse Effects of Anthracyclines

Anthracyclines, although effective in treating various cancers, come with several side effects. They are known to cause moderate to strong nausea, which has been mitigated to some extent with newer anti-emetic medications. Hair loss, particularly on the scalp, is common after treatment with drugs like doxorubicin or daunorubicin, and methods like 'cold caps' have limited efficacy in reducing this effect. Extravasation, or leakage of these drugs outside a vein, can lead to tissue damage and slow healing. [37] Stomatitis, a condition characterized by mouth sores, can occur more frequently with prolonged anthracycline infusion. Myelosuppression, including low white blood cell and platelet counts, is a common dose-limiting side effect. While certain patients with hepatic dysfunction may tolerate standard dosages, reducing doxorubicin doses is generally recommended in such cases to prevent excessive myelosuppression. Additionally, anthracyclines like epirubicin have been associated with acute leukemia in some cases. Patients who have received radiation therapy prior to anthracycline treatment may experience a "recall phenomenon," where latent radiation effects are reactivated, potentially causing inflammation and fibrosis in previously irradiated areas. [30]Considering these side effects, it's recommended to use radiation therapy as the final step in a treatment plan involving anthracyclines to minimize potential toxicity.

2.2.3 Anthracycline-Induced Cardiotoxicity

Cardiotoxicity induced by anthracyclines represents a major cause of morbidity and mortality. Clinically, it is characterized by the onset of heart failure or the detection of left ventricular dysfunction in individuals exposed to these drugs, with left ventricular ejection fraction being the most commonly used measure for assessment.

Although anthracyclines remain among the most widely prescribed and effective anticancer agents the significant risk of life-threatening cardiotoxicity continues to limit their overall usefulness. [36] Even after more than four decades of research, the exact pathogenic mechanisms behind anthracycline-induced cardiotoxicity have not been fully understood. [36] Survivors of sarcoma have a much higher risk of experiencing cardiovascular events compared to individuals in the general population, and these events often occur at a younger age.[34] It is unclear whether the mechanism(s) responsible for anthracycline-induced cardiotoxicity are the same as those that are responsible for killing tumor cells. [1] Anthracyclines cause cardiomyocyte death, leading to gradual patchy myocardial necrosis and, ultimately, multifocal myocardial fibrosis. This sequence is associated with the progression through various stages of heart failure, starting from asymptomatic cardiomyopathy, advancing to clinical heart failure, and ultimately culminating in end-stage heart disease.[37,38]

The molecular mechanisms that underlie AIC are complex and involve multiple pathological processes as changes in myocardial cell function and pathological cell death, involving mitochondrial dysfunction, inhibition of topoisomerases, disturbances in iron ion metabolism, degradation of myofibrils, and oxidative stress. [37]

CHAPTER 3

3.1. Oxidative Stress

Oxidative stress occurs when there's an imbalance between the production and accumulation of reactive oxygen species (ROS) in cells and tissues and the biological system's ability to detoxify these reactive products. While ROS play essential physiological roles like cell signaling and are typically generated as by-products of oxygen metabolism, external stressors such as UV radiation, ionizing radiation, pollutants, heavy metals, and xenobiotics like anticancer drugs can significantly increase ROS production. This imbalance leads to oxidative stress, causing damage to cells and tissues.[39,42]

Reactive oxygen species (ROS) are highly reactive chemical species containing oxygen, including the superoxide (O_2^-) and the hydroxyl (OH^-) anions, and hydrogen peroxide (H_2O_2). [40] ROS are metabolic by-products generated by biological systems.Various cellular processes such as protein phosphorylation, activation of transcription factors, apoptosis,

immune responses, and cell differentiation depend on controlled ROS production, which needs to be maintained at a low level. Mitochondria are the primary source of ROS production, occurring during both normal physiological processes and pathological conditions.[40,42]

The consequences of oxidative stress include lipid peroxidation, membrane damage, and the activation of proteases, nucleases, and protein kinases. [2] Oxidative stress biomarkers encompass products of lipid peroxidation like malondialdehyde (MDA) and advanced oxidation protein products (AOPP) related to protein oxidation. [45]Extensive lipid peroxidation within biological membranes can cause disruptions in structural integrity, a loss of fluidity, a decline in membrane potential, and increased permeability to ions. Additionally, enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) also function as biomarkers indicating oxidative damage. SOD is particularly crucial as a primary defense mechanism in the antioxidant system, catalyzing the dismutation of superoxide radicals (O2•−) into molecular oxygen (O2) and hydrogen peroxide (H2O2). The combined action of catalase (CAT) and glutathione peroxidase (GPx) then neutralizes the hydrogen peroxide in all vertebrates. [41,42]

Cells deploy various antioxidant defense systems that are in place to counteract the accumulation of reactive oxygen species (ROS) by scavenging and converting ROS into non-toxic molecules.[44] These systems encompass both enzymatic and non-enzymatic mechanisms. Enzymatic defenses include catalase, glutathione peroxidase (GSHPx), superoxide dismutase (SOD), and glutaredoxins (Grxs). Non-enzymatic antioxidants comprise vitamins E and C, beta-carotene, ubiquinone, lipoic acid, urate, and reduced glutathione (GSH).[39,40]Reduced glutathione (GSH) is a critical low-molecular-weight thiol-containing peptide found in most living cells and represents a key natural antioxidant. It functions as a scavenger of electrophilic and oxidant species either directly or through enzymatic catalysis. GSH serves as the cosubstrate of GSHPx, facilitating the reduction of peroxides and the production of oxidized glutathione (GSSG).[1]

Cardiovascular disease continues to be a leading cause of morbidity and mortality.[39,42] CVD encompass various pathologies affecting the heart or blood vessels, and oxidative stress plays a crucial role in their development.[2]Concerning vascular functions, an overabundance of reactive oxygen species (ROS) oversees the release of factors that influence either vasoconstriction or vasodilation, resulting in damage to endothelial cells (EC), hyperplasia of vascular smooth muscle cells (VSMC), and structural remodelling. Maintaining proper regulation of vascular tone is crucial for cardiovascular health. In this context, oxidative stress plays a significant role as a trigger for endothelial cell dysfunction.[2]

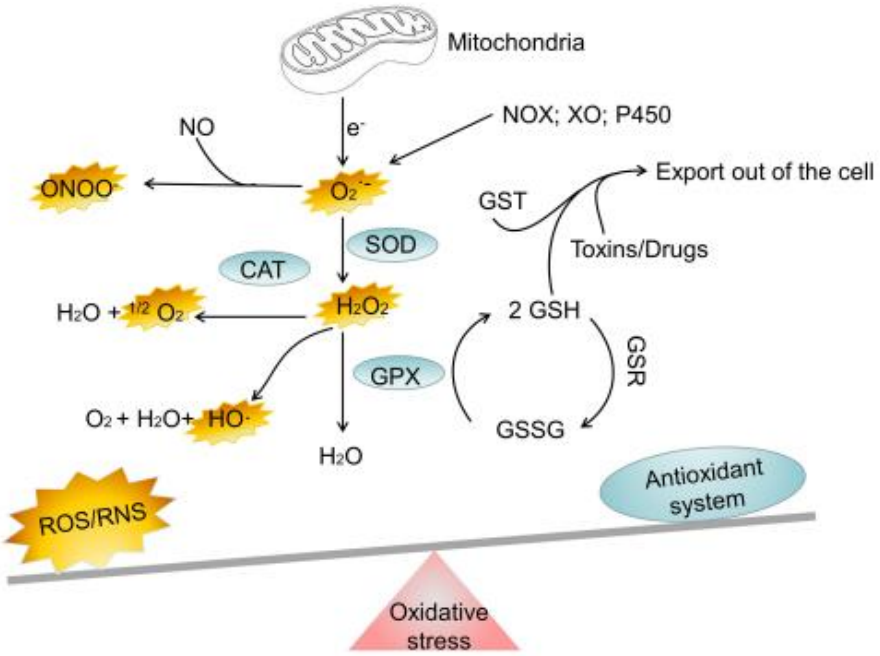

Figure 4 : The major oxidant and antioxidant systems. [43]

3.2. Reduced Glutathione

Reduced glutathione (GSH) is a water-soluble tripeptide with the structure of γ-glutamyl-cysteinyl-glycine, appears in all cells that plays a key role in maintaining our cellular oxidation-reduction (redox) status and regulates the activity of several enzymes.[47] Is highly significant as a thiol compound in organisms, serving as a catalyst, reductant, and reactant in biochemical processes. [51] In organs regularly exposed to toxins like the liver, kidney, lungs, and intestines, GSH is present in substantial amounts, often in millimolar concentrations. However, in bodily fluids, GSH concentrations are lower, typically in the micromolar range. GSH is involved in various cellular processes such as amino acid transport, protein synthesis, DNA synthesis, and cellular detoxification. The liver is the main source of plasma GSH. Viable cells like hepatocytes and macrophages release this thiol as part of their physiological functions, providing antioxidant protection to the extracellular environment.[52]
Glutathione exists in cells in 2 states: reduced (GSH) and oxidized (GSSG). Oxidized glutathione is actually 2 reduced glutathiones bound together at the sulfur atoms.(Figure 5) [51]
The redox status of cells is determined by the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Healthy cells at rest typically exhibit a high GSH/GSSG ratio (>100), whereas cells exposed to oxidative stress often have a lower ratio (1 to 10).[49] The GSH/GSSG ratio is the most vital redox couple in animal cells, playing essential roles in antioxidant defense , nutrient metabolism, and the regulation of pathways crucial for overall

body homeostasis. Glutathione also acts as a thiol buffer, maintaining sulfhydryl groups of many proteins in their reduced state.[51]

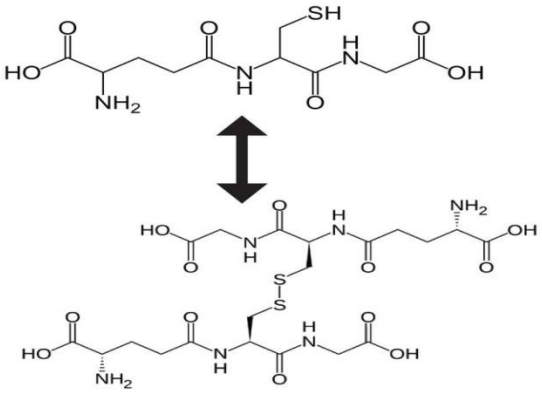

Figure 5: Reduced and oxidized form of glutathione. [51]

Glutathione is exclusively produced in the cytosol and actively transported into mitochondria. There are three main ways in which GSH becomes available in cells:[51]

- De novo synthesis through a two-step process involving the enzymes glutamate cysteine ligase (GCL) and glutathione synthetase, which requires ATP.
- Regeneration of oxidized GSSG back to reduced GSH via glutathione reductase, which requires NADPH.
- Recycling of cysteine from conjugated glutathione via gamma-glutamyltranspeptidase (GGTP), also requiring NADPH.

All three processes involved in glutathione - synthesis, regeneration, and recycling - demand energy. Their rates are predominantly influenced by several factors. Firstly, the cellular concentration of cysteine acts as the limiting factor in de novo glutathione synthesis. Secondly, GSH feedback inhibition plays a role in regulating the activity of glutamate cysteine ligase (GCL). Lastly, upregulation of de novo glutathione synthesis occurs in response to reduced GSH levels caused by oxidative stress, inflammation, or exposure to xenobiotics. This upregulation primarily involves increasing cysteine availability through the recycling of oxidized glutathione (GSSG).[51,52]

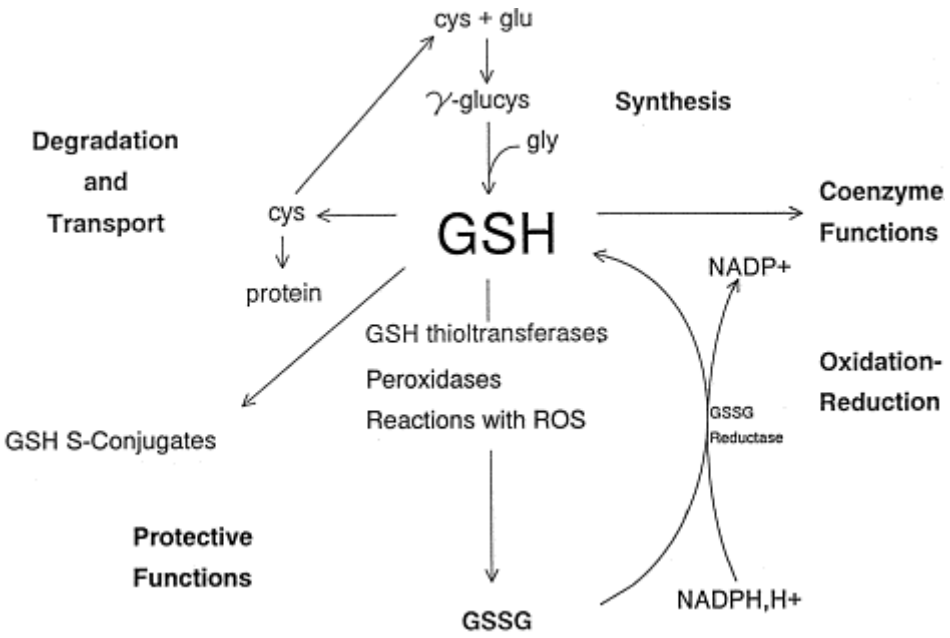

Figure 6: Overview of glutathione (GSH) metabolism.[115]

Deficiency in glutathione contributes to oxidative stress, potentially playing a significant role in the aging process and the development of various diseases.[49] GHS can react with various free radicals, including the hydroxyl radical, hypochlorous acid, superoxide, and peroxynitrite radical, and also reduces hydrogen peroxide. This makes GSH the primary cellular defense against oxygen-reactive species. The second line of defense involves antioxidant enzymes that utilize glutathione as a cofactor.[50] Cellular levels of antioxidants adjust to the levels of oxygen and oxyradicals, allowing cells to protect themselves against heightened free radical production. Excessive production or ineffective removal of free radicals can lead to cellular damage. Thus, assessing plasma glutathione levels can be valuable in evaluating the impact of xenobiotics as inducers or inhibitors of detoxification systems.[49]

Many diseases result from an imbalance between the production rate of oxidants (such as free radicals or reactive species) and the activity of cellular antioxidant systems. Glutathione plays a central role as an antioxidant defense, regulating pathways crucial for cellular homeostasis. It acts not only as a detoxifier of endogenous and exogenous compounds but also participates in processes like cell proliferation, apoptosis, gene expression, immune system regulation, and cell compound metabolism. Disturbances in GSH homeostasis are associated with the etiology and/or progression of various diseases beyond those related to cardiovascular disorders. [50]

Cardiovascular diseases (CVD) are closely linked to redox imbalances, including conditions like hypertension and atherosclerosis. The increased accumulation of reactive oxygen species is associated with variations in antioxidant enzyme genes such as glutathione peroxidases and glutathione S-transferases, which are connected to a higher risk of vascular diseases.

Polymorphisms in glutathione peroxidase genes, for instance, are associated with an elevated risk of coronary artery disease, stroke, and cerebral thrombosis. In smokers, the increased susceptibility to coronary heart disease is linked to polymorphisms in glutathione S-transferase genes.[50]

GSH plays a vital role in the cardiovascular system as a crucial antioxidant. It helps restore intracellular redox balance and prevents the inactivation of nitric oxide produced by the endothelium. This function is particularly significant in individuals with coronary spastic angina, as it prevents aberrant vasomotor reactivity. [51,52] Additionally, studies have shown that cardiac and systemic glutathione deficiency is associated with the functional status and structural cardiac abnormalities observed in patients with cardiac diseases. These findings suggest the potential development of a blood glutathione test as a new diagnostic tool for identifying asymptomatic patients with structural cardiac abnormalities.[27]

3.3. Glutathione Peroxidase

Glutathione peroxidase is an enzyme found in the cytosol that plays a crucial role in reducing hydroperoxides. In red blood cells (erythrocytes), GSH-Px is highly effective as an antioxidant, particularly in combating oxidative stress. Additionally, it performs important functions in phagocytic cells.[64] Glutathione peroxidase catalyzes a reaction where the reduced form of glutathione (GSH) interacts with hydrogen peroxide or lipid peroxides. This process aids in the detoxification of these molecules by forming a glutathione bridge with another glutathione molecule (GSSG) in its oxidized form. Hydrogen peroxide is further detoxified by catalase and glutathione peroxidase. The glutathione redox cycle is crucial for reducing intracellular hydroperoxides.[64]

The presence of glutathione peroxidase (GPx) was first discovered by Mills in 1957, initially observed in mammalian erythrocytes. It is notably effective in endothelial cells, particularly in lung tissue. Approximately 60–75% of the enzyme activity is concentrated in the cytoplasm of eukaryotic cells, with 25–40% located in mitochondria. The enzyme's activity is prominently observed in erythrocytes and liver cells.[65,56,64] GPx is critically important as it protects lipids from peroxidation within cells. Its role in the cytosolic compartment of cells is especially significant, contributing to the maintenance of cell structure and function.

The glutathione peroxidase family consists of various isozymes (GPX1-8) found in different subcellular locations and tissues.

Among these, GPX1-4 and 6 are selenocysteine-containing proteins. [61] Their active center consists of a conserved tetramer made up of selenocysteine residues (Sec), glutamine (Gln), tryptophan, and asparagine.In contrast , the other three GPXs have cysteine as an active site

[59]. The presence of selenocysteine (Sec) as a catalytic group is believed to facilitate a swift reaction with hydrogen peroxide, resulting in the rapid reduction of glutathione (GSH).[59] GPX1 and GPX4 emerge as the principal antioxidant enzymes within the GPX family, thanks to their extensive distribution throughout various tissues and organs.[59]Hence, the primary function of GPX1-4 is to mitigate oxidative stress by catalyzing the reduction of H2O2 or organic hydroperoxides. Moreover, they have the capability to inhibit inflammation and oxidant-induced programmed cell death processes, such as apoptosis, necroptosis, and pyroptosis.[57,61,62]They are highly expressed in the cytoplasmic and mitochondrial compartments of cardiomyocytes and constituting a crucial defense mechanism within the heart. [58,59] GPX2 and GPX3 demonstrate antioxidant effects in intestinal epithelial cells and plasma, respectively, while GPX5-8 lack significant antioxidant capacity.[55] It appears that GPx5 and GPx6 originated from a tandem duplication event involving GPx3.GPx7 and GPx8 are CysGPxs with relatively low GPx activity compared to other members of the glutathione peroxidase family. Surprisingly, over 700 CysGPx-homologous sequences have been identified across all domains of life, indicating a wide distribution of these enzymes. It has become evident that only a small portion of GPxs are selenoproteins.

Generally, peroxidases can disrupt regulatory processes in various ways. They may compete with redox-regulated proteins for hydroperoxides, which serve as signaling molecules. They can deactivate enzymes that rely on hydroperoxides for activation. They might undergo temporary inactivation, thereby preserving hydro peroxides for signalling purposes. They could utilize hydro peroxides to form Se-thioylated homo- or heterodimers or polymers. They can detect H2O2 or ROOH and specifically transmit the oxidation equivalents to target proteins, thereby enhancing the efficiency and specificity of the broadly acting low-molecular-weight oxidant H2O2. [57]

According to a previous study, in individuals with coronary artery disease, reduced activity of red-cell glutathione peroxidase 1 is independently linked to a higher risk of cardiovascular events. Glutathione peroxidase 1 activity could provide additional prognostic value beyond traditional risk factors. Moreover, enhancing glutathione peroxidase 1 activity may decrease the likelihood of cardiovascular events. [63]

There’s a well-established connection between selenium deficiency and heart disease, particularly highlighted in Keshan disease. This endemic cardiomyopathy is prevalent in regions of China where soil selenium levels are low. Keshan disease is characterized by mitochondrial insufficiency and reduced activity of GPx-1, underlining the importance of selenium and GPx-1 in maintaining heart health. [65] In general, a deficiency in GPx-1 contributes to endothelial dysfunction, which can lead to various cardiovascular issues. Conversely, overexpression of GPx-1 is protective, likely due to its role in modulating

hydrogen peroxide levels within endothelial cells. This modulation helps to preserve nitric oxide (NO), a crucial molecule for maintaining vascular health and function. [65]

3.4. Superoxide Dismutase

Superoxide dismutases (SODs) are widely distributed metalloenzymes that serve as the primary defense mechanism against reactive oxygen species (ROS). [72] They play a crucial role in the detoxification process of superoxide radicals produced within biological systems. This detoxification involves catalyzing the dismutation of superoxide radicals into hydrogen peroxide (H2O2), which is further converted into water (H2O) and oxygen (O2) with the help of catalase and peroxidase enzymes.[72,80] This process helps prevent the production of peroxynitrite and further damage to cells. SOD plays a vital role in maintaining the redox potential of cells and protecting them from reactive oxygen species (ROS), particularly during infections caused by intracellular pathogens. [80]

The evolutionary lineage of the superoxide dismutase (SOD) predates the split between eubacteria and archaea bacteria. [83] It is an ancient protein that is found universally across all living organisms, and it serves a critical function in protecting cells against the damaging effects of superoxide radicals. This defense mechanism is particularly important under extreme environmental pressures, highlighting the essential role of SOD in cellular survival and adaptation. [80]

As aforementioned, SOD catalyzes the conversion of the two molecules of virulent oxygen free radical (O^{-}) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) by using two equivalents of H^+ ions.(Figure 8)

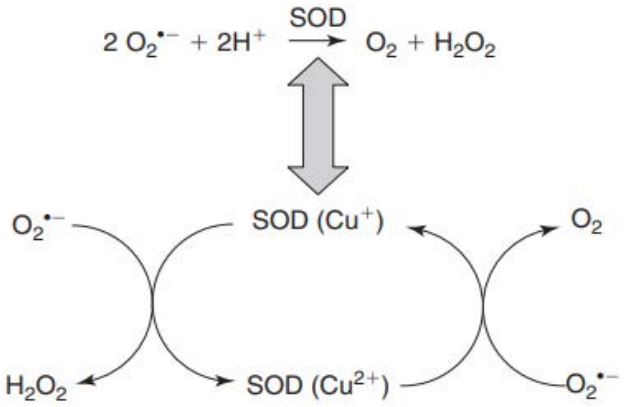

Figure 8: Schematic illustration of the biological process of $O_2^{\bullet-}$ dismutation into O2 and H2O2 catalyzed by Cu, Zn-SOD via a cyclic oxidation-reduction electron transfer mechanism. [83]

The superoxide dismutase (SOD) enzyme family is classified based on various factors, and one is on the metal ion present in their active sites. SODs generally have a metal cofactor in their catalytic center and are divided into three primary groups: copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD), and iron (Fe-SOD). The genes coding for SODs containing Mn-SOD, Fe-SOD, and Cu/Zn-SOD are designated as sodA, sodB, and sodC, respectively.
Nickel (Ni)- and iron-zinc (Fe/Zn)-containing isoforms have also been detected in various bacteria. [82] While FeSOD is primarily found in prokaryotes with a few exceptions in protozoan parasites, both MnSOD and CuZnSOD are present in both prokaryotic and eukaryotic organisms. These isoforms have been distinguished based on their different sensitivities to cyanide (CN) and hydrogen peroxide (H2O2). CuZn-SOD is highly sensitive to CN and H2O2, Mn-SOD shows resistance to CN and H2O2, and Fe-SOD is unaffected by CN but sensitive to H2O2. Furthermore, both Mn-SOD and Fe-SOD are inhibited by chloroform-ethanol, whereas CuZn-SOD remains unaffected by this inhibition.[81,82] The different forms of SODs are unequally distributed throughout all biological kingdoms and are located in different subcellular compartments.[72]
In humans, there are are three SOD isoforms: SOD1 (copper, zinc [Cu-Zn]-SOD) which is a soluble enzyme found in the cytoplasm and the space between mitochondrial membranes, SOD2 (Mn-SOD) that exists in the mitochondrial matrix and SOD3 (extracellular [EC]-SOD) that released into the extracellular space. [73,74] In seminal plasma, both SOD-1 and SOD-3 play important roles and are necessary for proper sperm functioning.[83]
The pathogenic mechanism of heart failure (HF) is complex and has not fully clarified, however, accumulating evidence indicates that the increased oxidative stress seems to have an important role to cardiac ventricular and vascular remodeling and leads to the progression of heart failure. [67,68,69]Oxidative stress refers to an imbalance between the body's antioxidant defense system and the production of reactive oxygen species.[70] The superoxide dismutase (SOD) family are the first-line antioxidant enzymes, which are responsible for modulating oxidative stress. [66]
The predominant isoform, responsible for over 70%, of the total SOD activity in the human cardiovascular system, is the extracellular superoxide dismutase (Ec-SOD) or SOD3.
Previous researches have shown a correlation between the activity of extracellular superoxide dismutase (Ec-SOD), endothelian function and the long-term outcomes in patients who suffer from chronic heart failure with cardiomyopathy.[66,71] The mechanisms, by which EC-SOD protects against fibrosis and tissue injury in many organs, such as heart, have been investigated but remain uncertain. EC-SOD, as an antioxidant enzyme, eliminates superoxide and modify oxidant balances within organs' tissues playing a crucial role throughout the cardiovascular system and potentially impacting cardiac morphology.[75]Extracellular superoxide dismutase possesses a matrix-binding domain that targets heparan sulfate

proteoglycans, such as syndecan-1, and is found on the surfaces of epithelial and endothelial cells, as well as within the heart tissue.[75,76] This is crucial for the ability of EC-SOD to defend against oxidants.[76,77] Moreover, studies conducted on the heart have shown that EC-SOD prevents oxidative injury after myocardial infarction and could also contribute to cardiac remodeling.[78,79]

3.5. Malondialdehyde

Malondialdehyde (MDA) is an organic compound that is derived from the peroxidation of polyunsaturated fatty acids (PUFAs) and arachidonic acid metabolism.[85] Specifically is a highly reactive three-carbon dialdehyde mostly existing in the enol form.[84] Lipid peroxidation plays a significant role in the development of various tissue injuries, especially those caused by exposure to toxic substances. Thus , MDA , as one of the most recognized secondary products of lipid peroxidation, serves as a marker of cell membrane injury. Elevated levels of lipid peroxidation products have been linked to various chronic diseases in both humans and model systems.[85] The high reactivity and toxicity of MDA underline that it is more than a biomarker; it has been proven to be a mutagenic substance.[89]

According to a survey plasma levels of MDA are significantly elevated in patients with chronic heart failure, and this is strongly correlated with the chronicity of the heart failure condition. Additionally, plasma MDA levels are associated with the presence of cardiovascular risk factors that can induce tissue damage.[86]The validation of elevated MDA levels as independent predictors of outcome appears to hold significant value for risk stratification in patients with chronic heart failure (HF).[87]

MDA can be found both blood platelets and serum.[88] The laboratory detection and quantification of MDA rely on its ability to react with two equivalents of thiobarbituric acid, resulting in the formation of a fluorescent red derivative. This derivative can then be assayed using spectrophotometry.[85] Measuring MDA through spectrophotometry using the TBARS assay is straightforward, but it lacks specificity because other aldehydes can produce light-absorbing products similar to MDA. ELISA assays, however, offer enhanced specificity compared to TBARS, demonstrating good performance in this regard.[90]

MDA serves as a biomarker for assessing oxidative stress across various biological samples, including blood, urine, and exhaled breath condensate (EBC), in patients affected by a wide spectrum of diseases. These diseases include cancer, cardiovascular disorders, pulmonary conditions, and neurodegenerative diseases..[90]

Oxidative stress primarily affects lipids, especially those with multiple carbon-carbon double bonds like polyunsaturated fatty acids. During oxidative stress, oxidants remove hydrogen

atoms from these lipids, forming unstable lipid radicals (L-). Subsequent oxygen insertion generates lipid peroxyl radicals (LOO-), which then extract hydrogen from other lipid molecules, continuing the reaction and producing more stable compounds called lipid hydroperoxides (LOOHs).[87] This process, known as lipid peroxidation, can lead to cyclization and cleavage of both lipid hydroperoxides and peroxyl radicals, resulting in the formation of secondary products. MDA is the primary and extensively researched compound resulting from lipid peroxidation, known for its mutagenic and toxic properties. It can also be formed enzymatically as a byproduct during the synthesis of thromboxane A2. Once formed, MDA can undergo metabolism by different enzymes, especially aldehyde dehydrogenase at the mitochondrial level. Alternatively, it can form covalent bonds with proteins and nucleic acids, resulting in the creation of DNA-protein crosslinks and various adducts that harm biomolecules.[87]

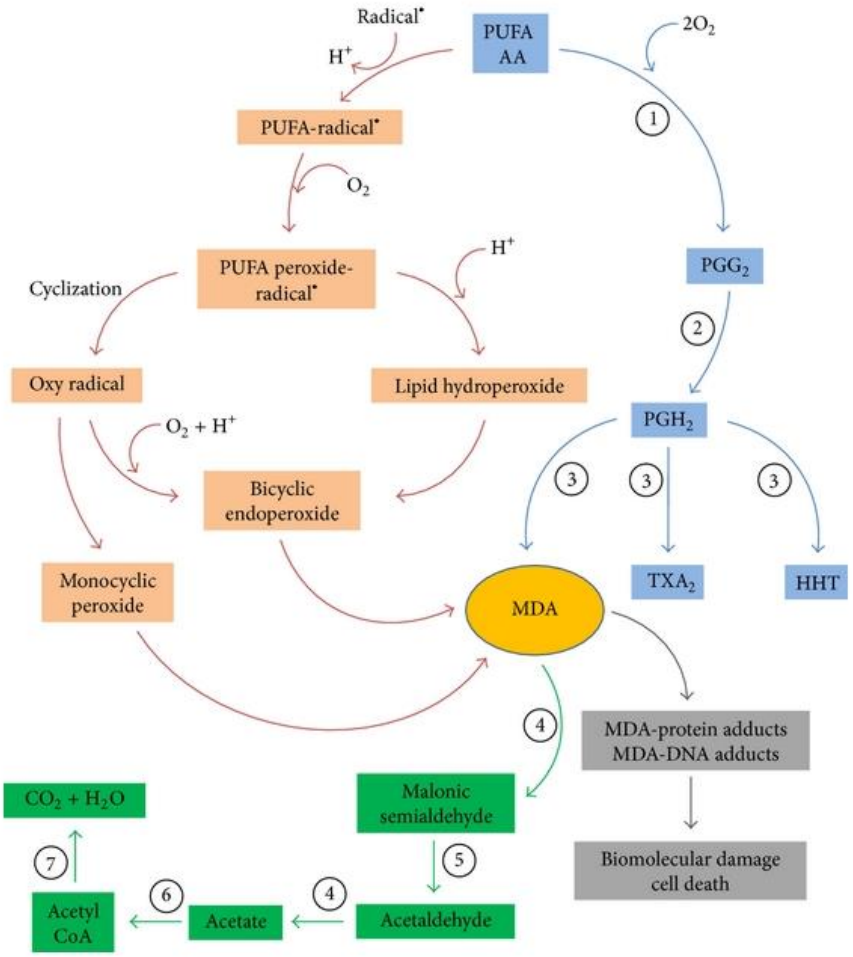

Figure 9 : MDA formation and metabolism. [89]

3.6. Catalase

Hydrogen peroxide is one of the most long-lived and freely diffusible reactive species which acts as a weak oxidizing and reducing agent. Although hydrogen peroxide is not very reactive, is the progenitor of many other ROS (reactive oxygen species). Research has shown that it is oxidatively modify glyceraldehyde-3-phosphate dehydrogenase by oxidation of the labile essential thiol groups at the active site of this enzyme.[91] It is important to be mentioned that this molecule plays an indirect role in most of the cellular injuries. The formation of ·OH radical, in the presence of transition metal ions such as Fe2+ by means of the Fenton reaction, is one the most important products. Many enzymes such as catalase, glutathione peroxidase, and other peroxidases (e.g. cytochrome c peroxidase and NADH peroxidase) are able to neutralize hydrogen peroxide. Catalase seems to be a key enzyme which uses hydrogen peroxide, a nonradical ROS as it's substrate. The aforementioned enzyme neutralize the composition of hydrogen peroxide that is very important for the maintainance of an optimum level of the molecule in the cell. This is also essential for various cellular signaling processes. The importance of catalase can be gauded from its direct and indirect involvement in numerous diseases and infections.[92]

Catalase is one of the most significant antioxidant enzymes which is primarily located in the peroxisomes of mammalian cells in various aerobic organisms.[92]It forms a tetrameric structure composed of four identical subunits, each arranged in a tetrahedral configuration and weighing around 60 kDa. Within its active center, catalase contains a heme group and NADPH. There are three distinct types of catalase based on variations in their sequence and structure. The first type is the monofunctional heme-containing enzyme, which is the most common and widespread among aerobic organisms. The second type is the bifunctional catalase-peroxidase, which is less common in nature and also contains a heme group. This enzyme shares structural and sequence similarities with plant peroxidases. The third type belongs to the Mn-containing catalase group, which does not have a heme group. [99]

The overall reaction for catalase involves the degradation of two molecules of hydrogen peroxide to water and oxygen (reaction 1). This catalytic reaction occurs in two stages, and the specific details of each stage depend on the type of catalase being considered. In the first stage, the heme group within catalase undergoes oxidation by the first molecule of hydrogen peroxide, leading to the formation of an oxyferryl species.[102] This species includes the removal of one oxidation equivalent from the iron atom and one from the porphyrin ring, resulting in the creation of a porphyrin cation radical (reaction 2). In the subsequent stage, this radical intermediate, referred to as compound I, is reduced by a second molecule of hydrogen peroxide. This reduction process regenerates the enzyme to its resting state while producing

water and oxygen (reaction 3).[102]Catalases can also function as peroxidases, utilizing suitable organic compounds as electron donors. During the peroxidase reaction, compound I is converted to compound II (reaction 4). Compound II can then be further oxidized by another molecule of hydrogen peroxide, resulting in the production of the inactive compound III,(reaction 5).For catalases that bind to NADPH, there is a mechanism to prevent enzyme inhibition caused by the appearance of compound III. This mechanism involves blocking or releasing NADPH to facilitate the generation of compound II, thereby maintaining the enzyme's activity.[103]

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (1)$$

$$Enz\,(Por{-}Fe^{III}) + H_2O_2 \rightarrow Cpd\ I\ (Por^{+\bullet}{-}Fe^{IV} = O) + H_2O \quad (2)$$

$$Cpd\ I\ (Por^{+\bullet}{-}Fe\,IV = O) + H_2O_2 \rightarrow Enz\,(Por{-}Fe^{III}) + H_2O + O_2 \quad (3)$$

$$Cpd\ I\ (Por^{+\bullet}{-}Fe\,IV = O) + AH_2 \rightarrow Cpd\ II\,(Por{-}Fe^{IV}{-}OH) + AH^{\bullet} \quad (4)$$

$$Cpd\ II\,(Por{-}Fe^{IV}{-}OH) + H_2O_2 \rightarrow Cpd\ III\ (Por{-}Fe^{III}{-}O2^{-\bullet}) + H_2O \quad (5)$$

Figure 10: Steps In Catalase Reaction.[102]

Catalase exhibits two enzymatic activities depending on the concentration of hydrogen peroxide (H2O2). In conditions of high H2O2 concentration, catalase functions catalytically, facilitating the removal of H2O2 by converting it into water (H2O) and oxygen (O2) through a catalatic reaction. Conversely, under conditions of low H2O2 concentration and in the presence of a suitable hydrogen donor such as ethanol, methanol, phenol, or other compounds, catalase acts peroxidically. In this peroxidatic reaction, it continues to eliminate H2O2 while simultaneously oxidizing its substrate. [101]

Catalase deficiency or malfunction is linked to various diseases, including diabetes mellitus, vitiligo, cardiovascular diseases, Wilson disease, hypertension, anemia, certain dermatological disorders, Alzheimer's disease, bipolar disorder, and schizophrenia.[100]

Catalase plays a crucial role in regulating cellular hydrogen peroxide levels , and its activity in hydrogen peroxide breakdown protects cells from oxidative damage. Reduced catalase activity has been observed in patients with schizophrenia and those with atherosclerosis.[92]

Studies have indicated that catalase, an essential enzyme, plays a significant role in mutagenesis, inflammatory conditions, and the inhibition of apoptosis. These processes are

commonly linked with oxidative stress conditions, where catalase's activity has been observed to have notable impacts.[92]

There is a substantial evidence indicating that reactive oxygen species (ROS) play an important role in unfavorable alterations in myocardial structure and function, leading to the progression of heart failure. [93]According to Qin, F. et al, catalase prevents the development of overt heart failure and it's overexpression attenuates myocardial oxidative stress. This is very important because high levels of oxidative stress in the myocardium have been observed in many animal models of heart failure (HF), including pressure overload [94,95] myocardial infraction[96]rapid pacing[97] as well as in human with HF.[98]

CHAPTER 4

4.1. Material and Methods

According to the Preferred Reporting Items for Systematic Review and Meta-analyses (PRISMA) [104], a comprehensive search in the PubMed electronic database was systematically conducted to identify all the original research studies taken place between March 1st, 2020 and October 15th, 2023. The research strategy employed included the following terms either in the title or the abstracts :

"*Cardiotoxicity*" AND "*rats*" AND "*anthracycline*" AND "*oxidative stress *"

"*Cardiotoxicity*" AND "*rats*" AND "*daunorubicin*" AND "*oxidative stress *"

"*Cardiotoxicity*" AND "*rats*" AND "*doxorubicin*" AND "*oxidative stress *"

"*Cardiotoxicity*" AND "*rats*" AND "*epirubicin*" AND "*oxidative stress *"

As a whole, a total of 105 published manuscripts concerning animal studies were included in the systematic review. Following the exclusion of duplicates, the retrieved studies underwent additional assessment to ascertain their relevance to the subject matter and eligibility. Subsequently, a thorough examination was carried out to extract all relevant data. Only data from rat species was evaluated. All citation types other than original research studies, such as review articles, was excluded. Furthermore, articles containing data from irrelevant organs (e.g., brain , kidney) were excluded , as well as those where anthracycline had not been administered.

Oxidative stress has been demonstrated to significantly contribute to the pathophysiology of cardiac remodeling and heart failure (HF) by causing subtle alterations in intracellular pathways and redox signaling, but causes cellular dysfunction and damage at higher levels.[67] The searches targeted oxidative stress, particularly focusing on malondialdehyde (MDA) as a lipid peroxidation marker in cardiac tissue as well as three endogenous enzymes-Superoxide

dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx)-and the antioxidant glutathione (GSH) in cardiac tissue. Incorporating the supplementary 40 studies from the preceding puplication [9], the total number of articles increases to 145, as illustrated in the diagram (Fig. 1).

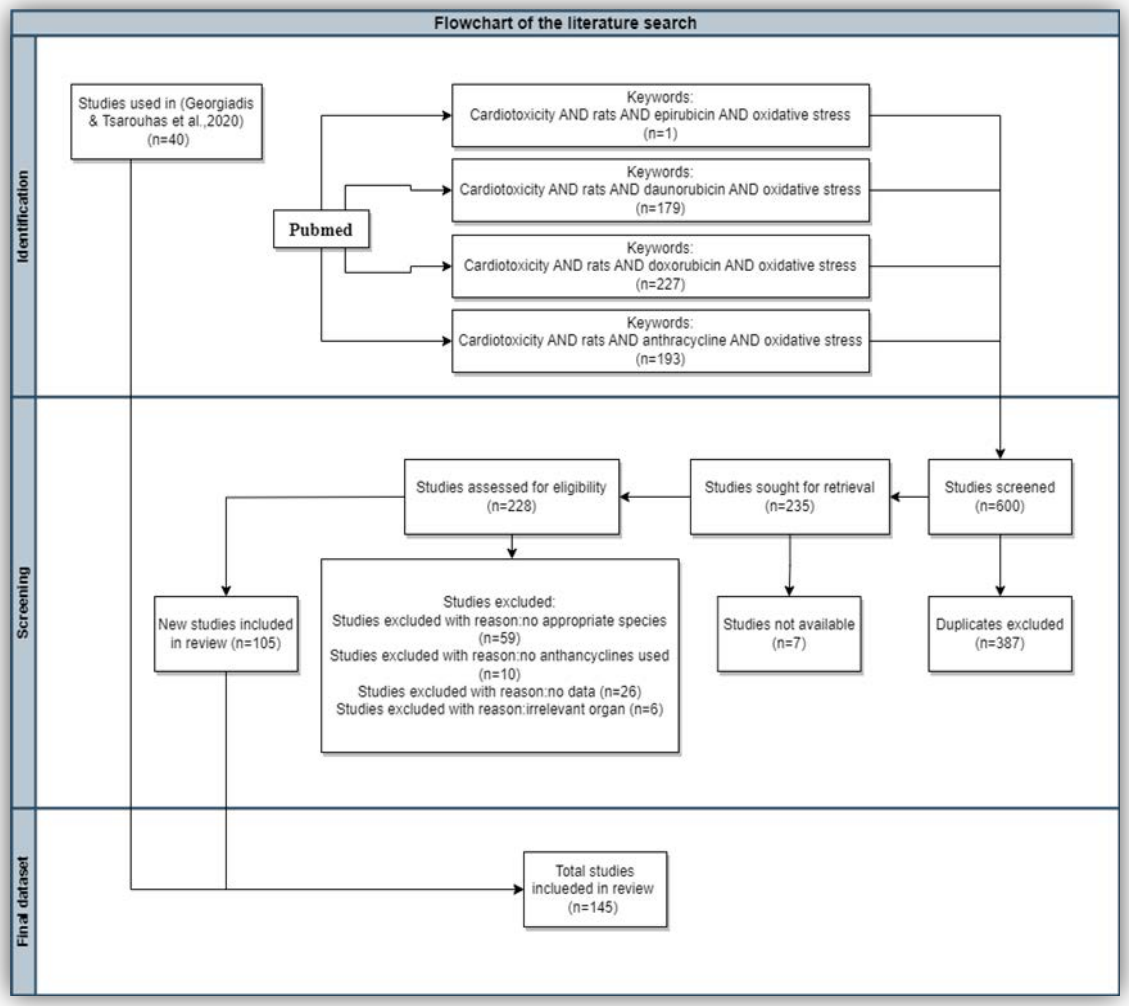

Figure 11. Prisma flow chart (literature search) for the present study design.

CHAPTER 5

5.1. Results

In the comprehensive review encompassing 145 studies, it has been consistently observed that exposure to anthracyclines leads to the suppression of key biomarkers such as catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GSH-Px) indices. Simultaneously, there is an evident increase in malondialdehyde (MDA) values, indicating heightened oxidative stress levels.

In the figures 12, 13, 14, 15, 16, we present an extensive comparison of the overall changes in biomarkers of oxidative stress, including GSH, GSH-Px, CAT, SOD, and MDA, within the cardiac tissue of rats experiencing anthracycline-induced cardiotoxicity, in contrast to their healthy counterparts. Our analysis reveals a significant increase in MDA levels, ranging notably from 20% to 200%. Conversely, there is a noticeable decrease in GSH-Px levels, ranging from 10% to 70%, and in GSH levels, showing a decline from 20% to 80%. Additionally, we observed a substantial decrease in SOD levels, ranging from 30% to 80%, and in CAT levels, ranging from 20% to 70%.

In particular, when examining MDA levels, there is a distinct difference, as illustrated in figure 17, between the MDA values detected in cardiac tissues and the mean control values obtained from all observations.

There is a clear dependence of all the five values in the cardiac tissue upon the anthracycline exposure of rats leading to cardiac dysfunction. It is very encouraging that there is a concurrent decrease and/or increase of the values when the rats develop cardiotoxicity which can provide supportive lines of evidence in a weight of evidence approach for assessing cardiotoxicity in a regulatory framework.

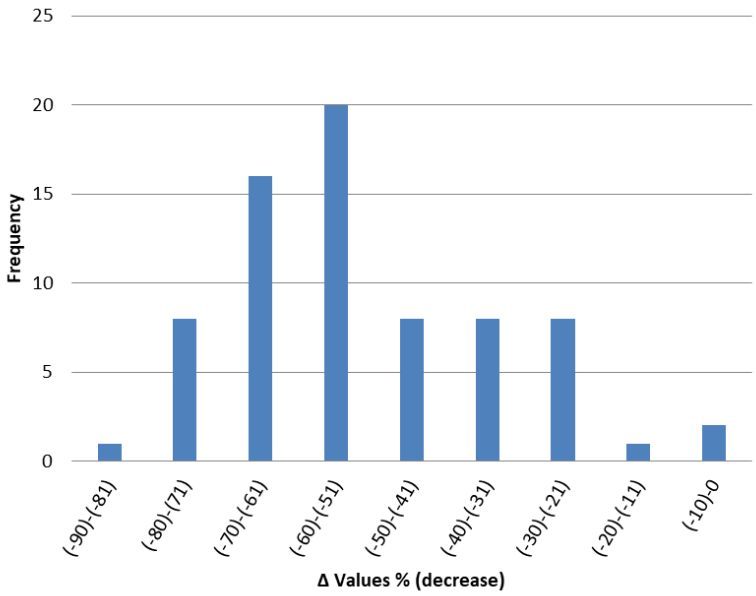

Figure 12. Percentage decrease in cardiac tissue GSH (%Δ values [(values of rats exposed to anthracyclines) - (values of control rats)] x 100) in rats exposed to anthracyclines compared to control animals, as reported in the reviewed studies. %Δ values were calculated in order to overcome the diversity of measuring units used in the literature.

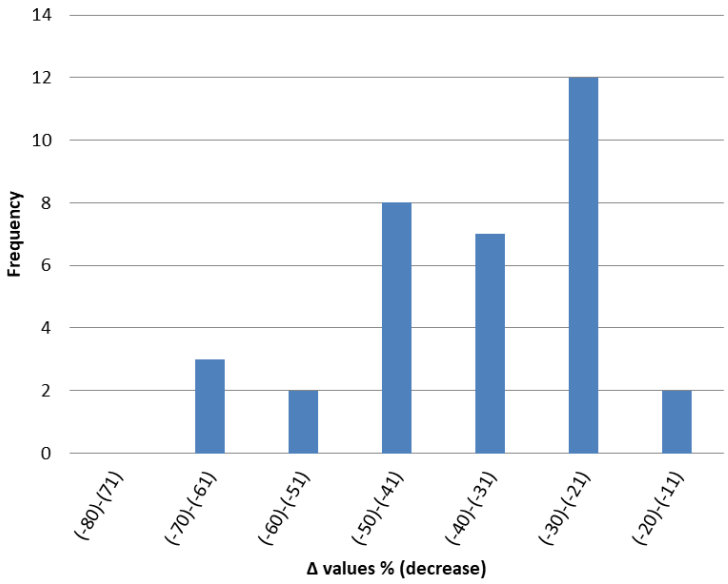

Figure 13. Percentage decrease in cardiac tissue GSH-Px (%Δ values [(values of rats exposed to anthracyclines) - (values of control rats)] x 100) in rats exposed to anthracyclines compared to control animals, as reported in the reviewed studies. %Δ values were calculated in order to overcome the diversity of measuring units used in the literature.

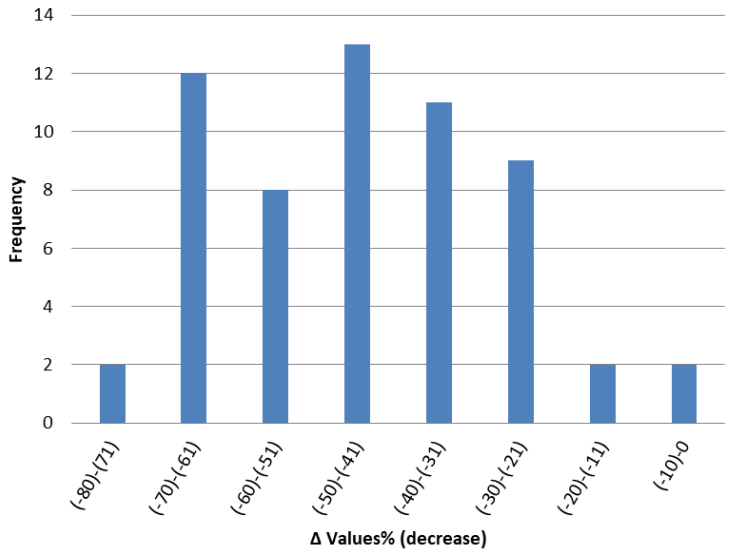

Figure 14. Percentage decrease in cardiac tissue CAT (%Δ values [(values of rats exposed to anthracyclines) - (values of control rats)] x 100) in rats exposed to anthracyclines compared to control animals, as reported in the reviewed studies. %Δ values were calculated in order to overcome the diversity of measuring units used in the literature.

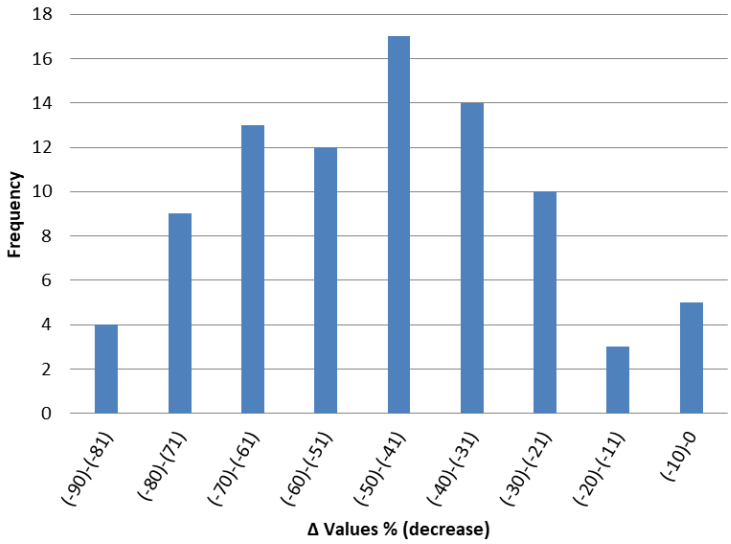

Figure 15.Percentage decrease in cardiac tissue SOD (%Δ values [(values of rats exposed to anthracyclines) - (values of control rats)] x 100) in rats exposed to anthracyclines compared to control animals, as reported in the reviewed studies. %Δ values were calculated in order to overcome the diversity of measuring units used in the literature.

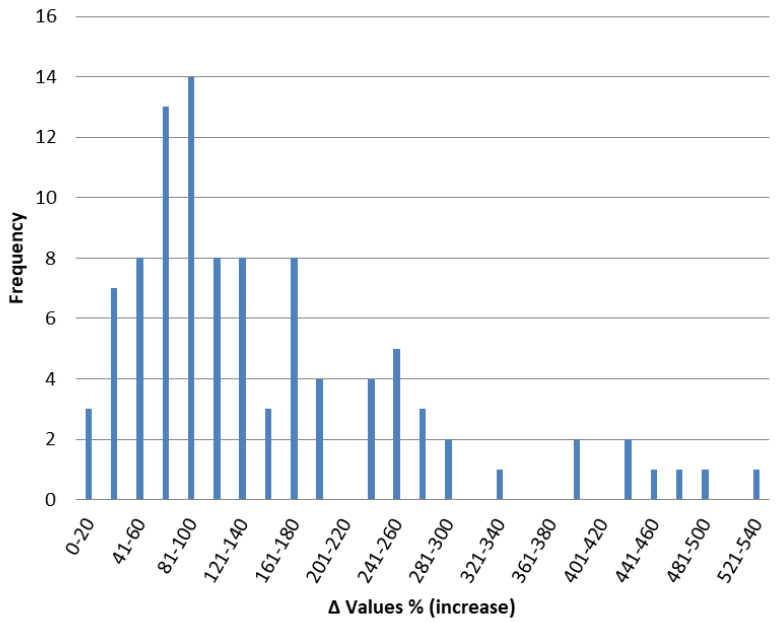

Figure 16. Percentage increase in cardiac tissue MDA (%Δ values [(values of rats exposed to anthracyclines) - (values of control rats)] x 100) in rats exposed to anthracyclines compared to control animals, as reported in the reviewed studies. %Δ values were calculated in order to overcome the diversity of measuring units used in the literature.

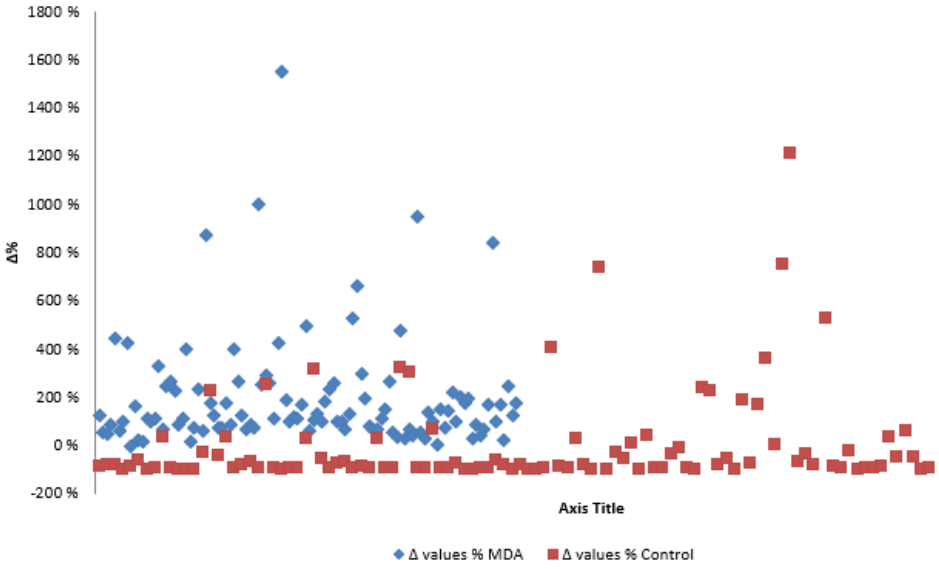

Figure 17. Difference between the Δ% observed between the control and MDA Δ% values of the reviewed studies (blue points) compared to the Δ% values of the control values and the mean control of all the reviewed studies (red values).

CHAPTER 6

6.1. Discussion

Myocardial fibrosis emerges as a pivotal event in anthracycline-induced cardiotoxicity, evident in both animal models and human studies.Recent research evaluating biopsy and autopsy material has revealed the presence of interstitial and replacement fibrosis in patients undergoing cancer therapy. [54] Another crucial event is the disorganization and de-arrangement of myocardial tissue, likely stemming from myocardial cell necrosis. Preserving the architecture of myocardial tissue is paramount, as its loss correlates directly with the decline in left ventricular function seen in patients with cancer-related cardiotoxicity. Cardiomyocyte necrosis and replacement fibrosis often coincide, yet another critical factor is thought to intervene in the changes affecting the heart's cytoskeleton, profoundly altering its mechanical properties.[39] At the cellular level, cardiomyocyte necrosis and apoptosis are hallmark features of cancer-related cardiotoxicity, accompanied by cell vacuolization upon histopathological examination of tissue samples.[108] These irreversible cellular changes align with the well-known effects of anthracycline cardiotoxicity, underscoring the significance of early detection efforts in clinical settings to address the permanent insults caused by anthracyclines.

Therefore, histopathological findings related to anthracycline cardiotoxicity can be divided into crucial findings strongly predictive of subsequent loss of function and findings not firmly associated with them. Myocardial necrosis and vacuolization in histopathological evaluations should be deemed significant, particularly when observed frequently. Myocardial disorganization and fibrosis, encompassing replacement and interstitial types, should be considered pivotal events closely tied to functional decline, especially when the myocardial cytoskeleton is affected.

To arrive at a comprehensive conclusion, it is essential to include the findings related to histopathological findings and inflammation indices conducted by other members of our team, running in parallel with my own research about oxidative stress biomarkers. The present review summarizes the range of key biochemical indices associated with inflammation and oxidative stress, including cTnT, cTnI, TNFα, IL-6, IL-1β, GSH, GSH-Px, CAT, SOD, and MDA, as well as the histopathological findings used to characterize anthracycline cardiotoxicity in rats. Additionally, it provides an overview of the normal values of these indices as presented in the respective studies.

The graphical representations clearly show a distinct separation between normal values and those suppressed due to anthracycline administration for the aforementioned indices. This

finding is crucial and promising, as it, when combined with echocardiographic indices and histopathological data, can substantially reduce uncertainty and significantly enhance the reliability of weight-of-evidence assessments for potential cardiotoxicity in humans resulting from chemical exposure. Consequently, these results underscore the necessity for more centralized research, ideally coordinated by a regulatory agency, to effectively establish a set of classification criteria for cardiotoxicity.

Currently, cardiotoxicity is not categorized as a separate hazard class of chemical substances in existing international regulations when assessing toxicity. Therefore, effects observed in animal studies are primarily evaluated at the tissue level. Consequently, chemicals other than pharmaceutical agents are classified as cardiotoxic only after significant effects are observed in humans, typically based on epidemiological studies.In a previous review of our research team [105], the cardiac pathology and function impairment due to exposure to pesticides revealed that several cardiovascular complications have been reported in animal models including electrocardiogram abnormalities, myocardial infarction, impaired systolic and diastolic performance and histopathological findings, such as haemorrhage, vacuolization, signs of apoptosis and degeneration [32] Furthermore, there is substantial evidence linking short- and long-term exposure to anabolic androgenic steroids with a range of cardiovascular complications. These complications can be identified through the use of echocardiography or biochemical markers. [7,10,105]

All these published data clearly indicate the necessity of establishing regulatory criteria for evaluating cardiotoxicity as an intrinsic property of chemical substances well in advance. It is crucial to characterize the risk of exposure to such chemicals through a well-developed regulatory network based on animal models, as it is the case for other human health hazard classes, such as carcinogenicity. Establishing regulatory criteria will enable international organizations to identify cardiotoxic effects early and classify chemicals to prevent long-term cardiovascular complications. Specific classification criteria should be developed based on anatomical, histopathological, echocardiographic, and biochemical parameters in animals, designed to exclude confounding factors in the development of observed cardiotoxicity. The results of the present study show promise in identifying echocardiographic criteria in rats for establishing cardiotoxicity. Further studies and meta-analyses are necessary to assess other species commonly used in research and explore the possibility of early recognition of cardiotoxicity, potentially through monitoring biochemical markers based on an understanding of the mode of action.

The diagnostic methods discussed in this manuscript have been widely utilized for several years. However, it is important to note that in recent years, novel biomarkers of target organ toxicity have gained significant applicability. Specifically, tumor suppressive and oncogenic pathways have been found to involve microRNAs (miRNAs)[106]—noncoding RNAs that

repress the expression of target mRNAs in a post-transcriptional manner, affecting processes such as apoptosis, differentiation, and cancer.[107] The measurement of plasma miRNAs and messenger RNAs (mRNAs) helps elucidate the ongoing physiological processes in cells and tissues that package and release miRNAs into cell-free space. Moreover, miRNAs offer a non- or minimally-invasive approach, enhancing animal welfare. From a technological perspective, they are considered ideal for quantitative analysis due to their standardization, rapidity, and robustness.[108]

The markers mentioned undergo significant changes early on after their release from tissues into the plasma during toxic events, exhibiting tissue-specific expression.[109] These advantages have sparked increased research interest in circulating miRNAs as promising biomarker candidates. They have the potential to play an essential role in human health risk assessment. Another crucial aspect is the tissue-specificity and early release of circulating miRNAs following tissue injury, at a stage when damage may still be reversible.

Another significant novel biomarker is the proto-typic oncogene c-MYC. There is potential for miRNAs linked to c-MYC to be utilized in human health risk assessment. Additionally, Fatty Acid-Binding Protein (FABP) acts as a carrier for long-chain fatty acids in the blood and plays a crucial role in lipid metabolism. The heart type isoenzyme of FABP is found in the heart and skeletal muscles, and the clinical utility of free FABP is comparable to that of myoglobin.[110] It is noteworthy to emphasize that in a cohort of 19 cancer patients who received immune checkpoint inhibitors therapy (ICIs), FABP levels were elevated without a significant reduction in left ventricular ejection fraction (LVEF). This observation suggests that there might be a more sensitive biomarker capable of detecting subclinical myocardial damage related to ICIs, compared to traditional cardiac biomarkers.[111]

Acknowledging the existence of the aforementioned biomarkers, it is important to note that our current research project aims to facilitate the use of biochemical markers in the hazard assessment of cardiotoxicity. Therefore, we chose to limit the review focus to more frequently used and traditional biomarkers that are already abundant in the literature and for which available data are more potent. Additionally, the experience gained from risk assessment for newly emerged hazard classes, such as endocrine disruptors, has shown that assessors, especially for regulatory purposes, often rely on older data for animal welfare reasons. However, it is crucial to emphasize that in recent years, these newly identified biomarkers have been extensively implicated in research across different pathways. Therefore, it should be further investigated in a separate project how they could be applied to the hazard assessment of cardiotoxic chemicals.

Advancements in LV function quantification methods have expanded beyond two-dimensional echocardiography (2DE) to include techniques like 3-dimensional echocardiography (3DE), cardiovascular magnetic resonance (CMR), and strain speckle-

tracking echocardiography. However, these methods require sophisticated technology and advanced medical and technical training compared to 2DE. Historically, multiple-gated acquisition scan (MUGA) has been a preferred method for serial assessment of LVEF during cancer therapy. LVEF determined by MUGA scan is more accurate and correlates better with other 3D imaging modalities like CMR than echocardiography. Nevertheless, the main limitation of MUGA scan is the exposure to radiation, which accumulates with serial scans during chemotherapy.[112] Regarding monitored parameters of cardiac function, global longitudinal strain (GLS) has emerged as a significant topic with a role in predicting cardiovascular outcomes compared to LVEF. Abnormal GLS indicates subclinical left ventricular systolic dysfunction, which could be highly valuable for early recognition of cardiotoxicity and setting regulatory criteria. GLS imaging remains underutilized in detecting subclinical cardiac dysfunction in breast cancer patients undergoing chemotherapy.[113]

In conclusion, there is a pressing need for more focused research efforts from both scientists and regulators to further enhance the weight-of-evidence exercise and describe the hazard of cardiotoxicity caused by chemicals as a regulatorily recognized toxicological class relevant to humans. The updated roadmap[114] proposed in this manuscript for identifying regulatory criteria from animal studies includes the following steps:

1. Meta-analysis of each line of scientific evidence (e.g., histopathological, biochemical, echocardiographic indices etc.) recognized by animal species after exposure to well-established cardiotoxicants to humans (e.g., anthracyclines) in order to identify threshold values or range of normal and/ or altered values due to exposure;
2. Validation of the above described evidence in animals exposed to other alleged cardiotoxic substances (e.g., AAS and pesticides);
3. Establishment of mechanisms of action based on information either of known or alleged cardiotoxicants and association thereof with the parameters introduced as scientific evidence in the development of classification criteria;
4. Introduction of novel indices and in silico methods.

CHAPTER 7

7.1. References

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