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In depth review analysis aiming to establish scientific regulatory criteria for the classification of chemical substances as cardiotoxicants according to relevant EU legislation

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In depth review analysis aiming to establish scientific regulatory criteria for the classification of chemical substances as cardiotoxicants according to relevant EU legislation

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Establishment of scientific regulatory criteria for the classification of chemical substances as cardiotoxicants

To my wife, Maria

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Abstract

Cardiovascular diseases are among the most significant causes of mortality in humans. Chemical substances toxicity and risk for human health are regulated at a European level through a well-developed regulatory network, but cardiotoxicity is not described as a separate hazard class. Currently, when assessing chemicals toxicity, cardiac effects if monitored and detected in animal studies, mainly on the tissue level, are considered by the authorities, but cardiotoxicity, as such, is not described as a separate hazard class of chemical substances through the available regulations, both at a European level and world-wide. Therefore, chemicals other than pharmaceutical agents are recognised to be cardiotoxic after having exerted such deleterious effects on humans, based on epidemiological studies.

We investigated the published literature in order to conduct an in-depth review of the cardiac pathology and function impairment due to exposure to different group of chemicals, such as pesticides and anthracyclines based on both animal and human data. Then we evaluated two important echocardiographic indices, namely ejection fraction and fractional shortening, in the literature concerning anthracycline administration to rats as the reference laboratory animal model. Finally, we performed an in-depth review analysis of several biomarkers reported to be altered in animal models after anthracyclines administration in order to investigate which of them could potentially be used as biochemical criteria in a weight of evidence approach in conjunction to the echocardiography indices and the histopathology findings.

The majority of human data on cardiotoxicity of pesticides (organophosphates, organothiophosphates, organochlorines, carbamates, pyrethroids, dipyridyl herbicides, triazoles and triazines), comes from poisoning cases and epidemiological data. Several cardiovascular complications have been reported in animal models including electrocardiogram abnormalities, myocardial infarction, impaired systolic and diastolic performance, functional remodelling and histopathological findings, such as haemorrhage, vacuolisation, signs of apoptosis and degeneration. Our research summarises for the first time the various side-effects on the cardiovascular system reported either in animal models (in vivo and ex vivo experiments) or in humans (epidemiological studies, case reports) after exposure to pesticides. In addition, the underlying mechanisms of the adverse outcomes are investigated in correlation with the mode of action of the various pesticides was reviewed. More than 40% of the

studies reviewed reporting cardiotoxicity deal with pesticides acting through inhibition of carboxyl ester hydrolases, particularly acetylcholinesterase (AChE). The most prominent side effect reported in this mode of action is oxidative stress induced in the myocardial tissue (ca 30%), which is also common in all mode of actions reviewed (ca24%). One third of the effects noted due to exposure to pesticides that alter the function of voltage-gated sodium channels are electrical disorders, which account for 14% of the total number of disorders discussed. Myocardial dysfunction accounts for ca 15% of the disorders observed and coronary artery disease for almost 8% of the disorders, with a universal distribution in all modes of actions.

Anthracyclines are used in cancer chemotherapy (e.g., leukaemia, lymphomas, stomach, uterine, ovarian, bladder and lung cancers) and they are isolated from *Streptomyces* bacterium. Clinically, the most important anthracyclines are doxorubicin, daunorubicin, epirubicin and idarubicin. Anthracyclines, are considered as well-established cardiotoxic compounds causing myocardial suppression. Cardiotoxicity in terms of impairment of cardiac function is largely diagnosed by echocardiography and based on objective metrics of cardiac function. In our research, we focused on the evaluation of two important echocardiographic indices, namely ejection fraction (EF) and fractional shortening (FS), in the literature concerning anthracycline administration to rats as the reference laboratory animal model. The normal and suppressed values of the two main echocardiographic indices discussed, %EF and %FS, respectively, have been identified. Reported baseline (normal) %EF values in rats vary (55%-96.5%). In 78.2% of the studies reviewed, normal values range from 70 to 90%. High %EF values (>90%) are reported in 14% of the studies. In contrast, normal %FS values present even higher variability (25%-84.2%). The majority (66.7%) of the values, though, are reported to be within the range of 40 and 60%. The suppressed %EF values reported from rats after anthracycline administration vary from 31% to 91%. EF values 50-80% are reported in 72.3% of the studies reviewed. Suppression of the %EF due to anthracycline administration varies from 10 to 40% compared to the normal values in more than two thirds of the studies reviewed (71.7%). On the other hand, suppressed %FS values ranging from 14% to 71.8%, present a more narrow distribution (%FS values 20-50% in 84.6% of the studies).

We performed an in-depth review analysis of several biomarkers reported altered in animal models after anthracyclines administration in order to investigate which of them could potentially be used as biochemical criteria in a weight of evidence

approach. The statistical analysis of the cardiac enzymes mainly, but of the biomarkers of oxidative stress, reveal a similar pattern from healthy rats to rats with cardiotoxic manifestations due to anthracycline exposure known to be relevant to humans.

All these published data suggest clearly that there is a need to establish regulatory criteria for assessing cardiotoxicity as an inherent property of a chemical substance well in advance and 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. Regulatory established criteria will enable international organizations to early identify cardiotoxic effects and classify chemicals in order to avoid long-term cardiovascular complications.

Classification should be based on:

a. Anatomical and histopathological criteria,

b. Echocardiographic criteria (e.g. LVEF, LVFS), and/or other cardiac imaging modalities (e.g. MRI) and

c. Biochemical criteria, of generic nature (e.g. circulating oxidative stress mark-ers), of more specific nature (e.g. oxidative stress markers of the cardiac tissue) and heart specific biomarkers (e.g. cardiac enzymes).

Περίληψη

Οι καρδιαγγειακές παθήσεις είναι από τις πιο σημαντικές αιτίες θνησιμότητας. Η τοξικότητα των χημικών ουσιών και ο κίνδυνος για την ανθρώπινη υγεία ελέγχονται σε ευρωπαϊκό επίπεδο μέσω ενός καλά ανεπτυγμένου νομοθετικού δικτύου, αλλά η καρδιοτοξικότητα δεν περιγράφεται ως ξεχωριστή κατηγορία κινδύνου. Επί του παρόντος, κατά την αξιολόγηση της τοξικότητας των χημικών, οι καρδιακές επιδράσεις (εάν παρακολουθούνται και ανιχνεύονται σε μελέτες σε ζώα, κυρίως σε επίπεδο ιστών), λαμβάνονται υπόψη από τις αρχές, αλλά η καρδιοτοξικότητα, αυτή καθαυτή, δεν περιγράφεται ως χωριστή κατηγορία κινδύνου χημικών ουσιών μέσω των διαθέσιμων κανονισμών, τόσο σε ευρωπαϊκό όσο και σε παγκόσμιο επίπεδο. Ως εκ τούτου, οι χημικές ουσίες, εκτός των φαρμακευτικών ουσιών, αναγνωρίζονται ως καρδιοτοξικές με βάση επιδημιολογικές μελέτες.

Πραγματοποιήσαμε μια εις βάθος έρευνα της δημοσιευμένης βιβλιογραφίας και πιο συγκεκριμένα προβήκαμε στην ανασκόπηση της καρδιακής παθολογίας και της υπολειτουργικότητας λόγω έκθεσης σε διαφορετικές ομάδα χημικών ουσιών, όπως τα φυτοφάρμακα και οι ανθρακυκλίνες, βασιζόμενοι σε δεδομένα τόσο ζώων όσο και ανθρώπων. Στη συνέχεια, αξιολογήσαμε δύο σημαντικούς ηχοκαρδιογραφικούς δείκτες, πιο συγκεκριμένα το κλάσμα εξώθησης και το κλάσμα βράχυνσης της αριστερής κοιλίας, σχετικά με τη χορήγηση ανθρακυκλίνης σε αρουραίους ως μοντέλο αναφοράς. Τέλος, πραγματοποιήσαμε μια εις βάθος ανάλυση διαφόρων βιοδεικτών που αναφέρθηκαν διαφοροποιημένοι σε αρουραίους μετά τη χορήγηση ανθρακυκλινών, προκειμένου να διερευνήσουμε ποιος από αυτούς θα μπορούσε ενδεχομένως να χρησιμοποιηθεί με σκοπό τη θέσπιση βιοχημικών κριτηρίων που σε συνδυασμό με τους δείκτες ηχοκαρδιογραφίας και τα ιστοπαθολογικά ευρήματα θα μπορέσουν να χρησιμοποιηθούν σε μια προσέγγιση ανάλυσης δεδομένων με βάση την εγκυρότητά και σχετικότητά τους.

Για τα φυτοφάρμακα (οργανοφωσφορικά, οργανοθειοφωσφορικά, οργανοχλωρικά, καρβαμιδικά, πυρεθροειδή, διπυριδυλοζιζανιοκτόνα, τριαζόλες και τριαζίνες), η πλειονότητα των δεδομένων για καρδιοτοξικότητα προέρχεται από περιπτώσεις δηλητηρίασης και επιδημιολογικά δεδομένα. Αρκετές καρδιαγγειακές επιπλοκές έχουν αναφερθεί σε ζώα, όπως ανωμαλίες στο ηλεκτροκαρδιογράφημα, έμφραγμα του μυοκαρδίου, μειωμένη συστολική και διαστολική απόδοση, λειτουργική αναδιαμόρφωση και ιστοπαθολογικά ευρήματα, όπως αιμορραγία,

κενοτοπίωση, σημεία απόπτωσης και εκφυλισμού. Η έρευνά μας συνοψίζει για πρώτη φορά τις διάφορες παρενέργειες στο καρδιαγγειακό σύστημα που αναφέρθηκαν είτε σε ζώα (*in vivo* και *ex vivo*) είτε σε ανθρώπους (επιδημιολογικές μελέτες, αναφορές περιστατικών) μετά από έκθεση σε φυτοφάρμακα. Επιπλέον, διερευνήθηκαν οι υποκείμενοι μηχανισμοί των δυσμενών επιπτώσεων σε συσχέτιση με το μηχανισμό δράσης των διαφόρων φυτοφαρμάκων. Περισσότερο από το 40% των μελετών που εξετάστηκαν αναφέρουν ότι η καρδιοτοξικότητα αφορά φυτοφάρμακα που δρουν μέσω της αναστολής των υδρολασών των καρβοξυλικών εστέρων, ιδιαίτερα της ακετυλοχολινεστεράσης (AChE). Η πιο εμφανής ανεπιθύμητη ενέργεια που αναφέρεται σε αυτόν τον μηχανισμό δράσης είναι το οξειδωτικό στρες που προκαλείται στον ιστό του μυοκαρδίου (περίπου 30%), το οποίο είναι επίσης κοινό σε όλους τους μηχανισμούς δράσης που εξετάστηκαν (περίπου 24%). Το ένα τρίτο των επιπτώσεων που σημειώνονται λόγω της έκθεσης σε φυτοφάρμακα είναι ηλεκτρικές διαταραχές που αλλάζουν τη λειτουργία των διαύλων νατρίου, οι οποίες αντιπροσωπεύουν το 14% του συνολικού αριθμού των διαταραχών που συζητήθηκαν. Η δυσλειτουργία του μυοκαρδίου αντιπροσωπεύει περίπου το 15% των διαταραχών που παρατηρούνται και η στεφανιαία νόσος για σχεδόν το 8% των διαταραχών.

Οι ανθρακυκλίνες χρησιμοποιούνται στη χημειοθεραπεία του καρκίνου (π.χ. λευχαιμίες, λυμφώματα, καρκίνοι στομάχου, ωοθηκών, ουροδόχου κύστης και πνευμόνων) και απομονώνονται από το μύκητα *Streptomyces*. Κλινικά, οι πιο σημαντικές ανθρακυκλίνες είναι η δοξορουβικίνη, η δαουνορουβικίνη, η επιρουβικίνη και η ιδαρουβικίνη. Οι ανθρακυκλίνες αποτελούν αποδεδειγμένα καρδιοτοξικές ενώσεις. Η καρδιοτοξικότητα όσον αφορά τη μείωση της καρδιακής λειτουργίας διαγιγνώσκεται σε μεγάλο βαθμό με ηχοκαρδιογραφία και βασίζεται σε αντικειμενικές μετρήσεις της καρδιακής λειτουργίας. Στην έρευνά μας, εστιάσαμε στην αξιολόγηση δύο σημαντικών ηχοκαρδιογραφικών δεικτών, δηλαδή του κλάσματος εξώθησης και του κλάσματος βράχυνσης της αριστερής κοιλίας, στη βιβλιογραφία σχετικά με τη χορήγηση ανθρακυκλίνης σε αρουραίους ως μοντέλο αναφοράς. Οι φυσιολογικές και οι κατασταλμένες τιμές των δύο κύριων ηχοκαρδιογραφικών δεικτών που συζητήθηκαν, %EF και %FS, αντίστοιχα, έχουν ταυτοποιηθεί. Οι αναφερόμενες τιμές αναφοράς (φυσιολογικές) %EF σε αρουραίους ποικίλλουν (55%-96,5%). Στο 78,2% των μελετών που εξετάστηκαν, οι φυσιολογικές τιμές κυμαίνονται από 70 έως 90%. Υψηλές τιμές %EF (>90%) αναφέρονται στο 14% των μελετών. Αντίθετα, οι κανονικές τιμές %FS παρουσιάζουν ακόμη μεγαλύτερη μεταβλητότητα (25%-84,2%).

Ωστόσο, η πλειονότητα (66,7%) των τιμών αναφέρεται ότι κυμαίνεται μεταξύ 40 και 60%. Οι κατασταλμένες τιμές %EF που αναφέρθηκαν από αρουραίους μετά τη χορήγηση ανθρακυκλινών κυμαίνονται από 31% έως 91%. Οι τιμές EF 50-80% αναφέρονται στο 72,3% των μελετών που εξετάστηκαν. Η καταστολή του %EF λόγω χορήγησης ανθρακυκλίνης κυμαίνεται από 10 έως 40% σε σύγκριση με τις φυσιολογικές τιμές σε περισσότερα από τα δύο τρίτα των μελετών που εξετάστηκαν (71,7%). Από την άλλη πλευρά, οι κατασταλμένες τιμές %FS που κυμαίνονται από 14% έως 71,8%, παρουσιάζουν μια πιο στενή κατανομή (τιμές %FS 20-50% στο 84,6% των μελετών).

Πραγματοποιήσαμε μια εις βάθος ανάλυση διάφορων βιοδεικτών σε ζωα πριν και μετά τη χορήγηση ανθρακυκλινών, προκειμένου να διερευνήσουμε ποιοι από αυτούς θα μπορούσαν ενδεχομένως να χρησιμοποιηθούν ως βιοχημικά κριτήρια σε μια προσέγγιση ανάλυσης δεδομένων με βάση την εγκυρότητά και σχετικότητά τους. Η στατιστική ανάλυση των τιμών των καρδιακών ενζύμων κυρίως, αποκαλύπτει παρόμοιο μοτίβο από υγιείς αρουραίους σε αρουραίους με καρδιοτοξικές επιπτώσεις λόγω έκθεσης σε ανθρακυκλίνες.

Όλα τα δεδομένα υποδηλώνουν σαφώς ότι υπάρχει ανάγκη να θεσπιστούν ρυθμιστικά κριτήρια για την αξιολόγηση της καρδιοτοξικότητας ως εγγενούς ιδιότητας μιας χημικής ουσίας και να χαρακτηριστεί ο κίνδυνος έκθεσης σε τέτοιες χημικές ουσίες μέσω ενός ρυθμιστικού δικτύου βασισμένου σε ζωικά μοντέλα, όπως συμβαίνει αντίστοιχα και με άλλες τάξεις κινδύνου για την ανθρώπινη υγεία. Τα κριτήρια θα επιτρέψουν στους διεθνείς οργανισμούς να εντοπίζουν έγκαιρα τις καρδιοτοξικές επιδράσεις και να ταξινομούν τις χημικές ουσίες προκειμένου να αποφευχθούν μακροχρόνιες καρδιαγγειακές επιπλοκές. Η ταξινόμηση πρέπει να βασίζεται σε:

1. Ανατομικά και ιστοπαθολογικά κριτήρια,
2. Ηχοκαρδιογραφικά κριτήρια (π.χ. LVEF, LVFS) και/ή άλλες τεχνικές απεικόνισης της καρδιάς (π.χ. μαγνητική τομογραφία) και
3. Βιοχημικά κριτήρια, γενικής φύσεως (π.χ. δείκτες οξειδωτικού στρες του κυκλοφορικού συστήματος), πιο ειδικής φύσεως (π.χ. δείκτες οξειδωτικού στρες του καρδιακού ιστού) και ειδικοί βιοδείκτες για την καρδιά (π.χ. καρδιακά ένζυμα).

CHAPTER 1

General Introduction

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

Chemicals, through the different steps from their production to their handling, transport and use, are a real danger for human health and the environment. People of any ages, from children to elderly, using many different languages and alphabets, belonging to various social conditions, including illiterates, are daily confronted to dangerous products (chemicals, pesticides, etc.).

To face this danger, and given the reality of the extensive global trade in chemicals and the need to develop national programs to ensure their safe use, transport and disposal, it was recognized that an internationally-harmonized approach to classification and labelling would provide the foundation for such programs. Once countries have consistent and appropriate information on the chemicals they import or produce in their own countries, the infrastructure to control chemical exposures and protect people and the environment can be established in a comprehensive manner.

The new system, which was called "Globally Harmonized System of Classification and Labelling of Chemicals (GHS)", addresses classification of chemicals by types of hazard and proposes harmonized hazard communication elements, including labels and safety data sheets. It aims at ensuring that information on physical hazards and toxicity from chemicals be available in order to enhance the protection of human health and the environment during the handling, transport and use of these chemicals. The GHS also provides a basis for harmonization of rules and regulations on chemicals at national, regional and worldwide level, an important factor also for trade facilitation.

While governments, regional institutions and international organizations are the primary audiences for the GHS, it also contains sufficient context and guidance for those in industry who will ultimately be implementing the requirements which have been adopted (UNECE, 2021)(GHS, 2017).

Table 1. Human health hazard classes according to GHS

Hazard Class	Associated Hazard Category
Acute toxicity	Categories 1-4 (with 1 being the most dangerous)
Skin corrosion	Categories 1A, 1B, 1C, and 2
Skin 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, 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

1.2. Classification, Labelling and Packaging (CLP) Regulation

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.

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), and since 1 June 2015, is the only legislation in force in the EU for classification and labelling of substances and mixtures. CLP is legally binding across the Member States and directly applicable to all industrial sectors. It requires manufacturers, importers or downstream users of substances or mixtures to classify, label and package their hazardous chemicals appropriately before placing them on the market. One of the main aims of CLP is to determine whether a substance or mixture displays properties that lead to a hazardous classification. In this context, classification is the starting point for hazard communication. When relevant information (e.g. toxicological data) on a substance or mixture meets the classification criteria in CLP, the hazards of a substance or mixture are identified by assigning a certain hazard class and category. The hazard classes in CLP cover physical, health, environmental and additional hazards. Once a substance or mixture is classified, the identified hazards must be communicated to other 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.

CLP sets detailed criteria for the labelling elements: pictograms, signal words and standard statements for hazard, prevention, response, storage and disposal, for every hazard class and category. It also sets general packaging standards to ensure the safe supply of hazardous substances and mixtures. In addition to the communication of hazards through labelling requirements, CLP is also the basis for many legislative provisions on the risk management of chemicals (ECHA, 2021).

Table 2. Human health hazards according to CLP Regulation (ECHA, Introductory Guidance on the CLP Regulation, v.3, Jan. 2019)

Hazard Class	Associated Hazard Category
Acute toxicity	Categories 1-4 (with 1 being the most dangerous)
Skin corrosion/ irritation	Categories 1, 1A, 1B, 1C, and 2
Serious eye damage/ eye irritation	Categories 1 and 2
Respiratory or Sensitization (Skin or Eye)	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, 2, and additional category for effects on or via lactation
Specific target organ toxicity: single exposure	Categories 1 and 2 and category 3 for narcotic effects and respiratory tractirritaion, only
Specific target organ toxicity: repeated exposure	Categories 1 and 2
Aspiration hazard	Category 1

1.3. Occupational Safety and Health Administration (OSHA) of the U.S. Department of Labor

In March 2012, the Occupational Safety and Health Administration (OSHA) of the U.S. Department of Labor, revised its Hazard Communication Standard to align it with the United Nations GHS, Revision 3. The revision to the Hazard Communication Standard (HCS) built on the existing standard, by requiring chemical manufacturers and importers to follow specific criteria when evaluating the hazardous chemicals and when communicating the hazards through labels and safety data sheets (SDSs).

OSHA's Hazard Communication Standard is designed to protect against chemical-source injuries and illnesses by ensuring that employers and workers are provided with sufficient information to anticipate, recognize, evaluate, and control chemical hazards and take appropriate protective measures. This information is provided through SDSs, labels, and employee training. In order for SDSs, labels, and training to be effective, the hazard information they convey must be complete and accurate. Thus, it is critically important to obtain comprehensive and correct information about the hazards associated with particular chemicals (OSHA, 2016).

1.4. Classification of Hazardous Chemicals under the Work Health and Safety (WHS) Act, Australia Regulations

With regard to Work Health and Safety (WHS) Act and after 31 December 2016 all workplace chemicals must be classified according to the GHS and labels and SDS must be in accordance with the GHS as implemented under the WHS Regulations. This timeline is illustrated in the following diagram, including the relevant documents to use for classification, labelling and SDS (WHS, 2012).

1.5. Aim of the thesis and methods

When relevant toxicological data on a substance or mixture meets the classification criteria, the hazards of a substance or mixture are identified by assigning a certain hazard class and category. The hazard classes cover physical, environmental and human health hazards

As it is observed from the examples of different international regulations above, cardiotoxicity is not described as a separate hazard class and no definite criteria are set in order to classify a chemical as cardiotoxic. For example, in the CLP hazard class of STOT, on the contrary, criteria have been developed for toxic damage to the liver, the kidneys, the hematopoietic system, the various glands, like the thyroid gland, etc. Cardiotoxocity has been mainly linked to side effects of pharmaceuticals and it could

be diagnosed many years post-exposure at the time of clinical manifestations (Berardi et al., 2013; Germanakis et al., 2013; Madeddu et al., 2016; Vasilaki et al., 2016; Baggish et al., 2017). As a result, allegedly cardiotoxic substances or products face no market restrictions at a regulatory level. In general, cardiotoxicity testing is an unmet need in the current screening programs of environmental chemicals (Sirenko et al., 2017).

It is acknowledged that chemicals other than pharmaceutical agents are recognised to be cardiotoxic after having exerted such deleterious effects on humans, based on epidemiological studies. In a previous review of our research team, 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. In addition, there is evidence that short and/ or long-term exposure to anabolic androgenic steroids is linked to a variety of cardiovascular complications which could be identified by using echocardiography or biochemical markers. All these published data suggest clearly that there is a need to establish regulatory criteria for assessing cardiotoxicity as an inherent property of a chemical substance well in advance, and 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. Regulatory established criteria will enable international organizations to early identify cardiotoxic effects and classify chemicals in order to avoid long-term cardiovascular complications.

Classification should be based on:

a. Anatomical and histopathological criteria,

b. Echocardiographic criteria (e.g. LVEF, LVFS), and/or other cardiac imaging modalities (e.g. MRI) and

c. Biochemical criteria, of generic nature (e.g. circulating oxidative stress mark-ers), of more specific nature (e.g. oxidative stress markers of the cardiac tissue) and heart specific biomarkers (e.g. cardiac enzymes).

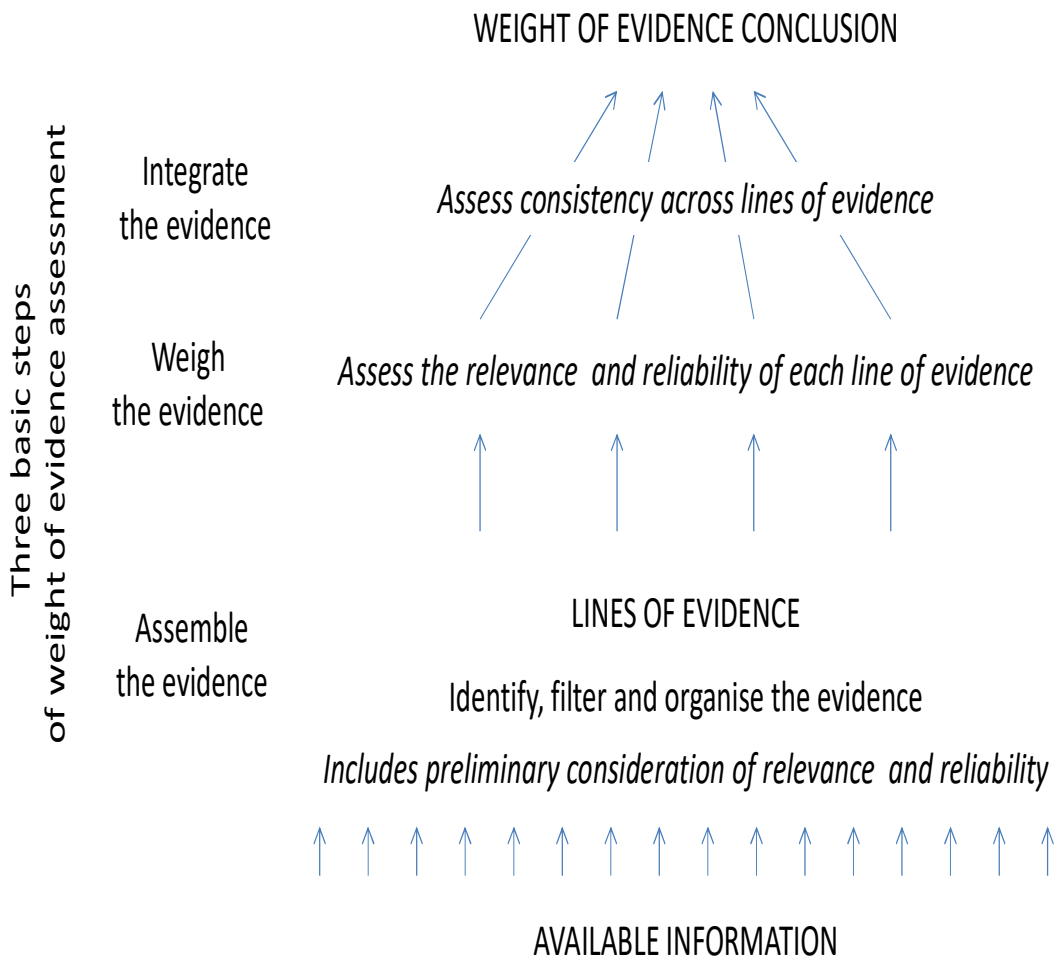

Figure 1. Diagrammatic illustration of weight of evidence assessment as a 3-step process which may occur at one or more points in the course of a scientific assessment (EFSA, 2017)

Based on animal data from anthracyclines, which are well-established cardiotoxic pharmaceuticals causing myocardial suppression to patients treated for cancer, a set of anatomical and histopathological, echocardiographic and biochemical criteria will be developed with applicability to chemicals in general and relevance to humans. Hence, concrete criteria on the cardiotoxicity assessment of substances in order to enable risk assessors to assess the cardiotoxicity endpoint and further classify it, will be established.

1.5.1. **Echocardiography indices**

The identification of most commonly used metrics of myocardial function in animal studies of anthracycline induced cardiotoxicity are presented, along with the range of these values differentiating normal cardiac function from animals with

pathological echocardiographic findings indicative of anthracycline cardiotoxicity as per author presentation.

PubMed electronic database was systematically searched to detect all original research studies published until March 1st 2020, according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement (11). The specific literature search strategy used was: (AND ("*rats*" OR "*doxorubicin* OR "*echocardiography*" OR "anthracycline" OR "*ejection fraction*")) either in the Title, or the Abstracts. The reference list of the retrieved studies was further evaluated for the relevance of the subject and the eligibility by screening the titles/abstracts of full papers. Animal data only from rat species were assessed, as it is evident from the search string. In total, 86 published manuscripts on animal studies were considered for the systematic review.

The first index is LV fractional shortening (FS) and is calculated by the formula FS(%) = (LV end-diastolic diameter [LVD_d] minus LV end-systolic diameter [LVD_s])/LVD_d × 100.

Left ventricle (LV) Ejection fraction (EF) is the second and more common, index of left ventricular contractility. EF can be calculated from the equation $EF(\%)=[(LVD_d^3 - LVD_s^{3)}/ LVD_d^3]x100$ (13) or from the equation EF (%) = (LVEDV-LVESV)/LVEDV x100, where LVEDV is the left ventricular end-diastolic volume and LVESV is left ventricular end-systolic volume.

1.5.2. **Biochemical Indices**

To monitor cardiotoxicity caused by anthracyclines, cardiac imaging is primarily used and secondarily, biochemical markers.

An in-depth review analysis of several biomarkers reported altered in animal models after anthracyclines administration is being performed by our research group, in order to investigate which of them could potentially be used as biochemical criteria in a weight of evidence approach together with other lines of evidence, namely the echocardiography indices discussed above. The indices for which values are being retrieved from the literature, in the framework of this project, are listed below:

Biomarkers of Oxidative stress

- Catalase (CAT)
- Malondialdehyde (MDA)

- Reactive oxygen species (ROS)
- Superoxide dismutase (SOD)
- Total antioxidant capacity (TAC)
- Total Oxidant Status (TOS)
- Glutathione (GSH)
- Glutathione peroxidase (GSH-Px)
- Lipid hydroperoxide (LH)

Biomarkers relevant to damage of the heart muscle

- Lactate dehydrogenase (LDH)
- Creatine kinase (CK)
- Creatine kinase-myocardial band isoenzyme (CK-MB)
- Cardiac troponin I (cTnI)
- Cardiac troponin T (cTnT)

Biomarkers relevant to increased ventricular blood volume and consequent response of cardiomyocytes to stretching

- Atrial natriuretic peptide (ANP)
- Brain natriuretic peptide (BNP)

Biomarkers of inflammation

- Interleukin-1 family members (IL-1)
- TNF alpha

1.5.3. **Histopathological criteria**

The last but not least criterion, which needs to be investigated and reviewed in the context of a weight of evidence approach are the findings from histopathological analysis of the heart tissue from animals exposed to well-established cardiotoxic chemicals. Histopathological data, when assessed properly can provide reliable information. More specifically, significant functional changes in the heart muscle noted at necropsy and/or at microscopic examination, but also morphological, reversible or not, changes which pro-vide evidence of marked heart dysfunction and cell death incapable of regeneration could be of relevance. Preliminary data in the literature are encouraging. For example, in rabbits exposed to anabolic steroids local fibrosis and a

mild chronic inflammation of cardiac tissue was observed [Germanakis et al., 2013; Madeddu et al., 2016; Vasilaki et al., 2016], while in rabbits exposed to the pesticides propoxur and diazinon the main histopathologic findings were fibrosis, hemorrhagic infiltration of myo-cardial tissues and degeneration of muscle cells, with no signs of inflammation. What is rather interesting in this case, is the significant persistence of various amounts of both pesticides studied in cardiac tissues, suggesting that the cardiac muscle cells were directly exposed to both pesticides [26]. Finally, clinical observations or small changes in heart weight with no evidence of organ dysfunction could also provide useful information.

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CHAPTER 2

Pesticides and cardiotoxicity. Where do we stand?

The work presented in Chapter 2 is included in the following article:

Georgiadis N, Tsarouhas K, Tsitsimpikou C, Vardavas A, Rezaeee R, Germanakis I, Tsatsakis A, Stagos S, Kouretas D, 2018. Pesticides and cardiotoxicity. Where do we stand? Toxicology and Applied Pharmacology; 353, 1–14. https://doi.org/10.1016/j.taap.2018.06.004

2.1. Introduction

The term “pesticides” is commonly used as a synonym for plant protection products. Pesticides are mainly used to keep crops healthy and protect them from diseases and infestation. However, pesticides could also have broader applications to cover also products like biocides, which are intended for non-plant uses to control pests and disease vectors, such as insects, rats and mice. (Food and Agriculture Organization of the United Nations, 2002)*”.*

Plant protection products contain at least one active substance, which could be either a chemical or micro-organisms (e.g. viruses). When grouped into chemical families, the dominant pesticides groups include organochlorines, organophosphates and carbamates. According to the Stockholm Convention on Persistent Organic Pollutants, nine out of the twelve most dangerous and persistent organic chemicals are organochlorine pesticides (United Nations Environment Programme, 2005; Gilden *et al.*, 2010). Organochlorine pesticides are chlorinated hydrocarbons. Representative and notorious compounds in this group include dichlorodiphenyltrichloroethane (DDT), methoxychlor, dieldrin, chlordane, toxaphene, mirex, chlordecone (Kepone), and gamma hexachlorocyclohexane (lindane) (Centers for Disease Control and Prevention, 2015). Nowadays, organophosphates and carbamates have replaced organochlorines world-wide. The chemical structures of the main classes of pesticides reviewed hereafter are presented in Figure 1.

Regulation (EC) No 1107/2009 and Regulation (EU) No 528/2012 lay down rules and procedures for approval of the usage of active substances in plant protection and biocidal products at European Union (EU) level and for the authorisation of plant protection and biocidal products in the European market.

Authorisation of plant protection and biocidal products is based on the risk assessment thereof for human health and the environment, based on the identified hazards and classification of their active substances according to the Classification, Labelling and Packaging (CLP) Regulation (EC) No 1272/2008). The CLP Regulation is based on the United Nations' Globally Harmonised System (GHS) and is the only legislation in force in the EU for classification and labelling of substances and mixtures. CLP requires manufacturers, importers or downstream users of substances or mixtures to classify, label and package their hazardous chemicals before placing them on the market. One of the main aims of CLP is to determine whether a substance or mixture displays hazardous properties that lead to a classification.

When relevant toxicological data on a substance or mixture meets the classification criteria in CLP, the hazards of a substance or mixture are identified by assigning a certain hazard class and category. The hazard classes in CLP cover physical, health, environmental and human health hazards. More specifically for human health hazards the classifications are listed below:

- Acute toxicity (oral, dermal, inhalation)
- Skin corrosion / skin irritation
- Serious eye damage / eye irritation
- Respiratory sensitisation
- Skin sensitisation
- Mutagenicity
- Carcinogenicity
- Toxicity for reproduction
- Specific target organ toxicity STOT (single exposure, SE)
- Specific target organ toxicity STOT (repeated exposure, RE)

- Aspiration hazard

In CLP Regulation, cardiotoxicity is not described as a separate hazard class and no definite criteria are set in order to classify a chemical as cardiotoxic. In the CLP hazard class of STOT, on the contrary, criteria have been developed for toxic damage to the liver, the kidneys, the hematopoietic system, the various glands, like the thyroid gland, etc. Cardiotoxocity has been mainly linked to side effects of pharmaceuticals and it could be diagnosed many years post-exposure at the time of clinical manifestations (Berardi *et al.*, 2013; Germanakis *et al.*, 2013; Madeddu *et al.*, 2016; Vasilaki *et al.*, 2016; Baggish *et al.*, 2017). As a result, allegedly cardiotoxic substances or products face no market restrictions at a regulatory level. In general, cardiotoxicity testing is an unmet need in the current screening programs of environmental chemicals (Sirenko et al., 2017)

There is a long-lasting discussion in the literature linking pesticides with several human pathologies, such as endocrine disruption, diabetes mellitus, Parkinson (Yan *et al.*, 2018; Paul *et al.*, 2018; Mesnage *et al.*, 2018; Hennig *et al.*, 2018; Adeyinka *et al.*, 2018; Hassani *et al.*, 2015; Clark, 2018). Nevertheless, from a regulatory point of view pesticides can be classified only to the hazard classes of the CLP Regulation, listed above. Since 2012, when the European regulatory framework for pesticides came into force, several hazards for human health and the environment have been officially recognized for 79 pesticide active substances that were evaluated to be placed on the European market. These results are summarised in Table 1 and Figure 1. Acute toxicity either orally or dermally or via inhalation is the most popular hazard identified, while for STOT RE the vast majority has to do with the liver and for STOT SE for respiratory irritation. Active substances identified as carcinogens have to be replaced and withdrawn from the market.

The current review summarises for the first time the various side-effects on the cardiovascular system reported either in animal models (*in vivo* and *ex vivo* experiments) or in humans (epidemiological studies, case reports) after exposure to organophosphates, carbamates, organothiophosphates, pyrethroids, organochlorines, dipyridyl herbicides (paraquat), triazines, triazoles, thiazoles. An effort is being made to classify these side effects into various classes of cardiotoxic disorders, based on the cardiovascular toxicity guidelines developed for cancer treatment (Zamorano *et al*, 2016), which are so far the only relevant guidelines for cardiotoxicity and refer to direct effects of the cancer treatment on heart function and structure, or may be due to accelerated development of cardiovascular disease, especially in the presence of traditional cardiovascular risk factors. In addition, the underlying mechanisms of the adverse outcomes are investigated in correlation with the mode of action of the various pesticides discussed.

2.2. Methods

In this evaluative literature review, the literature was screened up to April 2018 with due emphasis given to the past five years. MEDLINE and Embase databases were searched, using the following key words: pesticides, pesticide toxicity, pesticides exposure, cardiotoxicity, mode of action, cardiac effects, organophosphate & cardiotoxicity, organochlorine & cardiotoxicity, carbamates & cardiotoxicity, pyrethroids & cardiotoxicity, triazole & cardiotoxicity, triazines & cardiotoxicity, either in the title, abstracts, or in the text. The relevance of the subject and eligibility in all the publications detected was further evaluated based on the title and abstract. Human data and animal data from rodents were assessed. Data on zebrafish were excluded.

In general, the cardiovascular complications mainly as a results of cancer therapy can be divided into nine categories: myocardial dysfunction and heart failure (HF); coronary artery disease (CAD); valvular disease; arrhythmias, especially those induced

by QT-prolonging drugs; arterial hypertension; thromboembolic disease; peripheral vascular disease and stroke; pulmonary hypertension and pericardial complications (Zamorano *et al*, 2016). Having those categories in mind and based on the findings of the studies discussed in the present review, the following classes of cardiovascular disorders have been identified and used hereafter: (1) Myocardial dysfunction, (2) Electrical, (3) Biochemical (oxidative damage), (4) Anatomical, (5) Cytopathological, (6) Histopathological, (7) Biochemical (functional implications), (8) Coronary artery disease, (9) Biochemical (lipidemic profile and other), (10) Blood pressure disorders (hypertension, hypotension). The precise timing of the cardiovascular disorders associated with pesticide exposure is an issue to be addressed, while more than one of the classes described above can co-exist in a specific cardiovascular manifestation due to pesticide exposure. Whether a specific class precedes the others in a specific toxicity setting is also an issue for further study. Myocardial function suppression, for example, can be the result of an earlier myocardial insult that leads to cardiac remodeling. Biochemical disorders in the form of oxidative stress leading to membrane lipid peroxidation can provoque subclinical cardiovascular disease and precede myocardial echocardiographic findings. In many cases subcellular insults affect many organs, not only the heart. Heart has a limited regenerative capacity, though. Therefore, when aggregated toxicity exceeds a certain threshold of damage, a process of ventricular remodeling common to multiple forms of cardiac injury is initiated

Scientific uncertainties: The main uncertainty remains the fact that, according to the statistical meta-analysis performed by the authors (goodness of fit), not all classes of disorders/ side effects have been equally explored in each study analyzed in this report nor, in each mode of action of pesticides recognized, the same toxicity evaluation methods were performed.

2.3. Results

The mode of action of the most common classes of pesticides reviewed in the present study is summarised in Table 2. The more interesting and comprehensive published cardiotoxic effects of organophosphates (diazinon, chlorpyrifos, methidathion, malathion, profenofos, monoctotophos, dimethoate, and dichlorvos), organothiophosphates (phorate), carbamates (propoxur), pyrethroids (tefluthrin, fenpropathrin, cypermethrin, tetramethrin, prallethrin and permethrin), organochlorines (endosulfan and lindane), dipyridyl herbicides (paraquat), phosphides (aluminum phosphide), triazines (atrazine) and thiazoles (penconazole, itraconazole) on experimental animals and humans are summarised in Tables 3-7.

More than 40% of the studies reviewed reporting cardiotoxicity deal with pesticides acting through inhibition of carboxyl ester hydrolases, particularly acetylcholinesterase (AChE). The most prominent side effect reported in this mode of action is oxidative stress induced in the myocardial tissue (ca 30%), which is also common in all mode of actions reviewed (ca24%). One third of the effects noted due to exposure to pesticides that alter the function of voltage-gated sodium channels are electrical disorders, which account for 14% of the total number of disorders discussed. Myocardial dysfunction accounts for ca 15% of the disorders observed and coronary artery disease for almost 8% of the disorders, with a universal distribution in all modes of actions. A more detailed discussion follows.

2.3.1. **Inhibition of acetylcholinesterase (AChE) – Organophospathes**

Early studies using *in vivo* experimental models provide evidence for dose-dependent direct cardiotoxic effects of organophosphate pesticides. Even back in 1968,

experiments by Wolthuis et al. (Wolthuis and Meeter, 1968) showed that the organophosphate diisopropyl fluorophosphate (DFP) could induce cardiac failure in rats. At the same time, clinical observations (Kabrawala *et al.*, 1965) and abnormal electrocardiogram (ECG) patterns (Kiss and Fazekas, 1979) supported the early thoughts of organophosphates' cardiotoxicity in humans. Nevertheless, a meta-analysis on myocardial infarction incidences in licensed organophosphates and carbamates applicators, primarily farmers with pesticide licenses, in North Carolina and Iowa from the Agricultural Health Study, reported limited association between the lifetime use of 49 pesticides and fatal and nonfatal myocardial infarction (Mills *et al.*, 2009).

The vast majority of the cardiotoxic effects of organophosphates in animals are reported after subchronic/ chronic exposure (14 days to 12 months) (Table 3). In humans data boh form epidemiology and from acute poisoning point to coronary artery disease as the manin cardiotoxic outcome of organophosphates. It seems that there are complex and multifactorial pathways involved in the development of the above-mentioned cardiac toxicity, which also lead to disturbed cardiac rhythms and arrhythmias in organophosphate poisoning. Following acute exposure to organophosphates, acetylcholinesterase inhibition occurs and parasympathetic over-activity predominates (Roth *et al.*, 1993; Tsatsakis *et al.*, 1996a; Tsatsakis *et al.*, 1996b). It has been reported that selenium or vitamin E supplementation restored acetylcholinesterase and Na1K1-ATPase activity in the heart after exposure to organophosphates (Amara *et al.*, 2013). Long-term cardiac manifestations include QT prolongation, with or without simultaneous troponin increase, and in many cases without obvious anatomical or histopathological abnormalities (Shiyovich *et al.*, 2018; Yavuz *et al.*, 2004; Velmurugan *et al.*, 2013). In humans, though, fatally exposed to organophospahtes and with similar QT prolongation, ST- and T-abnormalities, histopathological evidence of focal necrosis

and regeneration were noted (Kiss and Fazekas, 1979). On the other hand, elevated levels of Creatine Kinase Isoenzyme MB (CK-MB) have been reported (Razavi *et al.*, 2013; Velmurugan *et al.*, 2013). Apoptosis, as depicted by Bax/Bcl2 ratio elevation (at both protein and mRNA levels), cytochrome c cytosolic release and caspase-3 activation in cardiac tissue, is suggested to be involved in the myocardial damage induced by organophosphates (Razzavi *et al.*, 2013). Uncommon arrhythmias may appear late after organophospate poisoning even after apparent clinical recovery and can be attributed to cardiotoxic effects, metabolic (acidosis) and electrolyte derangements or even to recovery and healing mechanisms of the damaged myocardium. Despite the usual lack of severe echocardiographic anomalies in organophosphate poisoning, patchy myocardial involvement has been found in histopathological analysis post mortem, suggesting a possible origin of late electrical anomaly (Anand *et al.*, 2009). It has been reported that following intoxication, the levels of free fatty acids increase and lipid homeostasis is altered (Kiss and Fazekas, 1979; Zaki *et al.*, 2012), which can contribute to the arrhythmogenicity. In addition, exposure to organophosphates, like profenofos, increases cytotoxicity enzymes activity, rendering them a possible cause for dysfunction of organs, such as the liver, kidney, heart and muscles (Zaki *et al.*, 2012). A generally distorted redox status has been reported for experimental animals exposed to organophosphates (Bas and Kalender, 2011; Yavuz *et al.*, 2004; Razavi *et al.*, 2015). It should be noted that the heart is particularly sensitive to peroxidative insults due to its limited antioxidant defenses that can enzymatically counteract hydroxyl radicals (Doroshow *et al.*, 1980). It has also been suggested that the developmental toxicity of several organophosphates, such as chlorpyrifos, extends beyond the nervous system to other organs' cell signalling cascades, which could be proven vital to cardiac and hepatic homeostasis (Meyer *et al.*, 2004). In two recent studies (Zafiropoulos *et al.*, 2014;

Koutroulakis *et al.*, 2014) residues of diazinon and chlorpyrifos were detected in the cardiac tissue and the amniotic fluid. Residues of pesticides in the heart is not expected, since heart is not directly involved in the toxicokinetics of pesticides, as it is the liver and the kidneys; distribution of the chemicals to the organs differs by their log K_{OW} and generally follows the blood flow path after the gastrointestinal tract (Dang *et al.*, 2017).

2.3.2. **Altered function of voltage-gated sodium channels – Pyrethroids**

Pyrethroid insecticides are known to affect the functions of sodium channels, which are present both in neuronal and cardiac cells. Pyrethroids are thought to be able to shift both voltage-dependent activation and inactivation of Na channels to hyperpolarized potentials (Trainer *et al.*, 1997; Spencer *et al.*, 2001). Cardiac myocytes are rich in sodium channels. The type I pyrethroid, tefluthrin, and the type II pyrethroids, fenpropathrin and α-cypermethrin (see Figure 1), modify the time course of sodium channel current {I(Na)} by altering the relative proportions of fast and slowly inactivating current and alter the voltage dependence of I(Na), prolong the ventricular action potentials and evoke after-depolarizations, indicating an arrythmogenic activity (Spencer *et al.*, 2001). Similarly, in a 28-year-old female having accidentally consumed prallethrin, metabolic acidosis and sinus arrest with escape junctional rhythm were developed, which persisted for 3 days, despite the correction of metabolic acidosis. Consequently, sinus rhythm with bradycardia was established, possibly due to pyrethroid effect on myocardial cells sodium channels. The human poisoning cases due to pyrethroids are limited, probably due to the high excretion rate of pyrethroids (Scheme and Team, 2005). Nevertheless, epidemiology could support a possible positive association between pyrethroids exposure and the risk of coronary heart disease (Han *et al.*, 2017). In the same context of ion signalling, cytosol calcium levels are important for

the cardiac muscle contractile state. A transient increase in cytosolic calcium is required for each cardiac cycle. It was found that early-life exposure to low doses of permethrin led to rats' cardiac muscle hypotrophy along with increased heart cells' calcium (de la Cerda *et al.*, 2002; Vadhana *et al.*, 2013). Nuclear factor E2-related factor 2 (Nrf2) is a transcription factor found in the cardiovascular system that controls the expression of a number of antioxidant genes and other cytoprotective phase-II detoxifying enzymes (Li *et al.*, 2009). Increased *Nrf2* gene expression levels were observed following pyrethroid exposure, especially in older animals (Vadhana *et al.*, 2013). Similar to organophosphates, ROS generation in heart tissues has been implicated in pyrethroid toxicity, as it was found that pyrethroids increase lipid peroxidation, alter the antioxidant capacity in heart cells' plasma membrane and induce oxidative DNA base modifications (Kale *et al.*, 1999; You *et al.*, 2000; Vadhana *et al.*, 2010). Permethrin has also been shown to accumulate in heart cells (0.1–0.2% of dietary intake) (Vadhana *et al.*, 2010).

2.3.3. **GABA-gated chlorine channel blockers – Organochlorines**

The Na^+/K^+ ATPase activity is crucial for the maintenance of the ionic balance and the resting electric potential of the cell membrane. It also serves as a signal transducer-regulator of cell enzymes and intracellular calcium levels acting in parallel with Ca^{2+} ATPase (Bers *et al.*, 2003). Lindane increased membrane-bound Ca^{2+} ATPase activity, while both Na^+/K^+ ATPase and Mg^{2+}ATPase activities were suppressed (Vijaya Padma *et al.*, 2013). Redox status of the myocardial tissue was also distorted by organochlorines, with lipid peroxidation being the most prominent effect (Vijaya Padma *et al.*, 2013; Kalender *et al.*, 2004). It is interesting that endosulfan affected myocardial cells from rats even at doses lower than LD_{50} values. It should be also considered that even at doses that are quite below the legally permitted limits of contamination/

exposure, combinations of chemicals may produce unpredicted toxicities necessitating evaluation of cardiotoxic effects of pesticides (at doses below the acceptable levels), when accidental co-exposure with other frequently used chemicals takes place (Tsatsakis *et al.*, 2016). In humans, organochlorines poisoning led to hypotension and electrocardiographic abnormalities, myocardial infarction and left ventricular myocardial dysfunction (Ramachandra and Rachel, 2013; Ozmen, 2011). Lindane accumulates in appreciable amounts in the heart and causes oxidative stress by modifying the scavenger enzymes activity (Ramachandra and Rachel, 2013). Chronic application of low doses of lindane shortened the action potential duration in rat papillary muscle. These effects were similar to those induced by hyperthyroidism (Sauviat and Pages, 2002).

2.3.4. **Cellular hypoxia, inhibition of cytochrome C oxidase – Aluminum Phosphide (AlP)**

Aluminum phosphide (AlP) reacts with water or acids to release phosphine: $AlP + 3\ H_2O \rightarrow Al(OH)_3 + PH_3$, $AlP + 3\ H^+ \rightarrow Al^{3+} + PH_3$. Phosphine is a strong inhibitor of electron transport chain in mitochondria, specifically mitochondrial complex IV (cytochrome-c oxidase) (Nath *et al.*, 2011). AlP reduced heart mitochondrial complexes II, IV and V activity, accompanied by increased lipid peroxidation and decreased ADP/ATP ratio in myocardial cells. ATP levels and ADP/ ATP ratio are thought to be important biochemical end-points for evaluating the status of mitochondrial respiratory chain performance (Hosseini *et al.*, 2013). AlP interrupted electron transfer between mitochondrial complexes and protons in mitochondrial inter-membrane space, which led to the loss of proton gradient and consequently led to mitochondrial permeability transition pore (MPT) opening and mitochondrial membrane potential (MMP) decline. Thus, mitochondrial membrane integrity was compromised and this outcome resulted in

cardiomyocytes death (Solgi *et al.*, 2015). Augmented oxidative stress (elevated ROS and plasma iron levels) along with myocardial energy ATP depletion and apoptosis induction in exposed animals has also been associated with the cardiotoxic effects of AlP (Baghaei *et al.*, 2016). In addition, clinical manifestations such as decrements in heart rate (HR) and blood pressure (BP) as well as ECG changes such as abnormal QRS complexes, QTc prolongation and ST height decrease are also reported (Abdolghaffari *et al.*, 2015). All the above effects refer to acute exposure. In humans, a characteristic feature of AlP poisoning is myocardial suppression and resistant hypotension (Bogle *et al.*, 2006; Chauhan *et al.*, 2015).

2.3.5. **Inhibition of photosynthesis – Atrazine, Paraquat**

Atrazine is a herbicide of the triazine class, which significantly decreased GSH and total thiol (T-SH) content both in serum and in cardiac tissue and significantly increased cardiac tissue Heme oxygenase-1 (HO-1) activity, while promoting oxidative DNA damage (Keshk *et al.*, 2014). Apoptosis is also present in atrazine poisoning, as caspase-3, the "point-of-no-return" in the apoptotic signaling cascade (Green and Amarante-Mendes, 1998), is increased in the heart tissue cytoplasm (Keshk *et al.*, 2014).

Oxidative stress, myocardial inflammation, apoptosis and endoplasmic reticulum (ER) stress are associated with paraquat cardiotoxic effects (Wang et al., 2017; Wang et al., 2014; We et al., 2010) after acute exposure. ER is an extensive intracellular membranous network participating in cellular functions such as Ca^{2+} storage, Ca^{2+} signaling and glycosylation (Cominacini *et al.*, 2015). Paraquat intoxication induced myocardial functional alterations and geometric transformation of the left ventricle including enlarged left ventricular end systolic diameter (LVESD) (Wang *et al*, 2014); however it did not induce significant changes in left ventricle (LV) posterior wall

thickness, septal thickness and LV end diastolic diameter (LVEDD) (Lei *et al.*, 2017). Therefore, myocardial contractility suppression seems to be the main myocardial effect of paraquat exposure.

2.3.6. **Blockage of cytochrome P450-dependent enzyme C-14 alpha-demethylase – Triazoles**

Very few studies focus on triazole cardiotoxicity mainly after subchronic/ chronic exposure (Table 7). The main cardiotoxic mechanism recognized after penconazole exposure is oxidative stress. The whole oxidative profile of the heart, including enzymatic (superoxide dismutase, GPx, and CAT) and non-enzymatic (MDA, protein carbonyls glutathione and vitamin C) parameters, along with the lipidemic profile was altered (Chaabane *et al.*, 2016). Paul and Rawal (2017) presented two case reports that developed acute systolic heart failure when itraconazole was used, as a medication. *Itraconazole* is an antifungal medication used to treat a number of fungal infections, such as aspergillosis, blastomycosis, coccidioidomycosis etc. When Itraconazole treatment was withdrawn in both cases, the first patient did not improve even months after cessation of therapy and was referred for heart transplant, whereas the second patient stabilized after a few weeks and his myocardial ejection fraction augmented on repeat echocardiographic testing.

2.4. Conclusion

Exposure to pesticides has been associated with several cardiovascular complications including electrocardiogram abnormalities, myocardial infarction, impaired systolic and diastolic performance, functional remodelling, histopathological insults, such as haemorrhage, vacuolisation, signs of apoptosis and degeneration, various biochemical complications, such as distorted lipidemic profile and increased systemic

and cardiac-tissue-specific oxidative stress and DNA alterations in cardiac cells that could lead to functional impairment. Very limited data point to retainment of pesticides (organophosphate, organochlorines) residues in the cardiac tissue. More research should be performed in this respect to verify if observed cardiotoxicity could be due to intense localised action.

In addition, several molecular pathways have been shown to be involved in pesticides cardiotoxicity. Organophosphates which significantly decrease serum acetylcholinesterase activity (Lopez-Carillo and Lopez-Cervantes, 1993) and organochlorines affect the redox status in the cardiac tissue and induce oxidative stress in a dose-dependent mode. Continuous exposure to organophosphates alters lipid metabolism and increases cytotoxicity enzymes activity, consequently leading to apoptosis (Zaki, 2012). The main mechanism involved in AlP cardiotoxicity is inhibition of cytochrome C oxidase in the myocardial cells mitochondria, resulting in decreased ATP production and induction of oxidative stress (Asghari *et al.*, 2017). Pyrethroids have been found to modify neuronal sodium channels as they induce a persistent, steady-state sodium current within depolarized membranes leading to cardiac hypotrophy (Bhaskar *et al.*, 2010), increase calcium release and enhance *Nrf2* gene expression levels in older animals. Cytosolic calcium levels are important for the contractile state of cardiac muscle. Permethrin also induced oxidative damage to purine bases in the heart cells (Vadhana *et al.*, 2010). Organochlorines affect myocardial cells in rats even at doses lower than LD_{50} values (Kalender *et al.*, 2004).

Pesticides toxicity and the risk they pose for human health are controlled at a European level through a well-developed regulatory network, but cardiotoxicity is not described as a separate hazard class. Specific classification criteria should be developed within the frame of Regulation (EC) 1272/2008 in order to classify chemicals as

cardiotoxic, if applicable, to avoid long-term cardiovascular complications. Classification should be based on anatomical, histopathological, echocardiographic and biochemical criteria both in animals and in humans developed in a way that could exclude cofounding factors in the development of the observed cardiotoxicity.

2.5. Tables

List of harmonised classification for human health and environmental hazards of pesticides (79) according to the CLP Regulation (Risk Assessment Committee opinions 2012 – April 2018)

Chemical Identification	CAS No	Classification
		Hazard Class and Category Code(s)
Pirimicarb (ISO)	23103-98-2	Carc. 2, Acute Tox. 3 (oral, inhalation) , Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Bendiocarb (ISO)	22781-23-3	Acute Tox. 2 (oral), Acute Tox. 3 (dermal, inhalation), Aquatic Acute 1, Aquatic Chronic 1
Fenoxycarb (ISO)	72490-01-8	Carc. 2, Aquatic Acute , Aquatic Chronic 1
Calcium phosphide; tricalcium diphosphide	1305-99-3	Water-react. 1, Acute Tox. 1(inhalation), Acute Tox. 2 (oral), Acute Tox. 3 (dermal), Eye Dam. 1, Aquatic Acute 1
Fenamiphos (ISO)	22224-92-6	Acute Tox. 2 (oral, dermal, inhalation), Eye Irrit. 2, Aquatic Acute 1, Aquatic Chronic 1
Ethephon	16672-87-0	Acute Tox. 3 (dermal), Acute Tox. 4 ((oral, inhalation), Acute Tox. 4, Skin Corr. 1C, Aquatic Chronic 2
Potassium (E,E)-hexa-2,4-dienoate	24634-61-5	Eye Irrit. 2
Dicopper oxide; copper (I) oxide	1317-39-1	Acute Tox. 4 (oral, inhalation), Eye Dam. 1, Aquatic Acute 1, Aquatic Chronic 1
Dicopper chloride trihydroxide	1332-65-6	Acute Tox. 3 (oral), Acute Tox. 4 (inhalation), Aquatic Acute 1, Aquatic Chronic 1

Copper flakes (coated with aliphatic acid)		Acute Tox. 3 (inhalation), Acute Tox. 4 (oral), Eye Irrit. 2, Aquatic Acute 1, Aquatic Chronic 1
Copper(II) carbonate--copper(II) hydroxide (1:1)	12069-69-1	Acute Tox. 4 (oral, inhalation), Eye Irrit. 2, Aquatic Acute 1, Aquatic Chronic 1
Copper dihydroxide; copper(II) hydroxide	20427-59-2	Acute Tox. 2 (inhalation), Acute Tox. 4 (oral), Eye Dam. 1, Aquatic Acute 1, Aquatic Chronic 1
Bordeaux mixture; reaction products of copper sulphate with calcium dihydroxide	8011-63-0	Acute Tox. 4 (inhalation), Eye Dam. 1, Aquatic Acute 1, Aquatic Chronic 1
Copper sulphate pentahydrate	7758-99-8	Acute Tox. 4 (oral), Eye Dam. 1, Aquatic Acute 1, Aquatic Chronic 1
Tebuconazole (ISO)	107534-96-3	Repr. 2, Acute Tox. 4 (oral), Aquatic Acute 1, Aquatic Chronic 1
Chlorophacinone (ISO)	3691-35-8	Repr. 1B, Acute Tox. 1 (oral, dermal, inhalation), STOT RE 1, Aquatic Acute 1, Aquatic Chronic 1
Isoxaflutole (ISO)	141112-29-0	Repr. 2, Aquatic Acute 1, Aquatic Chronic 1
Abamectin (combination of avermectin B1a and avermectin B1b) (ISO)	71751-41-2 [1] 65195-55-3 [2]	Repr. 2, Acute Tox. 1 (inhalation), Acute Tox. 2 (oral), STOT RE 1, Aquatic Acute 1, Aquatic Chronic 1
Acequinocyl (ISO)	57960-19-7	STOT SE 1, STOT RE 2, Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Sulcotrione (ISO)	99105-77-8	Repr. 2, STOT RE 2, Skin Sens. 1A, Aquatic Acute 1, Aquatic Chronic 1
Tralkoxydim (ISO)	87820-88-0	Carc. 2, Acute Tox. 4 (oral), Aquatic Chronic 2

Cycloxydim (ISO)	101205-02-1	Repr. 2
Carvone (ISO)	99-49-0 [1] 2244-16-8 [2] 6485-40-1 [3]	Skin Sens. 1
Tembotrione (ISO)	335104-84-2	Repr. 2, STOT RE 2, Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Clethodim (ISO)	99129-21-2	Acute Tox. 4 (oral), Skin Sens. 1, Aquatic Chronic 3
Anthraquinone	84-65-1	Carc. 1B
Warfarin (ISO)	81-81-2 [1] 5543-57-7 [2] 5543-58-8 [3]	Repr. 1A, Acute Tox. 1 (dermal, inhalation), Acute Tox. 2 (oral), STOT RE 1, Aquatic Chronic 2
Coumatetralyl (ISO)	5836-29-3	Repr. 1B, Acute Tox. 2 (inhalation), Acute Tox. 3 (oral), STOT RE 1, Aquatic Chronic 1
Difenacoum (ISO)	56073-07-5	Repr. 1B, Acute Tox. 1 (oral, dermal, inhalation), STOT RE 1, Aquatic Acute 1, Aquatic Chronic 1
Brodifacoum (ISO)	56073-10-0	Repr. 1A, Acute Tox. 1 (oral, dermal, inhalation), STOT RE 1, Aquatic Acute 1, Aquatic Chronic 1
Indoxacarb (ISO)	173584-44-6 [1] 144171-61-9 [2]	Acute Tox. 3 (oral), Acute Tox. 4 (inhalation), STOT RE 1, Skin Sens. 1B, Aquatic Acute 1, Aquatic Chronic 1
Benzoic acid	65-85-0	STOT RE 1, Skin Irrit. 2, Eye Dam. 1
Methyl 2,5-dichlorobenzoate	2905-69-3	Acute Tox. 4 (oral), STOT SE 3, Aquatic Chronic 2

Fenoxaprop-P-ethyl (ISO)	71283-80-2	STOT RE 2, Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Spirotetramat (ISO)	203313-25-1	Repr. 2, STOT SE 3, Eye Irrit. 2, Skin Sens. 1A, Aquatic Acute 1, Aquatic Chronic 1
Dodemorph acetate; 4-cyclododecyl-2,6-dimethylmorpholin-4-ium acetate	31717-87-0	Repr. 2, STOT RE 2, Skin Corr. 1C, Skin Sens. 1A, Aquatic Chronic 1
Fenpyroximate (ISO)	134098-61-6	Acute Tox. 2 (inhalation), Acute Tox. 3 (oral), Skin Sens. 1B, Aquatic Acute 1, Aquatic Chronic 1
Triflusulfuron-methyl	126535-15-7	Carc. 2, Aquatic Acute 1, Aquatic Chronic 1
Bifenazate (ISO)	149877-41-8	STOT RE 2, Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Bromadiolone (ISO)	28772-56-7	Repr. 1B, Acute Tox. 1 (oral, dermal, inhalation), STOT RE 1, Aquatic Acute 1, Aquatic Chronic 1
Tefluthrin (ISO)	79538-32-2	Acute Tox. 1 (inhalation), Acute Tox. 2 (oral, dermal), Aquatic Acute 1, Aquatic Chronic 1
Aclonifen (ISO)	74070-46-5	Carc. 2, Skin Sens. 1A, Aquatic Acute 1, Aquatic Chronic 1
Spiroxamine (ISO)	118134-30-8	Repr. 2, Acute Tox. 4 (oral, dermal, inhalation), STOT RE 2, Skin Irrit. 2, Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Fluazinam (ISO)	79622-59-6	Repr. 2, Acute Tox. 4 (inhalation),vEye Dam. 1, Skin Sens. 1A, Aquatic Acute 1, Aquatic Chronic 1
Bupirimate (ISO)	41483-43-6	Carc. 2, Skin Sens. 1B, Aquatic Chronic 1

Triflumizole (ISO)	68694-11-1	Repr. 1B, Acute Tox. 4 (oral), STOT RE 2, Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Fuberidazole (ISO)	3878-19-1	Carc. 2, Acute Tox. 4 (oral), STOT RE 2, Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Imazalil (ISO)	35554-44-0	Carc. 2, Acute Tox. 3 (oral), Acute Tox. 4 (inhalation), Eye Dam. 1, Aquatic Chronic 1
Dodemorph (ISO)	1593-77-7	Repr. 2, STOT RE 2, Skin Corr. 1C, Skin Sens. 1A, Aquatic Acute 1, Aquatic Chronic 1
Chlorsulfuron (ISO)	64902-72-3	Aquatic Acute 1, Aquatic Chronic 1
Etridiazole (ISO)	2593-15-9	Carc. 2, Acute Tox. 4 (oral), Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Pyridaben (ISO)	96489-71-3	Acute Tox. 3 (oral, inhalation), Aquatic Acute 1, Aquatic Chronic 1
Flumioxazin (ISO)	103361-09-7	Repr. 1B, Aquatic Acute 1, Aquatic Chronic 1
Epoxiconazole (ISO)	133855-98-8	Carc. 2, Repr. 1B, Aquatic Chronic 2
Penconazole (ISO)	66246-88-6	Repr. 2, Acute Tox. 4 (oral), Aquatic Acute 1, Aquatic Chronic 1
Fenpyrazamine (ISO)	473798-59-3	Aquatic Acute 1, Aquatic Chronic 1
Lenacil (ISO)	2164-08-1	Carc. 2, Aquatic Acute 1, Aquatic Chronic 1
Triadimenol (ISO)	55219-65-3	Repr. 1B, Lact., Acute Tox. 4 (oral), Aquatic Chronic 2

Terbuthylazine (ISO)	5915-41-3	Acute Tox. 4 (oral), STOT RE 2, Aquatic Acute 1, Aquatic Chronic 1
Quinolin-8-ol; 8-hydroxyquinoline	148-24-3	Repr. 1B, Acute Tox. 3 (oral), Eye Dam. 1, Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Thiacloprid (ISO)	111988-49-9	Carc. 2, Repr. 1B, Acute Tox. 3 (oral), Acute Tox. 4 (inhalation), STOT SE 3, Aquatic Acute 1, Aquatic Chronic 1
Cyanamide; carbamonitril	420-04-2	Carc. 2, Repr. 2, Acute Tox. 3 (oral, dermal), STOT RE 2, Skin Corr. 1, Eye Dam. 1, Skin Sens. 1, Aquatic Chronic 3
Cymoxanil (ISO)	57966-95-7	Repr. 2, Acute Tox. 4 (oral), STOT RE 2, Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Acetochlor (ISO)	34256-82-1	Carc. 2, Repr. 2, Acute Tox. 4 (inhalation), STOT SE 3, STOT RE 2, Skin Irrit. 2, Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Metazachlor (ISO)	67129-08-2	Carc. 2, Skin Sens. 1B, Aquatic Acute 1, Aquatic Chronic 1
Flufenoxuron (ISO)	101463-69-8	Repro Lact., Aquatic Acute 1, Aquatic Chronic 1
Tebufenpyrad (ISO)	119168-77-3	Acute Tox. 3 (oral), Acute Tox. 4 (inhalation), STOT RE 2, Skin Sens. 1B, Aquatic Acute 1, Aquatic Chronic 1
Proquinazid (ISO)	189278-12-4	Carc. 2, Aquatic Acute 1, Aquatic Chronic 1
Metosulam (ISO)	139528-85-1	Carc. 2, STOT RE 2, Aquatic Acute 1, Aquatic Chronic 1
Dimethenamid-P (ISO)	163515-14-8	Acute Tox. 4 (oral), Skin Sens. 1, Aquatic Acute 1, Aquatic Chronic 1
Flonicamid (ISO)	158062-67-0	Acute Tox. 4 (oral)
Sulfoxaflor (ISO)	946578-00-3	Acute Tox. 4 (oral), Aquatic Acute 1, Aquatic Chronic 1

Benzovindiflupyr (ISO)	1072957-71-1	Acute Tox. 3 (oral, inhalation), Aquatic Acute 1, Aquatic Chronic 1
Carbetamide (ISO	16118-49-3	Carc. 2, Repr. 1B, Acute Tox. 4 (oral), Aquatic Chronic 2

Most important Mode of Actions of groups of pesticides found in the literature (Shaon Kumar Das, 2013)

Type of pesticide	Mode of Action
	Inhibition of carboxyl ester hydrolases, particularly acetylcholinesterase (AChE)
Pyrethroids	Alter the function of voltage-gated sodium channels in insect neuronal membranes, thereby disrupting electrical signalling in the nervous system
	Ligand-gated ion channel activity (GABA-gated chlorine channel blockers)
Phosphines	Cellular hypoxia due to the effect on mitochondria, inhibition of cytochrome C oxidase and formation of highly reactive hydroxyl radicals (mitochondrial complex IV electron transport inhibitors)
	Block the cytochrome P450-dependent enzyme C-14 alpha-demethylase, which is needed to convert lanosterol to ergosterol
Triazines	Inhibition of primary events in photosynthesis in the chloroplast: binding to the D-1 protein in photosynthetic electron transport. This binding stops photosynthesis. Inhibition requires the presence of light and transpiration to move the chemical to foliage (Photosystem II (PSII) inhibition)
Dipyridyl	Photosystem I (PSI) inhibition: photosynthesis is affected leading to destruction of cell membranes; the specific effect is much faster than other desiccators (Summers 1980)

Cardiotoxic effects of organophosphates, carbamates and organothiophosphates in experimental animals and human data.

[#] Classes of cardiovascular disorders: (1) Myocardial dysfunction, (2) Electrical, (3) Biochemical (oxidative damage), (4) Anatomical, (5) Cytopathological, (6) Histopathological, (7) Biochemical (functional implications), (8) Coronary artery disease, (9) Biochemical (lipidemic profile and other), (10) Blood pressure disorders (hypertension, hypotension)

** Ubiquitin (Ub) is a small protein which covalently binds to lysine (Lys) residues of target proteins to presumably mark targeted proteins for degradation. At the same time, ubiquitylation regulates a number of biological processes, including DNA repair and replication, gene expression and apoptosis (Haglund and Dikic, 2005; Razavi et al., 2015)*

Cardiotoxic effects of pyrethroids in experimental animals and human data.

Subject	Substance/ Dose	Exposure Route/ Exposure Period	Evaluation Method	Signs of Cardiotoxicity (class of disorder[#])	Reference
Wistar rats and guinea pig	Tefluthrin Fenpropathrin Cypermethrin Tetramethrin (10 μM for each one)	Ex vivo study, direct action on Isolated heart ventricular myocytes	Electrophysiology	• Prolonged ventricular action potentials revoked after depolarizations (1) • Modified the time course of INa by altering the relative proportions of fast and slowly inactivating current (2) • Altered the voltage dependence of INa (2)	(Spencer *et al.*, 2001)
Male and Female 500-day-old rats	Permethrin (4 ml/kg equal to 1/50 of LD50)	Oral administration from the 6th to 21st day of life	Gene expression Calcium levels Heart surface area Histopathology	• Cardiac hypotrophy (4) • Increased calcium and Nrf2 gene expression levels in old age (3) The histological analysis of cardiac and skeletal muscle fibers demonstrated large areas of degenerating muscle fibers with evident loss of transverse striations and wide interfascicular spaces (6)	(Vadhana *et al.*, 2013)
Case Report 28-year-old Female	Prallethrin (20 ml of a preparation containing prallethrin (1.6%) and piperonyl butoxide (5%)	Intentional ingestion		• Metabolic acidosis (7) • Sinus arrest (2)	(Bhaskar *et al.*, 2010)
Epidemiological study, 72 CHD patients and 136 healthy individuals	Pyrethroids and pyrethroids metabolites	Exposure that led to urinary excretion		• Increased levels in Coronary Heart Disease (CHD) patients (8)	(Han *et al.*, 2017)
Freshly isolated rat heart cells (*in vitro*)	Permethrin (5, 10, 20 μM)		DNA damage assessed by comet assay	• Significant difference in % tail DNA between all concentrations of permethrin (7)	(Vadhana *et al.*, 2010)

[#] Classes of cardiovascular disorders: (1) Myocardial dysfunction, (2) Electrical, (3) Biochemical (oxidative damage), (4) Anatomical, (5) Cytopathological, (6) Histopathological, (7) Biochemical (functional implications), (8) Coronary artery disease, (9) Biochemical (lipidemic profile and other), (10) Blood pressure disorders (hypertension, hypotension)

Cardiotoxic effects of organochlorines and dipyridyl pesticides (paraquat) in experimental animals and human data.

Subject	Substance/ Dose	Exposure Route/Period	Evaluation Method	Signs of Cardiotoxicity (class of disorder[#])	Reference
New Zealand Rabbits	Diazinon (2.6 and 5.2 mg/kg/day) Propoxur (8.8 and 18 mg/kg/day) Chlorpyrifos (8.7 and 18 mg/kg/day)	Oral daily administration for 12 months	Echocardiography Biochemical and histpathological evaluations	• Reduced systolic and diastolic performances (1) • Increased oxidative stress of the cardiac tissues. Diazinon and propoxur increased lipid peroxidation in plasma (3) • Increased oxidative modifications in the genomic DNA content of the cardiac tissues (3) • Thin-walled left ventricles with reduced myocardial mass, impaired systolic (radial and longitudinal) and diastolic LV function assessed by M-Mode, PW and TDI Doppler (1) • Diazinon and propoxur retained in the cardiac tissue	(Zafiropoulos *et al.*, 2014)
Male Wistar rats	Methidathion (5 mg/kg)	Oral administration (5 days/ week) for 4 weeks	Biochemical and histpathological evaluations	• Increase of lipid peroxidation (3)	(Yavuz *et al.*, 2004)
Male Wistar rats	Chlorpyrifos (5.4 mg/kg equal to 1/25 of the oral LD50)	Oral administration for 4 weeks	Biochemical and histopathological evaluations	• Increased levels of MDA, SOD and CAT; decreased GPx and GST activities in heart (3)	(Bas and Kalender, 2011)
Male New Zealand rabbits	Chlorpyrifos (0, 125, 250 or 375 ppm in drinking water)	Oral administration for 90 days	Echocardiography	• significant decrease ($p < 0.05$) in HR, cardiac output (CO), left ventricular fractional shortening (FS), left ventricular ejection fraction (EF), percentage thickening of left ventricle posterior wall (PWT) (1) • Significant increase ($p < 0.05$) in left atrial diameter (LA), left ventricular internal diameter in end diastole (LVIDD), left ventricular end diastolic (EDV) and end systolic volumes (ESV) compared to controls (1)	(Cetin *et al.*, 2007)

Rats	Chlorpyrifos (1 or 5 mg/kg)	Subcutaneous injections on gestational days 9-12 or 17-20 or on postnatal days (PN) 1-4 or 11-14 Assessments done on PN60	Cardiac and Hepatic Cell Signalling	• Cell signalling cascades (7)	(Meyer *et al.*, 2004)
Sexually mature male white rats	Profenofos (47.5mg/kg for acute toxicity and 23.75mg/kg for subchronic toxicity)	Oral administration Acute intoxication: 120 hr Subchronic exposure : 14 days Withdrawal group: 14 days	Biochemical and Enzymes activities assays	• Alters lipid metabolism (9) • Increase the activities of cytotoxicity enzymes biomarkers (5)	(Zaki, 2012)
Female Sprague-Dawley rats	Diazinon (8, 10, 12 and 20 mg/kg) (oral LD50 = 300 mg/kg)	Oral administration for 3 weeks	Biochemical assays Histopathological evaluations	• Induced varying degrees of oxidative damage (3) • The histological analysis of cardiac and skeletal muscle fibers demonstrated large areas of degenerating muscle fibers with evident loss of transverse striations and wide interfascicular spaces (6)	(Abdou and ElMazoudy, 2010)
Adult female rats	Dimethoate (0.2 g/L drinking water)	Oral administration 30 days	Biochemical assays Histopathological evaluations	• Promoted oxidative stress with a rise in malondialdehyde, advanced protein oxidation, and protein carbonyl levels. An increase of glutathione peroxidase, superoxide dismutase, and catalase activities was also noted (3) • Derease in acetylcholinesterase and Na, K -ATPase activities (2) • Plasma levels of cholesterol, triglycerides, and low density lipoprotein-cholesterol increased and those of high density lipoprotein-cholesterol decreased (9)	(Amara *et al.*, 2013)
Male Wistar albino rats	Diazinon (20mg/kg)	Oral administration for 28 days	Biochemical assays	• A significant increase in cardiac MDA and NO content was observed compared with the control group, but cardiac antioxidants were significantly ($p \leq 0.05$) decreased (3)	(Abdel-Daim *et al.*, 2016)

Rats	Diazinon (15mg/kg/day)	Oral administration for 28 days	Biochemical assays	• Increased MDA level (3) • Lower level of reduced GSH (3) • Induction of apoptosis (5)	(Razavi *et al.*, 2013)
Adult male Wistar rats	Diazinon (15mg/kg/day)	Oral administration for 28 days	Biochemical assays	• Total ubiquitylation* of myocardial proteins was increased by 79% (7)	(Razavi *et al.*, 2015)
Male Wistar rats	Phorate (0.046, 0.092 or 0.184 mg/kg)	Oral administration for 14 days	Histopathological evaluations	• Congestion and hemorrhage, cardiac myofiber degeneration and round and shrunken focal degenerating myocytes (6)	(Saquib *et al.*, 2012)
Adult male Sprague–Dawley rats	Dichlorvos (0.25 LD50, 7.5 mg/kg), (0.35 LD50, 10.5 mg/kg), (0.5 LD50, 15.0 mg/kg), (0.75 LD50, 22.5 mg/kg), and (1.4 LD50, 42.0 mg/kg)	Intraperitoneal administration, evaluation at 2 and 6 weeks post-exposure	Echocardiography Histopathological evaluations	• QT prolongation without anatomical or histopathological abnormalities (2)	(Shiyovich *et al.*, 2017)
Wistar rats	Monocrotophos (MCP) (0.36 mg/kg/day equal to 1/50 oral LD50)	Oral administration for 3 weeks	Biochemical assays Histopathological evaluations	• Cardiac oxidative stress was conferred by accumulation of protein carbonyls, lipid peroxidation and glutathione production. The cardiac markers (cTn-I, CK-MB and LDH) were showed elevated expression in blood plasma, which signals the cardiac tissue damage (3) • The histopathology of the heart tissue authenticated the MCP induced tissue damage by showing signs of nonspecific inflammatory changes and oedema between muscle fibres (6)	(Velmurugan *et al.*, 2013)
Meta-analysis in the Agricultural Health Study, USA	Organophosphates Carbamates	Self-reporting use (1993-1997)		• Myocardial infarction mortality, insecticide use Hazard Ratio (HR)=0.93 (8) • Non-fatal myocardial infarction incidence, insecticide use HR=0.85 (8)	(Mills *et al.*, 2009)

Ex vivo study	Malathion (10, 15, 20 and 25 μg/ml)	Direct effect on human cardiac myocytes	MTT cell proliferation assay	• Toxicity in cardiac cells (5) LD50 calculated as 20 μg/ml	(Atale *et al.*, 2014)
41 patients at emergency care unit	Dichlorvos (dosage not specified)	Acute oral poisoning		• Sinus tachycardia (2) • ST-T changes (8) • Decreases in wall motion of the interventricular septum and left ventricle (reversible) (8) • Abnormal left ventricle perfusion (1)	(He *et al.*, 2011)

Subject	Dose	Substance/	Exposure Route/ Exposure Period	Evaluation Method	Signs of Cardiotoxicity (class of disorder#)	Reference
Male Wistar rats		Endosulfan 2mg/kg/day	Oral administration for 6 weeks	Measurement of antioxidant enzymes activities	•SOD, GPx, CAT activities and MDA level increased in the endosulfan-treated group heart tissue compared to control group (3) •Cytoplasmic edema and swelling and vacuolization of mitochondria of myocardial cells in endosulfan-treated group (6)	(Kalender *et al.*, 2004)
Male rats		Endosulfan 2 mg/kg/day	Oral administration for 28 days	Histopathological evaluations	•Sever congestion; Haemorrhages with interstitial oedema; Diapedesis of leukocytes; Myocardium showed different degrees of degeneration; Some of the myofibrils were found to be granular with pyknotic nuclei (6) •Thickening of wall of arteries (4)	(Jalili *et al.*, 2007a)
Case Report 50-years-old female	mentioned)	Lindane (dose not	Accidental ingestion		• NSTEMI (non-ST segment elevation myocardial infarction) (8) • Left Ventricular (LV) dysfunction (1)	(RamachandraBhat and Rachel, 2013)
Review article		Endosulfan	Poisoning		• Hypotension, (10) • ECG abnormalities (2)	(Ozmen, 2011)
Rats		Lindane (100 mg/kg)	Oral administration for 30 days	Biochemical analysis Histopathology	• Elevated activity for serum marker enzymes, lipid peroxidation (LPO), and membrane-bound Ca^{2}+ ATPase, with a concomitant decrease in the level of non-enzymatic antioxidant (GSH), enzymatic antioxidants such as SOD, CAT, GPx, and GST, and membrane-bound ATPases like Na+/K+ ATPase and Mg2+ ATPase in heart tissue (3) • Pathological changes(6)	(Vijaya Padma *et al.*, 2013)
Adult male wild-type (WT) and TLR4 knockout (TLR42/2) mice		Paraquat* 45 mg/kg	Intraperitoneal administration	Histopathological evaluations done after 48 hours	Myocardial functional and geometric alterations including enlarged left ventricular end systolic diameter (LVESD), reduced fractional shortening, decreased sarcomere shortening, maximal velocities of sarcomere shortening and relengthening associated with unchanged LV posterior wall thickness, septal thickness, LV end diastolic diameter (LVEDD), heart rate, sarcomere length, time-to-peak shortening and time-to-90% relengthening (1)	(Lei *et al.*, 2017)
Akt2 knockout mice		Paraquat* (45 mg/kg)	A single intraperitoneal injection	Echocardiography followed by isolation of cardiomyocytes for evaluation of mechanical properties of myocytes and intracellular Ca^{2+} levels. Aconitase and citrate synthase activities were measured in heart homogenates.	•Decreased intracellular Ca^{2+} release (2) •Significant decreases in fractional shortening, but no changes in other geometric parameters (1) •Increased myocardial apoptosis as reflected by upregulated Bax, downregulated Bcl-2 and elevated caspase-3 activity (5)	(Wang et al., 2017)
Cardiac-specific overexpression catalase mice and FVB littermates as wild type		Paraquat* (75 mg/kg)	A single intraperitoneal injection, examination48 h later	Echocardiography, edge detection, caspase-3 activity, immunoblotting	•Enlarged left ventricular (LV) end diastolic and systolic diameters; increased LV mass and resting myocyte length; reduced fractional shortening, cardiomyocyte peak shortening, and maximal velocity of shortening/relengthening; and prolonged relengthening duration in the FVB group (1) •increased apoptosis ablated by the catalase transgene (5 with underlying 3)	(We et al., 2010)

Wild-type and transgenic mice with overexpression of a mutant AMPK	Paraquat* (45 mg/kg)	A single intraperitoneal injection	Echocardiography followed by isolation of cardiomyocytes for evaluation of cell shortening/relengthening, intracellular Ca^{2+} transients, measurement of mitochondrial membrane potential	• Cardiac mechanical anomalies and compromised echocardiographic parameters (elevated left ventricular end-systolic diameter and reduced factional shortening) (1) • Suppressed cardiomyocyte contractile function, intracellular Ca^{2+} handling (2) • overt mitochondrial damage (loss in mitochondrial membrane potential) (2) • promoted phosphorylation of AMPK and autophagy, reduced cell survival (5)	(Wang et al., 2014)
Toll-like receptor 4 (TLR4) knockout (TLR4-/-) mice	Paraquat* (45 mg/kg)	A single intraperitoneal injection	Heart rate measurement followed by isolation of cardiomyocytes for evaluation of mechanical properties of myocytes and intracellular Ca^{2+} levels.	• No effect was observed in terms of diastolic, systolic and mean blood pressures following exposure to paraquat. • No effect was observed in cell length. • Paraquat significantly increased intracellular Ca^{2+} decay rate. (2)	(Wang et al., 2016)

*Paraquat is quaternary nitrogen dipyridyl pesticide with a Cl^- moiety

[#] *Classes of cardiovascular disorders: (1) Myocardial dysfunction, (2) Electrical, (3) Biochemical (oxidative damage), (4) Anatomical, (5) Cytopathological, (6) Histopathological, (7) Biochemical (functional implications), (8) Coronary artery disease, (9) Biochemical (lipidemic profile and other), (10) Blood pressure disorders (hypertension, hypotension)*

Cardiotoxic effects of phosphines in experimental animals and human data.

Subject	Substance/ Dose	Exposure Route/ Exposure Period	Evaluation Method	Signs of Cardiotoxicity (class of disorder#)	Reference
Male Wistar albino rats	Aluminum phosphide (AlP) 12 mg/kg (equal to LD50 value)	Oral administration; assessments were done 24hrs later	ECG, blood pressure (BP) and heart rate (HR)	• Changes in ECG patterns such as decrement of HR (2) • BP (10) • Abnormal QRS complexes, QTc and ST height. (2)	(Abdolghaffari *et al.*, 2015)
Male Wistar rats	Aluminum phosphide (AlP) 12 mg/kg	Oral administration; assessments were done 24hrs later	Measurement of biochemical and mitochondrial factors	• Oxidative stress (elevated ROS and plasma iron levels) (3)	(Baghaei *et al.*, 2016)
Male Wistar rats	Aluminium phosphide (AlP) 6 and 12 mg/kg	Oral administration; assessments were done 1, 2, 4, 8, 12 and 24 h later	Biochemical assays ECG Immuno-histochemistry	• Reducing of mitochondrial complexes (II, IV and V) followed by increasing lipid peroxidation and ADP/ATP ratio and declining mitochondrial membrane integrity that ultimately resulted in cell death (2,3) • Acute exposure (6 mg/kg) resulted in an increase in hydroxyl radicals and lipid peroxidation in a time-dependent fashion, suggesting an interaction of delivering electrons of phosphine with mitochondrial respiratory chain and oxidative stress induction(3) • Degeneration, fragmentation and loss of cross striation of the cardiac muscle fibers (4,6) • Showed marked caspase positivity in cardiac muscle with muscle fiber fragmentation and loss of cross striation (4,6)	(Solgi *et al.*, 2015)
41 year old Indian woman Review of 93 cases reported to the National Poisons Information Service (London) 1997-2003	10 g sachet of Fumino (aluminium phosphide - AlP) 56% w/w; United Phosphorus)	Deliberate ingestion		• Bilateral pulmonary infiltrates and ECG findings of sinus tachycardia (broad complex) • Hypoxia and metabolic acidosis (9) • Normal left ventricle (LV) size with moderately impaired LV function and cardiac index of 1.5 l/min/m^2	(Bogle *et al.*, 2006)
Case Report 17 year old Male	Aluminium phosphide 3 gr	Intentional ingestion		• Metabolic acidosis (9) • Myocardial depression (1)	(Chauhan *et al.*, 2015)

[#] *Classes of cardiovascular disorders: (1) Myocardial dysfunction, (2) Electrical, (3) Biochemical (oxidative damage), (4) Anatomical, (5) Cytopathological, (6) Histopathological, (7) Biochemical (functional implications), (8) Coronary artery disease, (9) Biochemical (lipidemic profile and other), (10) Blood pressure disorders (hypertension, hypotension)*

Cardiotoxic effects of triazines/triazoles pesticides in experimental animals and human data.

Subject	Dose	Substance/	Exposure Route/ Exposure Period	Evaluation Method	Signs of Cardiotoxicity (class of disorder#)	Reference
A report on two cases (one in early 30s and one in early 50s)		Itraconazole (triazole)	200 mg PO q8hr for 3 days, then 200 mg PO q12hr for 6-12 months		• Acute systolic heart failure (1) • Myocardial ejection fractions of 10-15% for the younger patient and 40-45% for the elder. (1)	Paul and Rawal, 2017
Male Wistar rats		Penconazole (triazole) 67 mg/kg	Intraperitoneal aministration every 2 days from day 7 until day 15	Biochemical assays	• Oxidative stress induction(3)	(Chaabane *et al.*, 2016)
Albino rats	mg/kg/day	Atrazine 400 (triazine)	Oral administration for 3 weeks	Biochemical assays	• Significantly decreased serum/cardiac tissue GSH and TSH levels; significantly increased cardiac tissue HO-1 activity; serum/cardiac tissue GPx and CAT activity, MDA and serum 8-OHdG level altered; significantly decreased cardiac tissue complex I activity (3)	(Keshk *et al.*, 2014)
				Histopathology Immuno-histochemistry	• Degeneration, fragmentation, and loss of cross striation of the cardiac muscle fibers (4,6) • Showed marked caspase positivity in cardiac muscle with muscle fiber fragmentation and loss of cross striation (4,6)	

[#] *Classes of cardiovascular disorders: (1) Myocardial dysfunction, (2) Electrical, (3) Biochemical (oxidative damage), (4) Anatomical, (5) Cytopathological, (6) Histopathological, (7) Biochemical (functional implications), (8) Coronary artery disease, (9) Biochemical (lipidemic profile and other), (10) Blood pressure disorders (hypertension, hypotension)*

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CHAPTER 3

What is considered anthracyclines cardiotoxicity in animal studies?

The work presented in Chapter 3 is included in the following article:

Nikolaos Georgiadis, Konstantinos Tsarouhas, Ramin Rezaee, Haritini Nepka, George E.N. Kass, Jean-Lou C.M. Dorne, Dimitrios Stagkos, Konstantinos Toutouzas, Demetrios A. Spandidos, Dimitrios Kouretas, Christina Tsitsimpikou, 2020. What is considered cardiotoxicity of anthracyclines in animal studies. Oncology Reports; 44(3), 798-818. https://doi.org/10.3892/or.2020.7688

3.1. Introduction

Chemotherapeutics cardiotoxicity is a major concern for clinicians treating different kinds of cancer, as it seriously affects their treatment options and the survival of the patient. The cut-off values for the identification of cardiotoxicity caused by chemotherapeutics in humans differ between the American and European guidelines: the definition considers a lower cut-off value of normality for the left ventricle ejection fraction (LVEF) of 50% in Europe (1) and 53% in the USA (2). Both Guidelines emphasize that a drop of LVEF compared to the patient's previous values is also required. This definition is crucial for patients and clinicians, as patients presenting this decline in cardio-imaging indices of cardiac function should be treated with angiotensin converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs) in combination with beta-blockers (3); nevertheless, modifications of anticancer treatment in such patients remain a matter of discussion among different specialists.

In animal studies, where new anticancer substances are evaluated and different agents are tested to overcome anticancer drugs cardiotoxicity, identification of the extent of cardiotoxicity is crucial and necessary for the evaluation of any favourable effects of the counteracting agent (4). In this regard, cardiac imaging is more often used at analogy to the clinical setting. Biomarkers and clinical signs of heart failure are also taken into consideration, but cardiac imaging in animal studies has gained momentum.

Anthracyclines is a class of drugs used in cancer chemotherapy isolated from *Streptomyces* bacterium. These compounds are used to treat many cancers, including leukemias, lymphomas, as well as breast, stomach, uterine, ovarian, bladder cancer, and lung cancers (5-7). The first anthracycline discovered was daunorubicin (trade name Daunomycin), which is produced naturally by *Streptomyces peucetius*, a species of actinobacteria. Clinically, the most important anthracyclines are doxorubicin, daunorubicin, epirubicin and idarubicin. Anthracyclines, which are considered as well-established cardiotoxic compounds causing myocardial suppression in a considerable number of patients, are also used in animal studies as an easy and low-cost method to introduce a model of dilated cardiomyopathy (8), as opposed to interventional research animal models of infarction and myocardial ischaemia (e.g. permanent ligation of the left anterior descending artery – LAD or cryo-pen application on the surface of the heart leading to cryo-scar ischemia). Different animal species and various anthracyclines dosing and administration schemes have been applied in the literature for the development of anthracyclines cardiotoxicity (9) and monitoring of the progress thereof, as well as testing different compounds/ schemes for ameliorating myocardial damage. To monitor

cardiotoxicity caused by anthracyclines, cardiac imaging is primarily used and secondarily, biochemical markers.

At the same time, other pharmaceutical compounds, such as anabolic steroids, along with everyday chemicals, such as metals and pesticides, have been implicated in adversely affecting cardiac pathology causing function impairment (10). Toxicity and risk for human health posed by chemicals are well controlled at a European level through a thoroughly developed regulatory network. Nevertheless, cardiotoxicity is not described as a separate hazard class and no specific classification criteria are available in order to legally classify chemicals well in advance as cardiotoxicand avoid potential long-term cardiovascular complications, which could significantly burden any national health system.

But, what is considered cardiotoxicity of anticancer agents and specifically anthracyclines when parameters of cardiac imaging are monitored in animal studies? Is there a uniformity in animal models of anthracyclines cardiotoxicity induction and most importantly, do all studies describe the same decline of myocardial function? Addressing these issues could be of wider use both in clinical medicine and practice, when assessing agents employed for salvation to cardiotoxic complications during oncology treatment, for example, as well as to regulators, when trying to establish reference values in echocardiographic function representing cardiotoxicity induced in animals by chemicals.

In the current in depth review, the identification of most commonly used metrics of myocardial function in animals studies of anthracycline induced cardiotoxicity are presented, along with the range of these values differentiating normal cardiac function from animals with pathological echocardiographic findings indicative of anthracycline cardiotoxicity as per author presentation.

3.2. Materials and methods

PubMed electronic database was systematically searched to detect all original research studies published until March 1st 2020, according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement (11). The specific literature search strategy used was: (AND ("*rats*" OR "*doxorubicin* OR "*echocardiography*" OR "anthracycline" OR "*ejection fraction*")) either in the Title, or the Abstracts. The reference list of the retrieved studies was further evaluated for the relevance of the subject and the eligibility by screening the titles/abstracts of full papers. The non-English citations (<5) were reviewed separately. Animal data only from rat species were assessed, as it is evident from the search string. All types of citations other than original research studies (e.g. review articles)

were excluded. Two authors (NG and CT) independently assessed the title and the abstract content (or both) of each record retrieved to decide which studies should be further evaluated and extracted all data. Disagreements were resolved through consensus or by consultation with a third author (KT). A final draft of the manuscript was prepared after several revisions and approved by all authors. In total, 86published manuscripts on animal studies were considered for the systematic review (Figure 1).

Despite the small size of the rat heart and the fast heart rate, echocardiography is systematically used in the evaluation of rat heart function (12). Data for 2 main indices of left ventricular contractility were extracted from the studies list.

The first index is LV fractional shortening (FS) and is calculated by the formula FS(%) = (LV end-diastolic diameter [LVD_d] minus LV end-systolic diameter [LVD_s])/LVD_d × 100.

Left ventricle (LV) Ejection fraction (EF) is the second and more common, index of left ventricular contractility. EF can be calculated from the equation EF(%)=[(LVD_d^3- $LVD_s^{3)}$/ LVD_d^3]x100 (13) or from the equation EF (%) = (LVEDV-LVESV)/LVEDV x100, where LVEDV is the left ventricular end-diastolic volume and LVESV is left ventricular end-systolic volume (12).

3.3. Results

A summary of the studies reviewed in the present report is presented in Table I.

In Figures 2, 3 and 4, 5 the normal and suppressed values of the two main echocardiographic indices discussed, %EF and %FS, respectively, are presented. Reported baseline (normal) %EF values in rats vary (55%-96.5%). In 78.2% of the studies reviewed, normal values range from 70 to 90%. High %EF values (>90%) are reported in 14% of the studies. In contrast, normal %FS values present even higher variability (25%-84.2%). The majority (66.7%) of the values, though, are reported to be within the range of 40 and 60%.

Exposure to anthracyclines suppresses both echocardiographic indices. In the 86 studies reviewed in the present report, Doxorubicin is almost universally used to induce cardiotoxicity, along with Daunorubicin and Epirubicin in two studies (Table 1). The structures of the three anthracyclines used are presented in Figure 6. Anthracyclines were administered with order of appearance either via intraperitoneal injection, intravenous injection or orally with the feed. The doses were administered once, twice, three times per week. The duration of the dose administration spans from one week to ten weeks. In most of the experiments, the benchmark for terminating the administration was the proof of cardiac toxicity. The echocardiography values suggest that there is no specific dose regime threshold which indicates the establishment

of the effect, but it is specific to each experiment and probably dependent to other factors such as age and general condition of the animals.

The suppressed %EF values reported from rats after anthracyclines administration vary from 31% to 91% (Figure 4). EF values 50-80% are reported in 72.3% of the studies reviewed. Suppression of the %EF due to anthracycline administration varies from 10 to 40% compared to the normal values in more than two thirds of the studies reviewed (71.7%) (Figure 7). On the other hand, suppressed %FS values ranging from 14% to 71.8%, present a more narrow distribution (%FS values 20-50% in 84.6% of the studies). As shown in Figure 7, a more equal distribution of the %FS suppression due to anthracyclines toxicity is observed with approximately one fourth of the studies reporting 20-30% and 30-40% suppression, respectively. It is evident from Figures 8-9 that normal and suppressed %EF and %FS values separate sufficiently well. The rat strain does not seem to influence either the normal or the suppressed %EF and %FS values (Figure 10).

Only 11 studies used an acute administration scheme, with 3-20 mg/Kg bw anthracycline single injection either intravenously or intraperitoneally. Most of the studies used a prolonged administration period, from 2 weeks (33 studies) up to 10 weeks, and cumulative doses ranging from 1 mg/Kg to 20 mg/kg bw. All dosage schemes were carefully selected to induce cardiotoxicity and did not seem to affect the suppression of %EF and %FS monitored.

3.4. Discussion

Myocardial contractility suppression due to anthracycline administration is of increasing interest and represents a major challenge in the clinical setting. At the same time in a preclinical stage serves as a model for the assessment of new both chemotherapeutic and cardioprotective agents to be introduced in clinical practice. The myocardial toxicity of anthracyclines is known to be affected by sex and age, along with a number of cardiovascular risk factors and comorbidities (99). It is found that anthracycline related congestive heart failure reaches 10% of patients older than 65 years at usual doses(100). While in early studies it was thought that EF cannot accurately predict congestive heart failure attributed to doxorubicin (100), current perspective is that anthracycline related cardiotoxicity is manifested by a progressive continuous decline in LVEF(1) and identifying subclinical myocardial dysfunction related to anthracycline treatment has great therapeutic implications (2).

Preclinical animal studies are essential in cancer chemotherapy research along with the evaluation of the cardiotoxic propensity of the chemotherapeutic agents. The current recommendations for prevention of cardiac events from cancer chemotherapies are largely

based on recommendations. The American Society of Clinical Oncology, for example, recommends active screening and prevention of modifiable cardiovascular risk factors, such as tobacco use, high blood pressure, high cholesterol, alcohol use, obesity and physical inactivity (101). A well characterized animal model for defining cardiotoxicity due to chemotherapy and the treatment thereof is of great importance for clinical practice, as it will enable physicians to base their decisions not only on epidemiology but also on observations developed using concrete data from animal studies.

In the present review, the range of the main echocardiographic indices, namely EF and FS, used in describing anthracycline cardiotoxicity in rats was summarized along with the normal values of the said indices presented in the respective studies. In the graphic representation, it seems that normal and suppressed due to anthracyclines administration values for the two echocardiographic indices are well separated. This provides a first evidence for the possibility of setting a cut-off point for defining anthracycline cardiotoxicity in rats with an in depth future meta-analysis.

In the current review a wide range of EF and FS decline due to anthracycline administration was observed. However, the trends of the said decline are easily identified, especially for FS values, thus rendering the establishment of minimum cut off values of decline feasible. The question remains, as it has also been identified for humans, whether the absolute suppressed values of EF and FS, combined or separately, or the % suppression caused by anthracyclines should be used to describe cardiotoxicity, and which of the two approaches could be more effective in prevention. In our study, it seems that setting a range for % suppression of EF and FS could be more efficient in identifying early cardiotoxicity by counteracting for the intra-individual variation of the absolute values.

In the current in depth review analysis, we did not identify differences between rat strains in terms of suppressed EF and FS values due to anthracycline administration. This is an interesting finding as it seems that the usual strains used in rat studies are equally prone to the cardiotoxic anthracycline potential. In animal models of genetically programmed hypertension and heart failure, it is found that doxorubicin administration did not lead to lower myocardial contractility compared to non-genetically modified strains (102). In addition, in the current systematic review, acute and chronic anthracyclines cardiotoxicity models were found equally potent in inducing cardiotoxicity based on echocardiographic indices evaluated.

Currently, when assessing chemicals toxicity, cardiac effects if monitored and detected in animal studies, mainly on the tissue level, are considered by the authorities, but cardiotoxicity, as such, is not described as a separate hazard class of chemical substances

through the available regulations, both at a European level and world-wide. Therefore, chemicals other than pharmaceutical agents are recognised to be cardiotoxic after having exerted such deleterious effects on humans, based on epidemiological studies. In a previous review of our research team, 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 (103). In addition, there is evidence that short and/ or long-term exposure to anabolic androgenic steroids is linked to a variety of cardiovascular complications which could be identified by using echocardiography or biochemical markers (10, 104, 105). All these published data suggest clearly that there is a need to establish regulatory criteria for assessing cardiotoxicity as an inherent property of a chemical substance well in advance, and 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. Regulatory established criteria will enable international organizations to early identify cardiotoxic effects and classify chemicals in order to avoid long-term cardiovascular complications. Specific classification criteria should be developed based on anatomical, histopathological, echocardiographic and biochemical criteria in animals developed in a way that could exclude confounding factors in the development of the observed cardiotoxicity. The results of the present study are promising in identifying echocardiographic criteria in rats for the establishment of cardiotoxicity. Further studies and meta-analyses are needed in order to evaluate other species, commonly used in research, and explore the possibility of early recognizing the onset of cardiotoxicity, possibly through biochemical markers monitoring based on understanding of the mode of action.

3.5. Tables

Treatment protocol and main findings of the studies that examined anthracyclines cardiotoxicity in rats reviewed in the present report

Publication	Number of animals/Rat strain/ Sex	Anthracycline administered	Anthracycline total dose	Duration	Summary of Findings	Calculations
Zhang H *et al.*, 2018 (14)	30/Sprague Dawley rats/ Male	Doxorubicin (brand name Adriamycin)	1 mg/kg	Daily doses for 2 weeks	Cardiac dysfunction (parameters monitored: diastolic left ventricular internal dimension, systolic left ventricular internal dimension, LVEF and LVFS)	Values calculated manually by the authors of this review
Tian XQ *et al.*, 2017 (15)	70/Sprague Dawley rats/ Male	Doxorubicin	3.0 mg/kg	Once a week for 6 weeks	Cardiomyopathy	Values provided in the manuscript
Andreadou I. *et al.*, 2014 (16)	90/Wistar rats /Male	Doxorubicin	18 mg/kg, intraperitoneally (ip)	6 equal doses for 2 weeks	Cardiomyopathy (parameters monitored: cardiac geometry, function and histopathology)	Values provided in the manuscript
Oliveira MS *et al.*, 2013 (17)	20/Wistar rats/ Male	Doxorubicin	5 mg/kg, ip	Once a week for 4 weeks	Ventricular dysfunction	Values provided in the manuscript

Hydock DS *et al.*, 2009 (18)	46/Sprague-Dawley rats / Male	Doxorubicin	10 mg/kg ip	Acute administration (bolus)	Parameters altered: LVFS and LVPWT	Values provided in the manuscript
Fernandez-Fernandez A *et al.*, 2014 (19)	36/Sprague-Dawley rats Wistar rats Fischer-344 rats/NM	Doxorubicin	18 mg/kg	Over 12 days	Cardiac function altered (LVFS, left ventricular developed pressure, contractility and relaxation, cardiac capillary permeability)	Values provided in the manuscript
Todorova VK *et al.*, 2011 (20)	27/Fisher 344 rats / Female	Doxorubicin	12 mg/kg (1.5 mg/kg each)	Twice per week for 4 weeks	Parameters monitored: • Plasma levels of troponin I • Left ventricle (LV) function, LV PWT, LV volume, LVEF, LVFS	Values provided in the manuscript
Vasić M. *et al.*, 2019 (21)	68/ Wistar rats/ Male	Doxorubicin	15 mg/kg ip	Every other day for 2 weeks	Parameters monitored: Echocardiography, serum cardiac troponins, heart rate variability and blood pressure variability	Values provided in the manuscript
Mathias LM *et al.*, 2019 (22)	64/ Wistar rats / Male	Doxorubicin	20 mg/kg ip	Acute administration	Altered LVFS	Values provided in the manuscript

				(a single injection)		
Wang X. *et al.*, 2015 (23)	40/ Sprague-Dawley rats/ Male	Doxorubicin (brand name Adriamycin)	15 mg/kg ip	Acute administration (a single injection)	Altered LVEF, LVFS and LV outflow	Values calculated manually by the authors of this review
Arozal W. *et al.*, 2010 (24)	25/ Sprague–Dawley rats / Male	Daunorubicin	3 mg/kg/day (18 mg/kg total dose)	Every other day for 12 days	Altered cardiac function (haemodynamic status and echocardiography)	Values provided in the manuscript
Argun M, *et al.*, 2016 (25)	4/0 10-week-old Wistar albino rats/ Male	Doxorubicin	4 mg/kg/dose to a cumulative dose of 16 mg/kg, ip	Twice a week for 2 weeks	Parameters monitored: • Serum BNP and C-type natriuretic peptide • LV functions by echocardiography and histological assessment	Values provided in the manuscript
Tatlidede E. *et al.*, 2009 (26)	32/ Wistar albino rats of both sexes	Doxorubicin	20 mg/kg, ip	Every other day for 2 weeks	Parameters monitored: BP and HR, echocardiography, Lactate dehydrogenase	Values provided in the manuscript

Razmaraii N. *et al.*, 2016 (27)	24/ Adult Wistar rats/ Male	Doxorubicin	2 mg/kg/ 48 h	Over a 12-day period	Parameters monitored: LVSP, LVDP, rate of rise/drop of LV pressure, LVEF, LVFS, contractility	Values provided in the manuscript
Gziri MM *et al.*, 2013 (28)	43/ Pregnant Wistar rats/ Female	Doxorubicin	10 or 20 mg/kg i.v.	On 18th day of pregnancy	Altered left ventricular function	Values provided in the manuscript
Oliveira LF *et al.*, 2017 (29)	29/ Adult Wistar rats/ Male	Doxorubicin	accumulated doses of 8(n= 8), 12(n= 7), and16 (n= 7) mg/kg, ip	Four weekly injections over 8 weeks	Myocardial fibrosis Altered left ventricular systolic function	Values provided in the manuscript
Carvalho PB *et al.*, 2016 (30)	64/ Wistar rats / Male	Doxorubicin	20 mg/kg, ip	Acute administration (a single injection)	LVEF monitored	Values provided in the manuscript
Stewart LK *et al.*, 2019 (31)	72/ Sprague Dawley rats/ Male	Doxorubicin	15 mg/kg, ip	Acute administration (a bolus injection)	Parameters monitored: LV septal and PWT, LVESd, LVEDd, mitral and aortic valve blood flow profiles, heart dimensions	Values provided in the manuscript

Polegato BF *et al.*, 2015 (32)	35/ Wistar rats/ Male	Doxorubicin	20 mg/kg, ip	Acute administration (a single dose)	Parameters monitored: LVFS, isovolumetric relaxation time and myocardial passive stiffness	Values provided in the manuscript
Lee KH *et al.*, 2017 (33)	20/ Sprague Dawley rats/ Male	Doxorubicin	Cumulative dose: 20mg/kg, ip	Once every two days for 6 times	Impaired LV function and performance	Values calculated manually by the authors of this review
Cheah HY *et al.*, 2017 (34)	29/ Wistar rats/ Male	Doxorubicin	5mg /kg, i.v.	Acute administration (a single dose)	Parameters monitored: BP, HR, LVED volume, other echocardiographic parameteres	Values provided in the manuscript
Li X. *et al.*, 2019 (35)	48/ Adult Sprague–Dawley rats /Male	Doxorubicin	Cumulative dose:16 mg/kg, ip	Over a 4-week period	Parameters monitored: serum BNP level LVEDd, LVESd, LVEF, LVFS	Values provided in the manuscript
Dundar HA *et al.*, 2016 (36)	28/ Adult Wistar albino rats /Female	Doxorubicin	15 mg/kg, ip	Acute administration (a single dose)	Parameters monitored: LVIDd and LVISd) via the parasternal long axis two-dimensional images. LVFS and LVEF	Values provided in the manuscript

Barış VÖ *et al.*, 2019 (37)	31/ Sprague–Dawley rats/ Male	Doxorubicin	25 mg/kg, ip	For 12-14 days	Parameters monitored: left ventricular ejection fraction (LVEF), LVFS and mitral lateral annulus (s') velocity + left ventricular end-diastolic and end-systolic diameters	Values provided in the manuscript
Lu PP *et al.*, 2016 (38)	60/ Sprague–Dawley rats /Male	Doxorubicin	2.5 mg/kg/week, ip	For 6 weeks	Parameters monitored: LVFS and LVEF	Values provided in the manuscript
O'Connell JL *et al.*, 2017 (39)	115/ Adult Wistar rats/ male	Doxorubicin	2.5 mg/kg, ip (cumulative dose 15 mg/kg) 2 mg/kg, ip (cumulative dose 18 mg/kg)	6 doses over a period of 2 weeks Once a week for 9 weeks	Parameters monitored: left ventricular systolic and diastolic dimensions and EF	Values provided in the manuscript
Chang SA *et al.*, 2015 (40)	71/ Sprague-Dawley rats/ *not mentioned (nm)*	Doxorubicin	3 mg/kg/day, intravenously (iv)	Once a week for 6 weeks	Parameters monitored: SWT and PWT, LVED dimensions, LVES dimensions, LVEF	Values provided in the manuscript

Teng LL *et al.*, 2010 (41)	46/ Sprague–Dawley rats /Male	Doxorubicin	2 mg/kg, ip	Once a week for 8 weeks	Parameters monitored: LVED dimensions, LVES dimensions, FS	Values provided in the manuscript
Kim YH *et al.*, 2012 (42)	61/ Sprague-Dawley rats/ Male	Doxorubicin	1.25 mg/kg, ip	Every other day for 1 month (16 times)	LV systolic/diastolic dysfunction	Values provided in the manuscript
Kondru SK *et al.*, 2018 (43)	24/ Wistar rats/ Male	Doxorubicin	2 mg/kg, ip	Once in a week for 5 weeks	Myocardial dysfunction	Values calculated manually by the authors of this review
Moriyama T. *et al.*, 2016 (44)	66/ Crl:CD(SD) rats / Male	Doxorubicin	2mg/kg, iv	once weekly, for 6 weeks	Parameters monitored: LVEDd, LVESd, LVFS	Values provided in the manuscript
Burdick J. *et al.*, 2015 (45)	20/ Crl:CD(SD) rats/ Male	Doxorubicin	2 mg/kg, ip	Once a week for 6 weeks	Parameters monitored: LVFS	Values calculated manually by the authors of this review

Ammar HI *et al.*, 2015 (46)	50/ Wistar rats / Male	Doxorubicin	2.5 mg/kg, i.p	3 times a week for 2 weeks	Parameters monitored: LVED dimensions) and LVSD dimensions, FS	Values calculated manually by the authors of this review
Calvé A. *et al.*, 2012 (47)	21 / Sprague-Dawley rats Female	Doxorubicin	3 mg/kg	Acute administration (on postnatal day 26th)	Parameters monitored: IVSd, LVPWd, LVIDd, LVISd	Values provided in the manuscript
Shen LJ. *et al.*, 2016 (48)	150/ Sprague-Dawley (SD) rat/ Male	Doxorubicin	1mg/kg, ip 2 mg/kg, ip (cumulative dose 12 mg/kg)	Twice a week Once a week for 6 weeks	Parameters monitored: LVESd, LVEDd, LVEF	Values provided in the manuscript
Wu Z. *et al.*, 2019 (49)	32/ Sprague-Dawley rat /Male	Doxorubicin	2.5 mg/kg, ip (cumulative dose 15 mg/kg)	Every second day for 6 times	Parameters monitored: LVEDP, LVESP and left ventricular pressure (±dP/dtmax), LVEF and LVFS	Values calculated manually by the authors of this review
Shoukry HS *et al.*, 2017 (50)	32/ Wister rats / Male	Doxorubicin	2.5 mg/kg, ip	2 weeks	Parameters monitored: LVIDd, LVIDs, LVFS and LVEF	Values calculated manually by

						the authors of this review
Niu QY *et al.*, 2016 (51)	26 / Sprague Dawley (SD) rats / Male	Doxorubicin	Each dose consisted of 1, 1, 2, 2, 3, 3, 4 and 4mg/kg, ip (cumulative dose 20 mg/kg)	For 2 weeks on days 1st, 3rd, 5th, 7th, 9th, 11th, 13th and 15th, respectively	Parameters monitored: IVSd, IVSs, LVPWd and LVPWs, LVIDd, LVIDs were measured on left ventricular long-axis areas. LVEF and LVFS	Values provided in the manuscript
Boutagy NE *et al.*, 2018 (52)	20/ Wistar rats (Crl:Wl) /Male	Doxorubicin	2.15 mg/kg, ip (cumulative dose 15 mg/kg)	Every 3 days for 21 days	Impaired systolic function and LV volumes and dimensions, Parameters monitored: echocardiographic variables (LVEF, global longitudinal strain, global radial strain, LVEDV, LVESV, relative PWT	Values calculated manually by the authors of this review
Lee PJ *et al.*, 2014 (53)	150/ Fischer rats / Male	Doxorubicin	2.5 mg/kg, ip (cumulative dose 15 mg/kg)	Every other day for 2 weeks	Altered LV function Parameters monitored: LVFS, LVEDd and LVESd, LV end diastolic volume (LVEDV), right basal ventricular diastolic	Values calculated manually by the authors of this review

					diameter (RVD1), and the RV fractional area change (RVFAC)	
da Silva MG *et al.*, 2012 (54)	52/ Wistar rats / Female	Doxorubicin	1.25 mg/kg, ip	Three times a week for 2 weeks	Parameters monitored: aorta-to-left atrial diameter ratio, LVESd, LVEF	Values calculated manually by the authors of this review
Mao C. *et al.*, 2017 (55)	160/ Sprague-Dawley rats / Male	Doxorubicin	2 mg/kg, ip	Once a week for 8 consecutive weeks	Parameters monitored: LVEDd, LVESd, LVPWT, interventricular septum thickness (IVST), LVEF, LVFS	Values provided in the manuscript
Deng B. *et al.*, 2017 (56)	42/ Sprague-Dawley rats/ Male	Doxorubicin (brand name Adriamycin)	2.5 mg/kg, ip (cumulative 15 mg/kg)	6 injections over 2 weeks	Parameters monitored: LV dimensions, LVFS, LVEF	Values calculated manually by the authors of this review
Bertinchant JP *et al.*, 2003 (57)	45/ Wistar rats/Male	Doxorubicin	1.5 mg/kg, iv, (cumulative dose 12 mg/kg)	Once a week for up to 8 weeks	Parameters monitored: LVEDd, LVESd and LVFS	Values provided in the manuscript

Sun R. *et al.*, 2017 (58)	70/ Sprague-Dawley rats / Male	Doxorubicin	2.5 mg/kg, ip	Once a week for 6 consecutive weeks	Parameters monitored: LVEF, LVEDd, LVESd and LVFS	Values provided in the manuscript
Guerra J. *et al.*, 2005 (59)	12/ SHR rats / Male	Doxorubicin	1.5 mg/kg, ip (cumulative dose 13.5 mg/kg)	Once a week for 9 weeks	Parameters monitored: LVEDd, LVESd and LVEF	Values provided in the manuscript
Gao Y *et al.*, 2017 (60)	90/ Wistar albino rats / Male	Doxorubicin	2 mg/kg, ip	Every 3 days for 30 days	Parameters monitored: The interventricular septal thickness at diastole, left ventricular internal diameter in diastole and systole, LVPWd at diastole, EF, FS	Values calculated manually by the authors of this review
Chen Y *et al.*, 2015 (61)	60 / Sprague-Dawley rats / Male	Doxorubicin	2.5mg/kg, ip	6 injections over 2 weeks	Parameters monitored: LVAW, LVPWT, LVIDd were measured in systole and diastole. EF, FS and LV volume at end-systole and end-diastole	Values calculated manually by the authors of this review

Li H *et al.*, 2016 (62)	56 / Sprague -Dawley rats /Male	Epirubicin	8 mg/kg, ip	Every five days for a total of three injections	Parameters monitored: LV dimensions and wall thickness, EF, FS	Values calculated manually by the authors of this review
Schwarz ER *et al.*, 1998 (8)	60/ Sprague-Dawley rats / Female	Doxorubicin (brand name Adriamycin)	2.5 mg/kg, iv	Once a week for 10 weeks	Left ventricular end-systolic and end-diastolic diameters, FS	Values provided in the manuscript
Leontyev S. *et al.*, 2013 (63)	46/ Sprague–Dawley/ Male	Doxorubicin	2.5 mg/kg, ip	Once a week for 9 weeks	LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) + Fractional shortening (FS)	Values provided in the manuscript
Merlet N. *et al.*, 2013 (64)	158 / Sprague-Dawley rats / Male	Doxorubicin	2.5mg/kg, ip (total 15 mg/kg)	6 injections over 2 weeks	LV end diastolic and systolic diameters (LVEDD and LVESD), diastolic posterior wall thicknesses (dPWth). + LV end diastolic and systolic volumes (LVEDV and VESV) to assess LV ejection fraction (LVEF),whereas LV shortening fraction (LVSF)	Values calculated manually by the authors of this review

Ozkanlar Y. *et al.*, 2014 (65)	40 / Sprague–Dawley rats / Male	Doxorubicin	2.5 mg/kg, iv	Once a week for 3 weeks	Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening(LVFS)	Values provided in the manuscript
Hong YM *et al.*, 2017 (66)	12/ Sprague-Dawley rats /Male	Doxorubicin (brand name Adriamycin)	5 mg/ week	Once a week for 3 weeks	Fractional shortening (FS) and ejection fraction + interventricular septal dimension diastole; left ventricular internal dimension diastole; left ventricular posterior wall dimension diastole; interventricular septal dimension systole; left ventricular internal dimension systole; left ventricular posterior wall dimension systole;	Values provided in the manuscript
Teraoka K. *et al.*, 2000 (67)	75 / Wistar rats/ Male	Doxorubicin (brand name Adriamycin)	1 mg/kg, ip (cumulative dose 15 mg/kg)	15 times over a period of 3 weeks	Left ventricular diameter of the systole LVDs + Left ventricular diameter of the diastole LVDd. + %fractional shortening	Values provided in the manuscript

Hamed S. *et al.*, 2006 (68)	130/ Wistar rats (Harlan) / Male	Doxorubicin	Cumulative dose of 15 mg/kg	3 weeks	Left ventricular (LV) diameter in systole (LVIDs) LVIDd, LV diameter in diastole; IVSd, intra ventricular septum in diastole LV posterior wall thickness in diastole (LVPWd)	Values provided in the manuscript
Gabrielson KL *et al.*, 2008 (69)	21/ Sprague-Dawley rats / Female	Doxorubicin	Cumulative dose of 15 or 7.5 mg/kg	Six or three weekly doses, respectively	interventricular septum diastole (IVSD) and left ventricular posterior wall thickness at end diastole (PWTED) + LV chamber diameters were measured at the end of diastole (LVEDD) and systole (LVESD). EF%	Values calculated manually by the authors of this review
Yu Q. *et al.*, 2008 (70)	63/ Sprague–Dawley rats/ Male	Doxorubicin	2.5 mg/kg, ip	Once a week for 6 weeks	LV shortening (LVFS) was calculated as (LVEDD-LVESD)/LVEDD 9 100, where	Values provided in the manuscript

					LVEDD is LV end-diastolic diameter and LVESD is LV end-systolic diameter +LV ejection fraction	
Bai J. *et al.*, 2014 (*in Chinese)* (71)	Rats	Doxorubicin	6 injections total 15 mg/kg)	within 2 weeks	LVEF; LVFS; LVEDD and LVESD	Values provided in the manuscript
Lu XL *et al.*, 2015 (72)	48/ Sprague-Dawley rats/ Male	Doxorubicin	1 mg/kg on the 2nd and 4th days, 2 mg/kg on the 6th and 8th days, 3 mg/kg on the 10th and 12th days, and 4 mg/kg on the 14th and 16th days, ip		left ventricular internal end-diastolic diameter (diastolic LVID)and the posterior wall end-diastolic thickness (diastolic LVPW) + LV diastolic volume (diastolic LVV) and function indexes (stroke volume, EF and FS)	Values calculated manually by the authors of this review
Wachtman LM *et al.*, 2006 (73)	30 / Sprague-Dawley rats / Female	Doxorubicin	2.5 mg/kg, iv	Once a week for a total of 6 doses	FS	Values provided in the manuscript

Zhang J. *et al.*, 2013 (74)	40 / Wistar outbred rats / Male	Doxorubicin (brand name Adriamycin)	2.5 mg/kg, ip (total 15 mg/kg)	Three times per week for one week. After a two-week interval, administration for another week. These steps were conducted six times	the left ventricular end-systolic diameter (LVSD), the left ventricular end-diastolic diameter (LVDD), the left ventricular end-systolic volume (LVSV) and the left ventricular end-diastolic volume (LVDV) + The left ventricular ejection fraction (LVEF) and the left ventricular shortening fraction (LVFS)	Values provided in the manuscript
Chen X. *et al.*, 2007 (75)	39/ Wister rats / Male	Doxorubicin	2.5 mg/kg,ip	Six times for 2 weeks	LV end diastolic diameter (LVEDD), LV end systolic diameter (LVESD)and ejection fraction (EF) + Fractional shortening (FS) + LV systolic pressure (LVSP), LV end diastolic pressure (LVEDP), LV maximum dP/dt and LV minimum dP/dt	Values provided in the manuscript

Ha JW *et al.*, 2005 (76)	60 /Wistar rats / Male	Doxorubicin (brand name Adriamycin)	2 mg/kg, iv	Once a week for 2, 4, 6 or 8 weeks, consecutively	Left ventricular (LV) performance LV dimensions (end-diastolic and end-systolic diameter) + EF	Values calculated manually by the authors of this review
Emanuelov AK *et al.*, 2010 (77)	40/ Sprague–Dawley rats / Male	Doxorubicin	2.5 mg/kg, ip (total 15 mg/kg)	Every second day for a period of 2 weeks	Left ventricular systolic pressure (LVSP) Diastolic and systolic LV wall thickness,LV end-diastolic diameter (LVEDD), and LV end-systolic diameter(LVESD) were measured + percent LV FS	Values calculated manually by the authors of this review
Lim SC, 2013 (78)	52/ Sprague-Dawley rats / Male	Doxorubicin	2.5 mg/kg, ip	Six times over 2 weeks	LVES dimensions, LVED dimensions, LVFS.	Values provided in the manuscript
Hydock DS *et al.*, 2008 (79)	147 / Sprague–Dawley rats/ Male	Doxorubicin	10 mg/kg, ip	Acute administration (bolus injection)	SWT during systole (SWs) and diastole (SWd), PWT and PWT during diastole (PWd), LVEDd, LVESd, FS	Values calculated manually by the authors of this review

Xiang P. *et al.*, 2009 (80)	37/ Sprague–Dawley rats/ Male	Doxorubicin	2.5 mg/kg, ip	Once a week for 6 weeks	Left ventricular end-diastolic dimensions (LVEDD) and left ventricular end-systolic dimensions (LVESD) + LV fractional shortening (% FS)	Values provided in the manuscript
Kenk M. *et al.*, 2010 (81)	94/ Sprague-Dawley rats /Male	Doxorubicin (brand name Adriamycin)	2.5 mg/kg, ip (total 15 mg/kg)	6 injections over 2 weeks	left ventricle internal diameter (left ventricle diastolic and systolic dimensions; LVDD and LVSD), left ventricle posterior wall (LVPW), and intra-ventricular septum (IVS) thickness at end diastole and peak systole. →left ventricular volume in diastole and systole (LVDV, LVSV), stroke volume (SV), EF,FS, and left ventricular mass (LV mass)	Values provided in the manuscript
Katona M. *et al.*, 2004 (82)	23/ Adult Wistar rats / Male	Doxorubicin (brand name Adriamycin)	2.5 mg/kg , ip	Three times a week for 2 weeks	Parameters monitored: LVDDd and LVSDd, FS, LAD, AOD	Values provided in the manuscript

Hydock DS *et al.*, 2011 (83)	49 / Sprague-Dawley rats / Female	Doxorubicin	1.5 mg/kg i.p of (cumulative 15 mg/kg).	Once a day for 10 consecutive days	septal wall thickness at systole (SWs) and diastole (SWd), posterior wall thickness at systole (PWs)and diastole (PWd), LV end-systolic diameter (LVDs) and LV end-diastolic diameter (LVDd), and FS	Values provided in the manuscript
Hou XW *et al.*, 2006 (84)	40/ Wistar rats / Male	Doxorubicin (brand name Adriamycin)	2.5 mg/kg, ip	6 times for 2 weeks	LV dimensions(end-diastolic diameter [LVDd] and end systolic diameter[LVDs]) + % FS of the LV	Values provided in the manuscript
Hydock DS *et al.*, 2011 (85)	74/ Sprague–Dawley rats / Male	Doxorubicin	1 mg/kg, ip (total 10 mg/kg)	Once a day for 10 consecutive days	septal wall thickness at systole (SWs) and diastole (SWd), posterior wall thickness at systole (PWs) and diastole (PWd), LV end-systolic diameter (LVDs) and LV end-diastolic diameter (LVDd).	Values provided in the manuscript

					+ FS, LV mass and relative wall thickness (RWT).	
Koh E. *et al.*, 2004 (86)	33/ Wistar rats / Male	Doxorubicin (brand name Adriamycin)	2mg/kg , iv	Once a week for 8 weeks	LV dimensions [the end-diastolic diameter (LVDd), end-systolic diameter (LVDs), the intraventricular septal thickness, and the LV posterior wall thickness] + % FS of LV atrial natriuretic peptide; brain natriuretic peptide;	Values provided in the manuscript
Carresi C. *et al.*, 2018 (87)	40 / Wistar rats / Male	Doxorubicin	2,5 mg/kg, ip	6 times for 2 weeks	LVESd; LVEDd; IVSs; IVSd, LVPWs and LVPWd; EF; FS;	Values provided in the manuscript
Ma H. *et al.*, 2017 (88)	190 / Wistar rats / Male	Doxorubicin	2,5 mg/kg, ip	6 times for 2 weeks	Left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) + FS + EF	Values provided in the manuscript
Zhang XJ *et al.*, 2017 (89)	26 / Sprague‑Dawley rats/ Male	Doxorubicin	4 mg/kg, ip (cumulative dose 16 mg/kg)	Twice per week for 2 weeks	Diastolic interventricular septum thickness (IVSTd), systolic	Values calculated manually by

					interventricular septum thickness (IVSTs), + EF + FS	the authors of this review
Sun XP *et al.*, 2017 (90)	32/ Sprague–Dawley rats /Male	Doxorubicin	20 mg/kg, ip 5.0 mg/kg, iv	Acute administration (single dose)	(LVEF) from end-diastolic volume (EDV)and end-systolic volume (ESV), + EDV and ESV + LVFS	Values provided in the manuscript
Zhu HJ *et al.*, 2016 (91)	50/ Adult Sprague-Dawley rats/ Male	Doxorubicin	2 mg/kg/week	6 weeks	ejection fraction	Values provided in the manuscript
Croteau E. *et al.*, 2014 (92)	12/ Fisher rats / Male	Doxorubicin	2 mg/kg, iv	Once a week for 6 weeks	Left ventricular function Left ventricle ejection fraction	Values provided in the manuscript
Ikegami E. *et al.*, 2007 (93)	14 / Sprague – Dawley / NM	Doxorubicin	2.5 mg/kg, ip	3 times a week for 2 to 6 weeks	left ventricular diastolic diameter (LVDd) and left ventricular fraction shortening (LVFS) + FS	Values provided in the manuscript
Hiona A. *et al.*, 2011 (94)	24 / Sprague Dawley rats / Female	Doxorubicin	cumulative dose of 25 mg/kg, ip	Once a week for 6 weeks	LVFS	Values provided in the manuscript

Tang DX *et al.*, 2013 (95)	40 / Sprague-Dawley rats / Male	Doxorubicin	2.5 mg/kg, ip	Once a day for a total of 6 times	Parameters monitored: LVEF, left ventricular internal diameter at end-diastole (LVIDd), left ventricular internal diameters at end-systole (LVIDs), left ventricular posterior wall at diastole (LVPWd), left ventricular posterior wall at systole(LVPWs), left ventricle %EF, and left ventricle %FS	Values provided in the manuscript
Migrino RQ *et al.*, 2008 (96)	31/ Sprague Dawley rats / Male	Doxorubicin	2.5 mg/kg, iv	Once a week for 10 or 12 weeks	FS monitored	Values provided in the manuscript
Liu Y *et al.*, 2018 (97)	24/ Sprague–Dawley rats / Male	Doxorubicin (brand name Adriamycin)	Each dose consisted of 1, 1, 2, 2, 3 and 3 mg/kg , ip (cumulative dose 12 mg/kg)	At 1st, 3rd, 5th, 7th, 9th and 11th day, respectively	Parameters monitored: interventricular septum thickness of systolic, IVSd, LVIDd, LVISd, LVPW, LVPWd, EF, FS	Values provided in the manuscript

Liu X. *et al.*, 2006 (98)	120/ Sprague Dawley Rats/*nm*	Doxorubicin	3.3 mg/kg, iv	Once a week for 4 weeks		Values provided in the manuscript

LV: Left ventricular; (LV)EF: (Left ventricular) ejection fraction; (LV)FS: (Left) ventricular fractional shortening; BNP: brain natriuretic peptide; PWT: Posterior wall thickness; AWT: Anterior wall thickness; SWT: Septal wall thickness; BP: Blood pressure; HR: heart rate; LVSP: Left ventricular systolic pressure; LVDP: Left ventricular diastolic pressure; LVEDd: Left ventricular end-diastolic diameter; LVESd: Left ventricular end-systolic diameter; LVEDV: Left ventricular end-diastolic volume; LVIDd: Left ventricular internal diastolic diameter LVISd: Left ventricular internal systolic diameter; LVPWs: left ventricular systolic wall thickness; LVPWd: left ventricular diastolic wall thickness; IVSd: Intraventricular septum in diastole; LAD: left atrial diameter; AOD: aortic diameter

Figure legends

Figure 1. Prisma flow chart (literature search) for the present study design

Figure 2. Normal (baseline) Left Ventricular Ejection Fraction (LVEF) values in rats before anthracycline administration as reported in 57 relevant studies reviewed in the present report

Figure 3. Normal (baseline) Left Ventricular Fractional Shortening (LVFS) values in rats before anthracycline administration as reported in 80 relevant studies reviewed in the present report

Figure 4. Suppressed Left Ventricular Ejection Fraction (LVEF) values in rats due to anthracyclines toxicity as reported in 54 relevant studies reviewed in the present report

Figure 5. Suppressed Left Ventricular Fractional Shortening (LVFS) values in rats due to anthracyclines toxicity as reported in 78 relevant studies reviewed in the present report

Figure 6. Chemical structures of the three anthracyclines used to induce cardiotoxicity in the studies reviewed in the present report

Figure 7. Percentiles distribution of % suppression of Left Ventricular Ejection Fraction (LVEF) and Left Ventricular Fractional Shortening (LVFS) due to anthracyclines toxicity as mentioned in the studies reviewed in the present report

Figure 8. Scatter plot of normal (baseline) and suppressed Left Ventricular Ejection Fraction (LVEF) values in rats due to anthracyclines toxicity as reported the studies reviewed in the present report

Figure 9. Scatter plot of normal (baseline) and suppressed Left Ventricular Fractional Shortening (LVFS) values in rats due to anthracyclines toxicity as reported in the studies reviewed in the present report

Figure 10. Normal and suppressed Left Ventricular Ejection Fraction (LVEF) and Left Ventricular Fractional Shortening (LVFS) values for the two main rat strains used in the studies reviewed in the present report

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CHAPTER 4

Cardiotoxicity of chemical substances: an emerging hazard class

The work presented in Chapter 4 is included in the following article (under review):

Georgiadis N, Konstantinos Tsarouhas, Jean-Lou C.M. Dorne, George E. N. Kass, Petroula Laspa, Konstantinos Toutouzas, Dimitrios Kouretas, Christina Tsitsimpikou. *J. Cardiovasc. Dev. Dis.* **2022**, *9*, x. https://doi.org/10.3390/xxxxx

4.1 Introduction

Human health risks and hazards from chemical substances are well regulated internationally. When the available data meet the established classification criteria for the different regulated hazards as they are defined by the international legislations, a certain hazard class and category is assigned accordingly. Classification criteria generally include well defined endpoints, reference/ threshold values from animal studies with relevance to humans, along with criteria for epidemiological and clinical data from various populations. Special provision with regards the criteria development for classification is given to vulnerable population groups, such as workers, pregnant women, children etc. The hazard classes in general cover physical, environmental and human health hazards. It should be noted that all chemical substances, pharmaceutical active substances and biocides included, fall within the scope of harmonised classification. More specifically for human health hazards the most important classification hazard classes are listed below:

- Acute toxicity (oral, dermal, inhalation)
- Skin corrosion / skin irritation
- Serious eye damage / eye irritation
- Respiratory sensitisation
- Skin sensitisation
- Mutagenicity
- Carcinogenicity
- Toxicity for reproduction
- Specific target organ toxicity (STOT) (single exposure, SE)
- Specific target organ toxicity (STOT) (repeated exposure, RE)
- Aspiration hazard

However, cardiotoxicity, is not defined as a stand-alone hazard and therefore there are no defined criteria for classification of substances as cardiotoxic. Hence, from a regulatory point of view identification and regulation of substances, which cause cardiovascular adverse effects cannot be enforced and among others an opportunity to strengthen the national health systems remains unused. It has been estimated that at least 23% of all cardiovascular pathologies could be attributed to environmental exposures, mainly chemicals. Nevertheless, the causative agents remain largely uncharacterized [1].

More specifically, in the Classification Labelling and Packaging (CLP) European Regulation (Reg 1272/2008/EC), cardiotoxicity may be partially covered within the STOT hazard class based only on expert judgment of the evaluator toxicologist, since specific criteria

are not available in order to assess findings from animal studies or early clinical manifestations in humans. For other human organs, STOT criteria have been developed within the framework of CLP for toxic damage caused by chemicals, such as lungs, liver, kidneys, endocrine system, etc. It must be noted that no animal testing method for cardiotoxicity is currently available in Organisation for Economic Co-operation and Development (OECD) Testing Guidelines and cardiovascular measurements are not included in the current evaluation programs of environmental chemicals[1]. Hence, potentially cardiotoxic substances or products are not restricted at a regulatory level.

Specific classification criteria should be used in a weight of evidence approach for the assessment of cardiotoxicity of chemicals, and thus, reduce cardiovascular adverse effects in the general population after exposure to chemicals.

Classification should be based on the following scientific evidence (findings), in a way that would reduce uncertainties:

a. Anatomical and histopathological data,

b. Echocardiographic data on contractility (e.g., LVEF, LVFS), documentation of cardiac frequency and/or implementation of other cardiac imaging modalities (e.g., MRI)

c. Biochemical data, of generic nature (e.g., circulating oxidative stress markers), of more specific nature (e.g., oxidative stress markers of the cardiac tissue) and heart specific biomarkers (e.g. cardiac enzymes)

d. Identification of pathways and mode of actions, which modulate the changes observed in different parameters after exposure to chemicals and

e. In silico data, such as adverse outcome pathways (AOPs), omics, in vitro, organs on a chip, physiologically based pharmacokinetic models (PBPK), etc.

The authors after having recognized the regulatory gap in describing cardiotoxicity as a separate hazard class for chemicals, try to describe a methodological approach (suggested roadmap) for collection of animal evidence to be applied in the future by regulators and scientists in order to identify cardiotoxic chemicals from a regulatory perspective and, consequently, endorse in the legislation measures to protect human health form relevant exposure. In this context, the authors discuss respective preliminary results obtained from their research group (published and unpublished data) on specific indices and biochemical markers showing that they should be further investigated in order to set regulatory criteria and highlight the need for targeted research to this end.

4.2 Current definition of Cardiotoxicity

Cardiotoxicity has so far been mainly linked to side effects to humans after use of pharmaceuticals and it can be diagnosed in individuals' post-exposure, at the time of established clinical manifestations, which at that stage could even be irreversible[2-6]. The most common class of drugs known for cardiotoxic side-effects is anthracyclines.

Anthracyclines are used in cancer therapy and they are isolated from Streptomyces bacterium. They are used for treating different cancers, including leukemias, lymphomas, as well as breast, stomach, uterine, ovarian, bladder cancer, and lung cancers[7-9]. The first anthracycline discovered was daunorubicin. It is produced by an actinobacterium, Streptomyces peucetius. Clinically, the most important anthracyclines are doxorubicin, daunorubicin, epirubicin and idarubicin. Anthracyclines are well-known to cause myocardial suppression in patients going through the aforementioned therapies. In this sense, they are used in animal studies as an applicable method to introduce a model of dilated cardiomyopathy[10].

Cardiotoxicity is not restricted to anticancer agents. Chronically administered drugs, such as neurologic/psychiatric agents, also represent a major problem because cardiotoxicity may become evident only after long-term accumulation of the drug or its metabolites. Strikingly, almost 10% of drugs in the last four decades have been recalled from the clinical market worldwide due to cardiovascular safety concerns [11]. Currently, assessing the cardiotoxicity potential is a crucial parameter in drug development.

In humans, cardiotoxicity describes the deterioration of myocardial function post cancer treatment manifested by myocardial dysfunction and in several cases overt symptoms of heart failure. Echocardiography is the standard method to ascertain the presence of cardiotoxicity which can be manifested as acute, early or late. The cut-off values of echocardiographic indices in humans for the identification of cardiotoxicity caused by chemotherapeutics differ between the American and European guidelines: the definition considers a lower cut-off value of normality for the left ventricle ejection fraction (LVEF) of 50% in Europe[12] and 53% in the USA[13]. It should be stressed that these scientific guidelines indicate that a drop of LVEF compared to the patient's previous values is also required. This is of high importance since patients presenting this decline in cardio-imaging indices of cardiac function should be treated with angiotensin converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs) in combination with beta-blockers[14]. However, there is a debate among the experts related to possible modifications of anticancer treatment in patients who meet the aforementioned criteria. The above-described definition of cardiotoxicity post-cancer treatment, for simplicity reasons, does not account for indices of myocardial injury or specific cases of cardiovascular disease of different origin, such as coronary artery disease, despite the growing evidence for an etiological association with cancer treatment[12]. Nevertheless, assessing drug-induced

cardiotoxicity risk including QT interval prolongation, for example, is considered nowadays an integral part of the standard preclinical evaluation of new chemical entities as defined by the International Conference of Harmonization Expert Working Group for all drugs in development [15].

4.3 Roadmap for identifying regulatory criteria on cardiotoxicity based on animal studies

The identification process for setting specific criteria for cardiotoxic chemicals within a regulatory framework should use both animal and human data elaborating the set of findings mentioned above. Human data always have precedence over animal data but are produced or collected after manifestation of the cardiotoxic effects in humans, usually years post-exposure. On the other hand, animal data could be produced in advance and if the appropriate criteria are set, the effects in humans can be accurately predicted based on toxic manifestations in animals.

In order to develop effective criteria, "standard" cardiotoxic substances should be used to produce reference values in animals. Anthracyclines, as explained previously, could represent such a "standard" chemical. Regarding dosage, several schemes have been applied in the literature for the development of cardiotoxicity by anthracyclines in animals, including various dose regimes, different anthracyclines, as well as different animal species [16]. Dosing schemes represent both acute and chronic toxicity and are equally relevant for classification purposes. To monitor cardiotoxicity caused by anthracyclines, cardiac imaging is primarily used and secondarily, biochemical markers. It is important to stress that for recognizing cardiotoxicity induced by anthracyclines, the specific doses, although recorded, were not essential, since the final effect on heart functioning has been of interest at this stage. At a later stage, dosing could be of im-portance in identifying the threshold of the adverse effects observed.

In this context, in a previous in-depth review and following a systematic literature search [17], the identification of most used measurements of myocardial function in rats of anthracycline induced cardiotoxicity are presented, together with the range of these values differentiating normal cardiac function from animals with pathological echocardiographic findings indicative of anthracycline cardiotoxicity as per author presentation. At this stage, statistically significant differences are hard to derive, since a meta-analysis has not yet been performed. Therefore, a more descriptive statistical approach of recognizing extreme values and overlapping ranges between exposed and control animals has been applied.

The first attempt to identify a standard animal model focused in rats. Nevertheless, more research is needed to study strain differences as well as different species (i.e., rabbits) with more relevance to humans. The abundance of available studies should also be taken into account, since using already existing data is consistent with animals' welfare principles.

After having established a “standard” cardiotoxic chemical for reference and having described a range of values representing toxicity for various parameters monitored, the applicability of the identified value ranges should be tested in different substances, such as anabolic androgen steroids (AAS), industrial chemicals, for example metals and pesticides, which have been implicated in adversely affecting cardiac pathology causing function impairment.

In addition, the possible mechanisms of cardiotoxicity should also be described and associated with the monitored parameters (histopathological, echocardiographic, biochemical) in a causative way. Hence, information on already described mechanistic findings of alleged cardiotoxic chemicals could be of relevance.

More specifically, the pathophysiological mechanism of AAS cardiotoxicity is justified since androgen receptors are located both in the endothelium of the vascular smooth muscles and in the myocardium. Although the mode of actions have not been entirely defined, anabolic steroid abuse has been causally linked to effects such as hypertension, myocardial ischemia, left ventricular hypertrophy, sudden cardiac death even in consumers younger than 30 years, heart attack or stroke[18].

With regard to the pesticides the literature data suggest that several modes of actions can contribute to cardiotoxicity. More specifically, the most distinguished ones are: inhibition of carboxyl ester hydrolases (Organophosphates/ Carbamates), altering the function of voltage-gated sodium channels in insect neuronal membranes, thereby disrupting electrical signaling in the nervous system (Pyrethroids), ligand-gated ion channel activity (GABA-gated chlorine channel blockers) (Organochlorines), cellular hypoxia due to the effect on mitochondria, inhibition of cytochrome C oxidase and formation of highly reactive hydroxyl radicals (mitochondrial complex IV electron transport inhibitors) (Phosphides), blocking the cytochrome P450-dependent enzyme C-14 alpha-demethylase, which is needed to convert lanosterol to ergosterol (Triazoles), inhibition of primary events in photosynthesis in the (Photosystem I and II inhibition) (Triazines, Dipyridyl)[19].

Besides, cardiotoxicity with different mechanisms of action has been observed in industrial chemicals. More specifically, metals (e.g., platinum), cause direct injury on the myocytes, and cause mitochondrial ultrastructural abnormalities and platelet activation and aggregation. Cobalt causes cardiotoxicity via interference with energy production and contractile mechanisms along with nutrition and hypothyroidism. On the other hand, mercury is inducing cardiotoxicity by glutathione depletion, production of ROS, and interruption in selenium-dependent endogenous enzymatic reactions. Nanoparticles (i.e. titanium, zinc, silver, carbon,

silica and iron oxide nano-materials) induce cardiotoxicity via oxidative stress and inflammation, cellular apoptosis and decreased cell proliferation, decreased heart rate and down regulation of genes, like Myocyte Enhancer Factor 2C and a homeobox-containing transcription factor NKX2.5, functioning in heart formation and development[20].

Another representative example of an industrial chemical and its relation to cardiotoxicity is Ethanol. Cardiotoxicity outcome involves apoptosis, alterations of the excitation–contraction coupling in cardiomyocytes, structural and functional alterations of the mitochondria and sarcoplasmic reticulum, changes in cytosolic calcium flows, changes in calcium sensitivity of myofilaments, alterations of mitochondrial oxidation, deregulation of protein synthesis, decrease of contractile proteins and dis-proportion between the different types of myofibrils, changes in the regulation of myosin ATPase, up-regulation of the L-type calcium channels, increase of oxidative stress, and induction of ANP (atrial natriuretic peptide) and p21 (cyclin-dependent kinase inhibitor) mRNA expression in ventricular myocardium[21].

4.4 Evaluation of preliminary results in order to identify classification criteria

4.4.1 Echocardiography indices

Georgiadis et al. (2020) recently published a relevant comprehensive report (on echocardiographic data from animal models showing cardiotoxicity with relevance to humans, reviewing anthracyclines[17]. The most common measured indices are Left ventricle (LV) fractional shortening (FS) and (LV) Ejection fraction (EF) which are in-dices of left ventricular contractility. In Figure 1 the normal and altered values of the two main echocardiographic indices of anthracycline-treated and control animals, %EF and %FS, respectively, are presented.

There is a clear distinction between the normal LVEF values and the altered ones. More specifically, the normal EF values are ranging from 81.5±6.9% while the altered values are ranging from 59.3±9.5%. It is clear that even in the extreme values there is no overlap. With regards to the LVFS values, the normal FS values are ranging from 50.17% with a deviation of 8.61% while the altered values are ranging from 33.66% with a deviation of 8.49%. In the case of LVFS there is small overlap between the normal and altered values (Figure 1).

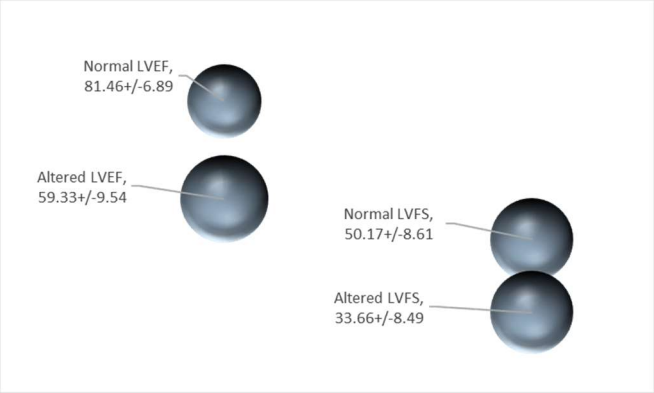

Figure 1. Mean LVEF and LVFS values from control animals (normal) and animals ex-posed to anthracyclines (altered) (data from [17] not previously published).

It should be highlighted that the usual strains used in the rat studies and reviewed in Georgiadis et al. (2020) are equally prone to the cardiotoxic anthracycline potential, regarding the drop of LVEF and LVFS observed.

4.4.2 Biochemical biomarkers

As a continuation of [17], an in-depth review analysis of several biomarkers al-tered in the specific animal models after anthracyclines administration, is being per-formed by our research group (data not yet published), in order to investigate which of them could potentially be used as biochemical criteria in a weight of evidence approach together with other lines of evidence, namely the echocardiography indices already presented. The indices for which values are being retrieved from the literature, in the framework of this project, are listed below:

Biomarkers of Oxidative stress

- Catalase (CAT)
- Malondialdehyde (MDA)
- Reactive oxygen species (ROS)
- Superoxide dismutase (SOD)
- Total antioxidant capacity (TAC)
- Total Oxidant Status (TOS)
- Glutathione (GSH)
- Glutathione peroxidase (GSH-Px)
- Lipid hydroperoxide (LH)

Biomarkers relevant to damage of the heart muscle

- Lactate dehydrogenase (LDH)
- Creatine kinase (CK)
- Creatine kinase-myocardial band isoenzyme (CK-MB)
- Cardiac troponin I (cTnI)
- Cardiac troponin T (cTnT)

Biomarkers relevant to increased ventricular blood volume and consequent response of cardiomyocytes to stretching

- Atrial natriuretic peptide (ANP)
- Brain natriuretic peptide (BNP)

Biomarkers of inflammation

- Interleukin-1 family members (IL-1)
- TNF alpha

Our preliminary results provided interesting findings. For example, for the im-portant clinically established specific cardiac enzyme, CK-MB, which is used to inform on adverse myocardial events with sufficient sensitivity and specificity, in a non-invasive way, the results are shown in Figure 2. The overall increase in CK-MB values of the rats exposed to anthracyclines at well-established cardiotoxic doses, compared to healthy rats, seem to follow the same pattern with respective echocardi-ographic measures. The vast majority (ca 80%) of the observed CK-MB values in an-thracyclines exposed rats show an increase from 50 to 200% compared to healthy rats (Figure 2). More detailed statistical analysis addressing significant associations is needed at a meta-analysis stage.

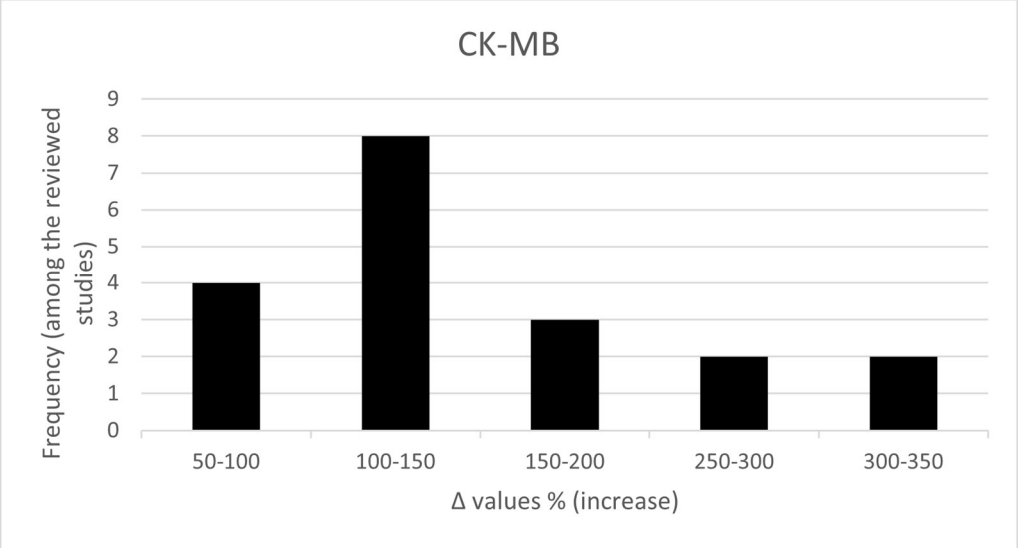

Figure 2. % Increase in CK-MB (%Δ values [(values of rats exposed to anthracyclines) – (values of control rats)]*100) in rats exposed to anthracyclines compared to control animals,

as reported in 19 relevant studies reviewed by Georgiadis et al 2020 [17] (data from Georgiadis PhD thesis not currently published).

%Δ values were calculated in order to overcome the diversity of measuring units used in the literature.

In addition, generic biomarkers of oxidative stress, both circulating and cardi-ac-tissue specific, which under certain circumstances could provide supporting evi-dence in hazard assessment of chemicals for cardiotoxicity are being reviewed. For example, there is a clear dependence of GSH values in the cardiac tissue upon the an-thracycline exposure of rats leading to cardiac dysfunction. More specifically, the overall change in cardiac tissue GSH seems to follow the changes reported for LVEF and shows a decrease ranging from 30 to 70% in the vast majority (83%) of rats with anthracyclines' caused cardiotoxicity compared to their healthy counterparts (Figure 3). However, it must be noted that whether biochemical markers can be combined with echocardiography and histopathology indices and findings in a scientific assessment is a complex issue and must be taken into account, when the lines of evidence are weighed in the context of a weight of evidence approach.

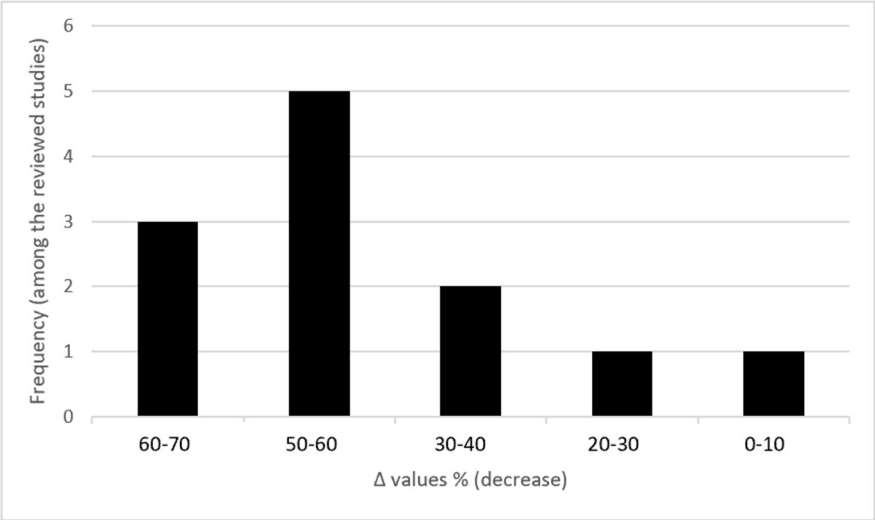

Figure 3. % Decrease in cardiac tissue GSH (%Δ values: [(values of rats exposed to anthracy-clines) – (values of control rats)]*100) in rats exposed to anthracyclines compared to control animals as they are reported in 12 relevant studies reviewed by Georgiadis et al (2020) [17] (data from Georgiadis PhD thesis not currently published).

%Δ values were calculated in order to overcome the diversity of measuring units used in the literature.

The statistical analysis both of the cardiac enzymes and of the biomarkers of ox-idative stress is on-going. Despite the fact that at the moment, for some markers the retrieved sample size is small, and consequently the statistical power of the analysis is limited, a similar pattern

of change is revealed between values of healthy rats and rats with cardiotoxic manifestations due to anthracyclines exposure. This is an important and encouraging finding which, when assessed together with echocardiographic in-dices and/or histopathological data, can significantly reduce the uncertainty and strengthen drastically the reliability of the weight of evidence assessment for possible cardiotoxicity in humans caused by chemicals.

Therefore, the preliminary results show that more centralized research, preferably coordinated by a regulatory agency is needed in order to effectively develop the set of classification criteria for cardiotoxicity.

4.5 Future perspectives and reflections

The diagnostic methods discussed so far in this manuscript are frequently used for several years. However, it must be noted that in the past years novel biomarkers of target organ toxicity have been widely used with significant applicability. More spe-cifically, tumor suppressive and oncogenic pathways have been found to involve mi-croRNAs (miRNAs) [22]. MicroRNAs which are noncoding RNAs that repress the ex-pression of target mRNAs in a post transcriptional way, including apoptosis, differen-tiation and cancer[23].

The measurement of plasma miRNAs and messenger RNAs (mRNAs) explain the ongoing physiologic processes in cells and tissues that package and release miRNAs into cell-free space. Moreover, miRNAs are non or minimally invasive, enhancing the animal welfare. Technologically, they are considered as ideal for quantitative analysis due to their standardization rapidness and robustness [24].

The aforementioned markers change significantly and early , after their release from tissues into the plasma during toxic events, which shows tissue-specific expression [25]. These advantages have increased the research interest for circulating miRNAs as promising biomarker candidates. They could hopefully play an essential role for hu-man health risk assessment. Another important element is the tissue-specificity and early release of circulating miRNAs upon tissue injury, when damage is still reversible.

Another important novel biomarker is the proto-typic oncogene c-MYC. It is be-lieved that miRNAs linked to c-MYC could be used in human health risk assessment.

Finally, Fatty Acid-Binding Protein (FABP) acts as a long-chain fatty acid carrier in blood and therefore has an essential role in lipid metabolism. The heart type isoenzyme is found in the heart and skeletal muscles and the clinical performance of free FABP is similar to that of myoglobin[26]. It is important to highlight that in a cohort of 19 can-cer patients, who underwent immune checkpoint inhibitors therapy (ICIs), FABP lev-els, were increased without

significant LVEF reduction, which could indicate that there might be a more sensitive biomarker to detect ICI-related subclinical myocardial damage than traditional cardiac biomarkers[27].

Acknowledging the existence of the aforementioned biomarkers, it must be noted that the current research project of our team aims to facilitate the use of biochemical markers in the hazard assessment of cardiotoxicity. Therefore, it was preferred to limit the review focus in more frequently used and traditional biomarkers that are already found in abundance in the literature and for which it is more potent to find available data. Moreover, the experience of risk assessment for newly appeared hazard classes, like endocrine disruptors, has shown that, especially for regulatory purposes, the as-sessors rely on older data for animal welfare reasons. In any case, it must be stressed that since in the recent years these newly identified biomarkers have been widely im-plicated in the research of different pathways, it should be further investigated in a different project how they could be applied in hazard assessment of cardiotoxic chemicals.

In the same line of progress, other methods than two-dimensional echocardiog-raphy (2DE) are currently available for LV function quantification, focusing on the early diagnosis of left ventricular dysfunction associated with chemotherapy, including 3-dimensional echocardiogram (3DE), cardiovascular magnetic resonance (CMR), and strain speckle-tracking echocardiogram. However, they require sophisticated tech-nology and advance medical and technical training compared with 2DE. Historically, multiple-gated acquisition scan (MUGA) has been one of the preferred methods for serial assessment of LVEF while on cancer therapy. LVEF determined by MUGA scan is more accurate and has more correlation with other 3-dimensional (3D) imaging modality (such as CMR) than echocardiography. The greatest limitation of MUGA scan has been the exposure to radiation, which will cumulate when performing the serial scans during the course of chemotherapy [28]. Regarding monitored parameters of cardiac function, global longitudinal strain (GLS) is a newly emerging topic, which has a significant role in predicting cardiovascular outcomes, compared to LVEF. Abnormal GLS is indicative of subclinical left ventricular systolic dysfunction, which for the purposes of regulatory setting of criteria for the early recognition of cardiotoxicity could be proven very useful. GLS imaging is underutilized in the detection of subclin-ical cardiac dysfunction in breast cancer patients receiving chemotherapy [29].

The last but not least criterion, which needs to be investigated and reviewed in the context of a weight of evidence approach are the findings from histopathological analysis of the heart tissue from animals exposed to well-established cardiotoxic chemicals. Histopathological data,

when assessed properly can provide reliable in-formation. More specifically, significant functional changes in the heart muscle noted at necropsy and/or at microscopic examination, but also morphological, reversible or not, changes which provide evidence of marked heart dysfunction and cell death in-capable of regeneration could be of relevance. Preliminary data in the literature are encouraging. For example, in rabbits exposed to anabolic steroids local fibrosis and a mild chronic inflammation of cardiac tissue was observed[3,5], while in rabbits ex-posed to the pesticides propoxur and diazinon the main histopathologic findings were fibrosis, hemorrhagic infiltration of myocardial tissues and degeneration of muscle cells, with no signs of inflammation. What is rather interesting in this case, is the per-sistence or accumulation of different quantities of both pesticides studied in cardiac tissues, showing that the cardiac muscle cells were directly exposed to both pesti-cides[30]. Finally, clinical observations or small changes in heart weight with no evi-dence of organ dysfunction could also provide useful information.

In conclusion, more focused research is needed both from scientists and regulators in order to facilitate even further the weight of evidence exercise and describe the hazard of cardiotoxicity caused by chemicals with relevance to humans as regulatory recognized toxicological class. More specifically, the roadmap suggested in the present manuscript for identifying regulatory criteria from animal studies to include into the regulation consists of the following steps:

1. Identification of the appropriate animal species and strain
2. Identification of the lines of scientific evidence (e.g., histopathological, biochem-ical, echocardiographic indices etc.) from animal studies with relevance to hu-mans
3. Meta-analysis of each line of scientific evidence recognized on animal species after exposure to well-established cardiotoxicants to humans (e.g., anthracy-clines) in order to identify threshold values or range of normal and/ or altered values due to exposure
4. Validation of the above described evidence in animals exposed to other alleged cardiotoxic substances (e.g. AAS and pesticides)
5. 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
6. Discussion and introduction of novel indices and in silico methods.

Abbreviations

AAS: anabolic androgen steroids; ALT: Alanine Transaminase; ANP: Atrial natriuretic peptide; AST: Aspartate aminotransferase; BNP: brain natriuretic peptide; CAT: Cata-lase; CK: creatine kinase; CK-MB: creatine kinase-myocardial band isoenzyme; cTnI: Cardiac troponin I; cTnT: Cardiac troponin T; ECHA: European Chemicals Agency; EFSA: European Food Safety Authority; FABP: Fatty Acid-Binding Protein; FS: frac-tional shortening; ICIs: Immune checkpoint inhibitors therapy; IL: interleukin; GSH: Glutathione; GSH-Px: glutathione peroxidase; HF: Heart failure; LDH: lactate dehy-drogenase; LH: lipid hydroperoxide; LV: left ventricle; MDA: malondialdehyde; miRNAs: microRNAs; ROS: reactive oxygen species; SOD: superoxide dismutase; TAC: Total antioxidant capacity; TNF-α: alpha-Tumor necrosis factor; TOS: Total oxidant status.

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CHAPTER 5

Unpublished results, General Discussion and Future Perspectives

5.1 Unpublished Results and discussion

5.1.1. **Echocardiography criteria**

Myocardial contractility suppression due to anthracycline administration is of increasing interest and represents a major challenge in the clinical setting. At the same time in a preclinical stage serves as a model for the assessment of new both chemotherapeutic and cardioprotective agents to be introduced in clinical practice. The myocardial toxicity of anthracyclines is known to be affected by sex and age, along with a number of cardiovascular risk factors and comorbidities (1). It is found that anthracycline related congestive heart failure reaches 10% of patients older than 65 years at usual doses (2). While in early studies it was thought that EF cannot accurately predict congestive heart failure attributed to doxorubicin (2), current perspective is that anthracycline related cardiotoxicity is manifested by a progressive continuous decline in LVEF (3) and identifying subclinical myocardial dysfunction related to anthracycline treatment has great therapeutic implications (4).

Preclinical animal studies are essential in cancer chemotherapy research along with the evaluation of the cardiotoxic propensity of the chemotherapeutic agents. The current recommendations for prevention of cardiac events from cancer chemotherapies are largely based on recommendations. The American Society of Clinical Oncology, for example, recommends active screening and prevention of modifiable cardiovascular risk factors, such as tobacco use, high blood pressure, high cholesterol, alcohol use, obesity and physical inactivity (5). A well characterized animal model for defining cardiotoxicity due to chemotherapy and the treatment thereof is of great importance for clinical practice, as it will enable physicians to base their decisions not only on epidemiology but also on observations developed using concrete data from animal studies.

In the present research, the range of the main echocardiographic indices, namely EF and FS, used in describing anthracycline cardiotoxicity in rats was summarized along with the normal values of the said indices presented in the respective studies. In the graphic representation, it seems that normal and suppressed due to anthracyclines administration values for the two echocardiographic indices are well separated. This provides a first evidence for the possibility of setting a cut-off point for defining anthracycline cardiotoxicity in rats with an in depth future meta-analysis.

In the current thesis a wide range of EF and FS decline due to anthracycline administration was observed. However, the trends of the said decline are easily identified,

especially for FS values, thus rendering the establishment of minimum cut off values of decline feasible. The question remains, as it has also been identified for humans, whether the absolute suppressed values of EF and FS, combined or separately, or the % suppression caused by anthracyclines should be used to describe cardiotoxicity, and which of the two approaches could be more effective in prevention. In our study, it seems that setting a range for % suppression of EF and FS could be more efficient in identifying early cardiotoxicity by counteracting for the intra-individual variation of the absolute values.

There is a clear distinction between the normal LVEF values and the altered ones. More specifically, the normal EF values are ranging from 81.5±6.9% while the altered values are ranging from 59.3±9.5%. It is clear that even in the extreme values there is no overlap.
With regards to the LVFS values, the normal FS values are ranging from 50.17% with a deviation of 8.61% while the altered values are ranging from 33.66% with a deviation of 8.49%. In the case of LVFS there is small overlap between the normal and altered values (Figure 1).

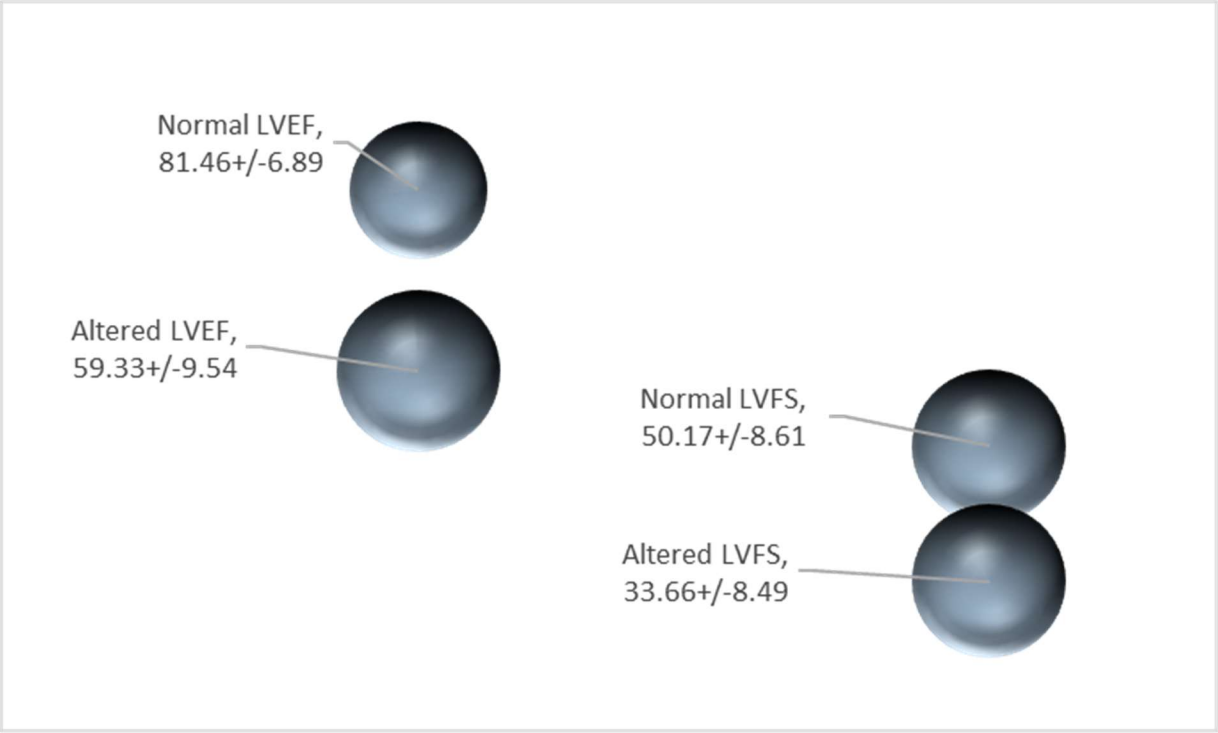

Figure 1. Mean LVEF and LVFS values from control animals (normal) and animals ex-posed to anthracyclines (altered).

5.1.2. **Biochemical criteria**

Oxidative stress refers to elevated intracellular levels of ROS that cause damage to lipids, proteins and DNA. Oxidative stress has been linked to a myriad of pathologies. However, elevated ROS are also signaling molecules i.e. redox biology that maintain

physiological functions (6). The TOS is usually used to estimate the overall oxidation state of the body (7). Similarly, the TAS is used to measure the overall antioxidant status of the body (8). SOD converts the superoxide anion (O2−) into hydrogen peroxide, which is ultimately detoxified by CAT and GSH - Px, with the water molecule as the end product (9). GSH is largely known to minimize the lipid peroxidation of cellular membranes and other such targets that is known to occur with oxidative stress. It is possible that a decrease in the amount of oxidative stress a cell is exposed to could increase health and performance. To protect against the deleterious effects of ROS, our bodies have a complex system of endogenous antioxidant protection in the form of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. The complete reduction of oxygen can be seen from the steps outlined below (10).

$O2 + e- \rightarrow O2-\bullet$ Superoxide radical

$O2-\bullet + H20 \rightarrow HO2\bullet + OH-$ Hydroperoxyl radical

$HO2 \bullet + e- + H \rightarrow H2O2$ Hydrogen Peroxide

$H2O2 + e- \rightarrow \bullet OH + OH-$ Hydroxyl Radical

GSH-Px is a cytosolic enzyme that catalyzes the reduction of hydrogen peroxide to water and oxygen as well as catalyzing the reduction of peroxide radicals to alcohols and oxygen (11).
CAT is one of the crucial antioxidant enzymes that mitigates oxidative stress to a considerable extent by destroying cellular hydrogen peroxide to produce water (12).
MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA. Malondialdehyde level is commonly known as a marker of oxidative stress and the antioxidant status in cancerous patients (13).
LHs are prominent non-radical intermediates of lipid peroxidation whose identification can often provide valuable mechanistic information, e.g., whether a primary reaction is mediated by singlet oxygen or oxyradicals (14).

In addition, generic biomarkers of oxidative stress, both circulating and cardiac-tissue specific, which under certain circumstances could provide supporting evidence in hazard assessment of chemicals for cardiotoxicity have been reviewed. For example, there is a clear correlation in the values of GSH with the anthracycline dosing scheme. More specifically, the overall change in cardiac tissue GSH seems to follow the changes reported in LVEF and shows a decrease ranging from 30 to 70% in the vast majority (83%) of rats with anthracyclines' caused cardiotoxicity compared to their healthy counterparts.

It must be noted that there is a significant pattern similarity in three of the more representative biomarkers of oxidative stress (i.e. MDA, CAT, TOS and SOT) as it is shown in figures 1,2,3,4,5 below.

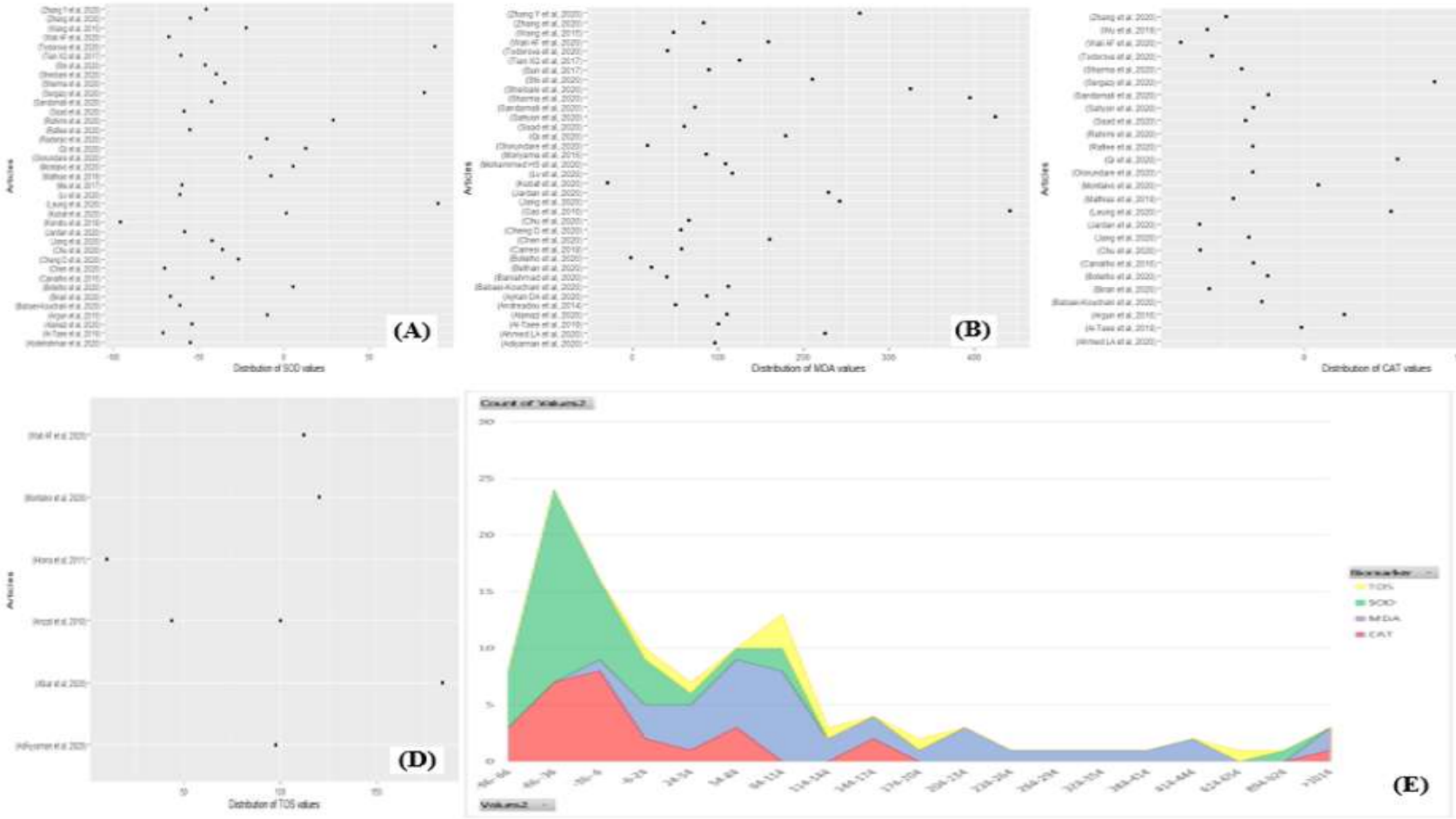

Figure 2. (A): % Increase in SOD (Δ values) in rats exposed to anthracyclines compared to control animals. (B): % Increase in MDA (Δ values) in rats exposed to anthracyclines compared to control animals. (C): % Increase in CAT (Δ values) in rats exposed to anthracyclines compared to control animals. (D): % Increase in TOS (Δ values) in rats exposed to anthracyclines compared to control animals. (E): Distribution of Δ values for SOD, CAT, TOS and MDA

With regard to Biomarkers relevant to damage of the heart muscle and more specifically for CTnT and cTnI there is an increase ranging from 0% to more than 500%. Moreover, very similar pattern is observed for LDH and finally we observed that CK-MB and CK react in similar manner to damages of the heart muscle (figures 3, 4, 5).

LDH is an enzyme found in almost all body tissues. It plays an important role in cellular respiration, the process by which glucose (sugar) from food is converted into usable energy for our cells. Extracellular activity of this enzyme increases under the condition of oxidative stress, since the cell integrity can be disrupted during the lipid peroxidation process (15).

CK has several functions in cellular energy metabolism. It catalyzes the reversible transfer of high-energy phosphate from ATP to creatine, facilitating storage of energy in the form of phosphocreatine. In muscle cells, this extra energy buffer plays a pivotal role in maintaining ATP homeostasis (16). Disruption of cell membranes due to hypoxia or other injury releases CK from the cellular cytosol into the systemic circulation. On this basis, elevated serum levels of CK have been used as a sensitive but nonspecific test for myocardial infarction. The poor specificity reflects the ubiquity of CK in many tissues other than the myocardium. CK is a dimeric molecule composed of two subunits designated M and B. Combinations of these subunits form the isoenzymes CK–MM, CK–MB, and CK–BB. A significant concentration of CK–MB isoenzyme is found almost exclusively in the myocardium, and the appearance of elevated CK–MB levels in serum is highly specific and sensitive for myocardial cell wall injury. The appearance of elevated CK–MB levels in serum is highly specific and sensitive for myocardial cell wall injury (17).

Troponin is a type of protein found in the muscles of your heart. Troponin isn't normally found in the blood. When heart muscles become damaged, troponin is sent into the bloodstream. As heart damage increases, greater amounts of troponin are released in the blood. Cardiac troponin T and troponin I are cardiac regulatory proteins that control the calcium mediated interaction between actin and myosin. Raised cardiac troponin concentrations are now accepted as the standard biochemical marker for the diagnosis of myocardial infarction (18).

Our results provided interesting findings. For example, for the important clinically established specific cardiac enzyme, CK-MB, which is used to inform on adverse myocardial events with sufficient sensitivity and specificity, in a non-invasive way, the results shows an overall increase in CK-MB values of the rats exposed to anthracyclines at well-established cardiotoxic doses, compared to healthy rats. More specifically, they seem to follow the same

pattern with respective echocardiographic measures. The vast majority (ca 80%) of the observed CK-MB values in anthracyclines exposed rats show an increase from 50 to 200% compared to healthy rats.

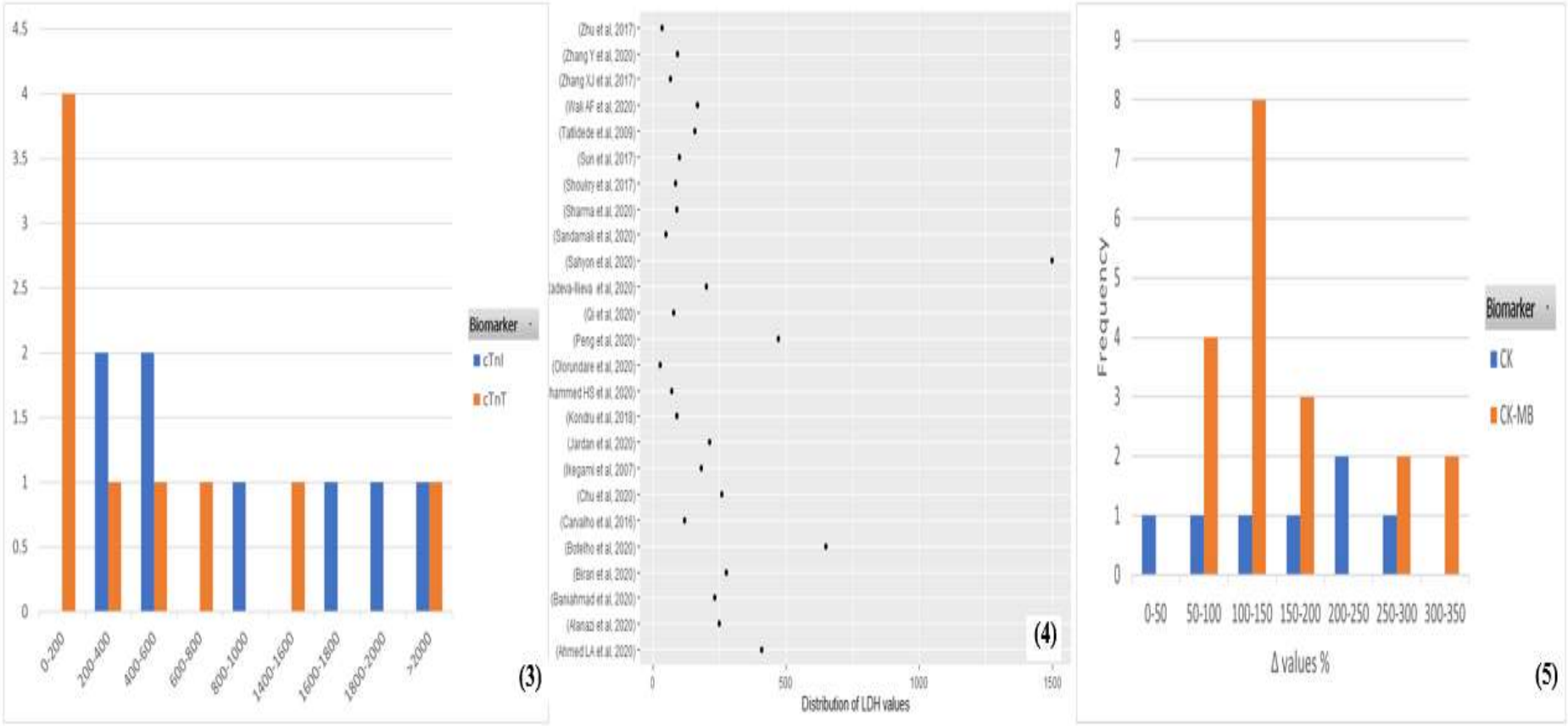

Figure 3. Distribution of %Δ values for cTnT and cTnI.

Figure 4. % Increase in LDH (Δ values) in rats exposed to anthracyclines compared to control animals.

Figure 5. Distribution of %Δ values for CK-MB and CK.

As for Biomarkers relevant to increased ventricular blood volume and consequent response of cardiomyocytes to stretching, two biomarkers have been investigated.

ANP or atrial natriuretic factor (ANF) is a natriuretic peptide hormone secreted from the cardiac atria that in humans is encoded by the NPPA gene. Natriuretic peptides (ANP, BNP, and CNP) are a family of hormone/paracrine factors that are structurally related. ANP acts acutely to reduce plasma volume by at least 3 mechanisms: increased renal excretion of salt and water, vasodilation, and increased vascular permeability (19).

BNP is a cardiac neurohormone biomarker that is secreted from the ventricles when they are under increased pressure and stress (20).

BNP shows a significant increase when ventricular blood volume increases and consequently cardiomyocytes respond to stretching (Figure 6).

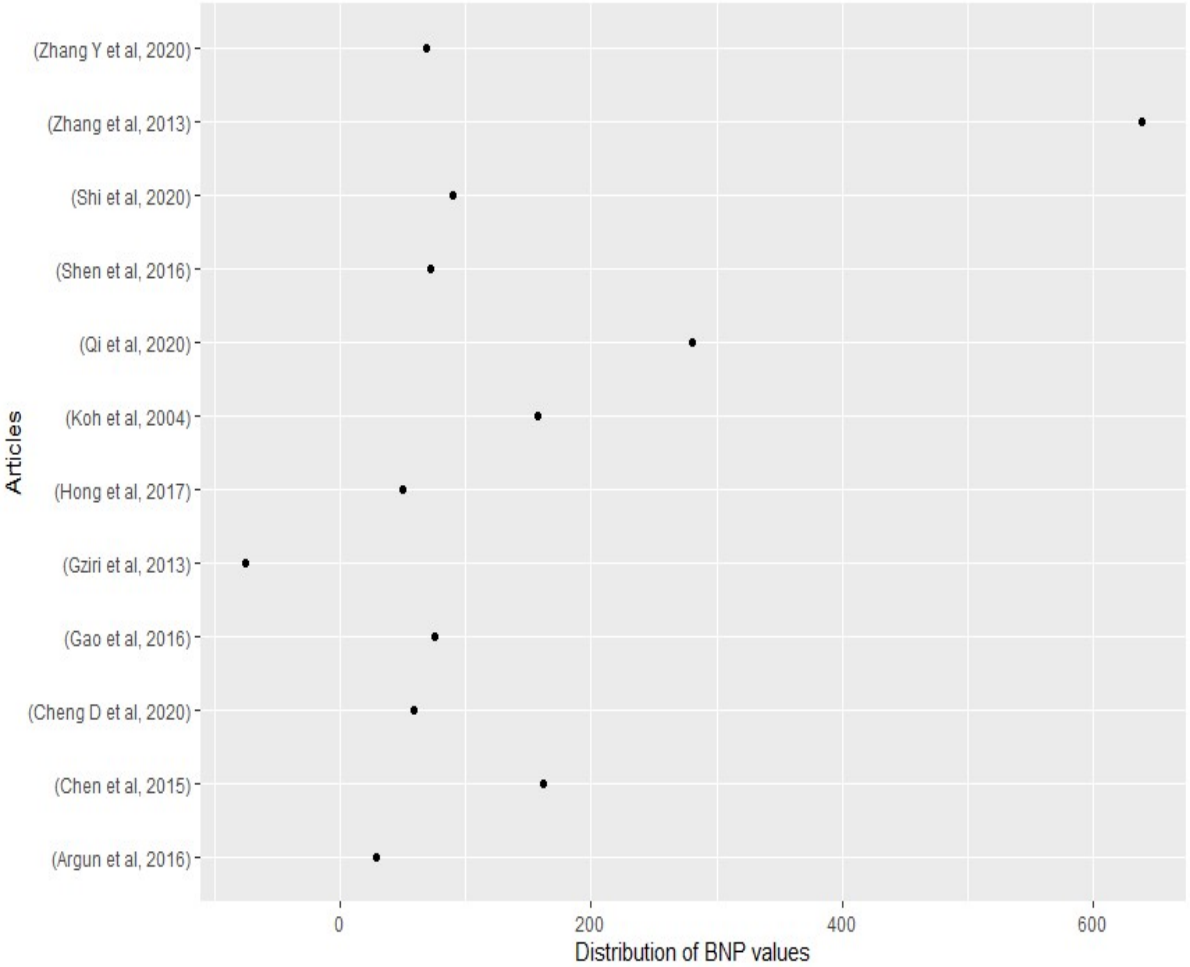

Figure 6. % Increase in BNP (Δ values) in rats exposed to anthracyclines compared to control animals.

Finally, with regard to the biomarkers of inflammation, IL-1 and TNF-a have been investigated for their behaviour to heart failure.

ILs play essential roles in the activation and differentiation of immune cells, as well as proliferation, maturation, migration, and adhesion. ILs regulate cell growth, differentiation, and

motility. They are particularly important in stimulating immune responses, such as inflammation. High levels of circulating cytokines correlate with the severity of HF, measured with the use of New York Heart Association's classification, and prognosis of the disease. In HF, there is an imbalance between pro-inflammatory and anti-inflammatory cytokines. Concentrations of several interleukins are increased in HF, including IL-1β, IL-6, IL-8, IL-9, IL-10, IL-13, IL-17, and IL-18, whereas the levels of IL-5, IL-7, or IL-33 are down-regulated. Concentrations of inflammatory mediators are associated with cardiac function and can be HF markers and predictors of adverse outcomes or mortality (21).

Tumour Necrosis Factor alpha (TNF alpha), is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signalling events within cells, leading to necrosis or apoptosis. The protein is also important for resistance to infection and cancers (22).

The inflamation biomarkers also show an increase. IL-6 seems to increase averagely from 0 to 200%, IL-1β from 30% and TNF-α from 0% to 390%.

Especially for TNF-a there is a distinctive pattern of increment to rats with heart failure (figure 7).

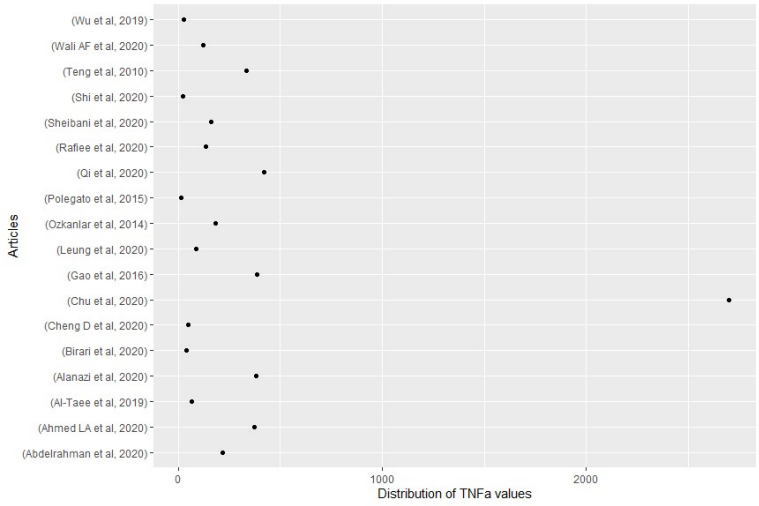

Figure 7. % Increase in TNF a (Δ values) in rats exposed to anthracyclines compared to control animals.

Acknowledging the existence of the several novel biomarkers, it must be noted that the current research project of our team aimed to facilitate the use of biochemical markers in the hazard assessment of cardiotoxicity. Therefore, it was preferred to limit the review focus in more frequently used and traditional biomarkers that are already found in abundance in the literature and for which it is more potent to find available data. Moreover, the experience of risk assessment for newly appeared hazard classes, like endocrine disruptors, has shown that, especially for regulatory purposes, the assessors rely on older data for animal welfare reasons. In any case, it must be stressed that since in the recent years these newly identified biomarkers

have been widely implicated in the research of different pathways, it should be further investigated in a different project how even they could be used in hazard assessment of cardiotoxic chemicals.

5.2 Future Perspectives

Currently, when assessing chemicals toxicity, cardiac effects if monitored and detected in animal studies, mainly on the tissue level, are considered by the authorities, but cardiotoxicity, as such, is not described as a separate hazard class of chemical substances through the available regulations, both at a European level and world-wide. Therefore, chemicals other than pharmaceutical agents are recognised to be cardiotoxic after having exerted such deleterious effects on humans, based on epidemiological studies. In a previous review of our research team, 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 (23). In addition, there is evidence that short and/ or long term exposure to anabolic androgenic steroids is linked to a variety of cardiovascular complications which could be identified by using echocardiography or biochemical markers (24, 25, 33). All these published data suggest clearly that there is a need to establish regulatory criteria for assessing cardiotoxicity as an inherent property of a chemical substance well in advance, and 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. Regulatory established criteria will enable international organizations to early identify cardiotoxic effects and classify chemicals in order to avoid long-term cardiovascular complications.

Despite the fact that for some markers the sample size is small, and consequently the statistical power of the analysis is limited, a similar pattern of change is revealed between values of healthy rats and rats with cardiotoxic manifestations due to anthracyclines exposure known to be relevant to humans. This is an important finding which, when assessed together with echocardiographic indices and/or histopathological data, can significantly reduce the uncertainty and strengthen drastically the weight of evidence assessment for possible cardiotoxicity in humans caused by chemicals, as assessed in animal models.

Specific classification criteria should be developed based on anatomical, histopathological, echocardiographic and biochemical criteria in animals developed in a way that could exclude confounding factors in the development of the observed cardiotoxicity. The results of the

present study are promising in identifying echocardiographic criteria in rats for the establishment of cardiotoxicity. Further studies and meta-analyses are needed in order to evaluate other species, commonly used in research, and explore the possibility of early recognizing the onset of cardiotoxicity, possibly through biochemical markers monitoring based on understanding of the mode of action.

It must be noted that in the past years novel biomarkers of target organ toxicity have been widely used with significant applicability. More specifically, tumor suppressive and oncogenic pathways have been found to involve microRNAs (miRNAs) (26). MicroRNAs which are noncoding RNAs that repress the expression of target mRNAs in a post transcriptional way, including apoptosis, differentiation and cancer (27).

The measurement of plasma miRNAs and messenger RNAs (mRNAs) explain the ongoing physiologic processes in cells and tissues that package and release miRNAs into cell-free space. Moreover, miRNAs are non or minimally invasive, enhancing the animal welfare. Technologically, they are considered as ideal for quantitative analysis due to their standardization rapidness and robustness (28).

The aforementioned markers change significantly and early , after their release from tissues into the plasma during toxic events, which shows tissue-specific expression (29). These advantages have increased the research interest for circulating miRNAs as promising biomarker candidates. They could hopefully play an essential role for human health risk assessment. Another important element is the tissue-specificity and early release of circulating miRNAs upon tissue injury, when damage is still reversible.

Another important novel biomarker is the proto-typic oncogene c-MYC. It is believed that, miRNAs linked to c-MYC could be used in human health risk assessment.

Last but not least, Fatty Acid-Binding Protein (FABP) acts as a long-chain fatty acid carrier in blood and therefore has an essential role in lipid metabolism. The heart type isoenzyme is found in the heart and skeletal muscles and the clinical performance of free FABP is similar to that of myoglobin (30). It is important to highlight that in a cohort of 19 cancer patients underwent Immune checkpoint inhibitors therapy (ICIs), FABP levels, were increased without significant reduction LVEF which indicates that they might be a more sensitive biomarker to detect ICI-related subclinical myocardial damage than traditional cardiac biomarkers (31).

The last but not least criterion, which needs to be investigated and reviewed in the context of a weight of evidence approach are the findings from histopathological analysis of the heart tissue from animals exposed to well-established cardiotoxic chemicals. Histopathological data, when assessed properly can provide reliable information. More specifically, significant functional changes in the heart muscle noted at necropsy and/or at microscopic examination, but also morphological, reversible or not, changes which provide

evidence of marked heart dysfunction and cell death incapable of regeneration could be of relevance. Preliminary data in the literature are encouraging. For example, in rabbits exposed to anabolic steroids local fibrosis and a mild chronic inflammation of cardiac tissue was observed (24, 25), while in rabbits exposed to the pesticides propoxur and diazinon the main histopathologic findings were fibrosis, hemorrhagic infiltration of myocardial tissues and degeneration of muscle cells, with no signs of inflammation. What is rather interesting in this case, is the persistence of different quantities of both pesticides studied in cardiac tissues, showing that the cardiac muscle cells were directly exposed to both pesticides (32). Finally, clinical observations or small changes in heart weight with no evidence of organ dysfunction could also provide useful information.

In conclusion, more focused research is needed both from scientists and regulators in order to facilitate even further the weight of evidence exercise based on animal data and describe the hazard of cardiotoxicity caused by chemicals with relevance to humans as regulatory recognized toxicological class.

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